The Pancreas: Biology and Physiology

EDITED BY
Fred S. Gorelick and John A. Williams

This book provides comprehensive and definitive coverage of the current understanding of the structure and function of the exocrine pancreas. While emphasis is on normal physiology, the relevant cell biological, developmental and biochemical information is also provided. Where appropriate, chapters also include material on functional changes in pancreatitis. All chapters are fully referenced and provide up to date information.

The book has been overseen and published by the American Pancreatic Association with Fred S. Gorelick and John A. Williams as Editors. It includes 26 chapters written by an international group of authorities; completed chapters are also presented in open access format on the Pancreapedia (www.pancreapedia.org). The book contains full-color images and summary diagrams that enhance readability and extend the detail provided in the text.

The Pancreas: Biology and Physiology is divided into four sections:

• Pancreatic Exocrine Structure and Function
  Anatomy, Bioenergetics, Cytoskeleton, Intracellular Signaling
• Acinar Cells
  Digestive enzyme synthesis, intracellular transport, Zymogen granules, Exocytosis
• Exocrine Pancreas Integrative Responses
  Hormonal and Neural Control of Protein and Fluid Secretion, Molecular mechanisms of fluid and bicarbonate secretion, regulation of growth and regeneration
• Pancreatic Islet and Stellate Cell Structure and Function
  Structure and vasculature of islets, regulation of islet secretion, Stellate Cells in health and disease

The book is designed to be a reference book for pancreas researchers but its clear and readable text will appeal to teachers, students and all individuals interested in the exocrine pancreas.

Cover image: Acinar cell with zymogen granule and exocytotic pit

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PREFACE

This book is designed to summarize the current state of knowledge on the structure and function of the exocrine pancreas. The focus is on the biology and physiology of the gland, although biochemistry, molecular biology, genetics, and pathophysiology are also considered. The work grew out of review entries from the Pancreapedia site (www.pancreapedia.org) and follows the publication in 2016 of the book *Pancreatitis*. Both books contain material published by the American Pancreatic Association under a Creative Commons Attribution-Noncommercial-NoDerivatives license and are available as open-access electronic files on the Pancreapedia site. To help fill the gaps, some new chapters were solicited and all authors of existing material were given the opportunity and encouraged to update. There are 26 chapters presented in four sections: “Pancreatic exocrine structure and function,” “Acinar cells,” Exocrine pancreas integrated responses,” and “Pancreatic islet and stellate cell structure and function.” Our goal is to make the information available to the Pancreas community worldwide at no cost while those who wish can purchase a modestly priced bound copy.

As with any compendium of this type, some readers may wonder why some chapters are present and other areas have been omitted. This is in part due to the editor’s choices but is also due in part to the fact that some areas come to an end or morph into other areas such as those that relate to a disease. There are a few areas without much recent progress where we just could not identify expert authors willing to devote time to summarizing that area. On the positive side, since the Pancreapedia is a living and expanding document, anyone seeing an area not present can volunteer to write an entry for the Pancreapedia.
ACKNOWLEDGMENTS

We thank all the authors who contributed their expertise and writing to a novel open-access form of publishing, the Pancreapedia. This book could not have been realized without the advice and assistance of the Michigan Publishing arm of the University of Michigan Library, especially Charles Watkinson and Jason Colman. We thank the other Pancreapedia editors, Ashok Saluja, Markus Lerch, and Stephen Pandol, for advice on selecting authors. We especially thank the students both graduate and undergraduate from the University of Michigan School of Information who organized and formatted material, including Emily Rinck, Ben Krawatz, Melissa Wu, and Juliana Lam. We especially thank M. Dolors Sans who took over this work on top of her own research for the last year and a half. We also thank the American Pancreatic Association, the National Library of Medicine, and the University of Michigan for their invaluable support.
Pancreatic Exocrine Structure and Function
Chapter 1
Anatomy and histology of the pancreas

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**Introduction**

The mandate for this chapter is to review the anatomy and histology of the pancreas. The pancreas (meaning all flesh) lies in the upper abdomen behind the stomach. The pancreas is a part of the gastrointestinal system that makes and secretes digestive enzymes into the intestine and also an endocrine organ that makes and secretes hormones into the blood to control energy metabolism and storage throughout the body.

It is worthwhile to mention a few definitions for key terms as used in the context of the pancreas:

**Exocrine pancreas**, the portion of the pancreas that makes and secretes digestive enzymes into the duodenum. This includes acinar and duct cells with associated connective tissue, vessels, and nerves. The exocrine components comprise more than 95 percent of the pancreatic mass.

**Endocrine pancreas**, the portions of the pancreas (the islets) that make and secrete insulin, glucagon, somatostatin, and pancreatic polypeptide into the blood. Islets comprise 1–2 percent of pancreatic mass.

Since we are dealing with a three-dimensional solid structure, the aphorism that “a picture is worth a thousand words” seems to pertain. Accordingly, this chapter will largely consist of images with extended legends. The images range from classic work of skilled medical artists to original drawings and photomicrographs from leaders in the study of pancreatic anatomy and structure. Text is interspersed as appropriate. Additional useful images are available online at other websites. We provide a list of some of these sites at the end with the references.

**Gross Anatomy**

*Figures 1–13* depict the gross anatomy of the pancreas and its relationship to surrounding organs in adults. It is customary to refer to various portions of the pancreas as head, body, and tail. The head lies near the duodenum and the tail extends to the hilum of the spleen.

When the terms anterior, posterior, front, and back are used, they pertain to relationships in the human, standing erect. Superior and inferior are used in the same context so that they mean toward the head and toward the feet, respectively. These usages obviously do not pertain in quadruped animals where dorsal, ventral, cephalad, and caudad are more useful terms.

Use of the terms left and right can be problematic. For example, the spleen is located in the upper portion of the abdomen on the left side of the body. When the abdomen is pictured from the front, this places the spleen on the viewer’s right-hand side. We will adopt the convention that right and left (unqualified) will be used in the first sense in the legends for gross anatomy (indicating the subject’s right and left side).

When we are designating location within an image, we will use “image right” and “image left” to denote relationships within the image.

Artwork in *Figures 3, 7–8, and 11–13* is by Jennifer Parsons Brumbaugh. These drawings were originally published in the AFIP Fascicle on pancreatic neoplasms and are used with permission of the publisher. Chapter 1 of the Fascicle is recommended as a source for additional detail regarding pancreatic anatomy and histology, and for discussion of the genetic control of pancreatic development.

The tail of the pancreas and spleen are in the left upper quadrant of the abdomen, and the head of the pancreas is in the right upper quadrant just to the right of the midline. If you place your right hand over your upper abdomen with fingers extending to the left over the lower portion of your rib cage and the tip of your thumb extended up over the lower portion of the sternum, then your pancreas lies behind your hand in the back (retroperitoneal) portion of the abdomen. This may be visualized by reference to the small image in the upper image right corner in *Figure 4.*

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The pancreas is about the size of the half of your hand that includes the index and third fingers excluding the thumb. The pancreas weighs about 100 grams and is 14–23 cm long.

- The head of the pancreas lies in the loop of the duodenum as it exits the stomach.
- The tail of the pancreas lies near the hilum of the spleen.
- The body of the pancreas lies posterior to the distal portion of the stomach between the tail and the neck and is unlabeled in this drawing.
- The portion of the pancreas that lies anterior to the aorta is somewhat thinner than the adjacent portions of the head and body of the pancreas. This region is sometimes designated as the neck of the pancreas and marks the junction of the head and body.

**Figure 1.** The gross anatomy of the human pancreas can vary. Figures 1A and 1B are two normal human pancreases from autopsies of adults. Both pancreases have been dissected to remove fat and adjacent organs. The two photos illustrate that there is considerable individual variation in the shape of the pancreas.

(A) This pancreas has a conspicuous uncinate lobe that curves down and to the left (arrow). This is an unusual configuration since the uncinate process usually fuses more completely with the dorsal pancreas adding mass to the head of the pancreas as seen in Figures 1B and 2.

(B) In this pancreas, the uncinate portion is fused to the remainder of the head. A probe (image left) has been put into the main pancreatic duct, and a second probe (vertical) is in the portal-superior mesenteric vein behind the pancreas. The diagonal groove in the tip of the tail (image right) marks the course of a branch of the splenic artery or vein.

**Figure 2.** Normal pancreas dissected to reveal the duct system. The pancreas is viewed from the front and a portion of the parenchyma has been dissected away to reveal: (1) the main (principal) pancreatic duct (Wirsung’s duct) with multiple branches; (2) the accessory duct (Santorini’s duct); and (3) the distal common bile duct. Although the regions are not labeled, we see the head of the pancreas, image left, and tail of the pancreas, image right. This drawing depicts a configuration that is intermediate to those shown in Figure 1 in regard to the degree of fusion of the uncinate process with the dorsal pancreas (drawing by Emily Weber for the Pancreapedia).

**Figure 3.** Anatomic relationships of the pancreas with surrounding organs and structures Several key relationships should be noted. Their recognition may be facilitated by also referring to Figures 4 and 5.

- The close proximity of the neck of the pancreas to major blood vessels posteriorly, including the superior mesenteric artery, superior mesenteric-portal vein, inferior vena cava, and aorta, limits the option for a wide surgical margin when pancreatectomy (surgical removal of the pancreas) is done.
Anatomy and histology of the pancreas

Figure 4. Cross-section of the upper abdomen at the level of the pancreas. Note that the plane of the transection is angled upward on the left as indicated in the drawing upper image right. The major organs except the liver (image left) and kidneys are labeled. Splenic flexure (image right) refers to the colon (used with permission. Copyright, American Gastroenterological Association).

- The common bile duct passes through the head of the pancreas to join the main duct of the pancreas near the duodenum as shown in Figure 2. The portion nearest to the liver lies in a groove on the dorsal aspect of the head (see Figure 7B).
- The minor papilla where the accessory pancreatic duct drains into the duodenum and the major papilla (ampulla of Vater) where the main pancreatic duct enters the duodenum are depicted, image left (image by Jennifer Parsons Brumbaugh; used with permission of the publisher).  

There is no anatomic landmark for the division between the body and tail of the pancreas, although the left border of the aorta is sometimes used to mark the junction. Hellman defined the tail as the one-fourth of the pancreas from the tip of the tail to the head, whereas Wittingen defined the junction between the body and tail as the point where the gland sharply narrowed. It would be difficult to define this point in the pancreases shown in Figure 1.

Embryology and Development

The pancreatobiliary anlagen appear at gestation week 5 in the human; fusion of the dorsal and ventral anlagen occurs during week 7. Full development of acinar tissue extends into the postnatal period. In mice, pancreatic development begins at embryonic day 8.5 (e8.5) and is largely complete by day e14.5.

Figure 5. CT scan of the upper abdomen at the level of the pancreas. This annotated CT scan is oriented with the abdominal wall at the top and the spine and muscles of the back at the bottom viewing the cross-section from below. Thus, the spleen is at the extreme image right and the liver is image left inside the ribs that appear as white ovals in the abdominal wall. Kidneys lie lateral to the spinal column with the tail of the pancreas nearly touching the left kidney (used with permission. Copyright, American Gastroenterological Association).

Figure 6. Mouse pancreas. The pancreas of an adult mouse is shown surrounded by the stomach (top), the duodenum and proximal jejunum (image left and bottom), and the spleen (image right). The duodenum wraps around the head of the pancreas (as demarcated by the line). Rodent pancreas is soft and diffuse compared with the human pancreas (photo provided by Catherine Carriere).
Figure 7. The arterial blood supply of the pancreas. The upper panel (A) is visualized from the front, and the lower panel (B) is seen from the back. The celiac trunk and the superior mesenteric artery both arise from the abdominal aorta. Both have multiple branches that supply several organs including the pancreas. The anastomosis of their branches around the pancreas provides collateral circulation that generally assures a secure arterial supply to the pancreas. Most of the arteries are accompanied by veins (not shown) that drain into the portal and splenic veins as they pass behind the pancreas as shown in B. The superior mesenteric vein becomes the portal vein when it joins the splenic vein (image by Jennifer Parsons Brumbaugh used with permission of the publisher²).

Figure 8. Lymph nodes draining the pancreas. This figure indicates the typical location of lymph nodes surrounding the pancreas. There is considerable individual variation in the location of lymph nodes and an image like this is idealized. Both, A and B, are anterior views. B includes some nodes that lie posterior to the pancreas (image by Jennifer Parsons Brumbaugh used with permission of the publisher²).

Figure 9. Pancreas-associated lymph nodes are assigned numerical codes (lymph node station numbers) that correspond to their anatomic location. This classification is used to denote the location of metastatic spread of pancreatic neoplasms or for other detailed studies. These station numbers are seldom used in Western publications and the image is provided primarily for reference (figure used with permission of the Japan Pancreas Association and the Kanehara publishers).

Eponymous names identify the anatomist, embryologist, or physician who is credited with first describing a structure. You may conclude that Wirsung, Santorini, and Vater were such scientists.

Histology and Ultrastructure

Figures 14–29 depict the histology of the exocrine pancreas at the light and electron microscopic levels. Most histologic images are from human tissue. Exceptions are usually noted in the legend. In Hematoxylin and Eosin (H&E)-stained sections, nucleic acids (DNA and RNA) stain blue; most proteins and carbohydrates stain pink.
Anatomy and histology of the pancreas

Figure 10. Nerves (yellow) serving the pancreas. The cross-sectional image (A) emphasizes the location of the celiac ganglia of the autonomic system lateral to the aorta while (B) emphasizes the rich nerve plexus that connects these ganglia to the pancreas. SMA (superior mesenteric artery). PL (plexus) (figure used with permission of the Japan Pancreas Association and the Kanehara publishers).

to red; fat is extracted by organic solvents used in tissue processing leaving unstained spaces. Sections for light microscopy are most often made from formalin-fixed paraffin-embedded tissue and the sections are usually 4 or 5 micrometers (μm) thick. Thinner (1-μm) sections of plastic-embedded tissues (prepared for electron microscopy) may also be used for light microscopy and a few such sections are also illustrated. For additional ultrastructural detail, the reader is referred to the chapter by Kern. 8

Duct System

The components of the duct system are the main pancreatic duct (duct of Wirsung), interlobular ducts that drain into the main duct throughout the pancreas as depicted in Figure 2, and intralobular ducts (sometimes called intercalated ductules) that link acinar tubules to the interlobular ducts. The intralobular ducts and ductules are ordinarily seen only at the level of light and electron microscopy. Enzymes from acinar cells are released into a bicarbonate-rich solution that is secreted by the CACs and ductal cells and flows from the acini and acinar tubules to the intralobular ducts, then into the interlobular ducts and main duct, and finally into the duodenum at the major or minor papillae. This duct system is illustrated in Figures 25–28.

Figure 11. (A) The figure reflects the embryonic development of the pancreas and biliary system in the human. The pancreas develops from two outgrowths of the foregut distal to the stomach. The ventral diverticulum gives rise to the common bile duct, gallbladder, liver, and the ventral pancreatic anlage that becomes a portion of the head of the pancreas with its duct system including the uncinate portion of the pancreas. The dorsal pancreatic anlage gives rise to a portion of the head, the body, and tail of the pancreas including a major duct that is continuous through the three regions. (B) The figure depicts the rotation of the ventral anlage to fuse with the dorsal anlage and fusion of the duct systems such that the main pancreatic duct is formed from portions of the ducts of both dorsal and ventral anlagen. The caudal portion of the head of the pancreas (uncinate) and the major papilla (ampulla of Vater) are derived from the ventral anlage. The minor papilla that drains the duct of Santorini is derived from the dorsal anlage (image by Jennifer Parsons Brumbaugh; used with permission of the publisher).
The anatomic variations depicted provide additional examples of individual differences in pancreatic anatomy seen in adults. These will be most easily understood by comparing Figures 11 and 12. It becomes apparent that the duct of Santorini is derived from the dorsal anlage, whereas the duct of Wirsung (the main duct of the pancreas) is derived from the fusion of duct systems of both dorsal and ventral anlagen and drains into the duodenum at the ampulla of Vater as depicted in A and B. The connection of the duct of Santorini to the duodenum may regress as depicted in A or persist as in B, C, and D. The duct systems of the two anlagen may fail to fuse as depicted in C giving rise to “pancreas divisum.” Rarely the duct systems may fuse but lose their connection to the ampulla as depicted in D. Pancreatic secretions then reach the duodenum through the duct of Santorini and the minor papilla (image by Jennifer Parsons Brumbaugh; used with permission of the publisher).

“Common channel” refers to the fused portion of the bile and pancreatic ducts proximal to entry into the duodenum. The common channel may be long as depicted in A or short as in B. Less often, there is no common channel because the ducts open separately into the duodenum as depicted in C. The common channel has received much attention because stones in the biliary tract (gallstones) may lodge in the common channel causing obstruction of both pancreatic and biliary duct systems. Such an obstruction is frequently regarded as the cause of acute pancreatitis (image by Jennifer Parsons Brumbaugh; used with permission of the publisher).

The integrity of the duct system is of key importance in preventing entry of the exocrine enzymes into the interstitial space where they may be activated and cause tissue damage manifest as pancreatitis. The main and interlobular ducts have thick dense collagenous walls. The connective tissue component of the duct wall becomes progressively thinner as the ducts branch and become narrower. Intercellular tight junctions between duct cells, CACs, and acinar cells...
Anatomy and histology of the pancreas

Figure 16. Acinar tissue, adult human pancreas (H&E). Acinar cells stain blue at their base because of the high content of RNA and the presence of nuclei. They are pink at their apex (lumenal aspect) where there is a high content of zymogen proteins (digestive enzymes). The nuclei of CACs are sometimes seen within an acinus (arrows).

Figure 18. Pancreas with acinar and centroacinar cells with a small intralobular duct (toluidine blue stain, 1-μm-thick plastic-embedded tissue). The presence of numerous round empty capillaries (arrows) in the interstitial spaces indicates that the pancreas was perfused with fixative. A small branching intralobular duct is evident at the top of the field. Blue ZGs are conspicuous in the acinar cells (micrograph contributed by James Jamieson).

Figure 17. Pancreatic tissue with acinar, centroacinar, and ductal cells (EM thick section). The acinar cells are larger than CACs and are easily identified because of the darkly stained zymogen granules (ZGs). The basal portion (B) of the acinar cells lies next to the interstitial space that contains vessels (V), nerves, and connective tissue. Nuclei (N) with nucleoli (n) are in the basal portion of the acinar cells. The Golgi (G) lies at the junction of the basal and apical (A) portions of the cell. CACs have less rough endoplasmic reticulum and no secretory granules. Their cytoplasm is more lightly stained. A small ductule (D) extends from image right to below center. This is a 1-μm-thick section of plastic-embedded tissue prepared for electron microscopy that was stained with toluidine blue (micrograph contributed by James Jamieson).

play a major role in preventing leakage of the duct system. These have not been well illustrated, although they can be seen in Figures 21 and 22 as dark, thickened zones in the adjacent cell membranes near the acinar or duct lumen. The chapter by Kern in The Pancreas provides excellent images and discussion of these tight junctions.

Interstitial Tissue

Interstitial tissue surrounds lobules of acinar tissue, ducts, and islets. The interstitium contains arteries, veins, capillaries, lymphatics, neural tissue, and stellate cells. Leukocytes may infiltrate the interstitium especially during pancreatitis or in reaction to neoplasms.

The pancreatic stellate cells (PSCs) are specialized connective tissue cells with characteristic structure (Figure 29). They secrete multiple components of the extracellular matrix and are activated by a multitude of factors including inflammatory mediators, alcohol and its metabolites, endotoxins, and cancer cell-derived factors.
Figure 19. Acinar and centroacinar cells (low-power electron micrograph). ZGs, rough endoplasmic reticulum (RER), and nuclei are all identifiable in the acinar cells. In addition, several small dense inclusions of variable structure are present in the cytoplasm (lower red arrow). These are residual bodies derived from degradation of acinar cell organelles by lysosomal enzymes. The formation of such residual bodies is called autophagy, and large complex membrane-bound structures reflecting this process are called autophagic vacuoles. Such “cellular debris” is sometimes extruded into the interstitium as seen near the top of the field (upper red arrow). Residual bodies are also sometimes extruded into the acinar lumen providing a pathway for “garbage” disposal into the intestine. An acinar lumen is indicated by a small black arrow that lies between two CACs left of center. Figures 21 and 22 show acinar lumens at higher magnification. ZGs vary in size from about 0.5 to 1.4 μm (micrograph contributed by James Jamieson).

Figure 20. Rough endoplasmic reticulum (RER) shown by high-magnification electron micrograph. The ribosomes adhere to the cytosolic surface of the membrane whereas the cisternal (luminal) side is devoid of ribosomes. Arrows in the cisterna (image left; red arrows) point toward the interior side of the endoplasmic reticulum. A few ribosomes appear to be free in the cytosol (micrograph by George Palade, contributed by James Jamieson).

Figure 21. Apical portions of acinar cells abutting two acinar lumens (electron micrograph). A portion of a CAC forms part of the wall of the lower lumen (image right lower corner). The arrow in this lumen points to the CAC that has multiple mitochondria in the cytosol. Microvilli are evident protruding into the lumen from both CAC and acinar cells. A second smaller acinar lumen is near the image left upper corner. ZGs are heavily stained, so it is not possible to distinguish their membranes. RER is also evident in the acinar cells (micrograph contributed by James Jamieson).

Figure 22. Apical domain of acinar cells is filled with zymogen granules (electron micrograph). The acinar cells abut a lumen near the center of the image. Microvilli protrude into the lumen. The section is lightly stained allowing visualization of the membrane of the ZGs. ZGs are typically about 1 μm in diameter. Tight junctions are present near the acinar lumen (arrows). A mitochondrion is evident upper image left and a smaller one is located lower image left (micrograph contributed by James Jamieson).
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Figure 23. Key elements of the acinar cell protein synthetic pathway show a close physical relationship (transmission EM). Many of the vesicles seen in the middle of the field are likely involved in the transport of newly synthesized proteins from the RER (image left) to the Golgi (right of center). Arrows mark budding of vesicles from the RER and indicate the direction of the transport by the vesicles to the Golgi and thence to the formation of ZGs (image right) (micrograph contributed by James Jamieson).

Figure 24. Steps of zymogen granule exocytosis at the apical membrane of the acinar cell are shown (transmission EM). Right of center there is a ZG with a hint of fusion of its membrane with the luminal cell membrane as an early step in secretion. To the left of this granule, there is a “cup” in the cell surface that apparently marks the site of excretion of a ZG after fusion of the membrane of zymogen granule with the luminal cell membrane. (The secretory process has been described in detail5, micrograph by George Palade, contributed by James Jamieson).

Figure 25. Main pancreatic duct, human (H&E). The lumen is lined by a single layer of cuboidal duct cells. The thickness of the collagenous duct wall is impressive and is probably accentuated because the lumen is empty and collapsed.

Figure 26. Interlobular duct, human (H&E). The lumen is lined by a single layer of duct cells. The collagenous wall is conspicuous but clearly thinner than that of the main duct. Near the center there is a smaller thin-walled intralobular duct joining the interlobular duct.

Endocrine Pancreas

Most islets (islets of Langerhans) that collectively comprise the endocrine pancreas are too small to be seen by gross examination, and thus they were not depicted in Figures 1–13. Islets vary greatly in size; ~70 percent are in the size range of 50–250 μm in diameter in humans with an average in the range of 100–150 μm.4 Smaller islets are dispersed throughout the acinar lobules and most larger islets lie along the main and interlobular ducts of the pancreas. Most islets are spherical or ellipsoid, but they can be irregular in shape—sometimes reflecting the pressure of an adjacent structure, often a duct, or limitation by a tissue plane. Several reports provide support for the presence of a

Activated PSCs function as fibroblasts, thus contributing to fibrosis associated with chronic pancreatitis and pancreatic ductal adenocarcinoma as discussed in detail by Mekapogu et al.10
higher population density of islets in the tail of the pancreas than in the head and body, although others find no difference. 11–14 In adult humans, the number of islets is calculated to be 500,000–1 million, whereas there are far fewer in smaller animals. 15,16 Islets comprise 1–2 percent of the pancreas in adults of most mammalian species. In addition to the islets, isolated islet cells may be found dispersed in the acinar lobules or in association with ducts.
Photomicrographs of islets follow (Figures 30–39). Several of these have been immunostained using antibodies to specific islet peptide hormones to demonstrate various islet cell types including β-cells (insulin), α-cells (glucagon), δ-cells (somatostatin), and pancreatic polypeptide (PP) cells. The PP cells are commonly regarded as the fourth most prevalent endocrine cell type in the islets. Most PP cells are in the portion of the pancreas derived from the ventral pancreatic anlage, that is, the uncinate process that is reported to comprise about 10 percent of the pancreas. In the portion of the pancreas derived from the dorsal pancreatic anlage, the majority of islet cells are β-cells (75–80%), followed by α-cells (about 15%), δ-cells (about 5%), and very few PP cells. In the uncinate process, there are few α-cells and many more PP cells. Stefan et al. present data from study of nondiabetic human pancreases showing that the PP cells comprise 54–94 percent of the volume of islets in the uncinate region, displacing most α-cells and some β-cells. These investigators provide data indicating that PP cells are the second most prevalent endocrine cell type overall in the pancreas among their 13 nondiabetic subjects.

Figure 31. Human islet (H&E). This islet is elongate and nearly triangular in this cross-section. A thin fibrous septum lies along its lower border. Although most islets are oval or round in cross-section, islets vary greatly in shape as illustrated here.

Figure 32. Exocrine pancreas with an islet, mouse pancreas (H&E). The top of a large islet abuts an intralobular duct that is slightly left of center (photomicrograph by Catherine Carriere).

Figure 33. Islet cells store each hormone in distinct locations (immunoperoxidase). Serial sections of an islet have been immunostained using antibodies to insulin (image left), glucagon (center), and somatostatin (image right). The presence of the hormones is indicated by the brown stain. The predominance of insulin-secreting β-cells is obvious. In the center and image right photos, the location of α-cells and δ-cells is primarily at the border of groups of β-cells (photos provided by Arief A. Suriawinata).

Figure 34. Triple immunolabeling of islet hormones shows the predominance of insulin-secreting cells and their distinct distributions. This islet was stained using antibodies to insulin, glucagon, and somatostatin to demonstrate beta cells (pink), alpha cells (brown), and delta cells (blue). The predominance of β-cells is obvious. α- and δ-cells are typically located at the periphery of clusters β-cells (image provided by Vincent A. Memoli and used with permission of the American Society of Clinical Pathology).
Figure 35. Mouse and human islets stained for glucagon (immunoperoxidase). These images show a minor species difference in the location of α-cells in mouse and human islets. In humans, α-cells appear within the islet, although they seem to be on the periphery of clusters of β-cells. Compare the staining in mouse islet to Figure 36, a mouse islet stained for insulin (micrographs provided by Susan Bonner-Weir).

Figure 36. Mouse islet stained for insulin (immunoperoxidase). Note that unstained cells are located in the periphery of the islet corresponding to the location of glucagon staining in Figure 35 (micrograph provided by Susan Bonner-Weir).

Online Resources

The following websites provide additional images of the pancreas. Some of the drawings are labeled in detail whereas others will challenge you to identify unlabeled structures. We recommend that you visit several of these after you review the text and images provided above. Due to the size of the files, it may take a minute or longer for some sites to open.

A drawing by Frank Netter similar to Figure 3 is posted online at:

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Figure 38. Human islet that was isolated during islet transplantation (electron micrograph). The α-, β-, and δ-cells are labeled. At the ultrastructural level, the cell types are distinguished primarily by differences in their granules. The α-cell granules are typically slightly larger than β-cell granules. δ-cell granules are typically less densely stained than the granules in α- and β-cells. At the image edge (12–1 and 9 o’clock) are interstitial (intercellular) spaces that were probably enlarged by perfusion during the islet isolation procedure. Multiple lipid vacuoles are in the cytoplasm of several islet cells—most notably in the central β-cell where lipid bodies lie at 4 and 11–12 o’clock around the nucleus. Scale bar = 4 μm (micrograph provided by Susan Bonner-Weir).

These two websites provide collections of drawings and photographs:

https://www.google.com/search?q=pancreas+anatomy&client=firefox-a&hs=kor&rls=org.mozilla:en-US:official&tbm=isch&tbo=u&source=univ&sa=X&ei=ne57Uq6gGtSr4AP7y4HQAw&ved=0CDEQsAQ&biw=1628&bih=953


Additional images of islets:


Acknowledgments

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References

Because this is an online publication, some references will be provided as website hyperlinks when this is appropriate. Several of the references are chapters in *The Pancreas: Biology, Pathobiology, and Disease*. Second Edition. Edited by V. L. W. Go et al. Raven Press Ltd. New York, 1993. These may be downloaded at http://journals.lww.com/pancreasjournal/Pages/the-pancreas_bio_pathobio_disease.aspx.

Introduction

The adult pancreas is comprised of at least 10 different cell types including those with endocrine function (α, β, δ, γ, and ε), exocrine function (acinar and duct cells), vascular cells, neurons, and mesenchymal cells that are activated in response to injury. The endocrine and exocrine cells are derived from a common endodermal population of cells that also give rise to the liver and parts of the stomach and small intestine. The process by which these cells are specified to take on mature pancreatic phenotypes and the extrinsic and intrinsic factors that guide these processes will be discussed within this chapter. For a more in-depth discussion on the specific aspects of pancreatic development, there are a number of exceptional review articles that focus on transcriptional regulation, epigenetic regulation, cell lineage tracing, distinction from liver tissue, and signaling pathways involved.1–7

Morphological Development—from Endoderm to Definitive Pancreas

Specification of the mouse pancreas is initiated from the distal part of the endodermal foregut and the proximal part of the midgut beginning at embryonic day (E) 8.5 based on the initial expression of pancreas-specific genes.8 By E9, the first morphological sign of pancreatic development is the condensation of mesenchyme over the endoderm that will give rise to the dorsal pancreatic bud, distal to where the stomach will form. In humans, this occurs just prior to 26 days of gestation.9–11 At E9.5, a clustering or anlage of cells emerges from the dorsal aspect of the gut tube at the point where the notochord comes in contact with the gut (Figure 1). This relationship between the notochord and endoderm is of critical importance (described below). Shortly after, the paired dorsal aorta move toward the midline and fuse, thereby displacing the notochord from the developing pancreas.12 Less than one day later in mice, or six days later in humans, a second ventrally located anlage appears, just distal to the developing liver, extending from the common bile duct. The two buds continue to develop independently, affected by different surrounding tissues, until approximately E13 (days 37–42 in humans), at which point, the rotational movement of the developing gut tube and elongation of both buds cause alignment and fusion into a single organ. In the majority of cases, one of the pancreatic ducts becomes patent, leaving a single common pancreatic duct, the Duct of Wirsung, for the organ. In about 10 percent of people, the two buds do not fuse leading to pancreatic divisum and the presence of a second duct, termed the duct of Santorini or accessory duct.13 Pancreatic divisum is the most common pancreatic developmental anomaly and, in some cases, is an increased risk for recurrent pancreatitis.14,15 By E15, clear acinar cell clusters and presumptive islets are apparent, and differentiation markers for all pancreatic cell types are expressed.

In general, pancreatic development can be separated into three time periods. The primary transition in mice encompasses E9–12 when specification of different pancreatic cell types takes place.16 During the primary transition, the pancreatic buds initially appear as stratified epithelium with a centrally located lumen (Figure 2). Cells within the anlage maintain multipotency, with the ability to give rise to endocrine, acinar, and duct cells. The number of multipotent pancreatic progenitor cells (MPCs) is determined during the primary transition and appears to dictate the final organ size.17 Beginning at E10.75, microlumen structures begin to form, with cells establishing an apical polarity eventually leading to duct formation.18 Integrin-based interactions between outer cells of the developing buds
and the adjacent basement membrane are required for initiation of branching.\textsuperscript{19} Minimal expression of pancreatic hormones and digestive enzymes is observed, although endocrine cells expressing multiple hormones exist, present as clusters within the dorsal bud. Whether these initial endocrine cells are maintained in the adult is still debatable.

The secondary transition occurs between E12 and E15 and is characterized by significant expansion and branching of the pancreatic bud, delamination of individual presumptive islet cells from the pancreatic epithelium, and enhanced expression of both hormones and digestive enzymes. At this point, most of the cells have been specified to become either endocrine, acinar, or duct cells, with only a small proportion of cells maintaining multipotency.\textsuperscript{20,21} The third stage of pancreatic development occurs after E15 and extends postnatally with continued maturation and expansion of the pancreas. While controversial, it appears that all cells within the pancreas after E15 are unipotent, determined to be either acinar, duct, or endocrine in nature. However, multipotency can be stimulated in the adult by injury.\textsuperscript{21–23} High-powered resolution of the developing pancreas reveals a relationship between cell location within the branching epithelium and the different cell types. Progenitor cells giving rise specifically to acinar cells are located predominantly at the tips of the branching duct network. Conversely, mature duct and endocrine cells are derived from the trunks of these branches (Figure 2). p120-catenin (p120ctn) mediates surface-tension cell sorting through heterogeneous expression. Low expression of p120ctn allows cells to migrate to tips, where they are influenced by their environment to become acinar cells, while high p120ctn expressing remain in the trunk and become duct or endocrine cells.\textsuperscript{24} A complete p120ctn deletion leads to defects branching and acinar differentiation.\textsuperscript{25} MPCs exist at the transition position between the tips and the trunks.\textsuperscript{20,26} Indeed, the location of these cells provided support for the hypothesis that centroacinar cells, which sit at the junction between acini and ducts, are pancreatic stem cells in the adult. However, studies examining centroacinar cells as stem cells have not supported such a role.\textsuperscript{27} As mentioned above, by E15, MPCs are rare. Evidence suggests that presumptive endocrine cells undergo a mesenchymal-epithelial transition process and coalesce to form the primitive islets of Langerhans.\textsuperscript{28,29} Further recruitment, proliferation, and maturation give rise to the mature islets postnatally. In mice, the islets consist of an inner core of β-cells, which secrete insulin, surrounded by a mantel of α (glucagon), δ (somatostatin), ε (ghrelin), and PP (pancreatic polypeptide) cells. In humans, these cells are positioned throughout the islets.

Differentiation of acinar cells is less overt. Acinar cells maintain attachment to the duct network and develop a classical epithelial phenotype based on their polarity and intercellular junctions. Expression of carboxypeptidase (CPA) is observed as early as E9,\textsuperscript{8} but CPA expression can be observed at early time points in MPCs as well as in acinar progenitor cells.\textsuperscript{20} More extensive expression of pancreatic enzymes begins around E12.5, and zymogen granules, the morphological hallmark of acinar cells, are first observed around E16.5. Acinar cells do exhibit polarity prior to birth based on the localization of tight and adherens junctions. However, the high degree of...
of organization within acinar cells is first observed following birth.\textsuperscript{30} At this point, mature acinar cell morphology has been obtained, while further proliferation and maturation at the molecular level continues to occur until after weaning, presumably due to alterations in dietary makeup.

**The Three Main Pancreatic Cell types Are Derived from a Common Progenitor Pool**

Elegant genetic lineage tracing experiments in mice have defined the progression of MPCs to individual unipotential populations as well as identified specific markers for this process. Mouse models were developed in which cre recombinase expression was driven in specific cell populations during development.\textsuperscript{31,32} Initially, these experiments involved spatially expressing \textit{cre recombinase} based solely on the promoter used and mating these \textquote{driver} mice to those containing a loxP-stop-loxP (LSL) site upstream of a reporter gene such as green fluorescent protein. Since this LSL site is downstream of a constitutively active and ubiquitous promoter, any cells derived from the initial expressing cells would be positive for the reporter gene. A number of expressing systems used in a system in which the \textit{cre} recombinase could be manipulated temporally through the addition of a tamoxifen-inducible tag linked to \textit{cre} (see Figure 3 for scheme). From this work, several observations could be made. First, prior to E12, MPCs exist that give rise to all cell types.\textsuperscript{20} Second, acinar progenitor cells are distinct from endocrine/duct progenitor cells indicating that duct cells have a closer developmental origin to endocrine cells.\textsuperscript{20} Third, MPCs do not normally exist within the adult but can be stimulated to reappear by injury to the pancreas.\textsuperscript{21} Fourth, acinar cells have the ability of dedifferentiation into duct-like cells and likely contribute to initial PanIN formation.\textsuperscript{33} These studies were also instrumental in defining that MPCs are located at the junction between tip and trunk cells of the branching pancreatic duct and can be identified by a specific combination of transcription factors (TFs). MPCs identified during the primary transition express pancreatic transcription factor 1A (PTF1A), pancreatic-duodenal homebox 1 (PDX1), Sry-related HMG box (SOX9), and NK homeobox 6.1 (NKX6.1). Hepatocyte nuclear factor 1β (HNF1β) defines epithelial cells in the trunk that give rise to endocrine and duct cells but not acinar cells,\textsuperscript{20,34} although there is some controversy as Wright’s group has suggested the HNF1β may actually be expressed in MPCs as well.\textsuperscript{21} Whether this difference reflects the time points examined or differences in analysis is unclear. During the secondary transition, PTF1A and NKX6.1 become mutually exclusive with PTF1A cells specifying an acinar cell phenotype and NKX6.1 found in duct/endocrine precursors.\textsuperscript{35}

**Developmental Relationships That Affect Pancreatic Development**

Specification of the pancreas is dictated by interactions with a number of structures developing in close proximity to the developing endoderm. As mentioned, the ventral pancreatic duct shares a common cellular origin with the liver and, therefore, signals must be present that dictate one fate from the other. Interestingly, it appears that the dorsal and ventral aspects of the endoderm are governed by either significantly different signaling events or are able to process specific signaling pathways, such as fibroblast growth factor (FGF) signaling, in different ways.\textsuperscript{36–39} This suggests that the default differentiation pattern differs for dorsal and ventral endoderm. Indeed, culturing ventral endoderm independent of other structures or signals results in pancreatic differentiation, based on the expression of both endocrine and exocrine cells\textsuperscript{40,41} (Figure 4). This suggests that the default pattern of differentiation for the ventral endoderm is pancreas, and that surrounding tissues in the embryo repress pancreatic development to become other organs. For example, signals from the cardiac mesoderm and the septum transversum, the structures that eventually give rise to the diaphragm and parts of the ventral mesentery, promote liver differentiation at
the expense of the pancreas. Conversely, culturing of the dorsal endoderm does not give rise to pancreas (Figure 4) suggesting that signals are required to promote differentiation and the default differentiation program is not pancreas.

Mesenchyme

Coalescence of the mesenchyme at the level where the dorsal pancreas will form is the first morphological sign of pancreatic development. Removal of the mesoderm, or the fibroblasts within the mesoderm, prior to pancreatic specification results in pancreatic agenesis. Mesoderm removal following specification results in a reduction of the total pancreatic size indicating an ongoing requirement for mesoderm signaling to attain complete organ development. Interestingly, culturing of pancreatic mesenchyme with other sections of the dorsal endoderm can promote pancreatic differentiation, while mesenchyme from other regions of the anterior-posterior axis does not have this ability. This suggests that the mesenchyme provides signals that promote pancreatic specification, yet limits differentiation, thereby allowing expansion of the organ. These signals come in a variety of sources.

Physical interactions between the mesoderm and developing pancreatic bud affect differentiation and maturation in multiple ways. Physical interaction is particularly important in specification of the exocrine pancreas. Glypicans and syndecans promote exocrine cell differentiation, and Notch signaling promotes PTFT1A’s function while repressing Neurogenin (NGN)-3 expression. PTF1A and NGN3 are key transcriptional regulators that promote exocrine and endocrine cell specification, respectively. Laminin-1, a key extracellular component within the mesenchyme, promotes pancreatic duct formation, and substrates that contain laminin-1, such as Matrigel, can promote duct formation from early pancreatic buds possibly through α6-containing integrin complexes. Furthermore, direct interaction between the mesenchyme and endoderm represses islet formation. Only after extensive branching excludes the mesenchyme from the developing ductal network can progenitor cells undergo epithelial to mesenchymal transition (EMT) and coalesce to form definitive islets. Finally, direct interactions between the mesenchyme and developing pancreatic bud maintain a progenitor-like state. Inhibition of EpiCAM (epithelial cell adhesion molecules) in pancreatic bud cultures induces premature endocrine cell maturation, suggesting Epi-CAM helps maintain a ductal endocrine progenitor cell type. In addition to physical interactions, secreted factors from the surrounding mesenchyme have also been identified that either promote or repress pancreatic specification and differentiation. Signaling through the TGFβ superfamily affects many steps in pancreatic differentiation. TGFβ signaling from the mesenchyme promotes endocrine differentiation from MPCs, and TGFβ treatment of pancreatic dorsal bud tissue leads to endocrine differentiation at the expense of exocrine tissue. Activin, another member of the TGFβ superfamily, also promotes endocrine differentiation while blocking branching and proliferation of the pancreatic bud and repressing exocrine differentiation. Inhibition of activin using follistatin promotes endocrine cell differentiation at the expense of endocrine tissue. Disruption of TGFβ signaling affects the secondary transition and once pancreatic fates have been determined, TGFβ signaling is required to maintain mature acinar cell fate as overexpression of dominant negative TGFβRII in the pancreas leads to acinar to duct cell metaplasia (ADM). Additionally, a number of fibroblast growth factors (FGFs) are secreted by the mesenchyme, which affect pancreatic development. FGFR4 represses endocrine differentiation and allows for expansion of the exocrine compartment, while FGFR7 and FGFR10 repress exocrine differentiation, thereby allowing proliferation and expansion of the pancreatic bud. Fgf10-/- mice exhibit pancreatic agenesis due to the inability to repress sonic hedgehog (SHH), a repressor of pancreatic differentiation (see below).

Figure 4. The dorsal and ventral pancreatic buds have different default patterns for development. The pancreas is derived from a ventral pancreatic bud (VPB) and a dorsal pancreatic bud (DPB) that first appear around E9.5 in mice. The ventral endoderm gives rise to liver tissue due to inhibitory signals from the septum transversum and cardiac primordia. In the absence of these signals, pancreatic development is the default pattern of differentiation. The dorsal endoderm requires inductive signals from the notochord to promote pancreatic development. In the absence of these signals, the dorsal endoderm will differentiate into nonpancreatic structures, such as the small intestine (figure is modified from reference 43).
Vascularity

At E8.5, de novo blood vessels coalesce from endothelial precursors immediately dorsal to the pre-pancreatic endoderm (reviewed in Risau et al.65). Paracrine signaling from these blood vessels is crucial in promoting early pancreatic organ specification, particularly in the dorsal pancreatic bud. The importance of blood flow was first identified in a genetically modified mouse model containing a targeted deletion of Cdh2, the gene encoding N-cadherin. Mice completely null for N-cadherin show dorsal pancreatic agenesis,66 initially suggested to be due to impaired interactions between the mesenchyme and pancreatic endoderm. However, restoring N-cadherin specifically in the heart rescued both heart and pancreatic development indicating that maintaining blood flow to the pancreatic anlage is required for dorsal pancreatic formation.67 Further studies showed that signals from the vasculature could be from both the circulating blood as well as the endothelial cells of the dorsal aorta and surrounding mesenchyme.68 However, within the pancreas E-cadherin does not play a role in maintaining exocrine function postnatally. Four days after birth, pancreas-specific Cdh1-null mice failed to gain weight and were hypoglycemic. Four days after birth, Cdh1-null mice displayed disrupted exocrine tissue architecture and integrity and activation of the Wnt and Yap signaling pathways.69

Interactions between the vasculature and endoderm also limit pancreatic differentiation. Overexpression of VEGF-A with the Pdx1 promoter leads to insulin production within the stomach. Conversely, overexpression of VEGF-A within the pancreatic anlage greatly enhances vasculature development and significantly reduces pancreas formation, specifically affecting exocrine pancreatic mass.70 Ex vivo cultures in which the developing pancreas was treated with VEGF showed increased vasculature and greatly reduced size of the pancreas, stalling epithelial cells in a progenitor-like state.71 Conversely, ablation of endothelial cells following pancreatic specification leads to more promiscuous differentiation of acinar cells, ultimately reducing the size of the pancreas as well. Therefore, the endothelium plays a dual role in dorsal pancreatic development. During the primary transition, the vasculature provides signals that specify pancreatic differentiation from the endoderm, yet maintain trunk progenitor populations in an undifferentiated state, preventing premature development of specification to an endocrine fate. During the secondary transition, the vasculature restricts exocrine differentiation.

Again, the signals derived from the vasculature are diverse. Lammert et al.70 showed that direct interaction with the aortic endothelial cells induced pancreatic bud-like structures and PDX1 expression in the adjacent endoderm. Endothelial cells are also required for PTF1A expression in the dorsal foregut. Conversely, development of the ventral pancreatic bud does not appear to require direct endothelial interactions41 but rather relies on paracrine factors for development. For example, sphingosine-1-phosphate, found in the blood, promotes recruitment to a pancreatic cell fate and induces proliferation of pancreatic mesenchymal and endothelial cells.67

Notochord and sonic hedgehog

A critical negative regulator of pancreatic development is SHH. This is evidenced by experiments in which SHH signaling is elevated either through a transgenic approach where the Pdx1 promoter drives SHH expression or by ablating the patched receptor (Ptc). Ptc represses smoothen, a downstream mediator of SHH signaling. Both Pdx1-Shh and Ptc−/− mice exhibit pancreatic agenesis.72 These experiments highlight the importance of repressing SHH at the initiation of pancreatic development. Forcing SHH expression, or the closely related Indian hedgehog (IHH), after pancreatic specification (E12.5) by using the Pax4 promoter, still leads to a significant loss of exocrine and endocrine tissue that is replaced by mesenchyme and stroma.73 Therefore, hedgehog suppression must be maintained to ensure proper pancreatic development. Indeed, activation of SHH signaling is observed during pancreatic injury correlating to acinar to duct cell metaplasia.74 Surprisingly, in zebrafish endoderm, ectopic SHH signaling expands the Pdx1-positive pancreas domain, while inhibiting hedgehog activity results in pancreatic agenesis indicating species differences in signaling pathways.75,76

While the mesenchyme expresses factors that promote SHH repression, including activin and FGFs, the notochord appears to be the primary source of these signals at the initiation of dorsal pancreas induction. The foregut expresses SHH along its length except at the point where the notochord comes in close proximity to the endoderm (Figure 4). Ectopic notochord engraftment represses SHH expression in the lateral and ventral endoderm, and SHH-neutralizing antibodies can induce pancreas gene expression in dorsal endoderm explants.77 However, blocking SHH in non-pancreatic endoderm does not lead to pancreatic development. This suggests that signals from the notochord are not instructive but, rather, permissive. In agreement with this permissive model, Shh−/− mice do not show expansion of the pancreas along the foregut, indicating that additional factors are required.

Ventral bud differentiation, cardiac mesenchyme, and septum transversum

The signals derived from the notochord and mesenchyme that permit pancreatic development are activin (TGFβ family) and FGFs. Surprisingly, these same factors repress ventral pancreatic differentiation, highlighting differences
in the developmental processes of the two pancreatic buds. A number of other findings highlight differences between ventral and dorsal pancreatic bud development. Hhex\(^{-/-}\) embryos fail to specify a ventral pancreas but the dorsal bud develops without issues.\(^{78}\) Alternatively, loss of Raldh2, which is required for endogenous retinoic acid signaling, prevents development of the dorsal pancreas while the ventral portion of the organ remains unaffected.\(^{79,80}\)

Since ventral pancreatic bud formation is the default pattern of ventral foregut differentiation, the factors that govern differentiation of this anlage initially repress pancreatic fate. Similar to the repression of SHH by FGFs and activin in the dorsal pancreatic bud, these factors repress pancreatic specification, thereby allowing differentiation of the liver. The ventral foregut endoderm lies in close juxtaposition to the cardiac mesoderm and septum transversum mesenchyme, and these tissues induce liver fate at the expense of the pancreas.\(^{37}\) Bone morphogenetic proteins (BMPs) also promote epigenetic reprogramming of ventral endoderm to a liver fate. In the absence of these structures or signaling molecules, the endodermal cells activate pancreatic differentiation pathways.\(^{42}\) Once liver development proceeds, the repressive signals are blocked allowing for pancreatic development from the more distal ventral endoderm to occur. In support of such a model, it has been suggested that Hhex\(^{-/-}\) fail to develop a ventral pancreas due to lack of proliferation of the endoderm, which would displace the putative pancreatic bud from influences of the cardiac mesoderm. Supporting this hypothesis, Hhex\(^{-/-}\) endoderm cultured in vitro is fully capable of expressing PDX1.\(^{78}\)

While these various developmental structures have a significant impact on pancreatic development, their effects are governed by the permissive nature of the developing endoderm. In the next sections, the TFs and the signaling pathways that dictate pancreatic development will be discussed.

### Transcription Factors Governing Pancreatic Development

In the previous sections, we discussed morphological events and extrinsic factors that regulate pancreatic development. In this section, we will discuss TFs that govern tissue and cell development. During pancreatic development, coordinated gene expression is critical for cell specification, expansion of the progenitor population, differentiation, and then final maturation and function. The TF network has been well characterized in the pancreas, especially regarding β-cell development, with many TFs exhibiting dual roles during development and adult function. In early development, specific TFs govern the establishment and maintenance of the MPCs, while in mature tissue, TFs affect cell-specific function. A complete description of each TF is not possible. Therefore, this section will focus on identifying TFs linked to factors that define MPCs or are involved in exocrine tissue development as more extensive reviews on TFs that affect endocrine and specifically β-cell development can be found elsewhere.\(^{81,82}\)

#### Transcription factors involved in specifying MPCs from endoderm

In the 1990s, two key TFs—PDX1 and PTF1a—were identified as critical for specification and differentiation of all cell types within the pancreas. Using genetically modified mice, both PDX1 and PTF1a were shown to be absolutely required for differentiation of both endocrine and exocrine pancreatic compartments indicating these factors are required for initial specification of MPCs from the endoderm. Since that time, several other TFs have been linked to specification of MPCs or shown to be required for expansion or maturation of the exocrine pancreas. Many of these proteins exhibit defined roles in development and mature cell function and have been implicated in various pathologies such as pancreatitis or pancreatic cancer. In this section, we have focused on their role in development.

**Pancreatic and duodenal homeobox 1**

Pancreatic and duodenal homeobox 1 (PDX1; alternatively known as insulin-promoting factor 1 (IPF1), or somatostatin-transactivating factor 1 (STF1)) is a homeobox containing TF. PDX1 is expressed in the developing mouse as early as E8.5 and maintained in all cells of the pancreas until E17.5, when it becomes restricted to islets.\(^{83,84}\) Initial expression of PDX1 appears to delineate a region of the developing gut that later gives rise to the stomach, small intestine, and bile duct, suggesting that it is involved in specifying this region of the gut tube to a subset of tissues.\(^{83}\) Single-cell RNA sequencing analysis of the dorsal and ventral pancreatic bud at E10.5 indicates PDX1 high- and low-expressing cells. PDX1-high cells from both ventral and dorsal pancreatic regions are pancreatic progenitors. PDX1-low cells from the dorsal pancreatic region are defined as premature endocrine lineages, while those from the ventral pancreatic region are progenitors for hepatic or extrahepatic lineages.\(^{85}\) Germline deletion of Pdx1\(^{-/-}\) in mice leads to an absence of all pancreatic tissues and neonatal lethality by postnatal day 5 due to hyperglycemia. Examination of the pancreas during development indicates that initial specification of the gut to become pancreas, as well as initial budding, still occurs in Pdx1\(^{-/-}\) mice. However, subsequent expansion and branching of the tissue does not occur, indicating that expansion and complete differentiation of the pancreas requires PDX1 activity.\(^{83,86,87}\) In the mature pancreas, PDX1 was initially described as limited to β- and δ-cells of the islets,\(^{84}\) only being reexpressed in acinar
cells upon pancreatic injury. However, recent studies have identified low levels of PDX1 in most mature acinar cells, and targeted deletion of Pdx1 in post-developmental acinar cells shows that PDX1 is required for maintaining this compartment of the tissue.

Heterozygous Pdx1 (Pdx1+/−) mice initially develop normally. However, over time Pdx1+/− mice develop insulin insufficiency, indicating a requirement for high levels of PDX1 in insulin production. Transient loss of PDX1 in adult tissue using a tet-inducible system supports the ongoing requirement for PDX1 in maintaining β-cell function, as loss of PDX1 leads to decreased insulin accumulation and apoptosis. In humans, homozygous mutation of PDX1 results in pancreatic agenesis, while heterozygous mutations of PDX1 are found in patients with maturity-onset diabetes of the young (MODY). Genetic analysis has identified the PDX1 gene as MODY4,106,107 and PDX1 can directly target and promote the expression of insulin and GLUT2 in β-cells.89

Pancreatic transcription factor 1A

Pancreatic transcription factor 1α (PTF1α; alternatively known as PTF1-p48 or p48) was first identified by its ability to bind combined E-box (CANNTG) and G-box (TGGGA) sequences found in promoters of digestive enzymes trypsin, cpa1, amylase, and elastase.93,94 Early in pancreatic development, PTF1α is coexpressed with PDX1 in MPCs.95 During the secondary transition, PTF1α becomes restricted to acinar progenitor cells.21 PTF1α is a basic helix-loop-helix (bHLH) TF that forms an unconventional protein trimer with ubiquitously expressed E proteins, such as E12 and E47,96 and recombinant binding protein J (RBPJ), a downstream mediator of NOTCH signaling. Combined, these factors make up the PTF1 complex, which specifies pancreatic acinar cells. However, while associated with RBPJ, these acinar cell progenitors do not undergo differentiation, thereby allowing for expansion of the progenitor population.97 Upon commitment to acinar cell differentiation, PTF1α preferentially interacts with the RBPJ-like (RBPJL) protein and nuclear hormone receptor NR5A2 to drive pancreatic differentiation of acinar progenitor cells.98,99

Targeted deletion of Ptf1a in mice results in postnatal lethality immediately after birth,52 likely due to neuronal functions, as PTF1α is also expressed in the neural tube and cerebellum.100 Ptf1a-deficient mice have no pancreatic tissue at birth with PTF1α-deficient cells instead contributing to the common bile duct and duodenum.101 Interestingly, the result of overexpressing PTF1α depends on developmental state. Forced PTF1α expression early in development in cells that would typically give rise to the duodenum and stomach leads to expansion of the pancreas, with increased differentiation of duct, islet, and acinar cell compartments.95 In ES cells, forced PTF1α expression requires MIST1 to activate digestive enzyme gene expression.102

Islet 1

Islet 1 (Isl1) is a member of the LIM/homeodomain family of TFs, expressed in a number of tissues during embryogenesis.103 In the developing pancreas, ISL1 expression is initially observed within the mesenchyme and later in pancreatic islets. Targeted deletion of Isl1 in mice leads to deficits in pancreatic development and provided initial evidence that the mesenchyme has a role in affecting pancreatic differentiation. Culturing putative mouse Isl1++ pancreatic endoderm with wild-type mesenchyme restored pancreatic differentiation confirming that ISL1 acts as non-cell autonomous signal in early pancreas development.104 While ISL1 is restricted to pancreatic islets in the adult, expressing Isl1 in acinar cells using an adenoviral-mediated approach induces tubular complex formation through acinar to ductal metaplasia, in which exogenous expression facilitated this process.105

Hepatocyte nuclear factors

The hepatocyte nuclear factors (HNFs) are a homeodomain-containing superfamily of TFs that affect multiple stages of development. HNF1b encodes a protein that binds DNA as a homodimer or heterodimer along with HNF1a. During endoderm development, expression of HNF6 activates HNF1b expression. HNF1b is critical for endoderm development with HNF1b+ cells giving rise to liver, kidney, pancreas, bile ducts, urogenital tract, lung, thymus, and gut.106 Mice with a germline deletion of the Hnf1b gene have a very similar phenotype to Ptf1a+− mice, with no pancreatic development observed at birth. Temporal and spatial deletion of Hnf1b throughout pancreatic development has demonstrated important roles in MPC expansion and acinar, ductal, and endocrine cell differentiation.108 In humans, mutations in the HNF1B gene result in renal cysts, diabetes syndrome, and noninsulin-dependent diabetes mellitus. Some individuals with HNF1B mutations display pancreatic exocrine dysfunction, pancreatic hypoplasia, and, in some cases, complete absence of the pancreatic body and tail suggesting specific loss of dorsal pancreatic development.109,110

Hepatic nuclear factor 6 (HNF6; also known as ONECUT1 or OC-1) binds DNA to elicit transcription of genes that are important throughout endodermal development. In pancreatic development, HNF6 expression begins prior to PDX1 in MPCs and is critical to several specific developmental functions depending on developmental time point.111–113 After the onset of Pdx1 expression, HNF6 is crucial for endocrine cell development. HNF6 induces HNF1B
expression, which promotes a wave of proliferation and initiates *Neurogenin 3* (*Ngf3*) expression. Following this increase in *Ngf3* expression, HNF6 expression becomes limited to duct cells and is very marginally expressed in acinar cells.\textsuperscript{113} *Hnf6*\textsuperscript{−/−} mice exhibit a hypoplastic pancreas with effects mostly limited to nonacinar cell compartments suggesting HNF6 is dispensable for acinar cell differentiation.\textsuperscript{113} However, in a follow-up study, the pancreatic area of *OC1*\textsuperscript{−/−} at postnatal day (P) 2 decreased due to apoptosis. RNA-sequencing of *OC1*\textsuperscript{−/−} exocrine-enriched samples showed decreased expression of *Mist1*, *Gata4*, *Nr5a2*, and *Ptaf1*, all TFs promoting the acinar cell phenotype,\textsuperscript{114} supporting a role for *OC1* in pancreatic development.

**Sex-determining region Y-box (SRY-box) containing gene 9 (SOX9)**

SOX9 is a member of the SRY-related, high mobility group box TFs. Early in development, between E9 and E12.5, SOX9 expression overlaps with PDX1 and marks MPCs. SOX9 is required for pancreatic cell fate and repression of intestinal differentiation through binding lineage-specific promoters and enhancers.\textsuperscript{115} A FGF10/SOX9/FGFR2 feed-forward loop maintains pancreatic organ identity in MPCs.\textsuperscript{116} While MPCs express SOX9, as cells begin to differentiate SOX9 expression decreases. By E14, SOX9 accumulation is restricted to a subpopulation of PDX1+ cells located within the epithelial cords.\textsuperscript{117} Pancreas-specific knockout of Sox9 using the *Pdx1-Cre* driver leads to stunted growth, elevated blood glucose levels, and death by postnatal day 4. Gross morphological assessment showed hypoplasia of both pancreatic buds beginning at E11.5, due to a reduction of PDX1+ cell proliferation and increase in cell death. Haplo-sufficient *Sox9* mice and individuals with campomelic dysplasia (CD), a semi-lethal skeletal malformation syndrome due to a heterozygous loss-of-function mutation in *SOX9*, have reduced islet cell mass, but the exocrine tissue remains unaffected.\textsuperscript{118,119} In the adult, SOX9 accumulates in some duct and centroacinar cells,\textsuperscript{118,120} and targeted deletion of *Sox9* in the adult pancreas of mice leads to a cystic phenotype and decreased expression of duct markers,\textsuperscript{121,122} indicating SOX9’s role in duct cell maintenance.

**GATA transcription factors**

GATA TFs are characterized by their ability to bind the DNA consensus site (A/T)GATA(A/G). GATA4 and GATA6 have ~85 percent homology, containing a highly conserved DNA-binding domain consisting of two zinc finger motifs.\textsuperscript{123} Initial expression of GATA4 and GATA6 occurs early in development and is associated with the development of many organs of endodermal and mesodermal origin.\textsuperscript{124} GATA4 and GATA6 are expressed between E9.5 and E15.5 in the developing pancreas. At E9.5, GATA4 and GATA6 are coexpressed throughout the pancreatic epithelium.\textsuperscript{125} As development continues, GATA4 accumulation becomes restricted first to the tips of branching ducts and then acinar cells by E16.5 where expression is continually reduced until E18.5 when it is undetectable in exocrine cells. However, in the adult, GATA4 is detectable in subsets of α- and β-cells.\textsuperscript{125} GATA6 expression overlaps GATA4 expression until E14.5–E15.5, when it then becomes restricted to the cells of the endocrine pancreas and duct epithelium.\textsuperscript{125} Transgenic mouse models in which GATA6 targets are repressed result in pancreatic agenesis. This is not the case when GATA4 targets are repressed suggesting GATA6 is critical for pancreatic development, whereas GATA4 is dispensable.\textsuperscript{125}

**Transcription factors promoting acinar cell differentiation**

**Nuclear Receptor 5a2 (Nr5a2)**

NR5A2, a member of the orphan nuclear receptor family, is critical for embryogenesis as germ line *Nr5a2* deletion results in early embryonic lethality.\textsuperscript{126} NR5A2 maintains expression of embryonic stem cell identity genes such as *Oct4* and *Nanog*\textsuperscript{127} as well as genes involved in self-renewal and growth.\textsuperscript{128} Temporal and spatial expression of *Nr5a2* determines its function, suggesting that NR5A2 function may link early endoderm development and endoderm differentiation.\textsuperscript{129} NR5A2 is expressed in the gastrointestinal endoderm and is required for the expansion of pancreatic epithelium. In pancreatic progenitor cells, NR5A2 promotes expression of genes involved in hepatic and pancreatic maturation.\textsuperscript{130,131} In zebrafish, null *Nr5a2* mutations have no exocrine pancreas while development of the endocrine pancreas remains largely unaffected.\textsuperscript{129} In the mature pancreas, NR5A2 regulates the expression of other protein complexes driving the assembly of digestive enzymes.

**Hairy and enhancer of split-1 (Hes1)**

*Hes1* is one of seven *Hes* gene family members, which encode proteins that heterodimerize with other bHLH proteins, normally leading to target gene repression. *HES1* is the key target of NOTCH signaling in pancreatic development. In the absence of NOTCH, RBPJ (also known as recombining binding protein Suppressor of Hairless) inhibits *HES1* expression. When NOTCH signaling is activated, *HES1* is expressed and represses expression of NOTCH ligands *Dll1* and *Jagged1* and the endocrine TF, *Ngf3*.\textsuperscript{135} In pancreatic progenitor cells, *HES1* expression inhibits the expression of *Ptaf1* and *Ngf3*. In *Hes1*\textsuperscript{−/−} mice, ectopic pancreatic tissue is found in the extrahepatic biliary system,
posterior stomach, and proximal duodenum. A conversion of the biliary system into pancreatic tissue has also been noted in Hes1<sup>−/−</sup>-deficient mice, suggesting HES1 restricts pancreatic differentiation from the endoderm. Subsequent studies showed that HES1 and SOX17 are part of a complex regulatory network that controls organ cell fate commitment of pancreatobiliary progenitors in the ventral foregut, by restricting ectopic pancreatic development. MIST1

MIST1 is a member of the bHLH family of TFs expressed at high amounts in mature acinar cells. Activated shortly after PTF1a in a subset of pancreatic cells at E10.5, it is unclear whether MIST1 marks only potential acinar cells or MPCs. By E14.5, MIST1 clearly marks committed acinar cells and is excluded from duct cells through the rest of development and in the adult pancreas. Deletion of Mist1 leads to loss of acinar cell organization and incomplete maturation of acinar cells. While still functional, Mist1<sup>−/−</sup> cells are more likely to undergo acinar-to-duct cell metaplasia, and these animals are more sensitive to pancreatic injury. Several targets of MIST1 transcriptional activity, including Cx32, Rab3d, and Atp2c2, have been identified. However, it is believed that MIST1 does not initiate the transcription of these genes but rather functions to enhance expression of genes required for regulated exocytosis. Therefore, MIST1 has been described as a scaling factor. Importantly, reactivation of MIST1 in adult Mist1<sup>−/−</sup> tissue restores expression and acinar cell organization, suggesting this is a primary role for MIST1 function. The regulation of MIST1 is less understood. While it is clearly downstream of PTF1a and PDX1 in the pancreas, MIST1 is expressed in other serous exocrine tissues, including salivary glands and chief cells, and no studies have shown direct targeting of MIST1 by PTF1a or PDX1. However, both NR5A2 and X-box binding protein 1 (XBP1) appear to directly regulate MIST1.

X-box binding protein 1

X-box binding protein 1 (XBP1) is a bHLH TF and a key mediator of the unfolded protein response (UPR) pathway, activated under conditions in which protein folding capacity of the cells is adversely affected. In nonstressed cells, XBP1 shows limited transcriptional ability. Upon activation of the UPR, inositol-requiring enzyme 1 (IRE1) becomes active and deletes a 26-bp sequence from the Xbp1 mRNA, producing spliced Xbp1 transcript that encodes a TF. sXbp1 targets genes involved in ER-associated degradation and increased protein folding as well as promoting apoptosis depending on the magnitude and length of the cell stress. Likely due to the heavy protein folding capacity required in the pancreas, sXBP1 is present and functional even under physiological conditions. Targeted deletion of Xbp1 during development results in embryonic lethality. Liver-specific rescue allows mice to survive for a short time postnatally to observe the phenotypic abnormalities that exist in other secretory organs. In the pancreas, Xbp1 deletion leads to incomplete development of acinar cells and as a result undergo apoptosis during development. Interestingly, XBP1 function is also required for maintenance of the acinar cell phenotype as induced deletion in adult cells leads to widespread apoptosis and regeneration specifically from acinar cells that escaped cre-mediated recombination. It appears that XBP1 targets several important factors required for acinar cell differentiation including Nr5a2 and Mist1.

Signaling Pathways Involved in Pancreatic Development

While many signaling pathways have been identified to regulate pancreatic development, we have focused on a few key pathways that have been identified in many studies.

β-catenin/Wnt

Wingless-related integration site (Wnt) signaling controls many embryonic developmental processes including body axis patterning, cell fate specification, and cell proliferation. Wnt signaling encompasses three different signaling pathways including the canonical or β-catenin-mediated pathway and two noncanonical pathways that involve planar cell polarity and calcium. Signaling through the canonical Wnt pathway regulates expression of key mediators in pancreatic development, while the noncanonical Wnt pathways regulate cell shape and intracellular calcium. In pancreatic development, several WNT ligands are expressed including WNT2b, WNT4, WNT5a, and WNT7b. Binding of WNT ligands to Frizzled (Fzd) receptors leads to Fzd interaction with Dishevelled (Dvl), at which point the WNT signaling pathway diverges into one of the three pathways mentioned above. Activation of the canonical pathway leads to the dissociation of β-catenin from the APC/Axin/GSK3β complex, leading to β-catenin’s translocation to the nucleus where it interacts with lymphoid enhancer factor (Lef-1)/T-cell factor (TCF) TFs to affect gene expression.

The timing of β-catenin expression and activation of Wnt signaling is critical in pancreatic development. Typically, WNT signaling maintains a progenitor-like state, thereby allowing expansion of the tissue prior to differentiation. Forced expression of WNT signaling prior
to pancreatic specification prevents liver and pancreas development.\textsuperscript{156,164} Detectable amounts of nuclear β-catenin are observed as early as E12.5–E14.5 in the pancreas, prior to the secondary transition.\textsuperscript{156} Activated β-catenin is critical during this time period as mice lacking β-catenin have smaller pancreata by E16.5.\textsuperscript{153} Targeted deletion of β-catenin results in the loss of exocrine pancreatic tissue along with increased tubular structures, increased parenchymal fibrosis, and inflammatory infiltrate. The smaller pancreatic size in β-catenin null animals is due to a decrease in proliferation of PTF1A-expressing progenitor cells and most likely not due to a loss of adherence junctions. These studies indicate that β-catenin is initially required for expansion of the pancreatic epithelium prior to differentiation as well as for terminal differentiation of acinar cells. Although the exocrine pancreas was abolished, a loss of Wnt signaling did not disrupt the development of fully functional islets.\textsuperscript{153}

**Retinoic acid**

Retinoic acid (RA) is generated through the metabolism of vitamin A by aldehyde dehydrogenases enzymes (ALDHs; \textsuperscript{165,166}). RA is a ligand for the nuclear hormone receptor—retinoic acid receptor (RAR)—which exists as a DNA-bound repressor complex in the absence of RA. Heterodimers of RAR and retinoid X receptors (RXR) change conformation upon binding of RA to RAR, allowing recruitment of additional transcriptional regulators, thereby altering the expression of RAR-associated genes.\textsuperscript{167}

In zebrafish, blocking RA signaling results in undetectable expression levels of endocrine (insulin) or exocrine (trypsin) markers and specifies initiation of pancreatic progenitors upstream of Pdx1.\textsuperscript{168} In Xenopus, pharmacological and genetic approaches to block RA signaling diminished Pdx1 expression only in the dorsal pancreas, suggesting that RA signaling might be required only for endocrine pancreatic development in Xenopus. However, RA inhibition at later developmental stages blocked expression of differentiated exocrine markers but retained Pdx1. Therefore, in Xenopus, RA signaling is not required for specification of ventral pancreas but is necessary for later exocrine cell differentiation.\textsuperscript{169}

In mice, retinaldehyde dehydrogenase 2 (Raldh2) is expressed between E8.75 and E12.5 in the dorsolateral mesenchyme that is in contact with the dorsal pancreatic bud. RA is synthesized in dorsolateral mesodermal cells, and the developing tissues responsiveness to RA follows a general dorsal to ventral gradient. Targeted deletion of Raldh2 in mice diminishes Pdx1 and Ptf1a expression in the presumptive dorsal pancreatic bud, while ventral pancreatic development is largely unaffected. Absence of Raldh2 during pancreatic development also decreases HB9 and ISL expression in the dorsal mesenchyme, supporting a mechanism by which dorsal bud agenesis may be due to deficient mesodermal signaling.\textsuperscript{79}

**Hippo signaling**

Hippo (or Salvador/Warts/Hippo (SWH)) signaling regulates organ size through controlling cell proliferation and apoptosis. Hippo signaling involves activation of mammalian Ste20-like kinases 1/2 (Mst1/2), also known as Hippo in drosophila, and Save family WW domain-containing protein 1 (SAV1), also known as Salvador in drosophila, complex. This leads to phosphorylation of large tumor suppressor 1/2 (LATS1/2). LATS1/2 maintains Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) in a cytoplasmic localization and inactive conformation. Therefore, upon LATS1/2 phosphorylation, dephosphorylated YAP and TAZ translocate to the nucleus where they interact with TAE domain family member (TEAD) 1–4 and alter gene expression that promotes proliferation and inhibits apoptosis.\textsuperscript{170} Pancreata of Mst1/2 double knockout mice have a decreased pancreatic mass due to a failure in exocrine cell maturation.\textsuperscript{171} Additionally, overexpressing YAP in later pancreatic development expands the duct network while decreasing the number of acinar and endocrine cells.\textsuperscript{171}

**NOTCH signaling**

The NOTCH pathway plays a role in dictating different developmental fates for closely associated cells. Since duct, exocrine, and endocrine cells arise from common epithelial compartments, NOTCH signaling has been suggested as a mechanism to ensure limited differentiation of endocrine cells. In neuronal development, NOTCH signaling triggers lateral inhibition, which prevents adjacent cells from having the same phenotype.\textsuperscript{172} In pancreatic development, cells expressing NGN3 are destined to become endocrine cells, and NGN3-expressing cells have enhanced expression of NOTCH ligands, Delta (DII), Jagged, and Serrated. NOTCH signaling is triggered by interaction of these ligands with NOTCH receptors on adjacent cells\textsuperscript{173} resulting in receptor cleavage and release of the NOTCH intracellular domain (NICD). Translocation of NICD to the nucleus leads to modulation of transcriptional regulators mindbomb and RBPJ,\textsuperscript{174} which enhances expression of HES1, a repressor of Ngn3. Therefore, lateral inhibition through increased HES1 has been suggested as a way of preventing widespread endocrine differentiation. In support of this theory, deletion of Dll or Hes1 in the pancreas during development increased endocrine differentiation at the expense of acinar cells.\textsuperscript{175,176} However, lateral inhibition cannot explain many of the effects of NOTCH signaling in pancreatic development, nor the final arrangement
of exocrine versus duct versus endocrine cells. NOTCH signaling is time dependent, and forced early expression of NOTCH signaling also reduces the acinar cell population, implying a role for NOTCH in the expansion of this population of cells before acinar differentiation.177

RBPJ activity can be modulated by NICD when it is present in the nucleus. RBPJ is a key component of the PTF1 complex, which includes PTF1a and the ubiquitous bHLH, transcription factor 3 (TCF3). During early pancreatic development, the PTF1 complex includes RBPJ, which allows for acinar cell expansion but limits differentiation.178 When RBPJ-like (RBPJL) replaces RBPJ in the PTF1 complex, the role of this complex switches from one that maintains a specified acinar cell fate to a complex that drives and maintains differentiated acinar cells.98 This RBPJ to RBPJL switch is consistent with targeted pancreatic RBPJL deletion, which decreases expression of genes involved in differentiated acinar cell processes such as digestive enzyme production, regulated exocytosis, or mitochondrial metabolism.179

NOTCH signaling has also been implicated in trunk cell (endocrine/duct progenitor) specification,26 duct cell differentiation, and endocrine lineage commitment.50,169,180 Centroacinar cells and a small percentage of duct cells seem to maintain HES1 expression181 suggesting that these cells might make up a pancreatic progenitor/stem cell population.

Conclusion

The plethora of genetically modified mouse models that allow for targeted and inducible deletion or activation of genes has greatly improved our understanding of exocrine pancreas development and provided insight into disease processes in which the developmental processes are not activated properly (e.g., pancreatic agenesis or insufficiency) or reactivated (e.g., pancreatic cancer). Understanding how these various signaling pathways and transcriptional factors work in concert to cause acinar cell differentiation is still an active area of discussion and many of the findings still need to be translated to human development. In particular, research will be needed to understand the impact of environmental factors (such as diet) on exocrine pancreatic disorders, both during development and diseases such as pancreatitis. Likely, these studies will identify the importance of epigenetic regulators or events that mediate the environmental impact on pancreatic development.

References


120. Kopp JL, Dubois CL, Schafer AE, Hao E, Shih HP, Seymour PA, Ma J, Sander M. Sox9+ ductal cells are multipotent progenitors throughout development but do not produce new endocrine cells in the normal or injured adult pancreas. Development 2011; 138:653–665.


144. Yoshiida H, Matsu T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Mol Cell 2001; 23:783–795.


Chapter 3

Bioenergetics of the exocrine pancreas: Physiology to pathophysiology

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Introduction

The major cell types defining exocrine functions of pancreas are pancreatic acinar and pancreatic ductal cells. Acinar cells secrete digestive enzymes, precursors of digestive enzymes (zymogens), and NaCl-rich fluid, while ductal cells secrete large volumes of bicarbonate-containing fluid and form a conduit for exocrine pancreatic secretion (reviewed in 1–3). Both exocytotic secretion of proteins and fluid secretion are energy-consuming processes. Furthermore, pancreatic acinar cells have high levels of protein synthesis4 supporting substantial protein export by secretion. The bioenergetics of this organ are designed to sustain these energy demands and adjust ATP generation when cells undergo shifts between resting and stimulated conditions. Disruption/insufficiency of ATP generation is an important feature of pancreatic damage in pathological conditions. In this chapter, we will consider both physiological adaptation of bioenergetics in exocrine pancreas and its disruption in acute pancreatitis.

Pancreatic acinar cells constitute more than 80 percent of the volume of the exocrine pancreas5 and are thus likely to make major contributions to bioenergetics parameters measured in experiments utilizing undissociated pancreas. Signaling mechanisms and bioenergetics of this cell type have been extensively investigated and a substantial part of this chapter therefore will be focused on the bioenergetics of pancreatic acinar cells. There has also been recently significant progress in characterizing bioenergetics changes in ductal cells, particularly in conjunction with the pathophysiology of acute pancreatitis; we will discuss this progress in section “Alteration of Bioenergetics in Pancreatic Pathophysiology.”

Mechanisms of ATP Generation

Information on the relative importance of different substrates (carbohydrates, amino acids, and fatty acids) for the bioenergetics of exocrine pancreas is somewhat limited. Pancreatic acinar cells express a range of amino acid transporters,6 allowing accumulation of amino acids against a significant concentration gradient; this is beneficial for prominent protein synthesis in this cell type and could also support utilization of amino acids as substrates for ATP production. Notably, in in vivo and in vitro experimental models of acute pancreatitis expression of several amino acid transporters was significantly downregulated.6 A study by S. Araya and colleagues highlighted a high rate of glutamine transport in pancreatic acinar cells and compared CO2 generation in response to addition of amino acids (glutamine and leucine), glucose, and palmitoyl.7 The study concluded that glutamine is the preferred energy source for pancreatic acinar cells and that these cells operate an efficient conversion of glutamine to glutamate followed by glutamate utilization (via α-ketoglutarate) in the Krebs cycle.7 Information on the contribution of other amino acids and fatty acids, however, is sparse. Glucose can support the bioenergetics of acinar cells, and glucose-containing extracellular solution has been frequently utilized in studies of glycolysis and oxidative phosphorylation in this cell type. The relative contribution of oxidative metabolism and glycolysis to the net ATP generation in exocrine pancreas and pancreatic acinar cells is still a subject for debate. Mitochondria occupy a significant proportion of cell volume in pancreatic acinar cells (approximately 8%). It is an early study by Bauduin and colleagues concluded that mitochondria are by far the major source of ATP production in acinar cells while glycolysis makes only minor contribution.8 This paper, however, reported that high concentration of oligomycin (inhibitor of the mitochondrial F1F0-ATP synthase: abbreviated to ATP synthase) reduced nucleoside triphosphate (presumably mainly ATP) content to approximately 41 percent of the control value, suggesting a significant contribution of glycolysis to ATP generation. However, the same authors reported that antimycin (inhibitor of the mitochondrial electron transport chain) reduced nucleoside triphosphate content to 16 percent and anaerobiosis to 8 percent.8 H. Kosowski and colleagues from...
W. Halangk laboratory reported a decrease of ATP content to approximately 21 percent of the control value following 30 minuses of anoxia. Experiments with anoxia and antimycin could be interpreted as evidence for a rather modest contribution of glycolysis to ATP generation in the acinar cells. However, these treatments are expected to trigger the reverse mode of ATP synthase, which can convert this enzyme into a powerful ATP consumer (i.e., in this mode the enzyme functions as an ATPase consuming ATP and transporting protons into the matrix; reviewed in 10–13). The discrepancy between the ATP levels retained in the presence of oligomycin and after the inhibition of mitochondrial electron transport chain by anoxia or antimycin could be explained by a reverse mode of ATP synthase. ATP consumption by this reverse mode could be potentially prevented by the F$_{1}$F$_{0}$-ATP synthase inhibitory factor 1 (IF1). However, it should be noted that normal pancreas contains relatively low levels of IF1 (e.g., in comparison with heart or pancreatic cancer tissue) and therefore the effect of the reverse mode could be significant. Consequently, experiments with oligomycin or combination of oligomycin with an inhibitor of the electron transport chain should provide a better estimation of contribution made by glycolysis to ATP generation of the acinar cells. The early estimation of 41 percent is consistent with more recent findings indicating a significant (at least 30–40 percent) contribution of glycolysis to ATP generation in acinar cells. Furthermore, the balance of glycolytic and oxidative ATP production depends on external factors. In particular, secretagogues that produce moderate Ca$^{2+}$ signals upregulate the rate of Krebs cycle and mitochondrial ATP generation, while application of insulin strongly potentiates glycolytic ATP production, which can sustain bioenergetics of acinar cells with damaged mitochondria.  

**Stimulus-Metabolism Coupling**

In the late 1970s, an important and surprising observation was made in experiments on isolated pancreatic acini; upon stimulation with secretagogues (caerulein, cholecystokinin, or carbachol), cellular ATP levels were stable or even slightly increased. It would be expected that the increased energy demands imposed by the stimulation of secretion should reduce ATP content. The findings were therefore paradoxical and suggested existence of an effective mechanism(s), capable of compensating and even overcompensating for the increased ATP usage in the stimulated cells, that is, indicative of an efficient stimulus-metabolism coupling in this cell type. Early evidence for such stimulus-dependent upregulation of bioenergetics came from the study by M. Korc and colleagues demonstrating strong and rapid upregulation of glucose uptake in isolated pancreatic mouse acini stimulated with secretagogues (see Figure 1A). Caerulein, CCK, and carbachol were tested and all produced clear and substantial increases in glucose uptake (2.5–3 times in comparison with control). Crucially, the increase in the glucose uptake could be mimicked by the Ca$^{2+}$ ionophore A23187. Furthermore, incubation of cells in the solution containing the Ca$^{2+}$ chelator EGTA suppressed the agonist-induced glucose uptake. The authors therefore concluded that intracellular Ca$^{2+}$ may “mediate hormone-stimulated glucose transport.” The authors’ suggested role of cellular Ca$^{2+}$ in the regulation of bioenergetics of the acinar cells was confirmed by the following 40 years of research. Important findings that characterized the role played by Ca$^{2+}$ signaling in regulating bioenergetics were made at the end of the previous century in Denton’s laboratory. These experiments revealed the ability of Ca$^{2+}$ to upregulate the rate of Krebs cycle; the effect of Ca$^{2+}$ was mediated by rate-limiting dehydrogenases of the Krebs cycle (reviewed in 22,23). At the single cell level, the relationship between cytosolic Ca$^{2+}$, mitochondrial Ca$^{2+}$, and NAD(P)H (an important reducing equivalent produced in Krebs cycle that supplies electrons for the respiratory chain) were first demonstrated in hepatocytes. This study utilized cellular autofluorescence to monitor the NAD(P)H responses and revealed fast upregulation of the NAD(P)H production by Ca$^{2+}$ transients. In isolated pancreatic acinar cells, relationships between Ca$^{2+}$ signaling (in cytosol and mitochondria) and NAD(P)H were first characterized by Voronina and colleagues. This study revealed fast and robust NAD(P)H transients associated with Ca$^{2+}$ responses (Figure 1B and C), consistent with NAD(P)H responses recorded from perfused pancreas.

Subsequently, rises of NAD(P)H in response to CCK-induced Ca$^{2+}$ transients were shown to occur in isolated human pancreatic acinar cells, confirming a similar stimulus-metabolism coupling to that previously elucidated in murine cells. Physiological concentrations of important secretagogues (e.g., CCK and ACh) induce oscillatory Ca$^{2+}$ responses in acinar cells (reviewed in 27–29). NAD(P)H responses were sufficiently fast to form oscillations (reflecting oscillations in the rate of the Krebs cycle) closely associated with the oscillations of cytosolic Ca$^{2+}$. Upon stimulation with Ca$^{2+}$-releasing secretagogues, pancreatic acinar cells generate local and global Ca$^{2+}$ transients (reviewed in 27,28). All global Ca$^{2+}$ transients were able to trigger NAD(P)H transients (i.e., increased rate of the Krebs cycle). The majority of local Ca$^{2+}$ responses were also able to trigger resolvable NAD(P)H responses except for the very short local Ca$^{2+}$ transients (less than 2.9 seconds) that are probably quenched by cytosolic calcium buffers before reaching the mitochondria. To upregulate activity of dehydrogenases of the Krebs cycle and accelerate its rate, Ca$^{2+}$ must enter mitochondrial matrix. The phenomenon and properties of mitochondrial Ca$^{2+}$ entry were
extensively characterized in the second part of the previous century; however, the molecular mechanism was only discovered in 2011.

Mitochondrial Ca\(^{2+}\) entry is primarily mediated by the mitochondrial Ca\(^{2+}\) uniporter complex\(^ {32,33}\) (reviewed in \(^ {34}\)). In mammals, this complex is composed of the mitochondrial Ca\(^{2+}\) uniporter (MCU, pore-forming protein localized in the inner mitochondrial membrane), its Ca\(^{2+}\)-dependent regulators MICU1/MICU2\(^ {35–38}\) and other associated and regulatory proteins (reviewed in reference \(^ {34}\)). An important function of MICU1/MICU2 is to provide a threshold Ca\(^{2+}\) concentration that limits Ca\(^{2+}\) entry into mitochondria at low cytosolic Ca\(^{2+}\) concentration. The voltage difference between mitochondria and cytosol is substantial (approximately 140 mV); it is sufficient to drive Ca\(^{2+}\) entry into mitochondria even at low resting Ca\(^{2+}\) levels. This would form a futile Ca\(^{2+}\) cycle necessitating continuous energy-dependent Ca\(^{2+}\) extrusion from mitochondria\(^ {35–37,39–41}\). At higher Ca\(^{2+}\) concentrations, MICU1 and MICU2 bind Ca\(^{2+}\) and undergo conformational change allowing Ca\(^{2+}\) entry through the MCU channel.\(^ {35–37,41}\) Threshold Ca\(^{2+}\) concentrations necessary for such “opening” of the MCU complex are usually significantly higher than the resting cytosolic Ca\(^{2+}\) and usually also higher than Ca\(^{2+}\) concentrations in the areas distant from Ca\(^{2+}\)-releasing channels in the cellular organelles or Ca\(^{2+}\) influx channels in the plasma membrane. Therefore, Ca\(^{2+}\) entry into mitochondrial matrix in many systems depends on the mitochondrial localization and specifically on the proximity of the organelles and their MCU complex to the Ca\(^{2+}\)-releasing channels (reviewed in \(^ {42}\)). In pancreatic acinar cells, mitochondria are found in three major groups—subplasmalemmal, perigranular, and perinuclear.\(^ {43–46}\) These mitochondria are conveniently localized to respond to Ca\(^{2+}\) release from IP\(_3\) receptors (IP\(_3\)Rs),\(^ {44}\) ryanodine receptors (RyR),\(^ {47}\) and store-operated Ca\(^{2+}\) (SOC) influx channels.\(^ {43,48}\) The mitochondrial Ca\(^{2+}\) uniporter is responsible for the bulk of Ca\(^{2+}\) entry into the mitochondrial matrix; however, other mitochondrial Ca\(^{2+}\) influx mechanisms have been reported (e.g., \(^ {49–51}\)). A recent study, utilizing MCU knockout (MCU KO) mice, demonstrated that the MCU complex is responsible for the major component of the mitochondrial Ca\(^{2+}\) entry in CCK-stimulated pancreatic acinar cells; however, very small mitochondrial Ca\(^{2+}\) signals were observed in CCK-stimulated acinar cells from MCU KO mice suggesting an alternative (and currently unknown) Ca\(^{2+}\) entry mechanism in pancreatic mitochondria.\(^ {17}\) These observations were consistent with the results of measurements of mitochondrial NAD(P)H and FAD responses. During the Krebs cycle, FAD is converted to FADH\(_2\); FAD is fluorescent and the decrease of its content/fluorescence in mitochondria suggests its reduction and could be interpreted (with caution) as an acceleration of Krebs cycle.\(^ {52}\) NAD(P)H and FAD

Figure 1. Stimulus-metabolism coupling in pancreatic acinar cells. (A) Changes in glucose uptake by pancreatic acini upon application of caerulein (adapted from Korc et al.\(^ {26}\)). (B) NADH oscillations in pancreatic acinar cells. Left panels show transmitted image of two pancreatic acinar cells and regions of interest for recording of fluorescence (corresponding to traces on the right panel). Lower left panel shows the distribution of NADH fluorescence (adapted from Voronina et al.\(^ {15}\)). (C) Correlation between cytosolic Ca\(^{2+}\) responses (revealed by negative deflections on the trace of Ca\(^{2+}\)-dependent Cl\(^{-}\) current) and changes of NADH fluorescence (adapted from Voronina et al.\(^ {15}\)). (D) Effect of mitochondrial Ca\(^{2+}\) uniporter (MCU) knockout on the NADH and FAD responses to CCK stimulation in pancreatic acinar cells (adapted from Chvanov et al.\(^ {17}\)).
responses associated with Ca\(^{2+}\) signals have been measured in a number of cell types (e.g., 24,53); in these studies, transient increases in NAD(P)H fluorescence were usually accompanied by decreases in FAD fluorescence. The amplitudes of both NAD(P)H and FAD responses to CCK stimulation drastically decreased in the acinar cells from MCU KO animals in comparison with wild-type (WT) mice\(^7\) (Figure 1D). This confirms the major role of Ca\(^{2+}\) signals and MCU-mediated mitochondrial Ca\(^{2+}\) responses in stimulus-metabolism coupling in pancreatic acinar cells. Notably, small NAD(P)H and FAD responses were retained in the cells from MCU KO mice; this is consistent with the retention of small mitochondrial Ca\(^{2+}\) signals in cells of these animals.\(^{17}\) Another important parameter reflecting cellular bioenergetics is oxygen consumption; in line with the observed upregulation of glucose uptake\(^{20}\) and NAD(P)H increase,\(^{16,17}\) oxygen consumption significantly increases in perfused pancreas\(^{25}\) and isolated acinar cells stimulated with Ca\(^{2+}\)-releasing secretagogues.\(^{17,25,54,55}\)

Importantly, this effect was diminished in acinar cells from MCU KO mice,\(^7\) again highlighting the role of mitochondrial Ca\(^{2+}\) in the stimulus-metabolism coupling of this cell type. The notion of the efficient stimulus-metabolism coupling in pancreatic acinar cells is consistent with the outcome of experiments involving ATP and creatine phosphate measurements in perfused rat pancreas, which indicated that “ATP and PCr remained largely unchanged” in spite of maximal stimulation of the organ with 100 pM CCK producing robust protein and fluid secretion.\(^56\) In this study, a higher concentration of CCK (1nM) produced a small decrease in ATP levels, which may be associated with tissue-level effects of supramaximal doses of this agonist and will be discussed in the role of bioenergetics in pancreatitis (see below). While studies of the relationships between Ca\(^{2+}\)-releasing secretagogues and bioenergetics, conducted on isolated acinar cells and perfused pancrea, reported stable or minimally changed ATP levels, despite upregulation of energy-consuming processes,\(^{15,20,21}\) results from experimental animal models, involving low “physiological” as well as supramaximal doses of secretagogues, are somewhat contradictory. In the study by R. Luthen and colleagues, all tested concentrations of caerulein (7 hourly intraperitoneal injections of caerulein each corresponding to 0.1, 1, 5, and 50 µg/kg body weight) produced significant and similar decreases in ATP concentration in mouse pancreata (by approximately 33% one hour after the first injection and 45% one hour after the last injection).\(^{57}\) It is conceivable that in intact tissue the rate of ATP production is limited by tissue oxygen levels and nutrient supply (e.g., due to changes of pancreatic microrcirculation, reviewed in\(^{58}\)), which are usually not restricted in single cell preparations. It should be also noted that other studies involving animal models and stimulation with secretagogues reported stable pancreatic ATP levels.\(^{56,59}\) This apparent contradiction emphasizes the importance of continuing investigations of stimulus-metabolism coupling at cellular and tissue levels as well as in animal models. The pathophysiology of acute pancreatitis is frequently associated with disruption of bioenergetics and loss of ATP in pancreatic tissue. In experimental models of acute pancreatitis, this is particularly clear for bile-induced and fatty acid-induced acute pancreatitis; the relevant studies will be discussed below.

**Metabolism—Signaling Feedback**

Several pathophysiologically relevant substances (including fatty acids, reactive oxygen species, asparaginase, bile acids, and nonoxidative alcohol metabolites) can deplete cellular ATP (see references 15, 18, 19, 60–64 and the next section). While Ca\(^{2+}\) signaling can influence metabolism and mediate efficient stimulus-metabolism coupling in pancreatic acinar cells, there is also a powerful effect of metabolism on signaling. Interesting early manifestation of such feedback mechanism came from study by A. Hofer and colleagues, which demonstrated inhibition of Ca\(^{2+}\) leak from the ER lumen by ATP depletion.\(^{65}\) Further studies identified the prominent influence of ATP depletion on ACh-induced Ca\(^{2+}\) oscillations in pancreatic acinar cells; a modest decrease in cytosolic ATP concentration (revealed by measuring luciferase bioluminescence in live cells) resulted in abrupt termination of Ca\(^{2+}\) oscillations \(^{66}\) and Figure 2A). The molecular phenomenon underlying this cellular response was identified in studies from the Yule laboratory that characterized ATP sensitivity of IP\(_3\) receptors responsible for the Ca\(^{2+}\) oscillations in acinar cells. All three types of IP\(_3\)Rs are expressed in pancreatic acinar cells;\(^67\) however, the expression of InsP\(_3\)R\(_3\) is relatively low\(^60\) and it is unlikely to play significant role in physiologically relevant Ca\(^{2+}\) responses. InsP\(_3\)R\(_2\) and InsP\(_3\)R\(_3\) are the major receptor subtypes in acinar cells.\(^67\) These are ATP-sensitive, and Ca\(^{2+}\) fluxes via these channels are inhibited by ATP reduction in mM or sub-mM range.\(^58,69\) Further study utilizing acinar cells from InsP\(_3\)R\(_2\) knockout mice revealed particular importance of this Ca\(^{2+}\)-releasing channel in determining ATP sensitivity of Ca\(^{2+}\) responses\(^68\) (Figure 2B). Another vital component of calcium signaling, Ca\(^{2+}\) influx via the plasma membrane, is also ATP-sensitive. In pancreatic acinar cells, Ca\(^{2+}\) influx is primarily mediated by the store-operated Ca\(^{2+}\) entry (SOCE) based on the interaction of the ER Ca\(^{2+}\) sensor STIM1 and the plasma membrane channel-forming protein Orai.\(^{48,70–73}\) A role for TRPC channels in SOCE has also been reported.\(^74\)

ATP depletion resulted in the efficient inhibition of the SOCE in pancreatic acinar cells.\(^66\) Inhibition of mitochondria and/or ATP depletion have been shown to suppress
SOCE entry in a number of other cell types and could be therefore considered as a general phenomenon not limited to the pancreatic acinar cell. The specific molecular mechanism responsible for such inhibition is still debated. Stimulus-metabolism coupling and metabolism-signaling feedback are schematically illustrated in Figure 3. As expected, ATP depletion also suppressed Ca\textsuperscript{2+} uptake into the ER lumen via sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) and Ca\textsuperscript{2+} extrusion via plasma membrane Ca\textsuperscript{2+} ATPase (PMCA). In pancreatic acinar cells, inhibition of SOCE by ATP depletion occurred in parallel with the inhibition of Ca\textsuperscript{2+} extrusion suggesting an important and hitherto unidentified link between the two processes. The inhibition of Ca\textsuperscript{2+} influx by ATP depletion is, however, incomplete and prolonged depletion leads to Ca\textsuperscript{2+} overload and damage/death of pancreatic acinar cells. Furthermore, some inducers of acute pancreatitis (e.g., bile acids and fatty acids) seem to be capable of generating an alternative Ca\textsuperscript{2+} influx pathway which is not inhibited by ATP depletion. A number of novel Ca\textsuperscript{2+} influx channels have been recently identified in pancreatic acinar cells and the effects of mitochondrial inhibition and ATP depletion on these channels are currently unknown. Identifying the relationships between mitochondrial function and Ca\textsuperscript{2+} influx is important for understanding the mechanisms underlying pancreatic acinar cell function and dysfunction.

**Figure 3. Simplified diagram of stimulus-metabolism coupling and metabolism-signaling feedback in pancreatic acinar cells.** The schematic illustrates the mechanisms of physiological adaptation of bioenergetics to modulate stimulation by secretagogues. In the diagram ΔΨ\textsubscript{m} indicates mitochondrial membrane potential (major component of the proton-motive force), ETC—electron transport chain, AAs—amino acids, and FAs—fatty acids. Dashed green lines illustrate the stimulating effect of physiological Ca\textsuperscript{2+} signals (Ca\textsuperscript{2+} oscillations induced by ACh or CCK) on the Krebs cycle and of insulin on glycolysis. Dashed black lines highlight the role of ATP in the regulation of InsP\textsubscript{3} receptors (InsP\textsubscript{3}Rs) and store-operated Ca\textsuperscript{2+} entry (SOCE). Both IP\textsubscript{3}Rs and the SOCE pathway are ATP-sensitive mechanisms. In resting unstimulated cells and those stimulated by physiological concentrations of secretagogues, ATP levels are sufficient to sustain both InsP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release from the endoplasmic reticulum and SOCE via the STIM/Orai mechanism. However, ATP depletion (e.g., triggered by the inducers of acute pancreatitis) can suppress the InsP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release and SOCE. This is also accompanied by the inhibition of Ca\textsuperscript{2+} leak from the ER, Ca\textsuperscript{2+} uptake into the ER by sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+} pumps, and Ca\textsuperscript{2+} extrusion by Ca\textsuperscript{2+} pumps of the plasma membrane (not shown). For the sake of clarity/simplicity we have omitted some Ca\textsuperscript{2+}-mobilizing second messengers and corresponding intracellular Ca\textsuperscript{2+}-releasing channels. (For detailed reviews of Ca\textsuperscript{2+} signaling in this cell type, see references 27 and 28.) We have also simplified the entry mechanisms of AA and FA derivatives into the Krebs cycle. Feedback regulation of the rate of the Krebs cycle by ATP, ADP, and NAD(P)H was also omitted on this diagram.
the recently identified Ca$^{2+}$ entry pathways and cellular bioenergetics could be the subject of productive future investigation. The pronounced effect of cellular metabolism on Ca$^{2+}$-dependent mitochondrial dysfunction is a core feature of acute pancreatitis. In contrast to the spatially restricted, oscillatory Ca$^{2+}$ signaling events that occur in response to physiological levels of secretagogues in isolated pancreatic acinar cells, supramaximal stimulation causes damaging global, sustained calcium elevations. The ability to cause overload of cytosolic Ca$^{2+}$ and thereby disrupt Ca$^{2+}$ homeostasis in acinar cells is a principal pathological feature common to known precipitants of acute pancreatitis, including bile acids, nonoxidative ethanol metabolites, and fatty acids (Figure 4A), and is an important trigger for mitochondrial damage that compromises cellular ATP production. The importance of altered energy levels within the pancreas relevant to its pathology has been recognized for many years, although in vivo studies have produced somewhat varied results. In the early 1990s, substantial falls of cellular ATP in the pancreas were detected in experimental AP models using noninvasive NMR spectrometry of 31P. Interestingly, there was heterogeneity in responses dependent on the model employed. Thus, whereas significant falls of ATP were detected in AP induced by fatty acid (oleic acid) infusion or regional ischemia, levels were unchanged in caerulein hyperstimulation and partial ductal obstruction models. In contrast, a contemporaneous study in rat pancreas showed that ATP levels were more than halved from controls in the caerulein AP model. A subsequent investigation by W. Halangk and colleagues supported these findings, demonstrating a reduction of phosphorylating respiration in acinar cells and fall of ATP to 57 percent in caerulein AP after 24 hours (using a Clark-type electrode). The authors concluded that caerulein-induced acute pancreatitis is accompanied by “a drastic and long-lasting reduction of the capacity for mitochondrial ATP production.” It should be noted that this phenomenon developed relatively slowly, that is, at early time points (up to 5 hours) the cellular ATP content reported was not significantly diminished from controls and therefore slower than other manifestations of experimental acute pancreatitis like vacuolization and intracellular trypsinogen activation. In contrast, a recent study found that high levels of L-lysine caused mitochondrial damage which occurred before activation of trypsinogen and NFkB, potentially via impairment of electron transport chain coupling or a disbalance between hydrolysis and synthesis of ATP. To date, the balance of evidence from in vivo models has demonstrated loss of pancreatic ATP as acute pancreatitis develops, indicating that early mitochondrial damage is a critically important factor in disease progression; variability between studies may reflect methodological differences, etiology, and/or severity of the experimental model and further comparative studies are warranted. The in vivo findings are therefore consistent with ATP measurements on isolated acinar cells showing rapid declines of ATP concentration in cells treated with fatty acid (POA) or bile acid (TLC-S). In addition, the bile acid chenodeoxycholate has been shown to decrease intracellular ATP levels in pancreatic ductal cells, with inhibition of both oxidative and glycolytic metabolism linked to impaired bicarbonate secretion. With respect to in vivo models, the bioenergetics of acinar and ductal cells in pancreatic tissue will be influenced by changes in local blood flow. This is particularly relevant to the pathophysiology of acute pancreatitis, which is associated with impairment of microvascular perfusion (reviewed in (58)). Inducers of acute pancreatitis have been shown to increase the permeability of pancreatic capillaries and reduce microvascular blood flow and pancreatic oxygen tension. Notably, different models of experimental acute pancreatitis are associated with distinct changes in the microcirculation, ranging from minimal responses and even modest increases of capillary perfusion in mild acute pancreatitis to drastic decreases of capillary perfusion associated with more severe models. The intrinsic abilities of the exocrine cells to adjust metabolism and signaling in response to secretagogues and the inducers of acute pancreatitis (discussed in the previous section) will be therefore “superimposed” on changes in oxygen supply and nutrient availability determined by the microcirculation.

The mechanisms underlying ATP depletion induced by acute pancreatitis precipitants have been addressed in detail using isolated pancreatic acinar cells. Confocal microscopy investigations demonstrated that Ca$^{2+}$ overload of acinar cells caused rapid depolarization of the mitochondrial membrane potential, accompanied by decreased NAD(P)H and increased FAD$^+$ levels, with a resultant cellular ATP fall leading to necrosis. When applied at lower concentration (200 µM) the bile acid TLCS caused predominantly oscillatory calcium rises, accompanied by NADH elevations, in accord with stimulus-metabolism coupling (as described above). However, sustained Ca$^{2+}$ elevations at higher concentration (500 µM) led to rises of mitochondrial Ca$^{2+}$ with dramatic decrease of NAD(P)H (Figure 4B). Similarly, the nonoxidative ethanol metabolite...
Figure 4. Pathological Ca\textsuperscript{2+}-dependent mitochondrial bioenergetic dysfunction in pancreatic acinar cells. (A) Correlation between palmitoleic acid (POA)-induced sustained cytosolic Ca\textsuperscript{2+} response (Fluo4) and mitochondrial depolarization (TMRM) (adapted from Criddle et al.\textsuperscript{87}). (B) Correlation between bile acid (TLCS: tauroliotholic acid sulphate)-induced sustained mitochondrial Ca\textsuperscript{2+} rises (Rhod2) and decrease of NAD(P)H (autofluorescence) in a doublet of pancreatic acinar cells (left panel) and corresponding traces (right panel) (adapted from Booth et al.\textsuperscript{62}). (C) Bioenergetics changes in response to POA in pancreatic acinar cells, shown as concurrent decrease of mitochondrial NADH and increase of FAD (autofluorescence: left panel) and depletion of ATP (Magnesium Green). (D) Provision of ATP via a patch-pipette prevents toxic POA-induced sustained Ca\textsuperscript{2+} elevation in pancreatic acinar cells, highlighting the vital importance of ATP-dependent Ca\textsuperscript{2+} pumps to Ca\textsuperscript{2+} clearance and prevention of cell damage (C, D) (adapted from Criddle et al.\textsuperscript{87}).

Palmitoleic acid ethyl ester (POAEE) and POA, produced by hydrolysis of the fatty acid ethyl ester,\textsuperscript{103,104} induced sustained elevations of cytosolic Ca\textsuperscript{2+} that resulted in mitochondrial dysfunction.\textsuperscript{87} Simultaneous measurements of cytosolic Ca\textsuperscript{2+} and ΔΨ\textsubscript{m} revealed the temporal relationship of these events; as cytosolic Ca\textsuperscript{2+} progressively rose there was a mirrored mitochondrial depolarization and rapid decrease of NAD(P)H\textsuperscript{105} (Figure 4A). The consequence
of mitochondrial impairment was a profound depletion of intracellular ATP (measured using Magnesium Green); this effect was maximal and not further increased by the protonophore CCCP\(^{87}\) (Figure 4C). Interestingly, a Ca\(^{2+}\)-independent action of POA was also detected, revealed by pretreatment with the Ca\(^{2+}\) chelator BAPTA, which prevented the loss of \(\Delta \psi_m\), although NAD(P)H decrease still persisted. The rundown of mitochondrial ATP production has dramatic effects on Ca\(^{2+}\) homeostasis in the pancreatic acinar cell, which appears particularly vulnerable to sustained cytosolic Ca\(^{2+}\) increases; unlike excitable cells such as cardiomyocytes, the contribution of the Na\(^+\)/Ca\(^{2+}\) exchanger to Ca\(^{2+}\) homeostasis in the pancreatic acinar cell appears weak or absent.\(^{106}\) The major routes for Ca\(^{2+}\) clearance, the SERCA and PMCA pumps, are fueled by cellular ATP and would be progressively impaired as energy levels fall during acute pancreatitis development.\(^{107,108}\) This is likely to result in a vicious cycle in which Ca\(^{2+}\)-dependent mitochondrial dysfunction perpetuates sustained cytosolic Ca\(^{2+}\) elevation, resulting in augmented necrotic cell death.\(^{109}\) The importance of maintaining ATP levels for acinar cell function was demonstrated by the provision of intracellular ATP via a patch pipette\(^{87}\); this prevented the damaging Ca\(^{2+}\) elevations induced by POA, confirming the crucial roles of the SERCA/PMCA for Ca\(^{2+}\) clearance in pancreatic acinar cells\(^{87}\) (Figure 4D). In accord, ATP provision prevented the sustained Ca\(^{2+}\) rises and consequent acinar cell necrosis induced by bile acid,\(^{62}\) while supplemental ATP was able to mitigate the detrimental effects of ethanol and its nonoxidative metabolites on CFTR in ductal cells that promote acute pancreatitis.\(^{110,111}\)

**How does ATP depletion occur?**

The precise mechanisms of ATP depletion are still unclear, although a crucial role of MPTP opening in mediating mitochondrial dysfunction has been identified in the pancreas,\(^{112–114}\) in common with other tissues such as heart, liver, and brain.\(^{115–117}\) The formation of this megapore causes a profound permeabilization of the inner mitochondrial membrane that allows movement of solutes <1.5 kDal in and out of the organelle. Although the exact composition of the pore is contentious and currently unresolved,\(^{118–122}\) prevention of MPTP opening is achieved via inhibition of the modulatory protein cyclophilin D (CypD); this protein is therefore considered an important target for acute pancreatitis therapy.\(^{113,123–126}\) Our group has shown that knockout and pharmacological inhibition of CypD ameliorated damage in isolated murine and human acinar cells induced by acute pancreatitis precipitants and was protective in multiple in vivo experimental models reflecting the principal etiologies (including biliary: TLCS-AP\(^{127}\) and alcoholic: FAEE-AP\(^{104}\)).\(^{113}\) Furthermore, CypD-sensitive MPTP formation mediated mitochondrial dysfunction in pancreatic ductal cells, indicating a common mechanism operating in multiple cell types implicated in the pathogenesis of acute pancreatitis.\(^{3,126}\) The principal trigger for MPTP opening is the sustained elevation of mitochondrial Ca\(^{2+}\)\(^{128}\); however, reactive oxygen species (ROS) have also been implicated in triggering MPTP channel formation.\(^{129,130}\) In human and murine pancreatic acinar cells, bile acid-induced effects are complex since sustained elevations of cytosolic and mitochondrial Ca\(^{2+}\) lead to sustained ROS increases; both Ca\(^{2+}\) and ROS formation are therefore likely to be important to the outcome of bioenergetics changes that underlie mitochondrial dysfunction in acute pancreatitis.\(^{62}\) Physiological and pathophysiological changes of acinar cell bioenergetics are schematically illustrated in Figure 5.

**Figure 5.** Diagram illustrating the effects of physiological and pathophysiological Ca\(^{2+}\) signals on mitochondrial bioenergetics that determine pancreatic acinar cell responses and fate. Physiological stimulation of pancreatic acinar cells with secretagogues cholecystokinin (CCK) and acetylcholine (ACh) elicits oscillatory Ca\(^{2+}\) signals that stimulate mitochondrial ATP production (stimulus-metabolism coupling) that fuels secretion. However, when subjected to stress, cellular bioenergetics are perturbed leading to instigation of cell death patterns. Precipitants of acute pancreatitis (AP), including bile acids, nonoxidative ethanol metabolites (fatty acid ethyl esters: FAEEs), fatty acids (FAs), and secretagogue hyperstimulation, cause sustained cytosolic Ca\(^{2+}\) elevations which raise mitochondrial matrix Ca\(^{2+}\). Ca\(^{2+}\)-dependent ROS generation in the mitochondria preferentially promotes apoptotic cell death, which may constitute a local protective mechanism whereby necrosis is avoided. However, as the level and/or duration of stress increases, a shift from apoptotic to necrotic cell death ensues. Ca\(^{2+}\)-dependent formation of the mitochondrial permeability transition pore (MPTP) is a primary event that results in mitochondrial depolarization, altered NADH/FAD levels, ATP fall, and necrotic cell death development. Elevated ROS levels also drive necrosis in a manner that is independent of the MPTP. Since clearance of cytosolic Ca\(^{2+}\) from the acinar cell is acutely dependent on ATP-requiring pumps (plasmamembran Ca\(^{2+}\)-ATPase (PMCA) and sarcos-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA)), the depletion of ATP evokes a vicious cycle that perpetuates Ca\(^{2+}\) overload and mitochondrial dysfunction. The figure is adapted from Criddle DN.\(^{131}\)
Role of ROS in bioenergetic alterations

Secretagogues and bile acids increase ROS generation in pancreatic acinar cells \(^62,132\) (reviewed in \(^131\)). In pancreatic acinar cells, augmented ROS production promotes apoptosis, \(^64\) which may be an endogenous protective mechanism that enables the cell to deal with cellular stress by avoiding necrosis. \(^62,64,133,134\) Necrosis is considered a largely uncontrolled form of cell death and in acute pancreatitis is associated with development of systemic inflammation \(^135\); accordingly, inhibition of caspases causes a more severe necrotizing pancreatitis. \(^133,136\) Our recent work has demonstrated that oxidative stress induced by exogenous H\(_2\)O\(_2\) or menadione, which generates intracellular ROS via redox cycling, \(^64\) caused bioenergetics changes that were independent of CypD-sensitive MPTP opening. \(^137\) Increasing levels of oxidants elicited an apoptosis to necrosis shift that was unaffected by knockout of CypD or pharmacological inhibition with cyclosporin A. This lack of sensitivity to CypD is consistent with evidence in primary hepatocytes showing that CypD deletion affected MPTP formation induced by Ca\(^{2+}\) but not by thiol oxidants \(^138\) and a model in which there is a regulated (Ca\(^{2+}\)-activated and cyclosporin-sensitive) and unregulated (cyclosporin-insensitive) MPTP. \(^139,40\) Therefore, counteracting excessive ROS in the pancreas with antioxidants might be desirable. However, despite many clinical trials for acute pancreatitis, results have generally been disappointing or inconclusive. \(^141\) A more recent approach to combat oxidative stress is the targeting of antioxidants to mitochondria in order to produce more localized, specific actions. \(^142\) For example, MitoQ, which accumulates in mitochondria due to its positively charged triphosphonium ion (TPP\(^+\)), \(^143\) prevented ROS-mediated damage in diverse cell types. \(^144-148\) Although efficacy in clinical investigations so far has been disappointing, \(^149,150\) in isolated acinar cells, MitoQ reduced H\(_2\)O\(_2\)-mediated ROS elevations yet afforded no protection against loss of mitochondrial membrane potential induced by CCK or bile acid. \(^151\) Furthermore, MitoQ treatment produced mixed effects in \textit{in vivo} acute pancreatitis models. When applied as a treatment after disease induction, it was unprotective in TILCS-AP but caused partial amelioration of histopathology scores in CER-AP, albeit without concomitant reduction of biochemical parameters (pancreatic trypsin and serum amylase). Interestingly, lung myeloperoxidase and interleukin-6 were concurrently increased by MitoQ in CER-AP, which may involve biphasic effects on ROS in isolated polymorphonuclear leukocytes, inhibiting acute increases but causing elevation at later time points. In pancreatic acinar cells, MitoQ exerted complex actions on mitochondrial bioenergetics that may be in part mediated by the targeting TPP\(^+\) moiety. \(^53\) Thus, MitoQ and the general antioxidant n-acetyl cysteine caused sustained elevations of basal respiration and inhibition of spare respiratory capacity, an important indicator of the cell’s ability to respond to stress, \(^152,153\) actions attributable to an antioxidant action. However, mitochondrial ATP turnover capacity and cellular ATP concentrations were also markedly reduced by both MitoQ and DecylTPP, a nonantioxidant control, suggesting nonspecific actions of the positively charged mitochondrial-targeting moiety. \(^154-156\) Such events were associated with a compensatory elevation of glycolysis and induction of acinar cell death. The results of this study also indicated that ROS exert a significant negative feedback control of basal respiration, potentially by effects on uncoupling proteins, thereby determining basal metabolic function. \(^157-159\) Increasing evidence points to important physiological signaling roles of ROS, \(^160-162\); therefore, an important consideration when addressing mitochondrial dysfunction by quenching free radicals is the potential consequences of disrupting normal physiological organelar function.

Inflammatory response: bioenergetics changes in blood cells

While most work elucidating bioenergetics changes related to acute pancreatitis has been carried out in pancreatic cells and tissues, changes in blood cells linked to the generation and propagation of a systemic inflammatory response are likely to be of significance. \(^163,164\) The bioenergetics changes in circulating leukocytes are only partially understood, not least because investigations encounter significant technical difficulties during isolation, including potential damage to cells that might induce artifacts. Previously, a modest (1.5-fold) increase in basal respiration was found in total peripheral blood mononuclear cells isolated from acute pancreatitis patients, although ATP production was unaltered. \(^165\) Our recent work has shown subset-specific bioenergetics alterations occur in leukocytes from acute pancreatitis patients that imply functional alterations linked to clinical disease progression. \(^166\) Separation of blood cell subtypes from acute pancreatitis patients was feasible for detailed bioenergetics evaluations using Seahorse XF flux analysis. The study found no difference in basal respiration in blood leukocyte subtypes between acute pancreatitis patients and healthy controls. However, when the mitochondria were challenged with a “stress test” (by manipulation of the respiratory chain with rotenone and antimycin (complexes I and III), oligomycin (ATP synthase), and FCCP (protonophore) \(^137,167,168\)), it was revealed that patient lymphocytes possessed decreased maximal respiration and spare respiratory capacity, indicating functional impairment. Furthermore, a diminished oxidative burst was evident in neutrophils from acute pancreatitis patients, compared to healthy controls, that may indicate compromised activity in this cell type, whereas this was enhanced...
in both monocytes and lymphocytes; further work is necessary to determine the bases for such altered bioenergetics in circulating blood cells and whether bioenergetics analysis might be predictive of acute pancreatitis severity and thereby assist prognosis.

**Therapeutic aspects: maintenance of ATP provision**

How may detrimental bioenergetics changes linked to development of acute pancreatitis be prevented? Recent attention has focused on inhibition of the Ca$^{2+}$ overload machinery that causes mitochondrial dysfunction. For example, preclinical studies in murine pancreatic acinar cells have shown that inhibition of Orai1, expressed in pancreatic acinar cells, prevented sustained Ca$^{2+}$ increases induced by fatty acid ethyl esters, coupled with maintenance of mitochondrial membrane potential and a reduction of necrosis. Prevention of the toxic effects of acute pancreatitis precipitants by Orai1 inhibitors GSK-7975A (GlaxoSmithKline) and CM4620 (Calcimedica) was further demonstrated in human pancreatic acinar cells, and the potential for acute pancreatitis treatment indicated from their efficacy in multiple in vivo experimental models (CER-AP, TLCS-AP, and FAEE-AP). More recently, important protective actions of the Orai1 inhibitor CM4620 on immune cells were shown that contribute to the beneficial outcomes in acute pancreatitis, and this agent is currently being evaluated in phase II clinical trials. Interestingly, a recent study has also identified the involvement of TRPM2 channels in Ca$^{2+}$ entry in pancreatic acinar cells and inhibition of these redox-sensitive channels may provide a novel approach to treat biliary acute pancreatitis. As mentioned above, prevention of the downstream effects of Ca$^{2+}$ overload induced by acute pancreatitis precipitants on mitochondria is considered a promising therapeutic strategy, with new MPTP inhibitors in development and evaluation in preclinical models. Despite a lack of clarity about the composition of the MPTP, most effort has been directed toward inhibition of the modulatory protein CypD, a prime target in multiple pathologies. Genetic deletion of CypD or pharmacological inhibition with cyclosporin A or its analogue NIM811 treatment effectively reduced histological damage and biochemical alterations in multiple acute pancreatitis models. Furthermore, since ATP supplementation ameliorated Ca$^{2+}$ clearance from the cytosol and prevented damage in acinar and ductal cells, there is potential to prevent necrotic damage in acute pancreatitis by boosting cellular energy provision. However, development of an effective means to achieve this in patients is challenging; ATP does not effectively cross cell membranes and is subject to degradation by enzymatic action. Nevertheless, studies using liposomes as a vehicle to deliver ATP have been shown to exert protective actions against damage in heart, liver, and brain. Notably a recent study showed that enhancing cellular energy production with galactose ameliorated asparaginase- and FAEE-induced acute pancreatitis, indicating potential therapeutic value of manipulating energy levels in acute pancreatitis patients. Currently, a multicenter randomized double-blind clinical (GOULASH) trial is being conducted to assess high- versus low-energy administration in the early phase of acute pancreatitis. Previously, enteral nutrition has been shown to significantly decrease mortality and frequency of multiorgan failure in severe acute pancreatitis and results of the GOULASH trial are awaited with interest.

**References**


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42. Tepikin AV. Mitochondrial junctions with cellular organelles: Ca\(^{2+}\) signalling perspective. Pflugers Arch 2018; 470:1181–1192.


44. Tinel H, Cancela JM, Mogami H, Gerasimenko JV, Gerasimenko OV, Tepikin AV, Petersen OH. Active mitochondria surrounding the pancreatic acinar granule region prevent spreading of inositol triphosphate-evoked local cytosolic Ca\(^{2+}\) signals. EMBO J 1999; 18:4999–5008.


57. Park HS, Betenhauser MJ, Won JH, Chen J, Yule DJ. The type 2 inositol (1,4,5)-trisphosphate (InsP\(_3\)) receptor determines the sensitivity of InsP\(_3\)-induced Ca\(^{2+}\) release to ATP in pancreatic acinar cells. J Biol Chem 2008; 283:26081–26088.


82. Plusczyn T, Westermann S, Rathgeb D, Feil G. Acute pancreatitis in rats: effects of sodium taurocholate, CCK-8, and...
108. Baggaley EM, Elliott AC, Bruce JI. Oxidant-induced inhibi-
106. Muallem S, Beeker T, Pandol SJ. Role of Na+
105. Criddle DN. The role of fat and alcohol in acute pancreatitis:
104. Diczfalusy MA, Bjorkhem I, Einarsson C, Hillebrant CG,
102. Knoefel WT, Kollias N, Warshaw AL, Waldner H, Nishioka
101. Kinnala PJ, Kuttila KT, Gronroos JM, Hankia TV, Nevalainen
100. Karch J, Molkentin JD. Identity of the elusive mitochondrial
99. Tomkotter L, Erbes J, Trepte C, Hinsch A, Dupree A,
98. Diczfalusy MA, Bjorkhem I, Einarsson C, Hillebrant CG,
97. Alexson SE. Characterization of enzymes involved in for-
96. Brini M, Carafoli E. Calcium pumps in health and disease.
95. Brini M, Carafoli E. Calcium pumps in health and disease.
94. Criddle DN. The role of fat and alcohol in acute pancreatitis:
93. N. Tomkotter, J. Trepte, A. C. Elliott, J. Hinsch, A. Dupree, A.
92. Tomkotter L, Erbes J, Trepte C, Hinsch A, Dupree A, Bockh
91. Tomkotter L, Erbes J, Trepte C, Hinsch A, Dupree A, Bockh
90. Tomkotter L, Erbes J, Trepte C, Hinsch A, Dupree A, Bockh
89. Tomkotter L, Erbes J, Trepte C, Hinsch A, Dupree A, Bockh
88. Tomkotter L, Erbes J, Trepte C, Hinsch A, Dupree A, Bockh
87. Tomkotter L, Erbes J, Trepte C, Hinsch A, Dupree A, Bockh
86. Sec on pancreatic microcirculation. Am J Physiol Gastrointest
85. Tomkotter L, Erbes J, Trepte C, Hinsch A, Dupree A, Bockh
84. Tomkotter L, Erbes J, Trepte C, Hinsch A, Dupree A, Bockh
83. Tomkotter L, Erbes J, Trepte C, Hinsch A, Dupree A, Bockh
82. Tomkotter L, Erbes J, Trepte C, Hinsch A, Dupree A, Bockh
78. Tomkotter L, Erbes J, Trepte C, Hinsch A, Dupree A, Bockh
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70. Tomkotter L, Erbes J, Trepte C, Hinsch A, Dupree A, Bockh


Cyclic Nucleotides and Their Biosynthesis

Cyclic nucleotides, like other nucleotides, are composed of three functional groups: a ribose sugar, a nitrogenous base, and a single phosphate group. There are two types of nitrogenous bases: purines (adenine and guanine) and pyrimidines (cytosine, uracil, and thymine). A cyclic nucleotide, unlike other nucleotides, has a cyclic bond arrangement between the ribose sugar and the phosphate group. There are two main groups of cyclic nucleotides: the canonical or well-established and the noncanonical or unknown-function cyclic nucleotides. The two well-established cyclic nucleotides are adenosine-3',5'-cyclic monophosphate (cyclic AMP) and guanine-3',5'-cyclic monophosphate (cyclic GMP). Both cyclic AMP and cyclic GMP are second messengers. The noncanonical cyclic nucleotides include the purines inosine-3',5'-cyclic monophosphate (cyclic IMP), xanthosine-3',5'-cyclic monophosphate (cyclic XMP), and the pyrimidines cytidine-3',5'-cyclic monophosphate (cyclic cCMP), uridine-3',5'-cyclic monophosphate (cyclic UMP), and thymidine-3',5'-cyclic monophosphate (cTMP). An overview of the noncanonical cyclic nucleotides is provided later.

A cyclase enzyme (lyase) catalyzes the formation of the cyclic nucleotide from its nucleotide triphosphate precursor (Figure 1). Cyclic nucleotides form when the phosphate group of the molecule of nucleotide triphosphate (ATP or GTP) is attacked by the 3' hydroxyl group of the ribose, forming a cyclic 3',5'-phosphate ester with release of pyrophosphate. This cyclic conformation allows cyclic nucleotides to bind to proteins to which other nucleotides cannot. The reaction is an intramolecular nucleophilic reaction and requires Mg$^{2+}$ as a cofactor, whose function is to decrease the overall negative charge on the ATP by complexing with two of its negatively charged oxygen. If its negative charge is not reduced, the nucleotide triphosphate cannot be approached by a nucleophile, which is, in this reaction, the 3’ hydroxyl group of the ribose. Soluble AC prefers Ca$^{2+}$ to Mg$^{2+}$ as the cofactor to coordinate ATP binding and catalysis.

Canonical Cyclic Nucleotide Signaling in the Exocrine Pancreas

Cyclic nucleotide signaling can be initiated by two general mechanisms. One mechanism is the binding of an extracellular ligand to a transmembrane G-protein-coupled receptor (GPCR). The receptor protein has seven transmembrane α-helices connected by alternating cytosolic and extracellular loops. The N-terminus is in the extracellular space, while the C-terminus is in the cytosol. The ligand-binding site is in the extracellular domain and the cytosolic domain has a heterotrimeric G protein-binding site. After a ligand binds to the GPCR, it activates a heterotrimeric G-protein, which is composed of three subunits: a guanine nucleotide-binding α-subunit and a βγ-heterodimer. Depending on which family the G protein is, it goes on to activate (G$\alpha$s protein subunit) or inhibit (G$\alpha$i protein subunit) the membrane-bound cyclase.

The second signaling mechanism involves the binding of a small signaling molecule to a soluble cyclase. The signal source can be either extracellular, such as nitric oxide (NO), or intracellular, such as bicarbonate. The signaling by an extracellular ligand is limited by its ability to cross the plasma membrane. In the cytosol, the signal molecule binds to the heme-binding domain of the soluble cyclase. The cyclase, in turn, increases the intracellular levels of cyclic nucleotides.

In the exocrine pancreas, adenylyl cyclases can be activated by either extracellular or intracellular signals. The extracellular signals can be a neurotransmitter, such as vasoactive intestinal polypeptide (VIP), a hormone, such as secretin, or a gas, such as NO. Intracellular signals include HCO$_3$⁻. The increase in the cyclic nucleotide levels modifies the activity of downstream effectors such as kinases, guanine-nucleotide-exchange factor (GEF),

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Chapter 4
Cyclic nucleotides as mediators of acinar and ductal function

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Cyclic nucleotides as mediators of acinar and ductal function

RNA-binding protein, ion channels, and phosphodiesterases, which are discussed later in this chapter.

Adenylyl Cyclase/Cyclic AMP Signaling

Cyclic AMP is formed from cytosolic ATP by the enzyme adenylyl cyclase. There are 10 isoforms of adenylyl cyclases; nine are anchored in the plasma membrane, with their catalytic portion protruding into the cytosol, and one is soluble.

Transmembrane AC

The nine transmembrane AC isoforms are each coded by a different gene (Figure 2). The human ADCY1 gene is located on chromosome 7 at p12.3, human ADCY2 gene on chromosome 5 at p15.3, human ADCY3 gene on chromosome 2 at p23.3, human ADCY4 gene on chromosome 14 at q12, human ADCY5 gene on chromosome 3 at q21.1, human ADCY6 gene on chromosome 12 at q12-q13, human ADCY7 gene on chromosome 16 at q12.1, human ADCY8 gene on chromosome 8 at q24, and human ADCY9 gene on chromosome 16 at q13.3. The transmembrane AC isoforms share a high-sequence homology in the primary structure of their catalytic site and the same three-dimensional structure. The AC structure is a pseudodimer that can be divided into two main regions, transmembrane and cytoplasmic regions, and further divided into five different domains: (1) the NH₂ terminus, (2) the first transmembrane cluster (TM1), (3) the first cytoplasmic loop composed of C1a and C1b, (4) the second transmembrane cluster (TM2) with extracellular N-glycosylation sites, and (5) the second cytoplasmic loop composed of C2a and C2b. The transmembrane regions (TM1 and TM2), whose function is to keep the enzyme anchored in the membrane, are composed of 12 membrane-spanning helices (6 membrane-spanning helices each with short interhelical loops). The cytoplasmic regions C1 and C2 are approximately 40 kDa each and can be further subdivided into C1a, C1b, C2a, and C2b. Both C1a and C2a are highly conserved catalytic ATP-binding regions, which dimerize to form a...
pseudosymmetric enzyme, which forms the catalytic site. ATP binds at one of two pseudosymmetric binding sites of the C1-C2 interface. Two amino acid residues, Asn1025 and Arg1029, of AC2 are conserved among the C2 domains and critical for the catalytic activity of AC; mutation of either residue causes a 30–100-fold reduction in the AC activity. A second C1 domain subsite includes a P-loop that accommodates the nucleotide phosphates and two conserved acid residues that bind to ATP through interaction with two Mg\(^2^+\); one Mg\(^2^+\) contributes to catalysis.

Figure 2. Schematic representation of the domain structure of the nine human transmembrane AC isoforms. The number of amino acid residues is reported on the side of each structure. Modification sites and domains are represented with different color. The transmembrane AC isoforms share a common structure composed of two cytosolic domains (C1 and C2) and six transmembrane segments organized in two tandem repeats. Both C1 and C2 domains contribute to ATP binding and formation of the catalytic core. **Abbreviations:** TM (transmembrane segments); DUF (domain of unknown function); ac (acetylation); P (phosphorylation site); ub (ubiquitination); S (serine); K (lysine); T (threonine); Y (tyrosine) (data obtained from PhosphoSitePlus).
Cyclic nucleotides as mediators of acinar and ductal function

whereas the second one interacts with nucleotide β- and γ-phosphates from substrate binding and possibly also for leaving-group stabilization. Both C2a and C2b are less conserved than the C1 domain.\textsuperscript{21,23} The C1b domain is the largest domain, contains several regulatory sites, and has a variable structure across the isoforms. However, the C2b domain is essentially nonexistent in many isoforms and has not yet been associated with a function.\textsuperscript{24} The overall domain structure of each human transmembrane AC isoform is shown in Figure 2 and a detailed comparison of the cytoplasmic domains (C1 and C2), transmembrane segments, acetylation, phosphorylation, and ubiquitination sites of each isoform is indicated. Figure 3 shows the three-dimensional model of AC and its relation to heterotrimeric G protein α subunit.

Without stimulation, the enzyme AC is constitutively inactive. There are at least two heterotrimeric G-proteins responsible for the regulation of transmembrane AC activity: G\textsubscript{s} and G\textsubscript{i}. When a secretagogue (e.g., secretin, vasoactive intestinal polypeptide) binds to its GPCR, it causes a change in the conformation of the receptor that stimulates the G\textsubscript{sa} subunit to exchange GDP against GTP, which causes GTP-G\textsubscript{sa} to detach from the G\textsubscript{bγ} subunits and bind to the two cytoplasmic regions of transmembrane AC.\textsuperscript{25} With GTP-G\textsubscript{sa} bound, AC becomes active and converts ATP to cyclic AMP in a process involving the release of water and a pyrophosphate. G\textsubscript{sa} has shown to play an important role in the exocrine pancreas because G\textsubscript{sa}-deficient mice show morphological changes in the exocrine pancreas, as well as malnutrition and dehydration.\textsuperscript{26} Certain isoforms of transmembrane ACs are also positively (AC2, AC4, AC5, AC6, AC7) or negatively (AC1, AC3, AC8) regulated by the G\textsubscript{bγ} subunits, which also bind to the two cytoplasmic regions of transmembrane AC.\textsuperscript{25}

When a GPCR is coupled to the heterotrimeric protein G\textsubscript{s}, GTP-G\textsubscript{sa} binds to adenylyl cyclase and, unlike GTP-G\textsubscript{ia}, GTP-G\textsubscript{sa} inhibits the activity of the enzyme, causing lower levels of cyclic AMP in the cells. In the pancreas, somatostatin binds to its SS2 receptor and causes activation of G\textsubscript{ia} subunit and inhibition of adenylyl cyclase.\textsuperscript{28,29} Once the concentration of the ligand is below activation levels, the G\textsubscript{a} subunit, which has an intrinsic GTPase activity, hydrolyzes GTP to GDP, reassociates with G\textsubscript{bγ} and becomes inactive. The cycle of GTP hydrolysis and inactivation occur within seconds after the G protein has been activated. Upon inactivation, G proteins are ready to be reactivated by another extracellular signal.

Transmembrane ACs are classified into four groups based on their regulatory properties (Table 1 and Figure 4):

- **Group I**, which consists of Ca\textsuperscript{2+}-stimulated isoforms: AC1, AC3, AC8.
- **Group II**, which consists of G\textsubscript{bγ}-stimulated isoforms: AC2, AC4, AC7.
Group III, which consists of G<sub>αi</sub>/Ca<sup>2+</sup>-inhibited isoforms: AC5, AC6.

Group IV, which solely consists of Ca<sup>2+</sup>-<sub>βγ</sub>-insensitive isoform: AC9.

The expression profile of the transmembrane AC isoforms in intact mouse pancreas, isolated pancreatic acini, and duct fragment has been established using RT-PCR. Five different transmembrane AC isoforms were identified in pancreatic exocrine cells: AC3, AC4, AC6, and AC9 mRNAs were expressed in isolated pancreatic acini and sealed duct fragments, whereas AC7 mRNAs were only expressed in duct fragments. Using real-time quantitative PCR analysis, the relative expression of each isoform in

Table 1. Regulatory properties of AC isoforms.

<table>
<thead>
<tr>
<th>AC isoform</th>
<th>MW (kDa)</th>
<th>basal pl</th>
<th>G&lt;sub&gt;α&lt;/sub&gt;</th>
<th>G&lt;sub&gt;αi&lt;/sub&gt;</th>
<th>G&lt;sub&gt;βγ&lt;/sub&gt;</th>
<th>FSK</th>
<th>Calcium</th>
<th>Protein Kinases</th>
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</thead>
<tbody>
<tr>
<td>Group I:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>AC1</td>
<td>123.37</td>
<td>8.77</td>
<td>(+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+, CaM) or (-, CaM kinase)</td>
<td>(+, PKCα)</td>
</tr>
<tr>
<td>AC3</td>
<td>129.08</td>
<td>6.15</td>
<td>(+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
<td>IV</td>
<td>(+, PKCα)</td>
</tr>
<tr>
<td>AC8</td>
<td>140.1</td>
<td>6.53</td>
<td>(+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+, CaM) or (-, CaM kinase II)</td>
<td>(+, CaM)</td>
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<tr>
<td>Group II:</td>
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<td></td>
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<tr>
<td>AC2</td>
<td>123.27</td>
<td>8.4</td>
<td>(+)</td>
<td>(=)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+, PKCα)</td>
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<td>AC4</td>
<td>120.38</td>
<td>7.31</td>
<td>(+)</td>
<td>(=)</td>
<td>(+)</td>
<td>(+)</td>
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<td>AC7</td>
<td>122.71</td>
<td>8.49</td>
<td>(+)</td>
<td>(=)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+, PKCα)</td>
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<td>AC5</td>
<td>139.12</td>
<td>6.9</td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
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<td>130.61</td>
<td>8.56</td>
<td>(+)</td>
<td>(-)</td>
<td>β1y2</td>
<td>(+)</td>
<td>Calc-inhibited</td>
<td>(-, &lt; 1 μM)</td>
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<tr>
<td>AC9</td>
<td>150.95</td>
<td>7.07</td>
<td>(+)</td>
<td>(-)</td>
<td>(=)</td>
<td>(=)</td>
<td>(+) or +, weak</td>
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<tr>
<td>AC10</td>
<td>186/48&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.99</td>
<td>(=)</td>
<td>(=)</td>
<td>(=)</td>
<td>(=)</td>
<td>Calc-stimulated</td>
<td>(=)</td>
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</table>

(+) AC activity is stimulated; (-) AC activity is inhibited; (=) AC activity is not modified. Data taken from 31-34. (a) The molecular weight (MW) and basal isoelectric point (pI) data was obtained from PhosphoSitePlus (Cell Signaling Technology, Inc.). (b) AC6 is directly phosphorylated by PKA at Ser674 and thereby inhibited. (c) 186 kDa (the full-length form) and 48 kDa (the truncated form).
Cyclic nucleotides as mediators of acinar and ductal function

Pancreatic acini and ducts compared with the intact pancreas was assessed: isolated pancreatic acini were shown to have higher transcript levels of AC6 compared with intact pancreas, whereas isolated duct fragments were shown to have higher transcript levels of AC4, AC6, and AC7 compared with intact pancreas.

Similar transcript levels of AC3 and AC9 were observed in pancreas, acini, and ducts. In conclusion, several adenylyl cyclase isoforms are expressed in pancreatic exocrine cells, with AC6 being highly expressed in both pancreatic acinar and duct cells.

**Soluble AC**

Soluble AC10 is unique in some many ways. The human ADCY10 gene is located on chromosome 1 at q24. It is not anchored in the plasma membrane. As indicated in Figure 5, the catalytic domains C1 and C2 are located at the N-terminus and connected by a ~68-residue linker that forms a death domain-like subdomain with the ~33-residue N-terminus of the protein. The C-terminal from this C1-C2 tandem of the full-length mammalian soluble AC comprises a ~1,100-residue C-terminal region without a transmembrane region. Its catalytic domain sequence is more closely related to some bacterial ACs than mammalian ACs. Unlike transmembrane AC, soluble AC has no transmembrane domain. For that reason, its location is in the cytosol, but it can be associated with certain cellular organelles, such as the nucleus, mitochondria, and microtubules. Unlike transmembrane ACs that are regulated by G proteins, forskolin, and calmodulin, among others, soluble AC is stimulated by HCO$_3^-$ ions. The HCO$_3^-$ ion induces a conformational change of the active site of soluble AC similar to that observed in transmembrane ACs upon stimulation with Gs. Using RT-PCR and Western blotting, soluble AC has also been identified in acinar cells. By immunohistochemistry using a soluble AC antibody, AC10 has been localized just below the apical region of the cell in the non-stimulated condition and, after treatment with the CCK analog caerulein, a punctuate intracellular pattern was seen.

In pancreatic acini, the activation of soluble AC with HCO$_3^-$ enhances secretagogue-stimulated cyclic AMP levels and inhibits secretagogue-stimulated zymogen activation and cell vacuolization.

**Intracellular targets of cyclic AMP**

All the protein targets described below have a cyclic nucleotide-binding domain (CNBD) that has been conserved across a wide range of proteins, including the bacterial transcription factor catabolite activator protein.

**Protein kinase A**

Cyclic AMP stimulates protein kinase A (PKA), which phosphorylates a number of cellular proteins by transferring a phosphate from ATP to a serine or a threonine located in sequence of residue of target protein. PKA contains two regulatory subunits, which possess the cyclic nucleotide-binding domain (CNBD), and two catalytic subunits, which are responsible for the Ser/Thr phosphorylation. Upon binding of cyclic AMP to the two regulatory subunits, the two catalytic subunits are detached from the regulatory subunits and become active. The steps implicated in the activation of PKA by cyclic AMP are described in Figure 6.

The presence of PKA in pancreas was first reported in acinar cells from guinea pig. PKA catalyzes the phosphorylation of regulatory proteins associated with the pancreatic exocytotic process. However, PKA does not appear to directly participate in pancreatic amylase secretion because the inhibitor of PKA, H-89, does not modify either basal or cyclic AMP-dependent secretagogues-stimulated amylase secretion from mouse pancreatic acini. Unlike in mouse acinar cells, in sealed mouse ducts PKA plays an essential role in the regulation of fluid secretion.

One of the important targets of PKA is the transcription factor cyclic AMP response element binding protein (CREB). Similar to other cell types, in pancreatic acini CREB phosphorylation at Ser133 increases upon PKA activation. The phosphorylation of CREB promotes the formation of a transcriptional complex on the cyclic AMP (cyclic AMP) response element (CRE) of certain promoters. The complex contains three proteins: (1) CREB, (2) the CREB-binding protein (CBP), and (3) CREB-regulated

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**Figure 5.** Schematic representation of the domain structure of the soluble AC isoform, AC10. The number of amino acid residues is reported on the side of structure. Modification sites and domains are represented with different color. Abbreviations: P (phosphorylation site); S (serine); K (lysine); T (threonine); Y (tyrosine) (data obtained from PhosphoSitePlus).
transcription coactivator 2. Its role is to stimulate the gene expression of certain proteins implicated in the regulation of metabolism, signaling, proliferation, differentiation, survival, and oncogenesis.

Other important targets of PKA are the cystic fibrosis transmembrane conductance regulator (CFTR), 1,4,5-inositol trisphosphate receptor (IP3R), A-kinase anchoring proteins (AKAPs), ERK 1/2, and some isoforms of phosphodiesterase.44

In pancreatic duct cells, PKA phosphorylates CFTR located in the apical membrane, on its regulatory domain, which then enables channel gating (opening and closing) and Cl− secretion.45 Cyclic AMP evokes Cl− currents through CFTR, which mediates fluid transport across the luminal surfaces of pancreatic epithelial cells.46 In pancreatic acinar cells, PKA phosphorylates only one of the three IP3R isoforms, IP3R-3.47,48 The phosphorylation of IP3R-3 by PKA causes IP3-induced Ca2+ release, which is decreased in terms of the magnitude and kinetics of Ca2+ release.48,49

Another important target of PKA are the A-kinase anchor proteins (AKAPs), which are a family of structurally related proteins consisting of more than 50 members.50 AKAP-150 has been shown to play a relevant role in the regulation of Na+/K+ ATPase pump activity in the homologous parotid gland.51,52 Cyclic AMP also phosphorylates and thereby increases the activity of phosphodiesterases PDE3, PDE4, and PDE5 through PKA-induced phosphorylation.18,53 Both PDE3 and PDE4 are cyclic AMP-specific PDEs, whereas PDE5 is a cyclic GMP-specific PDE.18

Exchange protein directly activated by cyclic AMP (Epac)

Cyclic AMP stimulates Epac.15 There are two isoforms of Epac: Epac1 and Epac2.54 Both isoforms are homologous proteins with Epac2 having an N-terminal extension. They share common domain structures within a N-terminal regulatory region and a C-terminal catalytic domain (Figure 7).55–57 The N-terminal regulatory region possesses one (Epac1) or two (Epac2) cyclic nucleotide-binding domains (CNBDs) and a DEP (Dishevelled, Egl-10, and Pleckstrin) domain responsible for its localization to the plasma membrane. The C-terminal region contains CDC25-homology domain, a Ras exchange motif (REM) domain required for stabilizing GEF activity, and the GEF domain, which exerts GEF activity toward the small G proteins Rap1 and Rap2.58 Epac1 is found in both pancreatic acini and ducts50,43,59 and participates in cyclic AMP-stimulated amylase secretion.43 Epac2 has a high affinity for Rap2.15 which is a small G-protein localized on zymogen granules as shown by both mass spectrometry and immunocytochemistry60 and implicated in pancreatic amylase secretion.43 In addition to its

Figure 6. Schematic representation of the activation of protein kinase A (PKA) by cyclic AMP. PKA is composed of four subunits: two regulatory and two catalytic. In the absence of cyclic AMP, the regulatory subunit inhibits the catalytic subunit. Upon binding of cyclic AMP to regulatory subunits, the regulatory subunits change conformation. The catalytic subunits become detached and able to phosphorylate target proteins in the cell.

Figure 7. Domain structure of Epac1 and Epac2. Both Epac1 (881 amino acids) and Epac2 (993 amino acids) contain two regions: regulatory and catalytic regions. The regulatory region has the cyclic nucleotide-binding domain (CNB) and the Desheveled-Egl-10-Pleckstrin,64 which is responsible for the membrane localization. The catalytic region has the CDC25-homology domain, which is responsible for the guanine-nucleotide exchange activity; the Ras exchange motif (REM), which stabilizes the catalytic helix of CDC25-HD; and the Ras-association (RA) domain, which is a protein interaction motif.
role in pancreatic amylase secretion, Epac regulates exocytosis in pancreatic beta cells. Incretin-induced insulin secretion is mediated by Epac2, the primary isofrom of Epac in pancreatic beta cells.61–63

Cyclic nucleotide-gated channels

Cyclic nucleotide-gated (CNG) channels are nonselective tetrameric cation channels that mediate Ca\(^{2+}\) and sodium influx in response to direct binding of intracellular cyclic nucleotides.65,66 The mammalian CNG channel genes fall into two different gene families. One subfamily consists of four members, CNGA1, CNGA2, CNGA3, and CNGA4, which represent the principal subunits that, except for CNGA4, form functional channels.17 The core structural unit consists of six transmembrane segments, designated S1–S6, and a cyclic nucleotide-binding domain (CNBD) near the C-terminal region. A pore region of ~20–30 amino acids is located between S5 and S6. The S4 segment in CNG channels resembles the voltage-sensor motif found in the S4 segment of voltage-gated K\(^{+}\), Na\(^{+}\), and Ca\(^{2+}\) channels. Both N-terminal and C-terminal regions are located in the cytoplasmic side and a glycosylated segment connecting S5 to the pore region is extracellular.17 The functional role of CNGs is well studied in retinal rod photoreceptors,67 sperm,68 central nervous system,69 and cardiac excitability.70 Studies of CNG channels in excocrine tissues have not been reported.

CNG channels belong to a heterogeneous gene superfamily of pore-loop cation channels that share a common transmembrane topology and pore structure. Other members of this superfamily are the hyperpolarization-activated cyclic nucleotide-gated channel (HCN),71 the ether-a-gogo (EAG), and human eag-related gene72 family of voltage-activated K\(^{+}\) channels.73 HCN channels are principally operated by voltage and permeable to both Na\(^{+}\) and K\(^{+}\). Opening of HCN channels causes hyperpolarization of the membrane. Unlike CNG, in which cyclic nucleotides are strictly required to open the channel in HCN, cyclic nucleotides facilitate the opening by shifting the voltage dependence of activation to more positive values.71 Cyclic AMP has shown to modulate HCN channel activity through a PKA-dependent mechanism.34,75

The basolateral voltage-activated K\(^{+}\) channels, which belong to the HCN channel subfamily, are necessary for the regulation of Cl\(^{-}\) secretion from pancreatic acini. In the rat pancreatic acinar cells, the presence of K\(^{+}\) channels in the basolateral membrane causes a membrane hyperpolarization, which provides the driving force for Cl\(^{-}\)-efflux. In addition, the efflux of K\(^{+}\) balances the K\(^{-}\) uptake by the Na\(^{+}\), K\(^{+}\) ATPase pump, and other cotransporters.76 The functional and pharmacological properties of these channels are conferred once KCNE1 coassembles with KCNQ1.77 Both KCNE1 and KCNQ1 genes are expressed in rodent pancreas.78,79 Cyclic AMP76,80 and carbachol81 increase the amplitude of the slowly activating voltage-dependent K\(^{+}\) channel current (\(I_{\text{Ks}}\)) in rat pancreatic acinar cells.

RNA-binding protein

The Ca\(^{2+}\)-regulated heat-stable protein of 24 kDa (CRHSP-24, also known as CARHSP1) is a serine phosphoprotein originally identified as a physiological substrate for the Ca\(^{2+}\)-calmodulin-regulated protein phosphatase calcineurin (PP2B).16 In pancreatic acini, cyclic AMP partially dephosphorylated CRHSP-24 on at least two sites82 through the activation of a phosphatase inhibited by calyculin A and okadaic acid, namely a PP2A or PP4.82

Regulation of adenylyl cyclase/cyclic AMP signaling

The cytosolic levels of cyclic AMP are modulated by regulating GPCR activity, G protein activity, adenylyl cyclase activity, and cyclic AMP degradation.

Receptor regulation

GPCRs can be regulated in several ways. One way is through phosphorylation of specific amino acids in their cytosolic domain. When these amino acids are phosphorylated, the receptor becomes desensitized. G-protein-coupled receptor kinases (GRKs) are proteins that specifically phosphorylate GPCRs. Two GRKs have been found in the pancreas: GRK583 and GRK6.84. PKA can also phosphorylate GPCRs. In mouse pancreatic acini, VPAC receptors appear to be regulated by PKA phosphorylation based on the inhibition of PKA activity using a PKA inhibitor (H-89) causing up to twofold increase in VIP-stimulated cyclic AMP formation.30

G-protein regulation

G-protein activity can be affected by various toxins, with the two best studied being cholera toxin and pertussis toxin. Cholera toxin in complex with NAD\(^{+}\) and GTP-bound ADP-ribosylation factor 6 (ARF6-GTP) catalyzes the ADP-ribosylation of the α-subunit of G\(_{i}\) protein and prevents it from hydrolyzing its bound GTP, thereby locking the G\(_{i}\) protein in the active state, which causes the continuous activation of transmembrane AC.85 In guinea pig pancreatic acini, cholera toxin increases cyclic AMP levels and amylase secretion.86 In mouse pancreatic acini, cholera toxin increases amylase secretion.87,88 Its effect is potentiated by cholecystokinin and is less marked than in guinea pig pancreatic acini.87 Unlike cholera toxin, pertussis toxin modifies the α-subunit of G\(_{i}\) protein and locks the G\(_{i}\) protein in the inactive state. The toxin catalyzes the ADP-ribosylation of a cysteine residue at position-4 from the C-terminal of the α-subunit of G\(_{i}\) protein, inhibiting the
interaction of this protein with the receptor and attenuating the intracellular transduction.\textsuperscript{89–91} In rabbit pancreatic acini, \textit{pertussis toxin} enhances CCK-induced cyclic AMP levels without affecting cholecystokinin (CCK)-induced Ca\textsuperscript{2+} mobilization or amylase secretion.\textsuperscript{92} In rat pancreatic acinar cells, although the pretreatment with either \textit{pertussis toxin} or \textit{cholera toxin} does not modify CCK-stimulated intracellular Ca\textsuperscript{2+} levels or phosphoinositide hydrolysis,\textsuperscript{27} \textit{pertussis toxin} increases the basal levels of cyclic AMP and amylase secretion.\textsuperscript{93} Regulators of G-protein signaling (RGS) molecules, which catalyze the GTP hydrolysis of heterotrimeric G-proteins, also play a critical role in regulating G-protein activity. RGS1, RGS2, RGS4, RGS16, and GAIP have been found in isolated pancreatic acinar cells using RT-PCR.\textsuperscript{94} Although their function in the regulation of G\textsubscript{s} activity in exocrine pancreas is still unknown, in olfactory neurons RGS2 decreases G\textsubscript{s}-stimulated cyclic AMP levels.\textsuperscript{95}

\textbf{Adenylyl cyclase regulation}

Adenylyl cyclase activity can be regulated by distinct intracellular signals. As previously indicated in Table 1, transmembrane ACs are classified into four groups: group I consists of Ca\textsuperscript{2+}-stimulated (AC1, AC3, AC8); group II consists of G\textsubscript{i}-stimulated (AC2, AC4, AC7); group III consists of G\textsubscript{s}/Ca\textsuperscript{2+}/PKC/PKA-inhibited (AC5, AC6); and group IV consists of Ca\textsuperscript{2+}-inhibited (AC9), which is forskolin-insensitive.\textsuperscript{27,33,96} Recently, AC9 activity has also been shown to be inhibited by Gai/o proteins and PKC.\textsuperscript{97} The Ca\textsuperscript{2+}-binding protein involved in the stimulatory effect of Ca\textsuperscript{2+} on the group I is calmodulin, which forms an active Ca\textsuperscript{2+}-calmodulin complex. Calmodulin is present in pancreatic acini and activated by CCK.\textsuperscript{98} The Ca\textsuperscript{2+}-calmodulin complex binds to the calmodulin-binding site present in the group I and increases its activity dramatically. AC9 is also stimulated by calmodulin.\textsuperscript{99} The Ca\textsuperscript{2+}-binding protein involved in the inhibitory effect of Ca\textsuperscript{2+} on AC9 is calcineurin, which is a serine/threonine protein phosphatase activated by CCK\textsuperscript{100} and involved in amylase secretion from rat pancreatic acini\textsuperscript{101} as well as caerulein-induced intracellular pancreatic zymogen activation.\textsuperscript{102}

Forskolin is a diterpene extracted from the root of the plant \textit{Coleus forskohlii} that directly activates all transmembrane AC isoforms, except AC9,\textsuperscript{20,103} by interacting with the two cytoplasmic domains (C1 and C2) that form the catalytic domain.\textsuperscript{104} The lack of effect of forskolin on AC9 may be accounted for by the residues Tyr1082 and Ala1112.\textsuperscript{105}

Unlike transmembrane AC, AC10 is not activated by either G protein or forskolin. Its activation is dependent on the HCO\textsubscript{3}– levels,\textsuperscript{106} though it can also be activated by divalent cations, such as Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, and Mn\textsuperscript{2+}.\textsuperscript{107} A combination of Ca\textsuperscript{2+} and HCO\textsubscript{3}– activates soluble adenylyl cyclase synergistically.\textsuperscript{107} AC10 is also activated by changes in intracellular pH.\textsuperscript{108}

The most common posttranslational modification of an AC isoform is the phosphorylation of a serine, threonine, or tyrosine residue (Figures 3 and 4). Phosphorylation of AC1 and AC3 by Ca\textsuperscript{2+}/calmodulin kinases inhibits the cyclase activity by blocking the binding site. Phosphorylation of ACs by either PKA or PKC causes an inhibition of the enzyme activity. Ubiquitination and acetylation are other modifications found in the human AC isoforms, though their consequences in AC activity are still unknown.

\textbf{Regulation of cyclic AMP degradation}

Cyclic AMP degradation is carried out by the enzyme phosphodiesterase,\textsuperscript{44} which is an exonuclease capable of hydrolyzing a phosphate ester and pyrophosphate bonds and, thereby, converting cyclic AMP into 5’AMP.\textsuperscript{109} Eleven PDE isoforms exist and each has unique biochemical properties. PDE1, which hydrolyzes both cyclic AMP and cyclic GMP, has been found in pancreatic acini using immunocytochemistry,\textsuperscript{109} whereas PDE7B, a cyclic AMP-specific PDE, has been found in whole pancreas using Northern blotting.\textsuperscript{110} PDE4, which is highly expressed in most immune and inflammatory cells and a cyclic AMP-specific PDE, is involved in the development of acute pancreatitis because the selective inhibitor rolipram attenuates the severity of acute pancreatitis in rats.\textsuperscript{111}

\textbf{Role for adenylyl cyclase/cyclase AMP pathway in pancreatic exocrine cells}

The exocrine pancreas is primarily composed of pancreatic acini and ducts. Pancreatic acini synthesize and release digestive enzymes into the duodenum, whereas pancreatic ducts release a HCO\textsubscript{3}–-rich fluid to neutralize the acidic chyme released from the stomach. In this section, the roles for adenylyl cyclase/cyclic AMP pathway are described.

\textbf{Pancreatic duct HCO\textsubscript{3}–-rich fluid}

Secretagogues, such as secretin and vasoactive intestinal polypeptide (VIP), increase cyclic AMP and stimulate HCO\textsubscript{3}– fluid secretion from pancreatic duct cells.\textsuperscript{112–114} An increase in the levels of cyclic AMP, through PKA phosphorylation, activates CFTR to recirculate chloride back into the glandular lumen and, thereby, depolarizes both luminal and basolateral membranes. Depolarization of the basolateral membrane increases the driving force of an electrogenic sodium-HCO\textsubscript{3}– cotransporter on the basolateral membrane leading to the entry of HCO\textsubscript{3}–, which is then secreted at the apical membrane via the Cl–/HCO\textsubscript{3}– exchanger.\textsuperscript{45} The AC6/cyclic AMP/PKA pathway has an important role in the physiological function of pancreatic
ducts because the VIP-stimulated expansion of the lumen observed in vitro in pancreatic ducts from WT mice upon VIP stimulation was absent in duct fragments from AC6-deficient mice. In vivo collection of pancreatic fluid also showed a decrease in fluid secretion from AC6-deficient mice. The secretory effect is highly dependent on PKA activation because in isolated pancreatic ducts from AC6-deficient mice PKA activation was abolished in response to VIP, secretin, and forskolin.

Several ion channels are affected by cyclic AMP/PKA pathway. PKA phosphorylates CFTR located in the apical membrane of the pancreatic duct cells. Elevation of intracellular cyclic AMP by stimulation with forskolin significantly inhibits the Na\(^+/\)K\(^+\) ATPase in isolated rat pancreatic acini and, as a consequence, an increase in pancreatic acinar fluid secretion; Epac mediates VIP-induced PKA activation, whereas PKA mediates secretin-induced PKA activation.

Pancreatic acini enzymatic-rich fluid

Early work showed that a number of compounds that increase cyclic AMP levels stimulate amylase secretion from pancreatic acini. Phosphodiesterase inhibitors, such as 3-isobutyl-1-methylxantine, increase pancreatic amylase secretion. Pertussis toxin catalyzes the ADP-ribosylation of a cysteine residue at position-4 from the carboxy-terminal domain of the \(\alpha\)-subunit of G\(_\text{i}\) protein, inhibiting the interaction of this protein with the receptor and impairing intracellular transduction. Treatment with pertussis toxin causes an increase in cyclic AMP levels and amylase secretion from rat pancreatic acini, where multiple pertussis toxin-sensitive G proteins have been found (e.g., G\(_{i1}\), G\(_{i2}\), G\(_{i3}\), and G\(_{i6}\)). Forskolin interacts with the two cytosolic domains C1 and C2 of transmembrane ACs, except AC9. Forskolin slightly stimulates amylase secretion in rat and potentiates the response to Ca\(^{2+}\)-dependent secretagogues. Recently, pancreatic acini from AC6-deficient mice showed a reduction in stimulated amylase secretion and PKA activity. Because this inhibition was only partial, it is likely that other AC isoforms expressed in pancreatic acini are also responsible for the secretory role of pancreatic acini. The result showing the deletion of AC6 does not affect the response to the Epac1 analog 8-pCPT-2’-O-Me-cyclic AMP on amylase secretion supports this hypothesis.

Glucagon-like peptide-1 (GLP-1) is a glucagon-like hormone secreted by intestinal L cells in response to nutrient ingestion that can act through its receptor (GLP-1R). GLP-1R is expressed in isolated mouse pancreatic acini and mediates GLP-1-induced amylase secretion from isolated mouse pancreatic acini through cyclic AMP/PKA pathway activation.

Both VIP and secretin, through the phosphorylation of p21-activated kinase (PAK) 4, but not PAK2, cause an activation of the Na\(^+/\)K\(^+\) ATPase in isolated rat pancreatic acini and, as a consequence, an increase in pancreatic acinar fluid secretion; Epac mediates VIP-induced PKA activation, whereas PKA mediates secretin-induced PKA activation.

Differentiation, transdifferentiation, and proliferation

Cyclic AMP plays an important role in differentiation, transdifferentiation, and proliferation of pancreatic cells. Isolated adult islets of Langerhans were able to transdifferentiate to duct epithelial-like cyst structures in presence of elevated cyclic AMP and a solid extracellular matrix (e.g., matrigel and collagen 1). The presence of intracellular cyclic AMP elevating factor, such as cholera toxin, was also required for the proliferation and maintenance of pancreatic epithelial duct cells. However, transforming growth factor-\(\beta\) (TGF-\(\beta\)), which is an important regulator of growth and differentiation in the pancreas, can activate PKA without affecting cyclic AMP levels in pancreatic acini. TGF-\(\beta\)-mediated growth inhibition and TGF-\(\beta\)-induced p21 and SnoN expression are mediated by PKA because both effects were blocked the PKA inhibitors H89 and PKI peptide. A physical interaction between a Smad3/Smad4 complex and the regulatory subunits of PKA has been shown in pancreatic acini.

Development of pancreatitis

Acute pancreatitis is an acute inflammatory disease of the pancreas. The disease appears to be initiated when a pathologic factor like alcohol or bile injuries the acinar cell and it responds by releasing inflammatory mediators and by activating digestive enzymes, especially proteinases, and restricting their secretion. These events initiate a cascade that leads to pancreatic inflammation and local and systemic tissue injury. The participation of cyclic AMP in the development of pancreatitis has been studied by the Gorelick lab. An early study showed that cyclic AMP-dependent secretagogues sensitize the pancreatic acinar cells tozymogen activation induced by caerulein, a CCK analog. The same research group in a subsequent work showed that cyclic AMP, by enhancing the release of pancreatic enzymes from the acinar cell, can overcome the acinar cell injury induced by high concentrations of carbachol, a cholinergic agonist. Recently, the inhibition of soluble AC by KH7 was shown to enhance the activation evoked by caerulein of two important digestive enzymes chymotrypsinogen and trypsinogen, as well as caerulein-stimulated amylase secretion from rat pancreatic acini. Together these studies suggest a complex role for cyclic AMP in acute pancreatitis in...
which it may enhance some pancreatitis responses while simultaneously lessening the effects of others.

**Role for adenylyl cyclase/cyclase AMP pathway in pancreatic cancer**

Of all the different cancers, pancreatic cancer is one of the major unsolved health problems. In 2020, the U.S. projected estimates report that the number of deaths from pancreatic cancer will be over 47,050. This is a 2.8 percent increase in estimated deaths from 2019. Unfortunately, the usual cancer treatment options do not have much of a positive effect. For that, it is of interest to develop new clinical strategies to increase the survival of patients with pancreatic cancer.

In 1996, receptors for VIP and β-adrenergic agonists (i.e., adrenaline and isoproterenol), which are functionally coupled to AC, were found in five human pancreatic adenocarcinoma cell lines BxPC-3, Hs 766T, Capan-2, Panc-1, and Capan-1 (Hs 766T and BxPC-3 were the most responsive, followed by Capan-2 and Capan-1, and finally Panc-1; MIA PaCa-2 cells did not respond to any of the agonists tested). Because high concentration of secretin was necessary to stimulate AC, the process of neoplastic transformation has either downregulated the expression of secretin receptors or led to a defect in the receptor itself. Later, the G \(_{\alpha_i}/AC/cyclic AMP pathway was shown to participate in the development of pancreatic pre-neoplastic lesions and the regulation of multiple pancreatic cancer cellular processes, including migration and invasion.

Support for the AC/cyclic AMP pathway in developing pancreatic pre-neoplastic lesions has come from a number of studies using forskolin as a stimulator of AC. From all the transmembrane isoforms present, AC3 was more highly expressed in the pancreatic tumor tissue, compared to the adjacent nontumor tissues, and in two pancreatic cancer cell lines, HPAC and PANC-1, compared to the normal pancreatic ductal cell line (hPDEC). AC3 was also the isoform responsible for the inhibitory effect of forskolin on cell migration and invasion in pancreatic cancer cell lines HPAC and PANC-1. The mechanism by which forskolin/AC3/cyclic AMP pathway inhibits cell migration and invasion involves the quick formation of AC3/CAP1 complex and sequestration of G-actin, which leads to an inhibition of filopodia formation and cell motility.

The stimulation of the AC/PKA pathway by forskolin and VIP increases expression and release of apomucin MUC5AC from pancreatic cancer cells SW1990. Because the coexpression of mutants \(G_{NAS}^{R201C}\) and \(KRas^{G12D}\) upregulates apomucins MUC1 and MUC5AC, but not MUC2, which resembles the expression of apomucins in human IPMNs, \(GNAS^{R201C}\) mutation, by \(G_{\alpha_i}/cyclic AMP/PKA pathway, might induce the expression of MUC5AC in IPMNs.

**Guanylyl Cyclase/Cyclic GMP Signaling**

Cyclic GMP is made from GTP through a catalytic reaction mediated by guanylyl cyclase (GC). Like AC, GC can be transmembrane or soluble. Unlike transmembrane ACs, all transmembrane GCs share a basic topology, which consists of an extracellular ligand-binding domain, a single transmembrane region, and an intracellular domain that consists of an extracellular ligand-binding domain, a single transmembrane region, and an intracellular domain that contains a juxtamembranous protein kinase-homology domain (KHD), a coiled-coil amphipathic α-helical or hinge region, and the catalytic GC domain at its C-terminal end. The function of the KHD is still unknown. Although it binds ATP and contains several residues conserved in the catalytic domain of protein kinases, kinase activity has not been found. In fact, it regulates the GC activity at the C-terminal end. The coiled-coil hinge region is involved in the process ofimerization, which is essential for the activation of GC domain.

There are at least seven transmembrane guanylyl cyclases: GC-A, GC-B, GC-C, GC-D, GC-E, GC-F, and GC-G (Figures 8 and 9). Only GC-A, GC-B, and GC-C have shown to regulate the function of exocrine pancreas.

**Transmembrane GC**

There are at least two groups of ligands for transmembrane GC: natriuretic peptides and guanylin and uroguanylin peptides.
Natriuretic peptides

There are three members of natriuretic peptide family: atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (Figure 10A). The actions of natriuretic peptides are mediated by the activation of three transmembrane receptor subtypes: natriuretic peptide receptor type A (NPR-A, also known as GC-A), type B (NPR-B, also known as GC-B) (Figure 10B), and type C (NPR-C). The last one (NPR-C) is not a transmembrane GC; it was initially considered to be a clearance receptor whose only function was to regulate the plasma concentration of natriuretic peptides. Later, NPR-C receptors have been found to be coupled to the inhibition of AC through α subunit of an inhibitory guanine nucleotide regulatory protein (Gαi1/Gαi2) and/or the activation of phospholipase C (PLC) through the βγ subunits of the same G protein150–153 in a number of tissues, including pancreatic acini.154 Because NPR-C receptors are not coupled to cGMP generation, NPR-C receptors are not further discussed in this chapter.

The NPR-A and NPR-B receptors, whose relative molecular mass is 130–180 kDa, have a similar structure that contains four domains: an extracellular ligand-binding domain, a single transmembrane domain, an intracellular tyrosine-like domain, an amphipathic region, and a GC catalytic domain. Upon ligand binding, the NPR-A and NPR-B receptors change their conformation, which results in GC activation and cyclic GMP generation.155,156

Guanylin and uroguanylin

Guanylin and uroguanylin are peptides secreted from the intestine, which influence electrolyte and fluid transport in the intestine and kidney, respectively.157,158 Their effects are mediated by the NPR-C receptor, which is predominantly expressed in the intestine.

Soluble GC

Soluble GC is a histidine-ligated hemoprotein that consists of two homologous subunits, α and β. The well-known isoform is the α1β1 protein; α2β2 subunits have also been identified.159,160 Each soluble GC subunit consists of four domains, an N-terminal heme-nitric oxide oxygen (H-NOX) domain (also called a SONO domain), a central Per-ARNT-Sim114 domain, a coiled-coil domain, and a C-terminal catalytic cyclase domain. The β1 subunit contains a N-terminal heme-binding domain, a Per/Arnt/Sim114 domain, a coiled-coil domain, and a C-terminal catalytic domain,161 as described in Figure 11.

Intracellular targets of cyclic GMP

Intracellular targets of cyclic GMP, like intracellular targets of cyclic AMP, have a cyclic nucleotide-binding domain (CNBD) in their structure.

Cyclic nucleotide-gated channels

Cyclic nucleotide-gated channels (CNGs) have been described above (see Intracellular Targets of Cyclic AMP). The physiological significance of cyclic GMP as activating agent of CNG has been described in photoreceptor cells and olfactory sensory neurons, where CNGs play an important role in sensory transduction.66 There are no reports of CNG function in an exocrine tissue.

Cyclic GMP-dependent protein kinase

The increase in the levels of cyclic GMP activates cyclic GMP-dependent serine/threonine protein kinase (PKG). Two genes prkg1 and prkg2 code for the two isoforms PKGI and PKGII.14 The human prkg1 gene is located on chromosome 10 at p11.2–q11.2 and has 15 exons. The N-terminus of PKGI is encoded by two alternative exons that produce the isoforms PKGIα and PKGIβ. The human prkg2 gene is located on chromosome 4 at q13.1q21.1 and has 19 exons. Its transcript yields a protein with an apparent mass of 87.4 kDa.14 Like PKA, PKG is composed of two functional domains: a regulatory domain and a catalytic domain. The regulatory domain is subdivided into the N-terminal domain and the cyclic nucleotide-binding domain (CNBD) containing the high and low cyclic GMP affinity binding
pockets. The catalytic domain contains the Mg\(^{2+}\)-ATP- and peptide-binding pockets. Upon binding of cyclic GMP to the two regulatory subunits, the two catalytic subunits are released from the regulatory subunits and become active.\(^{14}\) The substrates of this kinase are P240, P132, and phospholamban, though none of them is a specific PKG substrate.\(^{166,167}\) The intracellular levels of cyclic GMP are regulated by PDE enzymes, which hydrolyze cyclic GMP into 5'GMP.\(^{167}\) In pancreatic acinar cells from guinea pig, the presence of PKG activity has been reported.\(^{40}\)

**Role for guanylyl cyclase/cyclic GMP pathway in pancreatic exocrine cells**

The role of GC/cyclic GMP in the regulatory function of pancreatic exocrine cells is still controversial. One of the
first papers published on isolated pancreatic lobules from guinea pig and rabbit showed that carbamylcholine (carbachol), pancreozymin (now known as CCK), and caerulein all increased the levels of cyclic GMP without modifying the levels of cyclic AMP. The authors concluded that cyclic GMP is the second messenger involved in the process of stimulus-secretion coupling in the acinar cells of exocrine pancreas. Later, Ca\textsuperscript{2+} was shown to be an important mediator of the stimulus-secretion coupling process. Moreover, increased intracellular levels of cyclic GMP has a little or no effect on the stimulus-secretion coupling in pancreatic acinar cells. However, cyclic GMP has been involved in the Ca\textsuperscript{2+} entry across the cell membrane to replenish the intracellular Ca\textsuperscript{2+} stores.

The function of NO, the ligand for soluble GC, has been studied in the exocrine pancreas. NO can increase endogenous cyclic GMP and rat pancreatic secretory activity. NO triggers an increase in intracellular Ca\textsuperscript{2+} levels via cyclic GMP and inositol trisphosphate in pancreatic acinar cells. NO is localized in intrapancreatic ganglionic cells and efferent nerve fibers and implicated in the control of mesenteric circulation. NO inhibits pancreatic exocrine secretion in dogs and rats. NO production regulates cyclic GMP formation and Ca\textsuperscript{2+} influx in rat and guinea pig isolated pancreatic acini. Blocking NO production by chemical inhibitors of NO synthase, NG-monomethyl-L-arginine or NG-nitro-L-arginine, abolished cyclic GMP formation induced by the cholinergic agonist carbachol in a dose-dependent manner. NO has shown to have a protective role in acute pancreatitis.

The functions of two ligands for transmembrane GCs, natriuretic peptides, and guanylin have also been studied in exocrine pancreas.

**Natriuretic peptides**

All of the three receptors of natriuretic peptides are expressed in pancreatic acini, and both ANP and CNP increase intracellular levels of cyclic GMP in isolated pancreatic acini. However, the action of ANP and CNP on
Pancreatic secretion is not mediated by an increase in cyclic GMP. Indeed, ANP and CNP increase pancreatic fluid and protein output through the NPR-C receptor activation/Ca\(^{2+}\) release.\(^{154,182,183}\)

**Guanynin and uroguanylin**

In rat pancreatic acini, guanylin increases cyclic GMP levels, elicits a small amount of amylase secretion, and a small Ca\(^{2+}\) transient.\(^{11}\) Guanylin is localized specifically to the centroacinar cells and proximal duct cells and released luminally into the pancreatic ducts based on its presence in the pancreatic juice.\(^{188}\) Functional studies in two different human pancreatic duct cell lines revealed that guanylin is an intrinsic pancreatic regulator of Cl\(^{-}\) current activation in pancreatic duct cells via cyclic GMP. Using whole-cell patch-clamp, forskolin increased Cl\(^{-}\) conductance mediated by cyclic AMP, while guanylin increased Cl\(^{-}\) conductance mediated by cyclic GMP, but not cyclic AMP.\(^{185}\)

The existence of both membrane and soluble GCs in pancreatic acini suggests that there are two distinct sources of cyclic GMP located in different compartments, which could have different effects in pancreatic acini.

**Role for guanylyl cyclase/cyclic GMP pathway in pancreatic cancer**

Guanynin, uroguanylin, and GC-C are expressed at mRNA and protein levels in pancreatic cancer specimens and cancer cell lines, and uroguanylin inhibits pancreatic cancer cell proliferation in a concentration-dependent manner.\(^{186}\)

The transmembrane cell surface receptor GC-C has been identified in 60–70 percent of pancreatic cancer. An anti-GC-C antibody–drug conjugate TAK-264 (formally known as MLN0264) in patients with advanced or metastatic pancreatic cancer (NCT02202785) showed low efficacy and, for that, no further clinical investigation was undertaken.\(^{187}\)

PGK1 is expressed in pancreatic cancer cells and its inhibitor, DT3, causes cytotoxicity through necrosis and inhibits proliferation and migration of pancreatic cancer cells.\(^{188}\)

**Noncanonical Cyclic Nucleotides**

Cyclic IMP, cyclic XMP, cyclic CMP, cyclic UMP, and cyclic TMP are cyclic nucleotides whose function is well characterized (Figure 12). Using HPLC-MS/MS spectrometry, both cyclic CMP and cyclic UMP have been found in numerous cultured cell types and in human urine. Cyclic CMP and cyclic UMP concentrations are regulated by the cell proliferation status because growth arrest of cells resulted in preferential decrease of cellular cyclic CMP and cyclic UMP concentrations over cyclic AMP and cyclic GMP concentrations. Previous findings suggest that cyclic CMP and cyclic UMP could play a role as second messengers because cyclic CMP and cyclic UMP-hydrolyzing PDEs were found in mammalian tissues. Recently, soluble AC has shown to be responsible for the production of cyclic CMP and cyclic UMP in HEK293 and B103 cells because the soluble AC inhibitor KH7 decreased HCO\(_3^{-}\)-stimulated cyclic nucleotide levels in concentration-dependent manner. Forskolin, which is a stimulator of all transmembrane ACs except AC9, does not affect the levels of cyclic CMP and cyclic UMP. The authors conclude that soluble AC may likely have a distinct role in the regulation of cyclic nucleotide levels compared to soluble GC, membrane GC, and membrane AC.\(^{1}\) In RFL6 lung fibroblasts endogenously expressing soluble GC, NO-stimulated cyclic UMP formation was similar to cyclic GMP formation.\(^{189}\) In contrast to soluble GC, transmembrane GC does not induce cyclic UMP formation.\(^{190}\) Recently, cyclic CMP was found in several mouse tissues including pancreas as assessed by HPLC-MS/MS and HPLC-MS/TOF.\(^{191}\)
Unlike cyclic CMP and cyclic UMP, cyclic TMP, cyclic IMP, and cyclic XMP levels are very low to be detectable in cultured cell lines. Cyclic IMP levels increase in a hypoxic environment probably as a result of ATP deamination, which becomes ITP, and by soluble GC activity, ITP becomes cyclic IMP.

Noncanonical cyclic nucleotides have been studied so far in cardiovascular system, central nervous system, and reproductive system. A description of their roles in the regulation of these system can be found in reference 157. To the best of our knowledge, at the present there is no data available for the role of noncanonical cyclic nucleotides in the digestive system.

References
10. Ulrich CD, 2nd, Holtmann M, Miller LJ. Secretin and vasoactive intestinal peptide receptors: members of a unique...


73. Ficker E, Jarolimek W, Brown AM. Molecular determinants of inactivation and dofetilide block in ether a-go-go (EAG) channels.
Cyclic nucleotides as mediators of acinar and ductal function


Intermediate filament keratins are cytoskeletal components in epithelial tissues, including the exocrine pancreas. Keratin (K) intermediate filaments are highly conserved proteins that are expressed from early developmental stages up to the differentiated epithelial cells in adult individuals. A multitude of specific cellular functions have been identified for keratins expressed in simple epithelia, such as the pancreas, liver, lung, and intestine. These functions vary, depending on the cell and tissue type, as well as the developmental stage and changes in the cellular environment. Keratins are composed of dynamic, posttranslationally regulated cytoplasmic filaments built up of obligate heteropolymers of type I and type II keratins. In simple epithelia, the main keratins are type II K8 and K7, and type I K18, K19, and K20.

The exocrine pancreas comprises more than 85% of the pancreatic mass and consists of acinar and ductal cells. The acinar cells express mainly K8 and K18, which assemble into both cytoplasmic and apicolateral filaments, as well as minor levels of K19 and K20, which are confined to the apicolateral regions under basal conditions. Pancreatic duct cells express mainly K19 (type I) and K7 (type II). Pancreatic keratins respond quickly to cell stress by keratin phosphorylation and filament breakdown followed by keratin upregulation, de novo filament formation, and remodeling during the recovery phase in experimental exocrine pancreatic injury models. However, despite these dynamic stress responses, mutations or genetic deletion of K8 or K18 in humans or mouse models, only have modest effects on exocrine pancreatic health and stress tolerance. This is different from other simple epithelial tissues—most notably the liver—where K8, K18, and K19 mutations or deletions cause clear pathological outcomes. In contrast, overexpression of K8/K18 leads to pathological outcomes in the exocrine pancreas but not in the liver. These seemingly antagonistic outcomes in two cell types that have similar keratin expression patterns underscore the versatile and tissue-specific function of keratins. The biological reasons underlying the different susceptibilities of the exocrine acinar cells to keratin deficiencies, compared to other simple epithelial cells, are not fully understood but may, in part, be due to the increased levels of regeneration protein-II observed in the pancreas in several K8/K18-deficient mouse models.

Cytoskeletal Intermediate Filaments

The cytoskeleton is an organized network of proteins present in all cells. In eukaryotic cells, this network consists of three main filament systems: microtubules, microfilaments, and intermediate filaments (IFs). Among the main cytoskeletal filament groups, IFs are, as their name suggests, intermediate in size, measuring 10–12 nm in diameter; microfilaments are the smallest (6 nm) and microtubules the largest (25 nm). IFs comprise a large and diverse group of proteins that are ubiquitously expressed. They are divided into six different types, where type I and type II comprise different keratins, type III includes the muscle IFs desmin and vimentin expressed in mesenchymal cells and type IV neurofilaments, including nestin and α-internexin expressed, for example, in nerve cells, and synemin, expressed widely in muscle cells. Type V comprises the lamins, which are nuclear IFs found in all nucleated cells, including all epithelial cells. Finally, group VI comprises the highly specialized IFs phakinin and filensin, expressed only in the eye lens. The different types of IFs are typically expressed in a highly cell- and tissue-specific and/or in a developmentally specific manner.

Keratins, as well as all other IF proteins, have a basic structure of a central coil-coil α-helical rod domain, flanked by an N-terminal head domain and a C-terminal tail domain of variable length (Figure 1). Two IF molecules dimerize to form tetramers, which are the building blocks of these mechanically strong, yet flexible filaments. The IF assembly does not require ATP and IF filaments are nonpolar, in contrast to microfilaments and microtubules. Keratins, as well as other IFs, are dynamically regulated through various posttranslational modifications (PTMs), including

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Chapter 5
Intermediate filament keratins in the exocrine pancreas

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phosphorylation, O-linked glycosylation, ubiquitination, sumoylation, acetylation, and transamidation.\textsuperscript{7,8}

The keratin family includes 54 different functional keratin genes and proteins that are divided into two generic groups: type I, acidic keratins, and type II, neutral or basic keratins. Type I and type II keratins form obligate heteropolymers consisting of at least one of each type. Keratins have a molecular weight in the range of 44–66 kD and constitute the main cytosolic IFs in all epithelial cells such as pancreatic acinar, duct, and endocrine islet cells. The different types of keratins are further expressed in a cell- and tissue-specific manner (Figure 2). The human type I and type II keratin genes are clustered on the human chromosomes 17q21.2 and 12q13, respectively, with the exception of type I K18, which is located adjacent to type II K8 on chromosome 12q13.13.\textsuperscript{11} K8 and K18 are thought to be the closest descendants to an “ancestral keratin pair,” and the adjacent location of these two keratins may reflect an early divergence within the keratin gene family.\textsuperscript{12,13} K8/K18 is also the earliest keratin pair expressed in embryogenesis.\textsuperscript{11}

Simple layered epithelial cells found in the intestine, lung, liver, and pancreas predominantly express the type II keratin K8 (and to a lesser extent K7), type I keratins K18, K19, K20, and in a few epithelial cells, K23. The specific type I and type II expression pairs differ between different organs, as discussed below. Overall, K8/K18 is the predominant pair in the liver and pancreas, while K8/K19 heteropolymers predominate in intestinal epithelial cells. However, in hepatocytes, K8/K18 is the only keratin pair, whereas in the pancreatic acinar cells and in intestinal cells, K8 pairs with K18, K19, and K20.\textsuperscript{9,14}

Of all simple epithelial organs, liver is the most affected by keratin abnormalities.\textsuperscript{15} This disease susceptibility is probably due to that hepatocytes express only K8 and K18, with the exception of ductal cholangiocytes in the liver, which also express K7 and K19.\textsuperscript{9,10} Several liver disease-associated mutations of K8, K18, and K19 have been identified in humans.\textsuperscript{5,9,17} Mouse models expressing these mutations often phenocopy these humans disease,\textsuperscript{5,15} and are therefore important models for studying keratinopathies. Since K8 is the main type II keratin in most simple epithelia, the K8 null mouse as well as mice overexpressing wild-type K8 or disease-related K8 or K18 mutations are valuable for studying the roles of keratins in simple epithelial organs. The liver and colon are the organs most affected by these keratin deficiencies and they are also the most studied in this context.\textsuperscript{5} The exocrine pancreas is less affected by K8 deletion; the K8 null exocrine pancreas appears, in fact, to be modestly protected from

Figure 1. Keratin protein structure. All keratins are composed of a central helical rod domain, flanked by a head domain at the amino terminal (N-terminal) and a tail domain at the carboxy terminal (C-terminal) of the protein. The rod domain is segmented into four parts: 1A, 1B, 2A, and 2B, interlinked by short linker regions (L1, L12, L2). The peripheral sites of the rod domain (depicted in grey) contain highly conserved regions in keratins. The head and tail consist of nonhelical segments, which contain most of the sites for posttranslational modifications (PTMs), including multiple phosphorylation sites.\textsuperscript{8,9}

Figure 2. Keratin expression in epithelial tissues and the pancreas. Intermediate filament type I and type II keratin (gray box) proteins are divided in to skin keratins (light yellow box), hair keratins (yellow box), and simple epithelial keratins (green box). The main keratins in adult human exocrine pancreas acinar cells are K8, K18, with the addition of K19* in the centroacinar cells. K19 and K7 are expressed in mammalian exocrine ducts (rat ducts also express K20). Mouse acinar cells also express mainly K8 and K18 but in addition lower levels of K19* and K20** near the apicolateral membranes. The endocrine pancreatic cells (in mouse) express K8 and K18 and lower levels of K7. K7 and K19 are found in the embryonic pancreas.
experimental pancreatitis-induced injury. Experimental K8 and K18 overexpression, conversely, conveys age-associated atrophic changes to the exocrine pancreas, yet does not result in any known pathological anomalies in the liver. In this chapter, the expression and regulation of keratins in the exocrine pancreas during basal conditions and pancreatic injury as well as keratin-dependent regulation of cell stress-response proteins will be considered. Further, the implications of keratin defects on different types of pancreatic injury and the changes in keratin expression in pancreatitis and pancreatic cancer will be discussed.

Intermediate Filament Keratin Function, Regulation, and Disease Association

IFs, including keratins, were first recognized as structural components of cells, serving mainly as static mechanical support for the cells. Further investigations into the structure and function of keratins nevertheless revealed that they are, in fact, highly dynamic structures that assemble and disassemble very quickly, for example, by means of post-translational regulation in response to various stimuli. Phosphorylation is the most frequently occurring—and also the most studied—PTM in keratins. The PTM sites are mainly located on the head and tail domains of keratins and these domains typically contain multiple phosphorylation sites (Figure 1). Keratin PTMs enable rapid and dynamic regulation of the keratin filament solubility, as well as network assembly and organization. Keratin PTMs, moreover, regulate the association of keratins with multiple essential cellular proteins, such as the cytolinker protein plectin, the 14-3-3 adapter protein, as well as several protein kinases and phosphatases. Keratins attach to the desmosomes and hemidesmosomes through cytokinoker proteins, thus forming a cytosolic network that extends from the cell membrane to the nucleus, providing a flexible, yet mechanically stable cellular reinforcement.

Keratins are, as a consequence of their interaction with multiple cellular proteins, involved in diverse physiological processes, in addition to providing dynamically adaptable mechanistic reinforcement in the cell. The multitude of different proteins that interact with keratins, many of which are essential cell-signaling mediators, imply a central regulatory role for epithelial keratins in intracellular organization, cell signaling, maintaining cell polarity, regulating translation, and targeting proteins and organelles in the cell. Keratins also serve a vital role in the protection from the liver.

Keratin Expression in the Exocrine Pancreas in Humans and Murine Models

The exocrine pancreas consists of acinar cells that secrete digestive enzymes and a network of ducts that transport these enzymes from the pancreas to the small intestine. The acinar cells are pyramidal-shaped simple epithelial cells arranged in acini, whereas the duct cells are simple squamous or cuboidal-type epithelial cells. Keratins make up the IFs of the pancreas and comprise 0.3% of the total pancreatic proteins. The pattern of keratin expression...
differs between different species and developmental stages. The subcellular localization of the different keratins in the pancreas is moreover highly orchestrated and molded by the cell-specific conditions.

Under basal circumstances, adult mouse acinar cells express K8 and K18 with minor levels of K19 and K20 (Figure 2). K8 and K18 form cytoplasmic filaments throughout the acinar cell, including prominent keratin bundles (known as apicolateral filaments) running along the apical and lateral domains, in parallel to the F-actin layer closest to the cell membrane. In contrast, K19 and K20 are only observed apicolaterally under basal circumstances (Figure 3). K19 and K7 are the main keratins of exocrine pancreatic duct cells in mice, and K19, K7, as well as K20, in rats.

Outside the scope of this chapter, the mouse endocrine pancreas, consisting of islets of Langerhans, expresses mainly K8 and K18, but also some K7, which like K8, forms heteropolymers with K18. Additionally, K20 expression has been reported in neonatal rat endocrine pancreas.

Figure 3. Keratin expression in mouse exocrine pancreatic acinar cells. (A) K8 (a, green) and K18 (b, red) form cytoplasmic heterodimeric filaments (merged image of K8/K18 is shown in c) in acinar cells whereas K19 (d, green), here costained with K18 (e, red; merged image of K19/K18 in f), is observed apicolaterally. Nuclei are shown in blue and an acinus in e and f with basally located nuclei are outlined with a dotted line. Scale bar = 20 μm. (B) The schematic illustration (created with BioRender.com) shows acinar cell cytoplasmic and apicolateral K8/K18 (green) and apicolateral K19 and K20 (orange) filament localization under basal conditions (left) and the apicolateral as well as de novo K19/K20 cytoplasmic filament localization during regeneration after acinar cell injury (right).
In humans, K7 and K19 are expressed in all epithelial cells of pancreas during fetal development. Postnatally, differentiated human acinar cells only express K8 and K18, while K7 and K19 expression is retained in the pancreatic duct cells and K19 in centroacinar cells. K20 does not appear to be expressed under basal conditions in human pancreatic duct cells to any significant degree.

The physiological importance of these subtle interspecies pancreatic cell differences in keratin expression is not known. However, given the ubiquitous expression of K7 and K19 in early fetal development, retention of K19 in rodent acinar cells may be indicative of a lower level of cellular differentiation. Furthermore, K20 expression in the neonatal rat endocrine pancreatic cells is associated with cell proliferation and increased expression of mouse K19 and K20, with acinar cell regeneration. It may hence be speculated that the presence of K7, K19, and K20 expression in rodent pancreas reflects a lower level of cellular differentiation and perhaps higher degree of plasticity and regenerative capacity. This hypothesis is in line with the observations that pancreatic regeneration after injury, obesity, and during pregnancy is significantly higher in rodent than in human pancreatic cells.

The Effects of Experimental Keratin Mutations, Deletion, or Overexpression in the Exocrine Pancreas under Basal Conditions

The exocrine pancreas has a strikingly high tolerance to keratin absence or mutations, when compared to similar keratin deficiencies in the liver, which also predominantly expresses K8 and K18. The subject matter deserves consideration since these findings challenge a simplistic view of keratins as static stress protectors in all cells and bid for a more scrutinizing analysis of the mechanisms underlying the stress protein functions of keratins. Several transgenic mouse models that either lack or overexpress wild-type keratins, or that overexpress specific human keratin mutations, have been used to explore the role of keratins in the exocrine pancreas (summarized in Table 1).

**K8 and K18 null mice**

The only type II keratin expressed in acinar cells is K8. Since keratins are obligate heteropolymeric proteins and require both a type I and type II keratin to form stable filaments, it is expected that acinar cells in mice lacking K8 are entirely void of keratins. Indeed, the absence of both K8

<table>
<thead>
<tr>
<th>Genotype:</th>
<th>Wild-type</th>
<th>K8 null</th>
<th>K18 null</th>
<th>hk18 R86C overexpression</th>
<th>K18 glycosylation Deficient (S303/I494A)</th>
<th>Low &amp; modest overexpression</th>
<th>High overexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratin filament network and subcellular location</td>
<td>K8/K18 cytoplasmic, apicolateral filaments</td>
<td>Absent cytoplasmic filaments</td>
<td>Disrupted cytoplasmic filaments</td>
<td>Normal but ↑K18</td>
<td>Normal but ↑K8/K18</td>
<td>Normal but ↑K8/K18</td>
<td>Normal but ↑K8/K18</td>
</tr>
<tr>
<td>Phenotype:</td>
<td>Normal histology</td>
<td>Normal histology</td>
<td>Normal histology</td>
<td>Normal histology</td>
<td>Normal histology</td>
<td>Normal histology</td>
<td>Age-dependent pancreatic, dysplasia, fibrosis, ductal metaplasia, inflammation, acinar dedifferentiation</td>
</tr>
<tr>
<td>Reg-II level</td>
<td>Normal</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>References</td>
<td>18, 48, 52, 53</td>
<td>18, 52</td>
<td>18, 52</td>
<td>47, 52</td>
<td>32</td>
<td>19, 47</td>
<td>19</td>
</tr>
</tbody>
</table>
and K18 has been demonstrated by immunofluorescence labeling and immune-electron microscopic analysis in K8 null mice. In contrast, K18 null mice still express K8, since K8 forms heteropolymers with K19 in the absence of K18. However, under basal circumstances, K8/K19 keratin filaments are located solely to the apicolateral region of the acinar cells in K18 null mice, as K19 cannot compensate for cytoplasmic K18 filament formation under basal conditions. However, the histology of the K18 null pancreas is normal, apart from some polynuclear areas that bear a resemblance to the histological observations in the livers of K8 null and K18 null mice.\(^\text{18,35}\) The secretory response upon stimulation by cholecystokinin octapeptide (CCK-8) is normal in K8 and K18 null mice, but the acini are moderately less viable than in wild-type mice.\(^\text{18}\) The modest phenotype changes in K18 null mouse pancreas may be explained, at least in part, by the existence of the K8/K19 heteropolymers. However, in K8 null mice, the resistance to injury probably comes down to other compensatory mechanisms.\(^\text{18}\) One such suggested mechanism is the upregulation of regulatory protein II (Reg-II), which occurs upon stimulation by cholecystokinin octapeptide (CCK-8).\(^\text{55}\) This latter study used mice with different levels of keratin upregulation: human K18 overexpressing mice where keratin levels were only mildly increased, mouse K8 overexpressing mice with moderately upregulated K8, K18, K19, and K20, and mice that overexpressed both mouse K8 and human K18 (K8/K18 overexpressors), leading to a substantial keratin upregulation.\(^\text{19}\) Though the effects of keratin overexpression were less severe in the study by Toivola and colleagues, similar acinar cell anomalies were observed by Casanova et al., such as age-dependent atrophy and fatty vacuole formation—particularly in the mice with the highest levels of keratin overexpression. Hence, it is likely that the aggravated injury in the earlier study may have been due to a higher level of keratin upregulation.\(^\text{19}\)

Genetic overexpression of both K8 and K18 in pancreatic acinar cells changes the distribution and phosphorylation level of keratins and causes alterations in the quantity, size, and distribution of the amylase-containing zymogen granules in the acinar cells. The zymogen granules in K8/K18 overexpressing cells, as well as in human K8 overexpressing mice, were smaller in size but more numerous than in wild-type mice.\(^\text{19}\) Moreover, the granules were not retained in their characteristic apical location but instead diffusely localized in the cytoplasm in K8/K18 overexpressors.\(^\text{19,54}\)

In addition to these phenotypic differences, K8/K18 overexpressors also displayed increased keratin phosphorylation at K8 S79 and K18 S33, and thick K8/K18 bundles that localized not only around the apical lumen but also in perinuclear and cytoplasmic locations of the acinar cells.\(^\text{19}\)

Hence, overexpression of keratins in acinar cells appears to interfere with the intracellular organization of keratin and alter the exocrine function of the acinar cells. Interestingly, mice that overexpress a K18 S33A mutation (which inhibits its serine 33 phosphorylation) display keratin filaments that are retracted from the basal and nuclear region and instead concentrated in the apical region of acinar cells. However, these mice do not display abnormal pancreatic histology or disease.\(^\text{55}\)

These studies of the exocrine pancreas, using keratin overexpressor mice, further highlight the interrelationship between type I and type II keratins. Since K18 overexpression caused a minimal increase in keratin levels compared to K8 overexpression, it is evident that the level of type II keratins has a more profound effect on overall keratin regulation than type I keratins in the exocrine pancreas. Moreover, as the exocrine pancreas contains more than one type I keratin (K18, K19, K20), a deficiency or upregulation of one of these may be compensated, to a certain extent, by a down or upregulation of another type I keratin.

**Transgenic mice overexpressing wild-type keratins**

In contrast to keratin deletion, keratin overexpression is well tolerated in the liver, while in the pancreas it is associated with various pathological changes.\(^\text{19,54}\) In a study using transgenic mice overexpressing human K8, Casanova and colleagues reported severe age-dependent progressive abnormalities in the exocrine pancreas, demonstrated by a 30% loss of pancreatic mass, dysplasia, fibrosis, ductal metaplasia, inflammation, and dedifferentiation of acinar cells into duct cells.\(^\text{54}\) Moreover, Toivola et al. (2008) showed that the extent of pancreatic damage correlates with the level of keratin overexpression.\(^\text{19}\) This latter study used mice with different levels of keratin upregulation: human K18 overexpressing mice where keratin levels were only mildly increased, mouse K8 overexpressing mice with moderately upregulated K8, K18, K19, and K20, and mice that overexpressed both mouse K8 and human K18 (K8/K18 overexpressors), leading to a substantial keratin upregulation.\(^\text{19}\)

The contribution of specific keratin mutations for increased susceptibility to injury or disease has been analyzed with the help of transgenic mouse models, expressing human keratin mutations. Many of these experimental models, including human K18 R90C mice (which have a mutation equivalent to K14 R125C, found in epidermolysis bullosa simplex patients) as well as human K8 G62C mice (expressing a common human keratin variant mutation in liver disease patients), display early-onset liver inflammation, necrosis, and increased susceptibility to hepatotoxicity.\(^\text{15,36,56}\) The exocrine pancreas in these mice, on the contrary, appears far less affected by these mutations. Although the viability of the acini is somewhat compromised in K18 R90C mice displaying disrupted keratin filaments, these mice do not display increased pancreatic injury under basal circumstances.\(^\text{18}\) K18 R90C mice lack intact cytoplasmic keratin filaments (displaying only K8/
K18 dots in the cytoplasm), but they express apicolateral filaments in acinar cells, since K8 polymerizes with K19 and K20 that are located apicolaterally under basal conditions. This is in contrast with K18 R90C mouse hepatocytes, in which apicolateral keratin filaments are scarcer. This difference between hepatocyte and pancreatic keratins has been suggested as one reason underlying the lesser disease susceptibility of the pancreas compared with the liver in the K18 R90C transgenic mouse model.\(^{18}\)

**Keratins in Mouse and Cell Models for Pancreatic Injury**

Keratin networks are dynamic and respond swiftly to changes in the cellular environment. Several studies have investigated the roles of keratins in the exocrine pancreas by subjecting wild-type and K8- or K18-deficient mice to experimental pancreatitis models, including the caerulein model and the choline-deficient diet model\(^ {57}\) (summarized in Table 2). Typically, in these conditions, keratins are first rapidly broken down upon acinar cell injury but reform quickly and become highly upregulated during recovery. During the recovery process, keratins are also extensively phosphorylated, as is common in keratin stress responses. This dynamic process has been described after caerulein-induced exocrine pancreatic injury, which causes a rapid disassembly of the acinar cell keratin network within 1 hour of induction of injury, followed by a reassembly of keratin cytoplasmic filaments in the early recovery phase 7–24 hours after induction of injury. The cytoplasmic keratin network reforms during the recovery phase, and temporarily, strong *de novo* cytoplasmic filaments containing K19 and K20 become very prominent (Figure 3B), in addition to the K8/K18 cytoplasmic filaments.\(^ {18,32}\) This keratin upregulation is transcriptionally regulated since keratin mRNA levels also increase 48 hours after caerulein induction.\(^ {46}\)

Ultimately, in the late recovery phase (within 5 days), retrieval of the normal, baseline filament network structure occurs, and K19 and K20 filaments repossess their exclusively apicolateral localization. A similar keratin upregulation on protein and mRNA level, accompanied by *de novo*

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**Table 2. Experimental pancreatic injury phenotypes in wild-type and keratin transgenic mice.** H, human; m, mouse; pK, phospho-keratin; ↑ - ↑↑↑/+ - +++, level of increase from low to higher; CDDcholine-deficient diet pancreatitis model; CV, coxsackievirus; ND, not determined; STZ, streptozotocin; * endocrine pancreas phenotype.

<table>
<thead>
<tr>
<th>Mouse genotype: Disease model:</th>
<th>Wild-type</th>
<th>K8 null</th>
<th>K18 null</th>
<th>hK18 R90C over-expressor</th>
<th>K18 glycosylation deficiency (S30/31/49A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caerulein Susceptibility: Keratin phenotype:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degradation</td>
<td>(\uparrow pK8)</td>
<td>Absent</td>
<td>Degradation</td>
<td>(\uparrow pK8)</td>
<td>Rearrangements</td>
</tr>
<tr>
<td>↑K8/K19/K19/K20 cytoplasmic filaments</td>
<td></td>
<td></td>
<td>↑K8/K19 cytoplasmic filaments</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>CDD Susceptibility: Keratin phenotype:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degradation</td>
<td>(\uparrow pK8)</td>
<td>Absent</td>
<td>Degradation</td>
<td>(\uparrow pK8)</td>
<td>ND</td>
</tr>
<tr>
<td>↑K8/K19/K19/K20 cytoplasmic filaments</td>
<td></td>
<td></td>
<td>↑K8/K19 cytoplasmic filaments</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>CV B4-V (high virulence)</td>
<td>No lethality</td>
<td>(\uparrow) lethality</td>
<td>No lethality</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CV B4-P (low virulence)</td>
<td>Susceptible</td>
<td>Less susceptible, quicker recovery</td>
<td>Similar to Wild-type</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>STZ acute</td>
<td>Low lethality</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>*Prone</td>
<td>*Moderately resistant</td>
<td>*Islet cell necrosis</td>
<td>Low lethality</td>
<td>(\uparrow) Lethality</td>
<td>(\uparrow) Apoptosis</td>
</tr>
<tr>
<td>STZ chronic</td>
<td>Mild exocrine damage</td>
<td>↑ exocrine damage: oedema, hyperplasia, atrophy</td>
<td>↑ exocrine damage; oedema, hyperplasia, atrophy</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>*Moderate damage</td>
<td>*Moderately resistant</td>
<td>*K8 htz null mice more sensitive</td>
<td></td>
<td></td>
<td></td>
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cytoplasmic K19 and K20 filament formation, is seen 1–2 days after discontinuation of choline-deficient diet feeding, with keratin levels returning to baseline levels 7 days into the recovery phase. A similar transitory, recovery phase apical to cytoplasmic localization shift also appears in caerulein-treated K18 null mice, in which the keratin network under basal circumstances is exclusively apicolateral in acinar cells. In this model as well, the filaments revert back to their apicolateral localization later in the recovery phase. It is still unknown what regulates the induction of this remarkable transient K19 and K20 cytoplasmic filament formation, but it is suggested that it plays an important role in the recovery of acinar cells after injury. The existence of K19 filaments in K18 null mouse acinar cells has been postulated as a possible reason for the high tolerance to experimentally induced pancreatic injury in these mice. If this is the case, it is possible that K18 mutations may be more detrimental for human acinar cells, which lack K19 filaments. Interestingly, the keratin upregulation appears to be specific to regeneration after pancreatitis, since generalized stress models, such as heat or water immersion, do not alter keratin pancreatic expression levels. However, TGFβRII dominant-negative mutant mice, which develop a severe chronic pancreatitis phenotype similar to human K8 over-expressing mice, show highly increased K8 and K18 levels in the pancreas. Keratin gene transcription in the pancreas has not been extensively studied, but the K19 gene in pancreatic duct cells is regulated by PDX1, GKLF/KL4, and SP1, indicating an association between K19 expression and the developmental stage in the pancreas.

Interaction with the keratin-binding protein, epiplakin, contributes to the dynamics of pancreatic keratin remodeling after caerulein injury, as epiplakin-deficient mice display both a quicker keratin network breakdown after injury and impaired filament rearrangement, as demonstrated by the accumulation of keratin aggregates at the most severe phase of the cell injury. These defects might be caused by excessive hyperphosphorylation in the absence of epiplakin, emphasizing the role of dynamic keratin regulation in stress responses. In addition to the stress-induced keratin filament remodeling, keratins could also be involved in caerulein-induced inflammatory responses in the acinar cells, since caerulein-induced keratin upregulation is associated with nuclear factor-κB (NF-κB) activation.

The extensive rearrangement of keratin filaments in response to pancreatic injury in caerulein- and choline-deficient diet-induced pancreatitis suggests a protective role for keratins in these injury models. Yet intriguingly, apart from minor differences observed in certain disease parameters, K8 null (which lack acinar cell keratins entirely), K18 null (which express only K8/K19 acinar cell filaments), and K18 R90C mice are not overall more sensitive to choline-deficient diet or caerulein-induced pancreatitis, compared with wild-type mice. In fact, even with more prominent vacuolation, the histological damage in terms of inflammation and edema, is slightly lower in K8 null and K18 null mice after caerulein, compared to wild-type mice. Moreover, no differences in pancreas histology, serum amylase, or lipase levels were observed in K18 R90C mice compared with wild-type K18 over-expressing mice. These seemingly near dispensable effects of keratin deficiencies in the exocrine pancreas are puzzling. However, some potential underlying reasons for the relative stress tolerance of the exocrine pancreas in keratin-deficient mice can be extrapolated from existing animal studies. These include differences in the cellular localization of keratins in the acinar cells and hepatocytes, upregulation of cytoprotective regulatory proteins (discussed in the next section), and differences in the function of keratins in different organs. Further, the importance of keratins in pancreatic stress may also depend on the type and duration of the injury. For example, in a study where K8 null and K18 null mice were challenged with coxsackie B virus-induced pancreatitis, the vulnerability of keratin-deficient mice depended on the virulence of the coxsackie virus strain. When subjected to acute pancreatitis caused by the highly virulent coxsackie virus strain B4-V (CVB4-V), keratin null mice suffered significantly higher mortality compared with wild-type mice (40% mortality in K8 null mice and 0% in K8 wild-type mice). In contrast, K8 null recovered quicker than their wild-type counterparts from infection with a less virulent, B4-P coxsackie virus strain (CVB4-P). It has been proposed that this difference may come down to the effect of Reg-II-stimulated tissue regeneration, since Reg-II upregulation occurs after infection with CVB4-P virus, as well as in the caerulein- and choline-deficient diet pancreatitis models, but not CVB4-V in wild-type mice. Interestingly, Reg-II is upregulated in the K8 null pancreas under basal conditions and this upregulation may assist in the injury response to CVB4-P-induced pancreatitis.

In addition to CVB4-V coxsackie virus-induced pancreatitis, keratin-deficient animals also appear to be more sensitive to exocrine pancreatic injury induced by streptozotocin (STZ). STZ is a commonly used toxin for inducing type I diabetes in experimental animals. This toxin is taken up by β-cells through the glucose-transporter 2 (GLUT2) and causes acute β-cell destruction if administered at high doses and partial β-cell depletion and inflammation if administered at multiple low doses. Interestingly, K8 null mice develop widespread exocrine pancreatic edema, atrophy, vacuolization, and inflammation in response to the chronic stress induced by low-dose, STZ treatment (40 mg/kg/day, for five days), while wild-type animals display only modest exocrine damage after this treatment. Interestingly, K8 null β-cells are less sensitive to acute high-dose STZ (200 mg/kg/day), likely due to mislocalization of GLUT2,
which is needed for the uptake of STZ into β-cells. Yet, transgenic mice expressing a human K18 glycosylation-preventing (K18-Gly(-)) mutation suffer severe exocrine pancreatic injury after the same high-dose STZ treatment. The STZ treatment in this study induced multi organ failure in the glycosylation-deficient K18 mice, hence the toxic effects on the pancreas may have been exacerbated by the keratin mutation-induced liver deficiency in these animals. Taken together, these results demonstrate that the function of keratins, and their importance in protecting from cellular injury in the pancreas, may be crucially dependent on the disease mechanisms of a particular experimental model, the duration of the injury, and the type of keratin anomaly. Interestingly, in addition to keratin IFs, the type V intermediate filaments, nuclear lamins, may also contribute to pancreatic stress protection, since pancreas-specific conditional lamin A null mice show a spontaneous phenotype similar to chronic pancreatitis, and patients with mutations in lamin A have a higher risk of developing pancreatitis.

Dynamic stress-induced regulation of keratins has also been described in acinar cell cultures invitro, under various experimental stress conditions. For example, K23 mRNA was highly induced by the histone deacetylase inhibitors sodium butyrate and trichostatin during differentiation in the pancreatic cancer cell line AsPC-1, demonstrating the propensity for keratin de novo expression during stress conditions. In pancreatic carcinoma, PANC-1 cells, which express K8 and K18, caerulein treatment induced K8 S431 phosphorylation and a reorganization of the keratin filaments into a tight perinuclear network through activation of ERK and downregulation of PP2A and alpha 4, resulting in enhanced cell migration. Interestingly, similar K8 phosphorylation and perinuclear reorganization have also been observed in PANC-1 and A549 cells (human carcinoma alveolar cells) exposed to metastasis-enhancing bioactive lipid sphingosyl-phosphorylcholine, 12-Otetradecanoylphorbol-13-acetate (TPA), leukotriene B4 (LTB4), or shearing force.

In the endocrine pancreas, Reg-I seems to play an important role in islet regeneration, while in the exocrine pancreas Reg-II is the predominant Reg-protein. However, Reg-II is substantially upregulated in the exocrine pancreas during recovery from caerulein- or choline-deficient diet-induced pancreatitis, which indicates that it has an analogous function to that of Reg-I in the endocrine pancreas. Interestingly, in the caerulein model, Reg-II levels increase early on, while the K18 and K19 levels are at their highest later in the recovery phase, but in the choline-deficient model, Reg-II and K18 peak at around the same time.

The unexpectedly high resistance to pancreatic damage in keratin-deficient or mutant mice discussed above has encouraged analysis of compensatory factors in these mice that might explain why keratin deletion causes less damage in the exocrine pancreas compared to the liver, despite a similar keratin expression pattern. In a microarray analysis of K8 null mouse pancreas, several genes were indeed found up- or downregulated compared to wild-type control mice. Among the significantly upregulated genes were several members of the Reggene family, but particularly the Reg-II gene. Upregulation of Reg-II was also observed in other mice with keratin deficiencies, such as K18 null, K18 R90C transgenic mice, and phosphorylation-deficient K18 S52A transgenic mice. However, this upregulation was not seen in K19 null mice, which express normal, cytoplasmic, and apicolateral keratin filaments. It has thus been suggested that the resistance to pancreatitis in K8 null and K18 null mice, likely comes down to a compensatory protective effect of the Reg-II upregulation. This compensatory mechanism could explain the greater resistance of K8 null mice to the moderate pancreatic injury caused by infection with CVB4-P, which is accompanied by anti-apoptotic and cell regenerative responses, as well as to the acute injury by CVB4-V infections, which causes an upregulation of genes that favor apoptosis, metaplasia, and fibrosis.

The benefit of acinar cell Reg-II overexpression for recovery from experimental pancreatitis and diabetes has been questioned in a study by Li and colleagues, since transgenic acinar cell-specific overexpression of Reg-II neither protected the mice from streptozotocin-induced diabetes nor from caerulein-induced pancreatitis. In this study, Reg-II overexpression was moderate and roughly at a similar level to the spontaneous Reg-II upregulation after caerulein treatment. It is possible that the advantage of transgenic overexpression of Reg-II over an endogenic Reg-II upregulation in response to caerulein is not significant, if the experimentally induced overexpression levels do not exceed the endogenic stress-induced upregulation. Reg-II is nevertheless markedly upregulated in acinar cells upon injury and is hence evidently a stress response protein, albeit the precise function of Reg-II in pancreatic injury remains unclear. Apart from Reg-II upregulation, other factors that may contribute to the exceptional

Regenerating Protein II as a Cytoprotective Factor in Keratin-Deficient Exocrine Pancreas Models

The regenerating (Reg) gene was first isolated from islet β-cells in the pancreas. The protein encoded by this gene (now termed Reg-I) was found to have a significant stimulating effect on β-cells in the endocrine pancreas and was able to ameliorate diabetes in 90% of pancreatectomized rats and in nonobese diabetic (NOD) mice. It was later discovered that the Reg genes constitute a multigene family consisting of three types of genes (Reg-I, II, and III) that differ in expression pattern and functional characteristics, as reviewed in references.
Intermediate filament keratins in the exocrine pancreas

Keratins in Human Pancreatitis and Pancreatic Cancer

Keratins and other IFs among the cytoskeletal proteins are interesting to the medical field since they are associated with over 100 human diseases. Some keratin mutations are known to be directly causative of disease (e.g., several skin diseases), while mutations in simple epithelial keratins have been shown to predispose to various liver diseases. Moreover, since keratin expression and post-translational modifications are frequently altered upon cell stress, keratins are also used as diagnostic biomarkers for diseases. One example of such histopathologies is the liver disease-associated formation of keratin aggregates, known as Mallory Denk bodies in hepatocytes.

The prevalence of K8 mutations in human pancreatitis has been analyzed in a few studies, but no clear associations have been found. Two K8 variant mutations, Y54H and G62C, that predispose to cryptogenic liver disease have been studied in this context. K8 G62C was first found to predispose to chronic pancreatitis. A later study found no significant correlation between the frequency of either K8 G62C or K8 Y54H and acute or chronic pancreatitis or pancreatic cancer in a cohort of more than 2,400 patients. Similarly, K8 G62C mutations were found not to be associated with familial, sporadic, or alcoholic pancreatitis. However, a recent study has identified an association between the KRT8 gene and pancreatic cancer in a genome-wide association study in a Japanese population.

Changes in keratin expression levels during pancreatitis and pancreatic cancer have been analyzed in a few studies. K20 is a keratin that under normal circumstances has an expression pattern restricted to a few cell types and can therefore be used as a marker for certain cancers. As mentioned earlier, K20 expression has been reported in normal pancreatic duct cells in rats, but the expression in humans is very low in the pancreas under basal conditions. However, K20 expression in human pancreatic duct cells is markedly induced in metastatic pancreatic cancer cells, and detection of K20 in pancreatic tumors or in blood or bone marrow samples from patients with pancreatic duct cell carcinomas correlates with a worse prognosis. Furthermore, K19, which in humans postnatally is restricted to duct cells, can be observed in precancerous acinar-like cells before the appearance of metaplastic changes in cell morphology. Hence, K19 expression in neoplastic acinar cells is an early sign of metaplasia, which precedes the fibrotic changes related to metaplasia.

Conclusions and Vision

During the last decades, keratin IFs have emerged as important cellular regulators and stress proteins of epithelial cells in many different organs. As the complexity of keratin-related functions has been unraveled, it has become evident that the function of keratins depends on the type of keratins expressed in the cells and their subcellular localization, as well as on the specific cell type and the cell-specific functions and regulatory pathways.

The keratin expression in the liver and the exocrine pancreas is similar, yet the susceptibilities to keratin deficiency-related injury are evidently different. The published studies relating to the role of keratins in pancreas and liver cells have provided some clues that help to explain the differences in stress tolerance.

The dramatic upregulation of the injury-response protein Reg-II in the exocrine pancreas of K8 null and other keratin-deficient models is probably one factor that protects K8-deficient mice from exocrine pancreatic injury. Indeed, this type of protection appears to take place also in skin cells, where keratin-mutant keratinocytes are similarly prepared for injury through upregulation of basal-level JUN-kine activation and profuse activation of osmotic shock-induced stress pathways. Keratin deficiency may thus induce a chronic injury response that puts cells in an “alert state” facilitating a swift cellular response to stress. The activation or inhibition of the same cell-signaling molecule may, however, have different consequences in different organs or under different circumstances, due to the complexity of the cell signaling pathways and downstream effects. It has, for instance, been shown that activation of transcription factor NF-xB has a protective, anti-apoptotic effect in liver injury and it has been suggested that the sensitivity of K8 null liver cells to apoptosis is linked to a defective activation of this transcription factor. Activation of NF-xB nevertheless appears to have a negative effect on pancreatic cell survival in caerulein-induced pancreatitis, since it enhances the inflammatory response. It is interesting to speculate whether the resistance of K8-deficient mice to caerulein-induced pancreatitis may be associated with interference of the NF-xB activation in pancreatitis, but a link between K8 deficiency and impeded NF-xB activation has not yet been reported for the exocrine pancreas.

Keratins in the exocrine pancreas may at first appear redundant, given that keratin deficiency is remarkably well tolerated under basal conditions, as well as in some pancreatic injury models. The dynamic remodeling of keratins in response to exocrine pancreatic stress and the association of keratins with both Reg-II regulation and
inflammatory responses, such as NF-κB activation, however, contradict the notion that keratins lack a role in exocrine pancreatic injury responses. Rather than presenting an exceptional model organ in which keratins do not matter for stress tolerance, perhaps the exocrine pancreas really demonstrates the complexity of keratin-associated cell biology and the remarkable adaptability of the cells to counterbalance inherent weaknesses.

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References


44. Alam CM, Silvander JSG, Helenius TO, Toivola DM. Decreased levels of keratin 8 sensitize mice to streptozotocin-induced diabetes. Acta Physiol (Oxf) 2018; 224:e13085.


Acinar Cells
Chapter 6

Pancreatic acinar cell protein synthesis, intracellular transport, and export

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Introduction

A key function of the exocrine pancreas is the production of digestive enzymes. The pancreatic acinar cell synthesizes, stores, and secretes the proenzymes and enzymes needed to digest dietary proteins, carbohydrates, and lipids. Meeting these functional requirements necessitates that the acinar cell has very high rates of protein synthesis and export. Nascent proteins undergo folding, select modifications, concentration, segregation from other classes of proteins, and vectorial movement before reaching their final destination in secretory granules. These are concentrated in the apical pole of the acinar cell. Eating stimulates neural and hormonal pathways that mediate acinar cell zymogen granule exocytosis into the pancreatic duct. The acinar cell has been a model system for foundational studies of protein synthesis and export. After electron microscopy was developed, for example, cell biologists first visualized organelles and established their function by studying acinar cells (Figure 1). Here, we focus on acinar cell protein synthesis, trafficking, and processing in the pancreatic acinar cell necessary for its central role in producing digestive enzymes. We present results primarily obtained using rodent acinar cells, though the limited data from human acinar cells suggest the functions are likely the same as in rodents.

Protein Synthesis

The ribosome

The ribosome is the central element of the protein synthetic machinery. The eukaryotic ribosome is composed of two subunits: a large 60S unit containing 28S, 5S, and 5.8S rRNA and approximately 49 proteins and a small subunit at 40S, which includes 18S rRNA and about 33 proteins. The two subunits form a groove wherein new protein synthesis directed by messenger RNA takes place, aided by associated protein complexes to initiate, elongate, and terminate protein synthesis. Secretory proteins, including pancreatic digestive enzymes, contain a distinct N-terminal signal sequence which contains a 15- to 50-amino acid peptide that includes a hydrophobic core. Also known as a leader sequence, the signal sequence allows nascent proteins to cross the endoplasmic reticulum (ER) membrane by traversing the translocon, a large multiprotein channel. The signal sequence binds to the signal recognition particle (SRP) complex and brings the ribosome, attached nascent protein, and mRNA to the ER membrane SRP receptor. The SRP is then released and protein synthesis resumes with entry of the signal sequence into the translocon and its subsequent proteolytic cleavage from the nascent protein. In addition to soluble export proteins, the translocon also mediates the insertion of transmembrane proteins into the ER membrane and has a role in protein degradation.

The endoplasmic reticulum

The synthesis of new secretory proteins occurs on and in the ER. This organelle has two morphologically distinct compartments: the rough ER (RER) and smooth ER (SER), which have specialized functions. The RER has ribosomes on its cytoplasmic surface, which are easily seen by electron microscopy, whereas the SER lacks ribosomes (Figure 1). Between the RER and SER are regions termed transitional elements. Vesicular transport of nascent proteins from the ER occurs at sites in the transitional ER known as ER exit sites (Figure 2). The intracisternal space is formed by RER, transitional elements, and SER; this is the site in which nascent proteins begin to fold and undergo export. To accommodate high rates of secretory protein synthesis, a dense RER occupies much of the basal region of the acinar cell and extends apically. Regularly placed groupings of electron dense ribosomes (~30 nm) mark the sites of active protein synthesis on the cytosolic face of the ER membrane.

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The acinar cell ER shows regional functional specialization that also facilitates cell signaling. For example, the initial acinar cell cytoplasmic calcium signal that mediates protein secretion depends on an ordered, spatial release from distinct ER stores. In response to neurohumoral stimulation, the first calcium signal arises at the apex of the cell from select ER domains and is mediated by the inositol trisphosphate (IP3) receptor. Similarly, the propagation of the calcium wave throughout the acinar cell requires calcium release from ER ryanodine receptors which are distributed toward the base of the cell.

Distinct direct contacts between cell organelles have now been described in many tissues and include sites between the ER and mitochondria and ER and plasma membrane. These are known to be involved in lipid transfer and calcium signaling. In the acinar cell, apically distributed IP3 receptors (ER) that release calcium into the cytosol are found to closely approximate regulators of calcium entry into the cell (Orai1, STIM, and others) that are activated when depletion of ER calcium is sensed. These connections are thought to help coordinate both physiologic responses, such as protein secretion, and pathologic cell responses.

**Figure 1.** Electron micrograph of guinea pig pancreatic acinar cell showing compartments involved in nascent protein synthesis and storage. The vectorial features of this pathway from endoplasmic reticulum to secretory granule are evident. Nascent proteins are synthesized in about 5 minutes in the endoplasmic reticulum; then they move by transport vesicles to the Golgi complex, where they exit at about 20 minutes in condensing vacuoles (aka immature secretory granules), and mature into secretory granules (image from reference 4).

**Modifications and Processing of Nascent Proteins**

Nascent secretory proteins are subject to a variety of modifications; many of these occur within specific cellular organelles and are involved in forming the three-dimensional structure and covalent modifications necessary for proper protein function. Protein modifications often require the activity of accessory resident proteins that are concentrated in specific organelles.
Cleavage of the signal peptide

One of the first modifications of secretory proteins within the ER is the proteolytic removal of the signal peptide. The cleavage is mediated by a specific ER protease (signal peptidase) and occurs within the translocon after a large portion of the nascent protein has entered the ER cisterna. Removal of the signal peptide traps the nascent protein in the secretory pathway unless the protein misfolds.

Protein modifications and folding

Secretory proteins undergo posttranslational modifications within the ER that directs their folding into a tertiary structure that shields their hydrophobic residues in the interior of the molecule and forms their functional domains. Several major protein modifications, some of which are covalent, occur within the ER—including disulfide bond formation, glycosylation, and acetylation. Accessory ER resident proteins also direct the folding of nascent proteins.
**Disulfide bonds**

Disulfide bonds form by oxidative linkage of sulphydryl groups between two Cys residues. These bonds are necessary to form higher-order structures both within and between polypeptides and can occur either co- or posttranslationally. The ER redox environment is uniquely suited to promote disulfide bond formation; indeed, disulfide bonds are highly abundant among secretory proteins and rarely found in cytosolic proteins. It follows that changes in ER redox status affect protein folding and stability. For example, proteomic assessment of acinar ER proteins following ethanol feeding shows a shift toward increased oxidation, which suggests the likelihood of aberrant disulfide bonds—leading to protein misfolding and ER stress.8,9 That said, disulfide bonds can occur spontaneously under normal conditions, and so ER-resident protein disulfide isomerasers (PDIs) act to rearrange disulfide bonds and ensure their formation between the correct Cys residues. As such, PDIs are also considered chaperones (see below). One PDI family member specifically expressed in pancreatic acinar cells, termed PDIp, interacts with several digestive enzymes and proteolytic zymogens and prevents their aggregation.10–12

**Glycosylation**

Glycosylation describes the addition of carbohydrate chains called glycans to nascent proteins. Protein glycosylation in the ER is mediated by resident glycosyltransferases and glycosidases that generate a milieu of glycan moieties that can influence protein folding and function (reviewed in reference 13). One such modification is the addition of N-acetylgalactosamine (GlcNAc) to Asn residues, termed N-glycosylation. N-glycosylation of newly translated peptides is regulated by the oligosaccharyltransferase (OST) complex by recognition of an Asn-X-(Thr/Ser) consensus sequence and occurs cotranslationally via interactions between the OST and the translocon. Though N-glycosylation is understood to be critical for nascent protein folding, little is known about this particular modification in acinar cells. Studies in the early 1990s demonstrated the presence of N-glycosylation on human pancreatic elastase 1 (Ela1).14 It was later shown that N-glycosylation of bile salt-dependent lipase (BSDL) was essential for its expression, association with molecular chaperones, and secretion.15,16 More work has been done investigating the significance of O-glycosylation of acinar cell proteins, which occurs in the Golgi, including lysosome-associated membrane proteins and some digestive enzymes.

**Acetylation**

Studies over the last decade have identified a critical role for Nε-lysine acetylation and ER proteostasis—that is, the dynamic regulation of the biogenesis, folding, trafficking, and degradation of proteins within the ER. Posttranslational acetylation of proteins in the ER is regulated by the ER acetyl-CoA transporter, AT-1 (SLC33A1), and ER-resident acetyltransferases, ATase1 and ATase2 (NAT8B and NAT8, respectively), which utilize the luminal acetyl-CoA as acetylation substrate. In contrast to N-glycosylation, protein acetylation occurs only after successful protein folding and does not appear to involve a consensus sequence—though only outward-facing lysines are acetylated, suggesting a dependence on protein tertiary structure formation. Interestingly, the acetyltransferases interact with the OST complex to acetylate the targeted residues, suggesting a relationship between N-glycosylation and acetylation. The current view is that this acetylation identifies properly folded (i.e., glycosylated) proteins and promotes their entry into the secretory pathway, whereas improperly folded proteins cannot be acetylated appropriately and are thus subject to degradation. It has been recently shown that AT-1 expression increases at the onset of pancreatitis but falls as the disease progresses.17 Furthermore, studies involving mice expressing mutant AT-1S113R, a human mutation associated with hereditary spastic paraplegias, or pancreatic acinar-specific AT-1 deletion, show elevated ER stress, inflammation, and fibrosis consistent with a chronic pancreatitis-like phenotype, which unexpectedly includes enhanced trypsin activation.17 Prior analyses of the so-called “ER acetylome” in neuronal cells identified acetylated proteins that are of interest in pancreatic acinar physiology, including BiP (GRP78), LAMP2, and cathepsin D.18 Given the recently established role of AT-1 in pancreatic acinar homeostasis, investigating the acetylation state of acinar cell proteins will provide further insight into the functional significance of this modification on pancreatic outcomes.

**Molecular chaperones**

Although very small peptides can spontaneously fold unsustained, the folding of larger and more complex proteins in the ER is hindered by the available space and necessity of precise luminal conditions. ER-resident chaperones assist newly translated polypeptides by recognizing and interacting with key protein regions and shielding them from outside interference while the necessary modifications are made. Two main chaperone systems regulate this process in the ER: heat shock chaperones (e.g., BiP, aka GRP78, and GRP94), which interact with exposed hydrophobic regions; and lectins (e.g., calreticulin/calsemin), which interact with N-linked glycans. Evidence shows that these chaperones often cycle between association, dissociation, and reassociation with their targets until folding is complete. It follows that molecular chaperones are critical for pancreatic acinar cell function. In mice, GRP78+/−/− pancreas exhibits alterations in ER morphology, reduction in the expression...
of calreticulin and calnexin, and experiences increased disease severity in response to caerulein-induced pancreatitis. As previously mentioned, PDIs are also considered chaperones as they support protein folding by facilitating disulfide bond formation.

The unfolded protein response
To sustain high rates of ER protein synthesis and secretory trafficking efficiency, pancreatic acini rely on a robust ER stress response system to manage the accumulation of proteins in the ER lumen. Known as the unfolded protein response (UPR), this system is regulated by three ER transmembrane proteins that “sense” the load of unfolded proteins in the ER lumen: inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase R-like ER kinase. These sensors mediate signal transduction pathways that initiate cellular response mechanisms to alleviate the burden of ER stress (reviewed in references 21, 22).

The adaptive UPR
During the initial stages of ER stress, or when ER stress is mild, IRE1 dimerizes and auto-transphosphorylates, activating its RNase activity which processes the transcription factor X-box binding protein 1 (XBP1) into its spliced product (XBPs). Effects of IRE1 on other types of mRNA have also been reported. Additionally, ATF6 translocates to the Golgi using the COPII complex-coated vesicles and is cleaved by site 1 and 2 proteases to generate its active cytosolic form cATF6. Both XBP1s and cATF6 regulate the expression of genes encoding ER chaperones, ER biogenesis, and ER-associated degradation (ERAD); furthermore, cATF6 controls XBP1 expression.23,24,27-30 These actions serve to increase the ER folding capacity and secretary output to clear accumulated proteins and are collectively referred to as the adaptive UPR.

The pathological UPR
In the event the adaptive UPR cannot ameliorate existing ER stress, subsequent pathways with more profound consequences are activated. Similar to IRE1, PERK autophosphorylates in response to ER stress and activates its kinase activity, which in turn phosphorylates the translation initiation factor 2α (eIF2α), inhibiting global cap-dependent translation. This provides the ER a reprieve from translational protein input. Should these actions fail to alleviate ER stress, PERK activates the apoptotic protein CCAAT/enhancer binding protein homologous protein (CHOP) by increasing expression of activating transcription factor 4 (ATF4). This duality in PERK function is illustrated by studies showing the necessity of both PERK and ATF4 in normal pancreatic development and function, whereas loss of CHOP results in normal exocrine function and protects against the acceleration of pancreatitis. Sustained IRE1 signaling may also play a role in the pathological UPR by activating jun N-terminal kinase (JNK), which contributes to inflammation and apoptosis, though this pathway has not been fully studied in pancreatic acinar cells.

Regulated IRE1-dependent decay
Under conditions of sustained ER stress, the endoribonuclease activity of IRE1 may become broader, affecting the translation of mRNAs other than XBP1. Reports indicate a potential role for regulated IRE1-dependent decay (RIDD) in regulating insulin mRNA in pancreatic β cells under ER stress due to hyperglycemia. The physiological role of RIDD in exocrine pancreatic function and response to stress has not yet been explored.

Removal and degradation of accumulated proteins
ER-associated degradation (ERAD) is a cellular pathway that removes terminally misfolded proteins from the ER lumen or membrane and regulates their degradation. ERAD may occur by proteasomal (ERAD-I) or autophagic (ERAD-II) mechanisms. It is believed that ERAD-I mediates the disposal of monomeric proteins. The best characterized branch of ERAD-I is regulated by the adaptor protein suppressor/enhancer of Lin-12-like (Sel1L) and the dislocon channel HMG-coA reductase degradation protein (Hrd1) complex, which retrotranslocates misfolded ER proteins to the cytosol where they are subsequently ubiquitylated and targeted to the proteasome for degradation. Interestingly, inducible Sel1L knockout mice exhibit classic exocrine insufficiency as well as persistent ER stress/UPR and, curiously, significantly smaller zymogen granules. This study suggests a key role for Sel1L and ERAD-I in pancreatic acinar ER homeostasis.

ER homeostatic mechanisms utilizing autophagic/lysosomal pathways will be collectively referred to here as ERAD-II, including ER-phagy, reticulophagy, and/or

Pancreatic acinar cell protein synthesis, intracellular transport, and export
ER-quality control (ERQC) autophagy; whether these classifications represent similar or distinct processes is the subject of debate, which will not be dissected here. In contrast to ERAD-I, ERAD-II is proposed to degrade large protein aggregates and ER membrane rather than individual proteins (reviewed in reference 46). In mammals, autophagy-mediated ER degradation utilizes ER membrane-associated receptor molecules. To date, six receptors have been identified: RTN3L, FAM134B, SEC62, CCPG1, ATL3, and TEX264. Although the various stimuli and protein targets for these receptors have not been completely characterized, they all interact with the autophagy protein LC3 through LC3-interacting regions (LIRs), which mediates the selective removal of ER through the autophagy pathway. Cell-cycle progression gene 1 (CCPG1) was first identified as an ER-phagy cargo receptor in pancreatic acini that is induced by UPR signaling, and loss of CCPG1 leads to ER disordering and loss of polarity in acini.47–49

Initiation of ERAD-II may be regulated by acetylation. The autophagy regulatory protein ATG9 is the only membrane-associated autophagy protein that resides in the ER. Studies of AT-1 function identified acetylation sites on ATG9 on the luminal side that restricts its activity, and loss of AT-1 function spurs increased autophagy.37,50 These findings suggest that ATG9 may act as an ER lumen acetylation sensor wherein loss of acetyl-CoA availability, corresponding to accumulation of proteins, induces reticulophagy.

The extent to which ERAD-I and ERAD-II regulate the degradation of pancreatic acinar cell proteins under normal and disease states is unclear. Similarly, whether defects in either ERAD mechanism contribute to aberrant intracellular trypsin activation and/or pancreatitis pathology remains to be investigated.

Early Acinar Cell Secretory Protein Trafficking

Pancreatic acinar cell secretory proteins are translated into the highly expanded rough ER (Figures 1 and 2). Following folding and posttranslational modifications discussed above, soluble and membrane-associated secretory proteins adjacent to specialized ERES are packaged into coat protein complex II (COPII)-coated vesicles and directed to the ER-Golgi intermediate compartment (ERGIC). In the ERGIC, resident ER proteins are sorted back to the ER in COPII-coated vesicles, while secretory proteins are concentrated and delivered to the Golgi cisternae (cis-, medial-, and trans-Golgi), sometimes termed the Golgi stacks and/or Golgi ribbons (reviewed in 51–53). Proteins may undergo further posttranslational modifications during their sequential movement through the Golgi. Ultimately, secretory proteins are concentrated into condensing vacuoles within the trans-Golgi compartment where they bud off as precursors of secretary or zymogen granules (ZGs). It should be noted that condensing vacuoles (CVs) and immature secretory granules (ISGs) are often used interchangeably in acinar cell literature (Figures 2 and 3).

ER to Golgi trafficking

Anterograde transport of ER membrane and protein to the ERGIC begins with the formation of COPII vesicles at specialized ERES subdomains.52,54 Formation of the COPII coat is directed by the cytosolic proteins Sar1, Sec23, Sec24, Sec13, and Sec31.55 In brief, the GTPase Sar1 is GTP-loaded and activated by the guanine exchange factor Sec12 near the ERES, prompting the insertion of Sar1 into the ER membrane using an amphipathic helix. Sar1 membrane insertion facilitates the membrane deformation ultimately required for vesicle formation and budding. Membrane-associated Sar1 also binds to the heterodimer Sec23-Sec24. Sec 24 interacts with transmembrane ER-associated receptors to facilitate soluble cargo loading. Sequential formation of these complexes at the ERES promotes the addition of an outer layer composed of a heterotetramer of two Sec13 and two Sec31 subunits which self-assemble into cage-like structures morphologically similar but biophysically distinct to clathrin coats.56

In addition to the five core COPII regulatory proteins, a growing number of proteins that transiently interact during COPII coat formation and trafficking have been identified but will not be detailed here.53,55 Vesicle scission occurs through an unclear process, and the COPII vesicles—typically 60–80 nm in diameter—are directed to the ERGIC where they tether and undergo fusion via soluble n-ethylmaleimide sensitive receptor (SNARE) protein interactions.57

Although the acinar cell is known for its massive protein secretory capacity and many studies have been directed at understanding the mechanisms of COPII vesicle formation in other cells, few have addressed this pathway in pancreatic acinar cells. In 1999, Martínez-Menárguez et al. used immuno-electron microscopy on pancreas thin sections to demonstrate that the membrane-associated SNARE protein rBet1 is concentrated in COPII-coated vesicles; however, the soluble secretory proteins amylase and chymotrypsin were not, suggesting that soluble cargo loading is not receptor-mediated but occurs by nonselective transport.58 A later study in yeast followed the loading of soluble secretory proteins and COPII membrane proteins demonstrating receptor-mediated concentrative sorting of soluble proteins into COPII vesicles.59 Whether additional acinar secretory proteins other than amylase and chymotrypsin may be selectively loaded in COPII vesicles remains unknown. A more recent study demonstrated that pancreas-specific deficiency of the Sec23B isofrom, but not Sec23A, results in embryonic lethality due to acinar cell degeneration but, interestingly, did not disrupt the morphology of islets.60,61 Clearly, further investigation of COPII formation, loading,
and trafficking in normal acinar cells and the importance of this pathway during pathologic ER stress is warranted.

**ER-Golgi intermediate compartment**

The ERGIC, also known as the vesicular tubular cluster due to its morphology, acts as sorting compartment between the ER and Golgi apparatus (Figures 1 and 2). The most reliable marker of this compartment is ERGIC-53, a mannose-specific membrane lectin that functions as a cargo receptor for the transport of glycoproteins from the ER to the ERGIC. The ERGIC is formed and maintained by the continuous fusion of COPII vesicles arriving from the ER. Resident ER proteins are retrieved from the ERGIC back to the ER by their concentration and sorting into COPI-coated vesicles. In contrast to COPII coat formation, COPI coat proteins are composed of seven COP adapters that are assembled in response to activation of an ADP ribosylation factor (Arf1, Arf2, or Arf3) by GTP loading with guanine exchange factors. Activation triggers Arf myristoylation and membrane tethering identical to Arf function in clathrin coat formation (reviewed in reference 63). Cargo selection by COPI is directed by sorting signals present on cytoplasmic domains including di-lysine, KKxx, and KxKxx motifs present on many ER proteins. Likewise, the KDEL receptor, which functions to both retain ER resident proteins and recycle them from the ERGIC and cis-Golgi, interacts with KDEL-containing proteins, including ER chaperones, thereby concentrating them in COPI vesicles.57

Interestingly, although COPI buds are most numerous in the ERGIC, they are also found in cisternae at all levels of the Golgi stacks as well as a minor presence on ISGs, indicating that either COPI recycling takes place at all levels of the early secretory pathway or that COPI vesicles have additional functions in the Golgi (see below).58 Of note, an earlier study by Sesso et al. provided

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**Figure 3. Additional secretory pathways identified in acinar cells.** The classic regulated secretory pathway is represented by the formation of mature secretory granules from ISGs/CVs. Parallel secretory pathways are formed by the trafficking of membrane and secretory proteins through endosomal compartment intermediates prior to secretion, either directly from the early endosome in the minor-regulated pathway (MRP) or through the recycling endosome as in the constitutive-like pathway (CLP).
a three-dimensional reconstruction of the rough ER-Golgi interface in serial thin sections of rat pancreas. This was depicted as a series budding small vesicles that fused to form tubulovesicular elements that appeared to be interposed between the ER and cis-Golgi, although the nature of these tubulovesicles is uncertain, it seems plausible they represented what is now termed the ERGIC.65

Golgi complex
A number of theories have been postulated regarding Golgi formation, structural organization, and mechanisms of protein and membrane trafficking between the Golgi compartments.66–69 Golgi architecture in mammalian cells consists of a morphologically heterogeneous set of membrane-limited compartments with a characteristic stack-like appearance (Figure 1). Membrane and proteins generally flow or mature in an anterograde direction from cis- to trans-cisternae. The cis-cisternae are oriented toward the ER and are associated with small vesicle formation, whereas the trans-Golgi is oriented toward the apical plasma membrane and is associated with production of secretory granules. These regions are most commonly described as zones of the cis-, medial-, and trans-Golgi, with movement between compartments by vesicle fission and fusion and so-called noncompact zones that are interconnected by lateral tubules between cisternae.70,71 These interconnected regions appear as higher-order structures by light microscopy giving rise to the term “Golgi ribbon.” There are numerous small, coated vesicles oriented both close to and budding off from the rims of the Golgi cisternae, the majority identified as COPI-coated by immuno-electron microscopy. COPI coats are most abundant in the ERGIC and cis-Golgi, which decrease in number moving toward the trans-Golgi.70 Recent studies have described these as COPIb vesicles that mediate anterograde trafficking within the Golgi cisternae whereas the COPIa vesicles mediate retrograde recycling of ER proteins from the ERGIC and cis-Golgi, although the molecular determinants dictating COPIa versus COPIb coats are uncertain.57,68

The most distal trans-Golgi cisternae give rise to and are continuous with the trans-Golgi network (TGN), a series of branching tubules with many budding profiles and forming vesicles. The TGN is biochemically distinct from the rest of the Golgi complex, having a higher concentration of certain proteins like TGN-38 compared to the cis- and medial-Golgi. The TGN is highly dynamic in accordance with the rates of protein synthesis and secretory protein entry into the compartment. Multiple organelles have been shown to arise from the TGN including early, late, and recycling endosomes, as well as vesicles specifically destined for apical and basolateral plasma membrane or back to the ER.70 Unique to the TGN is large number of clathrin coats and associated clathrin-coated vesicles (Figure 3).

Clathrin coat formation at Golgi membrane is mediated by assembly of clathrin adaptor protein complexes AP1, AP3, and AP4; these multisubunit complexes associate with cytoplasmic domains of specific Golgi transmembrane proteins to nucleate clathrin coat assembly.72,73 In addition to heteromeric AP complexes, additional monomeric clathrin adaptors are present at the trans-Golgi including Golgiloczalized γ-ear-containing Arf-binding proteins (GGAs) and enthoprotin/epsinR56.74

Protein Modification in the Golgi Complex
The Golgi is a major site of protein modification including terminal glycosylation, proteolytic processing, and sulfation. The Golgi fulfills its multiple functions using several classes of processing enzymes. These are primarily membrane proteins and may be regulated by their distinct localizations within the Golgi complex. For example, galactosyltransferase is restricted to the two or three trans-most Golgi cisternae.75

O-glycosylation
Complex O-linked oligosaccharides are attached to an oxygen atom of serine or threonine by glycotransferases within the Golgi (as opposed to N-linked glycosylation in the ER). Lysosomal enzymes, including lysosome-associated membrane proteins 1 and 2 (LAMP1,2), are glycoproteins that are modified in this manner and are essential for the function and integrity of lysosomes.76 Furthermore, a study perturbing O-glycosylation in pancreas showed that a number of pancreatic digestive enzymes (e.g., bile salt-activated lipase, pancreatic triacylglycerol lipase, pancreatic alpha-amylase) are O-glycosylated, and that loss of O-glycosylation results in exocrine and endocrine insufficiency.77

Mannose-6-P modification
In the cis-Golgi, mannose-6-phosphate (M6P) residues are added to proteins to direct sorting into the endolysosomal pathway.75 These residues interact with the M6P receptors (MPRs) which are localized to the Golgi region of polarized cells, coated vesicles, endosomes, and lysosomes as identified by Brown and Farquhar in 1984.76 Although an early study found MPRs to be restricted mainly to the cis-Golgi stacks, later work in acinar cells demonstrated AP1-mediated clathrin-coated vesicle retrieval of MPRs from immature secretory granules (see below).78,79 Two distinct MPRs, a 46-kDa MRP46 (cation-dependent MPR) and a 300-kDa MPR300 (cation-independent MPR), have been identified. Although the majority of soluble acid hydrolases are modified with M6P residues allowing their recognition...
by MPRs, other soluble enzymes and nonenzymatic proteins are transported to lysosomes in a M6P-independent manner mediated by alternative receptors such as lysosomal integral membrane protein (LIMP-2) or sortilin.\(^86\) Sorting of cargo receptors and lysosomal transmembrane proteins requires sorting signals present in cytosolic domains which interact with components of clathrin coats or an adaptor protein complex. Additionally, phosphorylation and lipid modifications can further regulate signal recognition and trafficking.\(^86\)

**Trafficking from the Golgi Complex Outward**

**Condensing vacuoles and formation of zymogen granules**

The *trans*-Golgi is the site of condensing vacuole (or immature secretory granule, ISG) formation in pancreatic acinar cells. Early radiolabeling of acinar secretory proteins *in vivo* combined with electron microscopy radiographic analysis suggested the condensing vacuoles received secretory proteins directly transported on vesicular carriers from the so-called “ER transitional zone” that likely represented the ERES or ERGIC (Figure 1).\(^81\) Later, higher-resolution studies by Jameson and Palade in pancreatic slices from guinea pig stimulated with secretagogue revealed the presence of label in the Golgi stacks\(^82\)—although in his 1974 Nobel acceptance speech Palade still depicted the ER transitional compartment as a direct route to the condensing vacuoles.\(^7\) Though many studies have examined condensing vacuole formation, a comprehensive understanding of how secretory cargo are concentrated into condensing vacuoles, how enlarged vacuoles form, and the molecular mechanisms of condensing vacuole fission from the TGN remains uncertain but is likely to be driven at least in part by the acidification of the compartment.

The details of secretory granule formation in specialized secretory cells have received considerable attention over the last 30 years. Evidence derived from *in vitro* fusion assays in subcellular fractions of neuroendocrine cells supports that newly formed ISGs undergo homotypic fusion in a process mediated by syntaxin 6 and synaptotagmin IV.\(^83,84\) Most studies in acinar cells posit that ISGs formed in the TGN are the direct precursors of mature secretory granules. However, Hammel et al.\(^85\) using a detailed morphometric analysis, proposed that small Golgi-derived vacuoles fuse to form ISGs.\(^85\) They further propose that small granules undergo homotypic fusion to form larger granules, although the molecular and microscopic details of this theory are lacking. Ultimately, the immature granules further mature and become smaller by clathrin-coated vesicle-mediated removal of membrane and small amounts of digestive enzymes to the endosomal system resulting in concentration of the digestive enzyme content into a mature, electron-dense ZG.\(^79\) AP1-mediated clathrin coat formation directed by MPRs also removes some, but not all, lysosomal enzymes from ISGs.\(^79\)

Few studies have investigated the secretagogue regulation of post-Golgi ZG formation. Kostenko et al. demonstrated a role for the tyrosine kinase c-src in mediating Golgi morphology and secretory granule formation.\(^86\) Overexpression of c-src caused Golgi expansion whereas pharmacological inhibition reduced granule formation in cultured AR42J acinar cells and isolated acinar cells. These results provide the first known signaling pathway for acute Golgi-mediated ZG formation in response to secretory stimulation.

**Anterograde endosomal trafficking through the minor secretory compartment**

The great majority of digestive enzyme secretion is mediated by ZG exocytosis at the apical membrane (Figures 2 and 3). However, there are two additional and unique parallel secretory pathways identified in acinar cells from the pancreas and parotid glands known as the constitutive-like (CLP) and minor regulated (MRP) pathways as shown in Figure 3.\(^87–92\) Though these pathways only provide a small contribution to total protein secretion, they are likely important to acinar cell function. The CLP and MRP were identified by their rapid discharge (~2 hours) of newly synthesized secretory proteins in pulse-chase studies, whereas ZG proteins were secreted by ~10 hours under basal conditions.\(^85\) Secretion from the CLP and MRP is acutely inhibited by brefeldin A (BFA), an inhibitor of guanine nucleotide exchange factors for class 1 ADP-ribosylation factors that function in vesicle formation from *trans*-Golgi and endosomal compartments.\(^87\) The CLP and MRP were proposed to originate from vesicle fission at the TGN and ISGs and traffic through an endosomal intermediate, subsequently identified as the early endosome prior to secretion at the apical membrane.\(^93–96\) The MRP traffics from early or sorting endosomes directly to the apical membrane upon low-level secretagogue stimulation, whereas the CLP may subsequently enter an endosomal recycling compartment prior to exocytosis. More recent studies have identified that the endosomal, TGN peripheral membrane protein TPD52 and its associated proteins Rab5 and EEA1 play an important role in CLP trafficking in acinar cells.\(^96\) It was also demonstrated that ZGs containing the SNARE protein vesicle-associated membrane protein 8 (VAMP8) require an intact endosomal pathway expressing D52, Rab5, and EEA1 in order to mature and/or undergo exocytosis.\(^97\)

**Concentration of Nascent Proteins through the Secretory Pathway**

Concentration of acinar cell nascent secretory proteins occurs throughout the pathway but is not uniform.
Differences of over an order of magnitude have been observed for amylase, chymotrypsin, and procarboxypeptidase A, and occur at multiple compartments in the secretory pathway. The net effect of the enrichment mechanism is to concentrate soluble proteins such as amylase, trypsinogen, and chymotrypsinogen 10- to 20-fold between the ER lumen and the Golgi complex and even further enrichment as they move toward the ZG and may be as high as a hundred-fold over the ER lumen. Though the mechanisms of such concentration remain unclear and likely vary among compartments, the decreasing pH gradient within the Golgi complex (going from pH 7.0 to 6.0) may contribute to this effect. Selection could also occur through interactions between secretory protein moieties and receptors; for example, the putative Golgi receptor muclin may interact with sulfated, O-glycosylated zymogens as a means to concentrate them in budding compartments.

Secretory Granule Exocytosis
Regulated secretion arises from a storage pool that excludes newly synthesized secretory proteins that accumulate during the intestinal interdigestive phase. It is able to release 15–30 percent of the gland’s secretory protein content by classical regulated exocytosis and is maximally stimulated following ingestion of a meal when release of massive amounts of digestive enzymes and zymogens (considering zymogens as a category of digestive enzyme) are required at rates greater than can be attained by protein synthesis alone. ZG exocytosis does not appear to contribute to resting secretion.

The purpose of these distinct mechanisms of secretion is unknown, but they likely serve to both ensure that some digestive enzymes will be present in the small intestine at all times and provide a secretory response that is proportional to luminal nutrients. A minor regulated pathway could serve to increase enzyme secretion in response to smaller quantities of food than presented by a full meal. Finally, a novel role for the constitutive and minor regulated compartments is that they might deliver the t-SNARES necessary for ZG fusion with the apical membrane.

The release of ZG content into the lumen of the acinus requires fusion of the vesicular membrane with the apical plasma membrane. Four key steps are likely involved in this process: approximation of secretory granules in the apical region of the acinar cell, near the plasma membrane; tethering of secretory granules to the plasma membrane; docking and priming that involves SNARE proteins; and the final, calcium-dependent fusion event.

The initial step, movement of the secretory granule from its site of formation in the trans-Golgi to the apical region of the cell, likely requires active involvement of contractile elements, particularly actin and associated motor proteins, in movement of the ZG to its apical plasma membrane target. It should be noted that in the resting interphase between rounds of exocytosis, an apical actin terminal web presumably negatively regulates resting secretion in that an actin mesh is always found between ZGs and the apical plasma membrane. However, other actin roles have been proposed. For example, an additional actin pool, regulated by the actin-polymerizing formin mDia1, mediates the final movement of ZGs to the apical membrane. After reaching this most apical domain, the membrane of the ZG must recognize and become tethered to the apical plasma membrane prior to fusion. This implies that the actin meshwork beneath the apical membrane must be dissociated for close membrane apposition to occur, though additional steps, including overcoming fusion barriers, are needed before fusion can occur.

The role of microtubules in pancreatic acinar cell secretion is less clear. Microtubules are long, dynamic cytoskeletal structures composed of heterodimer polymers of α- and β-tubulin that undergo cycles of regulated polymerization and depolymerization. During polymerization, β-subunits of one tubulin heterodimer contact the α-subunits of the next dimer resulting in one end of the microfilament having the α-subunits exposed and the other end with β-subunits exposed; these ends are designated the minus (−) and plus (+) ends, respectively. Microtubule motor proteins, such as kinesins and dynein, associate with select intracellular cargo and utilize ATP to facilitate transport along the microtubules. Studies in the mid-1970s found that treatment of rodent pancreas both in vivo and ex vivo with the microtubule-destabilizing compounds vinblastine and colchicine significantly inhibited, but did not fully prevent, secretagogue-stimulated amylase secretion. Later work demonstrated that the minus ends of microtubules are anchored along the apical membrane and extend radially to the plus ends anchored in the basal cytoplasm. Both kinesin and dynein have been identified in acini, though their localization (on ZGs, Golgi, ER) and effects on secretion are debated. Interestingly, some studies show kinesin associating with ZGs in apical regions in response to secretory stimulation, an unexpected finding given that kinesins travel along microtubules in a minus-to-plus direction (i.e., anterograde in acini). Marlowe et al. speculate that organelles could contain both kinesin and dynein for bidirectional movement, and that kinesin could be involved in post-exocytosis membrane retrieval. Additional high-resolution studies are needed to characterize the population and polarization of microtubules at the acinar apex to understand how kinesin shapes the secretory response.

The SNARE hypothesis for membrane recognition and fusion, which appears to be a generalized mechanism for all cells examined, is particularly relevant for the pancreatic acinar cell where specific interactions between the ZG
membrane and the apical plasma membrane ensure that exocytosis of digestive enzymes and proenzymes occurs into the acinar lumen.116 Two populations of ZGs have been identified in pancreatic acinar cells, those enriched in the SNARE protein VAMP2 and those enriched in Endobrevin/VAMP8.97 According to the SNARE hypothesis for exocytosis, VAMPs on the granule membrane interact with a syntaxin isoform and a SNAP isoform on the plasma membrane. Acinar cell apical plasma membrane contains syntaxins 2 and 4 and, interestingly, both apical plasma membrane and ZGs express SNAP23 and SNAP29.117,118 Co-immunoprecipitation analysis revealed that VAMP2 ZGs interact with plasma membrane syntaxin 2 and SNAP23, whereas VAMP8 ZGs interact with apical membrane syntaxin 4 and SNAP23. ZG-ZG compound exocytosis that occurs during secretion was shown to involve VAMP8/syntaxin 3 and SNAP23 all of which are present on ZGs. A role for SNAP29 in acinar cell function has not been described.

To determine the roles of these VAMPs in the acinar cell, VAMP8 knockout mice together with adenoviral expression of tetanus toxin to selectively cleave VAMP2 were used to delineate the roles of VAMP2 versus VAMP8 ZG exocytosis during secretagogue stimulated secretion.97 Results supported that VAMP2 and VAMP8 are the primary ZG SNAREs mediating stimulated but not basal secretion. Moreover, measuring acinar cell secretion over time in a perfusion apparatus revealed that VAMP2 ZG mediated an early immediate phase of secretion that peaks at 2 minutes and begins to decline followed by VAMP8 ZG-mediated second prolonged phase of secretion that peaks at 5 minutes and decays over 20 minutes. A subsequent study identified that the VAMP8, but not the VAMP2-mediated pathway, was primarily inhibited during high cholecystokinin (CCK)-induced acute acinar pancreatitis and that knockout of VAMP8 prevented most of the high-dose CCK-mediated secretory inhibition and fully blocked the accumulation of active trypsin in acinar cells.119

ZGs have been shown to undergo exocytosis at the basolateral plasma membrane during acute pancreatitis.120 Evidence suggests that VAMP8 normally inhibits basolateral exocytosis; however, PKC-mediated phosphorylation of the SNARE accessory protein Munc18c allows VAMP8 to mediate basolateral exocytosis in a SNARE complex involving VAMP8/syntaxin 4/SNAP23.121 Presumably, release of digestive enzymes to the extracellular space enhances tissue damage thereby exacerbating disease progress.

A unique mechanism that has been observed in acinar cells is that of sequential compound exocytosis, whereby secretory granules first fuse with apical plasma membrane (primary granule fusion) and this is followed by sequential fusion of other (secondary and tertiary) granules onto these primary granules.118,122 Though the physiological meaning of compound exocytosis is not currently understood, it may facilitate more rapid and efficient release of ZG content since the acinar lumen is a small fraction of the total surface area limiting the number of granules that can fuse with the apical membrane at any one time. What triggers sequential granule fusion following primary granule fusion at the apical plasma membrane is unclear but may involve changes in pH, changes in phospholipid content, acquisition of different SNARE proteins, and so on, and needs further research.

References
41. Suyama K, Ohmuraya M, Hirota M, Ozaki N, Ida S, Endo M, Araki K, Gotoh T, Baba H, Yamamura K-I. C/EBP homologous protein is crucial for the acceleration of
49. Lahiri V, Klionsky DJ. CCPG1 is a noncanonical autophagy cargo receptor essential for reticulophagy and pancreatic ER proteostasis. Autophagy 2018; 14:1107–1109.
52. Kurokawa N, Nakano A. The ER exit sites are specialized ER zones for the transport of cargo proteins from the ER to the Golgi apparatus. J Biochem 2019; 159:915–921.


Protein synthesis plays a central role in provision of pancreatic digestive enzymes and the maintenance of the pancreas. Both the mRNA profile and relative content of newly synthesized proteins are dominated by digestive enzymes. In the mature pancreas, about 90 percent of protein synthesis has been estimated to be devoted to a mixture of about 20 digestive enzymes. Whether the acinar cell can regulate digestive enzyme synthesis independent of the synthesis of cellular structural proteins is unclear. Pancreatic protein synthesis is regulated to match digestive enzyme synthesis to dietary need. Although the identity of individual synthesized proteins and long-term regulation is primarily determined by transcriptional regulation, the short-term extent, or rate of protein synthesis, is regulated at the translational level, because it needs to be immediate, flexible, and reversible.

In general, the gastrointestinal tract, including the exocrine pancreas, atrophies in the absence of food, and protein synthesis that occurs in response to food intake is required to maintain normal function. Individual dietary components, like protein and amino acids, regulate pancreatic protein synthesis in mice and rats. In humans, feeding increases both the rate of secretion and synthesis of digestive enzymes, although the rate of zymogen turnover remains fairly constant during feeding and fasting. Pancreatic protein synthesis is also regulated by hormones such as cholecystokinin (CCK) and insulin, as well as by neural stimulation, all of which are influenced by food intake (Figure 1).

This review highlights how dietary elements and hormones affect intracellular effectors, such as mTORC1 and intracellular calcium concentration, to regulate certain steps of the pancreatic protein translational machinery, at the initiation and elongation levels. When stimulatory conditions are present, pancreatic protein synthesis is mainly stimulated through the mTORC1 pathway, but when there is an overstimulation of the pancreas, or a dietary imbalance, there is an increase on intracellular calcium concentrations, and cell stress, that trigger a signal which inhibits protein synthesis. These mechanisms coincide with stress in the endoplasmic reticulum (ER stress), resulting in unfolded/misfolded protein products retained in the ER and the stimulation of several cell survival and cell death mechanisms.

Protein Synthesis Associated Mechanisms and Their Regulation

The acinar cell of the exocrine pancreas has the greatest rate of protein synthesis of any mammalian organ, and it has long been used as a cell model to study the protein synthesis mechanisms in mammalian cells. Cooley et al. nicely reviewed the protein synthesis pathways to secretion in pancreatic acinar cells, and we refer the reader to their publication on the Pancreapedia, and in Chapter 6 of this book, for more detailed information.

Briefly, in eukaryotic cells, the ribosomes—composed of two subunits: large (60S) and small (40S)—are the central elements of the protein synthesis machinery and translate the sequence of an mRNA into the amino acid sequence of a protein. To initiate the process, the two ribosomal subunits are recruited together, with the mRNA and tRNA, by different proteins and eukaryotic initiation factors (eIFs) on the cytosolic site of the ER membrane and start the addition of the first amino acid to the peptide chain. This complex continues the addition of amino acids to form a polypeptide, by a process called elongation, assisted by eukaryotic elongation factors (eEFs) on the cytosolic site of the ER membrane and start the addition of the first amino acid to the peptide chain. This complex continues the addition of amino acids to form a polypeptide, by a process called elongation, assisted by eukaryotic elongation factors, and terminate the process by the help of termination factors (eTFs). The newly formed polypeptide chains will be inserted into the ER lumen through channels formed by proteins called translocons and are subject to several modifications. These include chaperoning and folding mechanisms that will yield mature proteins that are ready to be directed to their final destination.

Pancreatic acinar cells devote most of their protein synthesis capacity to synthesize, and secrete, digestive enzymes. These mechanisms are directed to match the need for digesting the amount and quality of the different dietary elements. Meal-to-meal regulation needs to be
immediate, reversible, and flexible, and this is achieved by posttranscriptional processes directed at the regulation of the mRNA translation into protein.

The ER is an exceptionally prominent organelle in the acinar cell and is a major site of protein synthesis and transport, protein folding, lipid and steroid synthesis, carbohydrate metabolism, and calcium storage.

**mRNA translation**

Translation of mRNA into protein can be divided into three phases: initiation, elongation, and termination. During initiation (Figure 2), methionyl-tRNA initiator tRNA and several initiation factors associate with the 40S ribosomal subunit to form the 43S preinitiation complex. This complex binds to mRNA and migrates to the correct AUG initiation codon followed by the attachment of the 60S ribosomal subunit. Key regulated initiation factors in this process are the guanine nucleotide exchange factor eIF2B which activates eIF2 and eIF4E which recognizes the 5’(m7G)-cap of the mRNA. eIF4E is present in cells largely bound to its binding protein (4E-BP1) and is released when 4E-BP1 is phosphorylated on multiple sites. eIF4E interacts with the scaffolding protein eIF4G and eIF4A to form the eIF4F complex. The binding of eIF4F to an m7G cap commits the translational apparatus to the translation of the mRNA, and the ribosome will seek the start codon to start translation. The activation of S6 kinase and the phosphorylation of the ribosomal protein S6 enhance the translation of a specific set of mRNAs. In the elongation process, amino acids from aminoacyl-tRNAs are added to the growing peptide in the order dictated by the mRNA bound to the ribosome (Figure 3).

In the termination phase, the completed protein is released from the ribosome and translocated into the ER lumen to continue its maturation steps, involving many chaperones and folding processes.

**Posttranslational steps and associated processes**

As mentioned above, a protein destined for secretion must undergo proper folding and modifications, with the
During initiation, the initiator Met-tRNAi binds to a free 40S ribosomal subunit in a reaction requiring GTP bound to eIF2 to form the 43S preinitiation complex. In the next step, the eukaryotic initiation factor 4E (eIF4E) recognizes the capped end of the mRNA and interacts with the scaffolding protein eIF4G to form the eIF4F complex. After this, the 43S preinitiation complex will join the eIF4F complex and the ribosome will seek the start codon to start the process. A GTPase-activating protein promotes the hydrolysis of the GTP bound to eIF2 and releases eIF2-GDP. Regeneration of active eIF2-GTP is mediated by the guanine nucleotide-exchange factor eIF2B. The dissociation of the initiation factors allows the addition of the 60S subunit of the ribosome to form the 80S initiation complex, which is competent to enter elongation.

The first step of elongation is the binding of an aminoacyl-tRNA to a vacant ribosomal A-site base pairing with the mRNA on the A-site. This process requires elongation factor 1A. The second step is peptidyl transference; the carboxyl end of the polypeptide chain uncouples from the tRNA molecule in the P-site and joins the amino acid linked to the tRNA molecule in the A-site by a peptide bond. The third step, translocation, is catalyzed by elongation factor 2 (eEF2) and involves the translocation of the peptidyl-tRNA in the A-site to the P-site as the ribosome moves exactly three nucleotides along the mRNA molecule. The elongation process will repeat this cycle of amino acid addition to elongate protein polypeptides.
aid of chaperones and folding enzymes such as protein disulfide isomerase (PDI) and the immunoglobulin heavy-chain binding protein (BiP). These interactions occur after nascent protein synthesis and translocation into the ER lumen.\(^1\) Despite all these specific and sophisticated controlled mechanisms, a fraction of proteins does not achieve native/mature and functional form and are either misfolded or aggregated.\(^17\) At this point, these proteins can (1) remain in the ER or (2) enter the ER-associated degradation (ERAD) pathway mediated by the proteasome. These steps ensure that misfolded proteins do not inadvertently enter the secretory pathway.\(^18\) Retention of misfolded proteins in the ER can induce ER stress\(^19,20\) and a coordinated adaptive program called the Unfolded Protein Response (UPR).\(^21,22\) The UPR activates specific mechanisms directed to retain balance and proper function of the ER and the cell by (1) inhibiting protein synthesis,\(^23\) (2) upregulating protein folding by enhancing translation of ER chaperone and folding enzymes, and (3) activating degradation pathways associated with the ER—the ERAD.\(^24\) If the balance is not restored, it can lead to cell death or apoptosis,\(^25\) thus, achieving normal function is critical for cell’s survival.

Three ER-resident transmembrane proteins have been identified as proximal sensors of ER stress: the kinase and endoribonuclease inositol-requiring element 1 (IRE1),\(^26\) the PKR-like ER kinase (PERK)\(^27\) that phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF2α) on its Ser51 residue and inhibits translation initiation,\(^27\) and the basic leucine-zipper-activating transcription factor 6 (ATF6).\(^28\) Phosphorylation of eIF2α by PERK inhibits general protein translation but allows preferential translation of mRNAs encoding several short upstream open reading frames like the mRNA for the activating transcription factor 4 (ATF4).\(^29\) IRE1α dimerization followed by autophosphorylation triggers its mRNase activity, which processes the mRNA encoding unspliced X box-binding protein 1 (XBP1u) to produce an active transcription factor, spliced (XBP1s), that controls the transcription of genes encoding proteins involved in protein folding.\(^30\) The activation of the UPR may lead either to cell survival, by triggering the synthesis of ER chaperone proteins such as BiP and protein disulfide isomerase (PDI), along with a decrease in general protein translation, or to cell demise. The latter, occurs through the activation of programmed cell death signals.\(^21,30\)

There are several connections to activation of ER stress response pathways and pathological human conditions.\(^31\) A malfunction of the ER stress response caused by aging, genetic mutations, or environmental factors can result in various diseases such as diabetes, inflammation, cancer, pancreatitis (shown below), nonalcoholic fatty liver disease, and neurodegenerative disorders including Alzheimer’s disease, Parkinson’s disease, and bipolar disorder.\(^32–34\) How ER stress response pathways play a role in these pathologies is an active area of research, and various components of the stress response pathways are being investigated as potential therapeutic targets.\(^31,35\)

**Main intracellular regulators**

**Intracellular calcium concentration**

Calcium is a widespread signaling molecule that can affect different processes, including localization, function, and association of proteins, either with other proteins, organelles, or nucleic acids. Ca\(^{2+}\), in addition to its role as an intracellular mediator of cell-surface humoral interactions, may function prominently in the regulation of posttranscriptional protein synthesis in a variety of eukaryotic cell types.\(^11,36\) Early studies hypothesized that the rate of protein synthesis could be modulated in intact cells by varying the concentration and subcellular distribution of intracellular calcium, and it was thought that it was controlled by free cytosolic calcium rather than the sequestered cation. However, Brostrom and Brostrom (1990)\(^37\) proposed that maintenance of optimal rates of protein synthesis depends on the amount of calcium sequestered in the endoplasmic reticulum rather than free cytosolic calcium, and several other studies in different cell types confirmed this hypothesis.\(^38–40\)

Maintenance of free versus bound Ca\(^{2+}\) balance in all cellular compartments is critical for many cellular functions. This balance can be achieved, in part, thanks to calcium-binding proteins located in the cytoplasm or in specific organelles that retain or release Ca\(^{2+}\) as needed. Some of these proteins (e.g., calnexin, calreticulin BiP, PDI) are located in the ER and, in addition to binding to calcium, function as chaperones and help with protein folding.\(^41\) Integration of Ca\(^{2+}\) signaling in the lumen of the cellular reticular network including the ER, the mitochondria, the nucleus, and the cytoplasm provides integrated mechanisms for responding to cellular stresses by activation of appropriate coping responses.

In response to signal transduction-generated inositol trisphosphate, calcium is released to the cytoplasm as part of the signal transduction cascade.\(^11,42\) Sequestered calcium can also be released experimentally by treating cells with calcium ionophores (A23187) or inhibitors of the microsomal calcium-dependent ATPase such as thapsigargin (Tg). All of these treatments disrupt protein folding in the ER and inhibit translation initiation, through an ER stress mechanism that involves phosphorylation of eIF2α by PKR and the inhibition of eIF2B activity.\(^21,37,43,44\) It has also been hypothesized that perturbation of the translocon, rather than suppression of protein processing, initiates the signal emanating from the ER culminating in eIF2α phosphorylation and translational repression. Therefore, sequestered Ca\(^{2+}\) from the ER can be seen as moderating the rate of
mRNA translation in many cell types, including pancreatic acinar cells.

The importance of maintaining Ca\textsuperscript{2+} homeostasis and appropriate adaptation to ER stress is underlined by the accumulating evidence that constant disturbing of Ca\textsuperscript{2+} homeostasis and chronic ER stress could lead to neurodegenerative disorders,\textsuperscript{45} diabetes,\textsuperscript{46,47} cardiac hypertrophy,\textsuperscript{48} or cancer.\textsuperscript{49}

The \textit{mTORC1} pathway

The \textit{mTORC1} pathway is essential for cells to maintain homeostasis, providing tight control between the synthesis and degradation of cellular components, and it is the allosteric target of the drug rapamycin that has clinical uses in organ transplantation, cardiology, and oncology.\textsuperscript{50} \textit{mTORC1} connects environmental cues (nutrient and growth factor availability as well as stress) to metabolic processes in order to preserve cellular homeostasis. Under nutrient-rich conditions, \textit{mTORC1} promotes cell growth by stimulating biosynthetic pathways, including synthesis of proteins, lipids, and nucleotides, and by inhibiting cellular catabolism through repression of the autophagic pathway.\textsuperscript{50} \textit{mTOR} is an evolutionary conserved serine/threonine protein kinase that belongs to the PI3K-related kinase (PI3K) superfamily. This atypical kinase is the basis of two structurally and functionally different complexes termed \textit{mTORC1} (mTOR complex 1) and \textit{mTORC2}.\textsuperscript{51} In addition to the \textit{mTOR} catalytic subunit, \textit{mTORC1} consists of regulatory-associated protein of mammalian target of rapamycin (Raptor) (a scaffold protein that is required for the correct subcellular localization of \textit{mTORC1}),\textsuperscript{52,53} mammalian lethal with Sec13 protein 8 (mLST8; also known as GßL) (which associates with the catalytic domain of \textit{mTOR} and stabilizes the kinase activation loop),\textsuperscript{52–54} and the two inhibitory subunits: proline-rich Akt substrate of 40 kDa (PRAS40)\textsuperscript{55} and DEPT domain containing \textit{mTOR}-interacting protein (DEPTOR).\textsuperscript{56} Both, DEPTOR and PRAS40 are inhibitory proteins; phosphorylation blocks this inhibition. PRAS40 represents an essential component for insulin activation of \textit{mTORC1}. Raptor is essential to \textit{mTORC1} function and its genetic deletion leads to loss of activity.\textsuperscript{1} \textit{mTORC2} contains, among other components, \textit{mTOR}, \textit{mLST8}, DEPTOR, the regulatory subunits mSin1, Rictor (rapamycin-insensitive companion of \textit{mTOR}),\textsuperscript{57} and regulates cell survival and proliferation primarily by phosphorylating several members of the AGC (PKA/PKG/PKC) family of protein kinases.\textsuperscript{58,59}

Several proteins have been identified as substrates for phosphorylation by \textit{mTORC1}. The first ones were proteins implicated in the control of mRNA translation, and this process remains one whose control by \textit{mTORC1} is best understood.\textsuperscript{60} \textit{mTORC1} functions to activate several steps in mRNA translation (phosphorylates S6 kinases, the inhibitory eIF4E-binding proteins (4E-BPs), and the eIF4G). Its signaling requires amino acids (the precursors for protein synthesis) to activate these translation steps. Leucine is the most effective single amino acid that can stimulate \textit{mTORC1} signaling.\textsuperscript{61} Finally, it has also been described that \textit{mTORC1} positively regulates translation elongation\textsuperscript{60} and the protein degradation process through the proteasome, to increase the intracellular pool of amino acids, which will influence the rate of new protein synthesis\textsuperscript{61} (Figure 4).

Deregulation of \textit{mTORC1} signaling increases the risk for metabolic diseases, including type 2 diabetes,\textsuperscript{62} cancer,\textsuperscript{63} and epilepsy.\textsuperscript{58} In the exocrine pancreas, stimulation of the \textit{mTORC1} pathway has been shown to be activated by several hormones and nutrients and leads the stimulation of acinar cell protein synthesis/digestive enzymes\textsuperscript{53} (Figure 4). \textit{mTORC1} activation and signaling in pancreas is usually demonstrated by phosphorylation of downstream mediators, ribosomal protein S6 and 4E-BP1.

Experimental Analysis of Pancreatic Protein Synthesis

Pancreatic protein synthesis has been traditionally measured by administering isotopically labeled amino acids with subsequent measurement of the incorporation of label into newly synthesized protein. For animal studies, this is usually a radioactive isotope, while human studies have most often used stable isotopes.\textsuperscript{8} Several animal studies have measured pancreatic protein synthesis after diet-induced stimulation,\textsuperscript{64,65} hormonal stimulation (insulin, CCK),\textsuperscript{66–74} or after acute pancreatitis.\textsuperscript{75–80} Among these studies, there are some discrepancies on the outcome of the results that can be due to the different ways of administering the labeled amino acid as a tracer and because some were lacking an accurate assessment of the specific radioactivity of the precursor amino acid at the site of protein synthesis.

The primary assumption was that the experimental treatment does not alter the relationship between the labeling of the sampled pool and that of the aminoacyl-tRNA, the direct precursor of protein synthesis, on \textit{in vivo} experiments, specially. For those studies, the readily accessible compartment pools, such as the intracellular free amino acids or plasma pools, to estimate precursor labeling were used. However, experimental conditions have the potential to alter precursor enrichment either by affecting the amount of labeled amino acid entering the cell or by affecting the contribution of unlabeled amino acids derived from protein degradation to the charging of aminoacyl-tRNA. In these cases, the problem of accurately determining precursor enrichment can be minimized by the \textit{flooding dose} technique, originally described by Garlick et al.\textsuperscript{1} and validated by Sweiry et al. in rat pancreas.\textsuperscript{81}
With the flooding dose technique, the labeled amino acid is injected, not as a tracer but contained in a large (i.e., much larger than the endogenous free amino acid pool) bolus of unlabeled amino acid, making the specific activities in all free amino acid compartments more alike than if the labeled compound is given as a tracer dose. Thus the labeling of aminoacyl-tRNA is less likely to be affected by experimental manipulations. In addition, the large amount of amino acid injected ensures that the specific activity in the free pools remains almost constant for a period of time after injection. Although various amino acids have been used, L-[\textsuperscript{3}H]-phenylalanine was chosen as a radioactive tracer for experiments in the pancreas, because phenylalanine transport across the basolateral membrane of the pancreatic acinar cells is not rate-limiting for protein synthesis, and because [\textsuperscript{3}H] can be easily quantitated by liquid scintillation counting (LSC). The flooding dose technique is advantageous not only because it is reliable but also because it can be used in unrestrained and unanesthetized animals and has been well validated in muscle, liver, and pancreas.

For in vitro studies, using isolated acini or pancreatic lobules, protein synthesis is measured by the incorporation of radioactively labeled amino acids, such as [\textsuperscript{35}S]-Methionine or [\textsuperscript{3}H]-Leucine, where the incorporation of these amino acids is linear during the experimental time because their intracellular pool is large and constant, due to the flooding of the extracellular space with...
an excess of amino acid, and therefore, there is no change on their specific activities.82

Stimulation of Pancreatic Protein Synthesis

Diet

Long-term regulation

Exocrine pancreatic adaptation to dietary changes has been observed in a variety of species.87,88 The content and secretion of the digestive enzymes, proteases, amylase, and lipases change in proportion to the dietary content of their respective substrates, protein, carbohydrate, and fat. Changes in content of specific enzymes take place over 5–7 days. Similar changes occur in the synthesis of specific digestive enzymes measured by the incorporation of radioactive amino acids.87,88 Changes in individual digestive enzymes occur at both the transcriptional and translational levels but the major effect results from changes in specific mRNA levels. Various hormones mediate many of these effects and in most cases their release is increased by the nutrients whose digestion they regulate.

Proteases: A high-protein diet (60–80% casein) increases the content of multiple proteases and the mRNA levels of trypsinogen, chymotrypsinogen, and proelastase in rodents.87,92 There are, however, differential effects on different isoforms of enzymes such as trypsinogen, and this increase is not mimicked by feeding the individual amino acids.93 This is consistent with a potential effect of dietary protein (but not amino acids) on CCK release and increasing its plasma levels. Feeding soybean trypsin inhibitor (SBTI), an indirect stimulant of endogenous CCK release, or infusing CCK, increased trypsinogen I and chymotrypsinogen B mRNA in rats.94 Other studies using isolated pancreatic lobules showed that synthesis of proteases following infusion of the CCK analog caerulein in vivo was greatly increased, compared to a small increase in translatable mRNA, suggesting a posttranscriptional regulation of their synthesis.93,96

Lipases: The content and synthesis of pancreatic triglyceride lipase increases in response to a high-fat diet (around 40–70% of calories as triglycerides).87 and this is accompanied by an increase in its mRNA.87,98 Secretin has been proposed as the mediator of the effect of dietary lipids.99 because fatty acids can stimulate secretin release, and infusion of secretin to rats, in vivo, induced an increase in the relative synthesis of pancreatic lipase.87

Amylase: The amount of carbohydrate in the diet has been shown to have significant effects on both pancreatic amylase content and amylase mRNA.87,88 The effects of carbohydrate are believed to be primarily mediated by insulin. In early studies with diabetic animals, amylase content, synthesis, and mRNA levels fall dramatically, while lipase increases moderately.88,100 Insulin administration restores the amylase synthesis, content, and mRNA levels in diabetic rats. However, insulin administration to normal rats either decreases or does not change pancreatic amylase levels. A more direct role for glucose on amylase synthesis, in addition to its indirect effects through insulin release, has been suggested.101 In fact, a dietary response sequence in the promoter of the amylase Amy2.2 gene has been identified that mediates dietary adaptation and the effect of insulin.102 The role of insulin in the regulation of pancreatic protein and digestive enzymes synthesis will be discussed in more detail later, in this review.

Short-term regulation

Meal stimulation: Only a few studies have evaluated the immediate regulation of the pancreatic translational synthetic machinery after food intake. Some early studies showed that fasting reduces total protein synthesis in the pancreas, and refeeding stimulates it.1,65,103 In humans, feeding increases both, the rate of secretion and synthesis of digestive enzymes, although the rate of turnover of zymogens remains fairly constant during feeding and fasting.8

In mice, acute food intake stimulates pancreatic protein synthesis (Figure 5A) and translational effectors, without increasing digestive enzyme mRNA levels (Figure 5B).2 These results indicate that acute feeding exerts a direct regulation of the mRNA translational machinery and a cellular reserve of untranslabeled mRNA, without changes in transcription.

The stimulation of total pancreatic protein synthesis in mice and rats is associated with the stimulation of the protein kinase B (PKB/Akt)/mTORC1 pathway and the phosphorylation of 4E-BP1 and ribosomal protein S6, downstream of mTORC1, as well as the formation of the eIF4F complex (Figure 6).2

Meal/food intake stimulation of pancreatic digestive enzymes triggers a series of stimulatory mechanism through dietary elements, hormones, and neural interactions (Figure 1) that are not completely understood, but this review summarizes what it is known about the most important ones.

Protein and amino acid stimulation: Protein and amino acids104 are essential components of both human and animal nutrition and are involved in the maintenance of general health and well-being.105 Amino acids are the building blocks of protein, cell structures, and tissues and can also act as regulators of protein metabolism and many physiological processes.106 A long-term deficiency of the essential AAs can lead to protein-energy malnutrition, manifested in humans as marasmus, or kwashiorkor.107 The therapeutic effects of amino acid when administered to patients, specially to infants, for growth as well as to maintain metabolism, have also been shown.108 AA supplementation specially benefits patients with chronic pancreatitis
Figure 5. Acute feeding in mice stimulates pancreatic protein synthesis without an increase in the mRNA for the digestive enzymes. (A) Pancreatic protein synthesis analyzed by the flooding dose technique, measuring Phe incorporation into pancreatic protein. Groups: Fed refers to animals fed ad libitum, with pancreas removed at 9–10 AM. Fasted refers to animals fasted for 18 hours, and re-fed refers to animals fasted for 18 hours and re-fed for 1, 2, 3, or 6 hours. Values are expressed as nanomoles of incorporated phenylalanine per milligram of protein per 10 minutes and are the means ± SE. *P < 0.05 versus fed group; #P < 0.05 versus fasted group. (B) Relative mRNA quantities of digestive enzymes and housekeeping genes in pancreas of animals fed ad libitum, fasted for 18 hours and re-fed for 2 hours. Values are the means ± SE. *P < 0.05 versus fed group, †P < 0.05 versus fasted group (from reference 2).

Figure 6. Effects of fasting and feeding on the eIF4F complex formation. eIF4F complex formation is measured as eIF4E and eIF4G coimmunoprecipitation. Data for coimmunoprecipitated eIF4E and eIF4G are expressed as percentage of fed group levels. Representative blots for eIF4G associated with eIF4E and total eIF4E are shown in the insets. *P < 0.05 versus fed group, #P < 0.05 versus fasted group (from reference 2).
and pancreatic cancer because they suffer from malnutrition.\textsuperscript{109} The branched-chain amino acids (BCAAs), leucine, isoleucine, and valine, in particular, are considered biological regulators,\textsuperscript{110} in part, because they stimulate insulin release, play an important role in energy homeostasis,\textsuperscript{111} and are involved in the stimulation of cell proliferation in certain types of cancer.\textsuperscript{112} On the other hand, it has also been shown that the deficiency of BCAAs in the diet improves metabolic health in mice\textsuperscript{113} and humans.\textsuperscript{114}

The exocrine pancreas synthesizes and secretes between 6 and 20 grams of digestive enzymes per day in humans\textsuperscript{115,116}, it is very vulnerable to protein deficiency because it requires optimal nutrition for enzyme synthesis.\textsuperscript{116} The effects caused by dietary protein deficiency in the pancreas were shown by Crozier et al.,\textsuperscript{117} in a study done in mice that mimicked the kwashiorkor syndrome. Mice were fed a protein-free diet for several days, resulting in pancreatic atrophy and the involvement of the mTORC1 pathway.

A high-protein diet\textsuperscript{6,118} and dietary amino acids\textsuperscript{7} have been shown to stimulate pancreatic protein synthesis at the translation initiation level in mice and rats and, at the same time, also stimulate pancreatic growth, because of their long-term treatment. Short-term effects of protein and AAs seem mainly to target the regulation of the protein synthetic machinery of pancreatic acinar cells through mTORC1 activation. BCAAs, especially leucine, can stimulate mTORC1 and the pancreatic protein synthesis machinery of rats, independently of CCK and insulin.\textsuperscript{5} Other studies also have shown the role of leucine in the exocrine pancreas of rats,\textsuperscript{7} pigs,\textsuperscript{119} and ruminants.\textsuperscript{120}

A short-term study in mice demonstrated that diets lacking protein or amino acids inhibited pancreatic protein synthesis (measured by the flooding dose technique) (Figure 7) and the amount of ribosomes engaged in translation on a given moment, by polysomal fractionation (Figure 8).\textsuperscript{4} Polysomal profiling analysis performed on pancreatic homogenates showed a significant increase in the polysomal fraction (i.e., more actively translated mRNAs into protein) in the control refed group (Figure 8B), compared to the control fasted group (Figure 8A). This was reflected in the analysis of the area under the curve of the polysomal fractions (Figure 8A and B). The groups lacking protein or leucine in the diet showed a very similar polysomal profile with a large reduction of the polysomal fraction, compared to the refed and fasted control groups (Figure 8C and D). The analysis of the area under the curve of these groups confirmed this reduction. At the same time, the analysis of the subpolysomal fraction indicated that refeeding with a 20 percent protein diet reduced the amount of free ribosomal subunits 40S and 60S, consistent with the increase in the 80S ribosomal unit and the polysomal fraction (Figure 8B). The lack of protein or leucine in the diet increased the subpolysomal peaks (area under the curve for both groups), indicating that there were more ribosomal subunits disengaged for translation (Figure 8C and D). These results confirm that total pancreatic protein synthesis was inhibited in the diets lacking protein or leucine, with a strong reduction of the mRNAs attached to the ribosomes for their translation into protein.

This inhibition was accomplished through different intracellular mechanisms: the protein-free diet inhibited pancreatic protein synthesis through a partial inhibition of the mTORC1 pathway, and of the elf2B activity; the leucine-free diet inhibition was caused by the inhibition of the elf2B and activation of the stress signals leading to the general control nonderepressible 2 (GCN2) kinase activation and elf2B phosphorylation, induced by the amino acid imbalance in the system and in the pancreas.\textsuperscript{4} This study shows that dietary amino acids are important regulators of postprandial digestive enzyme synthesis, and their deficiency could induce pancreatic insufficiency and malnutrition.

Amino acids regulate mRNA translation in the pancreas through multiple mechanisms. One mechanism involves activation of mTOR that controls several downstream effectors, including RNA polymerase I, S6K1, 4E-BP1, and the elongation factor 2 kinase (eEF2K) (inactive when phosphorylated). Amino acids, then, stimulate dephosphorylation (activation) of eEF2 that may also prevent activation of GCN2 by enhancing the removal of deacylated tRNA from the P-site on the ribosome (Figure 3)—a potential activator of GCN2 or elf2AK4 (elf2α kinase 4). GCN2 is induced during amino acid deprivation by a mechanism that involves uncharged tRNA binding to a regulatory region homologous with HisRS (histidyl-tRNA synthetase) enzymes.\textsuperscript{121,122} Phosphorylation of elf2 impedes recycling of elf2 to its active GTP-bound form (Figure 2), and the reduction in the levels of elf2–GTP reduces global translation, allowing cells to conserve resources and to initiate

![Figure 7](image-url)
a reconfiguration of gene expression to effectively manage stress conditions. A study using GCN2-deficient mice indicated an important effect of leucine deficiency in the translational machinery of the liver. Preventing activation of GCN2, amino acids preserve eIF2B activity, which promotes translation of all mRNAs, that is, global protein synthesis is enhanced.

BCAAs, specifically leucine, have been shown to stimulate pancreatic protein synthesis in rats, dairy goats, and calves. Our study demonstrated that BCAAs stimulate the activation of mTORC1, analyzed by the phosphorylation of 4EBP1 and S6K (Figure 9A and B) and the formation of the eIF4F complex, independently of the hormones cholecystokinin (CCK) (Figure 9C) and insulin.

Thus, dietary amino acids, as well as a mixed meal can stimulate the synthesis of digestive enzymes through the translational machinery by activating the mTORC1 pathway (Figures 5–9).

Cholecystokinin (CCK)

Regulation of translation initiation
As mentioned above, feeding stimulates pancreatic digestive enzyme synthesis at the translational level, and, in addition to dietary components, this process is also mediated by hormones such as CCK and insulin and neurotransmitters such as acetylcholine (Figure 1). There are no conclusive published studies about the role of postprandial CCK in the stimulation of pancreatic digestive enzyme synthesis, but unpublished results from our laboratory, using CCK-deficient mice in vivo, demonstrate that CCK is, in

Figure 8. Effect of the four different experimental treatments on pancreatic polysomal profiles. Each profile shows the peaks for the 40S and 60S ribosomal subunits, as well as for the whole ribosome (80S) and the different polysomes, in the polysomal fraction for representative fractionations. The graphs also show the calculations of the area under the curve for the subpolysomal and the polysomal fractions. The experimental groups were as follows: (A) fasted; (B) refed (20% protein); (C) refed protein-deficient diet (0% protein); and (D) refed leucine-deficient diet (no leucine). *P < 0.05 vs. control group. †P < 0.05 vs. control refed group (from reference 4).
part, necessary for the stimulation of pancreatic digestive enzymes synthesis after feeding a complete and balanced diet as well as for the stimulation of the mTORC1 pathway. On the other hand, there are multiple studies showing the involvement of CCK in the stimulation of pancreatic acinar cell protein synthesis and translational mechanisms, in vivo and in vitro.

In vivo: Bragado et al. demonstrated that CCK, either administered by intraperitoneal injection to rats, or by stimulating endogenous CCK release by intragastric administration of a trypsin inhibitor, induced a time- and dose-dependent phosphorylation of pancreatic eIF4E and its binding protein 4E-BP1 (or PHAS-I) as well as the formation of the eIF4F complex (Figures 2 and 4). These events occurred over a range of CCK doses from 0.2 to 5 μg/kg. The acute effects of endogenous CCK, released after gavaging a synthetic trypsin inhibitor, camostat (100 mg/kg), were similar. Thus, both exogenous and endogenous CCK activate translational initiation factors in vivo. The activation of translational machinery necessary for initiation of protein synthesis likely contributes to the normal postprandial synthesis of pancreatic digestive enzymes.

In vitro: CCK has a biphasic effect on enzyme secretion being stimulatory at physiological concentrations and inhibitory at higher concentrations, and follows the same biphasic pattern stimulating protein synthesis (Figure 10). Thus, CCK increases the rate of translation initiation and elongation processes at physiological concentrations. That this acute stimulation of protein synthesis is at the translational level is shown by the fact that it occurs without a change in mRNA levels and in the presence of actinomycin D. In these studies, the stimulation of protein synthesis occurred within 30 minutes and showed additivity between insulin and CCK. Increased synthesis of both digestive enzymes and structural proteins were observed, although differences between individual proteins suggested nonparallel translational effects. Additionally, CCK or its analogue caerulein activates several regulators of translation, like the S6 kinase (S6K), the phosphorylation of eIF4E, and the formation of the eIF4F complex, and the activation of the elongation processes and eEF2 appear to be regulated through a rapamycin-sensitive pathway and to be downstream of PI3K. Bragado et al. also showed that the expression and activity of p70s6k-p85s6k (S6K) was biphasic after CCK stimulation. Carbachol and bombesin, but not vasoactive intestinal peptide, also activated S6K. These studies concludes that the S6K stimulation by CCK is not mediated by PKC or mobilization of intracellular calcium but by PI3K. At the same time, it is shown that S6K is not involved in the secretion of digestive enzymes induced by CCK.

Other stimulants, besides CCK, stimulate total protein synthesis in pancreatic acinar cells at their physiological concentrations. Carbachol, insulin, and bombesin, in multiple species and preparations, all stimulate the synthesis of total protein, trypsinogen, chymotrypsinogen, lipase, and amylase by isolated acini.
Regulation of translation elongation

Stimulatory doses of cholecystokinin (CCK), bombesin, and carbachol increased elongation rates (measured as ribosomal half-transit time) in pancreatic acini in vitro (Figure 11A). At the same time, these secretagogues reduced elongation factor 2 (eEF2) phosphorylation (the main factor known to regulate elongation), and increased the phosphorylation of the eEF2 kinase (Figure 11B and C). The mTOR inhibitor rapamycin reversed the dephosphorylation of eEF2 induced by CCK, as did treatment with the p38 MAPK inhibitor SB202190, the MEK inhibitor PD98059, and the phosphatase inhibitor calyculin A. Neither rapamycin, SB202190, PD98059 nor calyculin A had an effect on CCK-mediated eEF2 kinase
phosphorylation. Therefore, translation elongation in pancreatic acinar cells is likely regulated by eEF2 through the mTOR, p38, and MEK pathways and modulated through PP2A.  

**Insulin**

The pancreas is composed of two separate organ systems—the exocrine pancreas, responsible for synthesizing the enzymes that will enter the intestine and participate in digestion, and the endocrine pancreas, composed of islets of Langerhans, responsible for synthesizing insulin and other hormones that enter the blood and regulate glucose levels and metabolism. The physical location of both systems in the same organ and the common vascular supply, including a portal blood system by which venous blood from islets can perfuse the exocrine tissue, seems likely to have a purpose. In fact, the exocrine pancreas and the pancreatic islets have a multitude of complex anatomical and functional interrelations that can affect one another.  

Among these, pancreatitis can induce diabetes, by damaging the endocrine cells of the islets, and diabetes is correlated with exocrine pancreas insufficiency. This indicates a role for insulin in the regulation of digestive enzyme synthesis and/or secretion.

For more detailed information on insulin secretion and action on pancreatic exocrine function, the reader is invited to check the reviews by Mann et al. on the Pancreapedia and in Chapter 25 of this book; and the one from Sans et al. also on the Pancreapedia and in Chapter 17 of this book.

Briefly, insulin is known to stimulate protein synthesis by translational effects in many tissues. Early studies described the presence of insulin receptors in pancreatic acinar cells and showed that insulin was involved in the incorporation of radioactive amino acids into total protein or specific enzymes of normal and diabetic rodents in vivo. Most of the studies showed a positive effect of insulin, but due to technical differences between studies, the results are difficult to interpret. When protein synthesis in mice was quantitated by autoradiography, there was more incorporation into peri-insular acinar cells than into tele-insular acinar cells, and this effect was lost after inducing diabetes with streptozotocin—implying that insulin had an effect on the peri-insular acinar cells. One study, with a different methodological approach, increased insulin secretion by treatment with sulfonylurea (a drug that stimulates insulin release) or by glucose infusion. Both treatments increased protein synthesis by 25–40 percent. When Zucker fatty rats were studied as a model of insulin resistance, overall pancreatic protein synthesis was reduced by nearly 50 percent. There was considerable difference in synthesis between different digestive enzymes separated by 2-D-gel electrophoresis. A morphological study of prolonged diabetes in rats showed gross abnormality in the acinar cell secretory pathway 28 days after STZ treatment that was partially reversed by insulin administration. However, shorter studies have not shown significant structural alterations 1 week after STZ other than the appearance of cytoplasmic lipid droplets.

More recent studies have been able to overcome some of the methodological issues by measuring the effects of insulin over short times to prevent changes in mRNA and under conditions where the precursor pool is large and constant. In vitro studies have used isolated pancreatic acini under dilute labeling conditions to keep the precursor pool constant. Insulin stimulates the incorporation of multiple different amino acids (leucine, methionine, phenylalanine) into protein, in isolated acini from diabetic rats. Similarly, insulin increased methionine incorporation into total protein and immunoprecipitated amylase in rat pancreas-derived AR42J cells. The effect of insulin on both cell types occurred over concentrations from 30 nM to 100 nM and was mediated by the insulin receptor. Insulin was also shown to have nonparallel effects on different digestive enzymes and structural proteins such as myosin and LDH.

Although the mechanism of insulin signaling is well studied in a number of target tissues, especially liver, fat, and muscle, only a few studies have been carried out in the exocrine pancreas. The main signaling pathway regulating protein synthesis is the mTORC1 pathway and this pathway has been documented in pancreatic acinar cells primarily in mediating the actions of CCK and acetylcholine to stimulate digestive enzyme synthesis. Insulin has been shown to be involved in protein synthesis in several studies and has nonparallel effects on translation of specific proteins. It also activates S6K and S6 phosphorylation downstream of TORC1 in rat and mouse acini. Akt is upstream of TORC1 and insulin activates the phosphorylation of Akt on S473 and T308 in rat acini. Bragado et al. showed that insulin stimulates protein synthesis in pancreatic acinar cells in vitro, through the PI3K/Akt/mTORC1 stimulated mechanisms (Figure 4). However, almost all of these studies were dependent on first inducing diabetes with streptozotocin to reduce endogenous levels of insulin, and diabetes effects could confound some of the results.

One recent study from our laboratory (to be published shortly) done in genetically modified mice in vivo shows, for the first time, that insulin signaling through its receptor in pancreatic acinar cells stimulates the synthesis of digestive enzymes after a balanced meal feeding. We developed a mouse model with a conditional insulin receptor (IR) KO in the pancreatic acinar cells: the pancreatic acinar cell insulin receptor knock out (PACIRKO) mice. It was obtained by crossing IR<sup>KO</sup>/<sup>Cre</sup> mice with tamoxifen-regulated Ela-Cre mice. The lack of insulin signaling to pancreatic acinar cells induced a 40 percent reduction in pancreas weight of PACIRKO mice as well as total pancreatic protein.
and digestive enzymes content. The Akt/mTORC1 pathway activity was significantly reduced in both PACIRKO and diabetic mice, compared to normal control mice. Total pancreatic fluid secretion was not changed, but protein concentration and content of several digestive enzymes was reduced in the PACIRKO mice, compared to diabetic models. The specific deletion of the insulin receptor gene induced a decrease of pancreatic digestive enzymes content, an increase in the size of pancreatic acinar cell lumen, as well as apoptosis and activation of regeneration mechanisms in the pancreas with time. This study demonstrates that insulin directly regulates the synthesis of digestive enzymes in a postprandial situation as well as the short- and long-term exocrine pancreatic homeostasis.

This effect of insulin stimulating the synthesis of pancreatic digestive enzymes has clinical implications related to type 1 diabetes, where insulin is reduced. A significant number of patients with diabetes suffer exocrine pancreatic abnormalities especially pancreatic exocrine insufficiency due to a reduction of the digestive enzymes, which occurs in about 50 percent of patients with type 1 diabetes. The decrease in pancreatic function correlates with the duration of both, type 1 and 2 diabetes. In other studies with type 1 diabetes patients, atrophy of exocrine tissue and pancreatic fibrosis has been reported. This pancreatic insufficiency during diabetes can affect physical development and metabolism in children and adolescent diabetic patients.

Exocrine pancreatic abnormality has also been seen in animal models of diabetes. Guinea pigs with spontaneous diabetes show fatty degeneration of pancreatic acinar cells and decreased digestive enzymes and bicarbonate secretion. Streptozotocin-treated diabetic rats show altered digestive enzyme gene expression and reduced amylase secretion without a decrease in the amount of fluid secreted. In isolated pancreatic acini, in vitro, there is a reduction of amylase secretion after STZ-induced diabetes due to reduced amylase pancreatic content. More clinically relevant, mouse models of type 1 diabetes like NOD mice or the Ins2Akita mouse model with insulin misfolding also show alterations in the exocrine pancreas.

From these studies, it can be concluded that in both animals and humans insulin (besides modulating the action of other hormones (CCK) or stimulators (ACh) is necessary for the synthesis of digestive enzymes, and that these functions are inhibited in T1D.

### Intracellular Calcium Levels Affecting Pancreatic Protein Synthesis

CCK and other G protein-stimulating pancreatic secretagogues inhibit pancreatic acinar protein synthesis at concentrations that hyperstimulate their receptors. All of them have a common intracellular mechanism, increasing intracellular calcium to high concentrations. High intracellular calcium levels have been shown to inhibit protein synthesis in other cell types by ER stress and activation of the UPR. On the other hand, it has also been shown that removing calcium from the media, or depleting calcium from inside the acinar cells, also inhibits pancreatic protein synthesis, indicating a need for calcium in the regulation of protein synthesis in pancreatic acinar cells.

### Calcium signaling is involved in the stimulation of pancreatic protein synthesis

Changes in physiological calcium levels inside pancreatic acinar cells act as a signaling mechanism that affects the activity of calcium-regulated enzymes. Calcineurin, also known as protein phosphatase 2B (PP2B), is a serine/threonine protein phosphatase that is highly regulated by Ca

Calcineurin is best known for driving the adaptive immune response by dephosphorylating the nuclear factor of the activated T-cells (NFAT) family of transcription factors. Therefore, calcineurin inhibitors, FK506 (tacrolimus), and cyclosporin A serve as immunosuppressants. FK506 and cyclosporin A (CsA) binding to their intracellular target proteins (12-kDa FK506-binding protein and cyclophilin A) inhibit calcineurin phosphatase activity. The use of FK506 and CsA has been used to implicate calcineurin in a number of cellular processes, including pancreatic endocrine and exocrine secretion. Moreover, study of the side effects of CsA and FK506 in organ transplant therapy has implicated calcineurin in the protein synthesis mechanisms of some tissues, including kidney and liver. However, in contrast to the apparent multitude of cellular substrates for the type 1 and 2A serine/threonine phosphatases, relatively few cellular targets of calcineurin have been described.

High concentrations of CsA were found to inhibit amylase secretion, but it is not clear whether FK506 inhibits pancreatic exocrine secretion or not (unpublished observations). Both immunosuppressants were found to block pancreatic growth in response to chronic elevation of CCK induced by feeding trypsin inhibitor to mice, indicating a role for calcineurin in pancreatic growth (see the review on pancreatic growth by John A. Williams in Chapter 22 of this book and in Pancreapedia). Since, protein synthesis is an obligatory requirement for growth of
all cells,\textsuperscript{185} calcineurin could also be involved in the regulation of pancreatic protein synthesis.

Using FK506 and CsA, we determined that calcineurin is involved in the CCK stimulation of pancreatic acinar cell protein synthesis and translational machinery,\textsuperscript{73} demonstrating the involvement of intracellular calcium in the stimulation of mRNA translation into protein. FK506 also inhibited protein synthesis stimulated by bombesin and carbachol (Figure 12A). FK506 did not significantly affect the activity of the initiation factor-2B or the phosphorylation of the initiation factor-2α, ribosomal protein S6, or the mRNA cap-binding protein eukaryotic initiation factor (eIF)4E. Instead, blockade of calcineurin with FK506 reduced the phosphorylation of the eIF4E-binding protein, decreased the formation of the eIF4F complex (Figure 12B), and increased the phosphorylation of eukaryotic elongation factor 2 (Figure 12C). From these results, it can be concluded that calcineurin activity and intracellular calcium are required for pancreatic protein synthesis, and this action may be related to effects on the formation of the mRNA cap-binding complex (Figure 1) and the elongation processes (Figure 2).\textsuperscript{73}

\textbf{High intracellular calcium levels inhibit pancreatic protein synthesis}

\textit{In vitro}

The inhibition of acinar protein synthesis and polysome size by CCK and the inhibitors of the microsomal Ca\textsuperscript{2+} ATPase, thapsigargin (Tg), and 2,5-di(tertbutyl)-hydroquinone (Bhq) occurs as a result of depletion of Ca\textsuperscript{2+} from the endoplasmic reticulum lumen, increasing intracellular cytoplasmic calcium.\textsuperscript{172} The combination of administering the intracellular Ca\textsuperscript{2+} chelator, BAPTA, with Tg and Bhq depleted the pools of [Ca\textsuperscript{2+}] without changing cytosolic [Ca\textsuperscript{2+}] but with the same inhibitory effect on protein synthesis and polysome formation, suggesting that depletion of intracellular Ca\textsuperscript{2+} stores, without changes in cytosolic [Ca\textsuperscript{2+}], decreases protein synthesis at translation initiation.\textsuperscript{172} It has also been demonstrated that removal of Ca\textsuperscript{2+} from the medium enhanced the inhibitory action of CCK on both protein synthesis and eIF2B activity as well as further increased eIF2α phosphorylation\textsuperscript{72} (Figure 13).

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure12.png}
\caption{(A) Effect of FK506 on pancreatic protein synthesis stimulated with different agonists. The incorporation of [\textsuperscript{35}S]methionine was analyzed in basal, CCK-stimulated (100 pM), bombesin (BBS)-stimulated (10 nM), and carbachol (CCh)-stimulated (30 μM) acini, with or without 100-nM FK506. In each experiment, \textsuperscript{35}S incorporation was normalized to control. *P < 0.05 vs. basal group; #P < 0.05 vs. its corresponding control stimulated group (CCK 100 pM; BBS 10 nM; CCh 30 μM). (B) Effects of FK506 on the formation of eIF4F and (C) on eEF2 phosphorylation. (B) Formation of the eIF4F complex, measured as coimmunoprecipitation of eIF4E and eIF4G. (C) Phosphorylation of eEF2 (Thr-56) in response to CCK in the absence or presence of 100 nM FK506. Insets: Representative Western blots for eIF4G and total eIF4E (B) and for phosphorylated and total eEF2 (C). *P < 0.05 vs. basal group; #P < 0.05 vs. control CCK-stimulated group (from reference \textsuperscript{73}).}
\end{figure}
In the same study, with rat acini, carbachol (CCh) and the ionophores A-23187 and Tg also inhibited eIF2B and protein synthesis, whereas bombesin and the CCK analog JMV-180, that do not cause depletion of intracellular calcium stores at high concentrations, were without effect (Figure 14).72

Previous studies have shown that eIF2B can be negatively regulated by glycogen synthase kinase-3 (GSK-3). However, GSK-3 activity, as assessed by its phosphorylation state, was inhibited at high concentrations of CCK, an effect that should have stimulated, rather than repressed, eIF2B activity. Therefore, CCK inhibits eIF2B activity only through eIF2alpha phosphorylation (Figure 13).**

**In vivo**

Inhibition of pancreatic protein synthesis in vivo normally occurs during the development of caerulein-induced acute pancreatitis (AP),56 but it has been shown that in some studies, total pancreatic protein synthesis is inhibited, whereas in other studies, protein synthesis is not affected. On the other hand, in a study done using minced rabbit pancreas samples, a decrease in protein synthesis was observed in response to stimulatory doses of CCK, and this was accompanied by a decrease in the number of polysomes.71 Most of the differences seen on the effects of CCK in vivo, ex vivo, or in vitro could be explained by methodological differences on the way of administering and analyzing the incorporation of AAs into protein.

In a caerulein-induced AP study,86 pancreatic protein synthesis was already reduced 10 minutes after the initial caerulein administration and was further inhibited after three and five hourly injections (Figure 15A). Caerulein inhibited the two major regulatory points of translation initiation: the activity of the guanine nucleotide exchange factor eIF2B and the phosphorylation of eIF2alpha (Figure 13).
Figure 15. Effect of caerulein-induced acute pancreatitis on pancreatic protein synthesis and on the first step of translation initiation (Figure 1), in mice. (A) l-[3H]phenylalanine incorporation into pancreatic protein in C57BL/6 mice. Values are expressed as nanomoles of incorporated phenylalanine per 10 minute per milligram of protein and are means and SE. *P < 0.05 vs. control (saline groups), (B) eukaryotic initiation factor (eIF)2B activity, and (C) eIF2α phosphorylation. The pancreas was removed 10 minutes, 30 minutes, and 1 hour after a single-dose injection of caerulein at 50 μg/kg and 1 hour after 3 hourly injections. The results are expressed as a percentage of pooled control levels. In (C) the insets show representative blots for eIF2α phosphorylation levels and total eIF2α. For (B) and (C), data shown are means and SE. *P < 0.05, #P < 0.01 vs. pooled control group (from reference 86).

factor eIF2B (with an increase of eIF2α phosphorylation) (Figure 15B and C) and the formation of the eIF4F complex due, in part, to degradation of eIF4G (Figure 16). This inhibition was not accounted for by changes upstream (caerulein activated Akt) or downstream of mTORC1. Caerulein also decreased the phosphorylation of eEF2, implying that elongation was not inhibited during AP. Thus, the inhibition of pancreatic protein synthesis in this model of AP results from the inhibition of translation initiation due to increased eIF2α phosphorylation, reduction of eIF2B activity, and the inhibition of eIF4F complex formation. This inhibitory effect appears to be calcium-dependent and suggests that pancreatic acinar cells adapt to this short-term stress by inhibiting the synthesis of pancreatic digestive enzymes. Although not yet fully studied, an ER resident kinase such as PERK is likely to mediate eIF2α phosphorylation and it has been shown to be important for the translational control and cell survival of pancreatic acinar cells. The inhibition of protein synthesis associated with high concentrations of CCK could therefore be an adaptive or protective mechanism in response to stress localized in the ER.

Several early studies showed that pharmacological inhibition of protein synthesis during AP helped prevent acinar cell damage. Later studies, on the contrary, have shown that a complete inhibition of pancreatic protein synthesis with cycloheximide during AP reduced pancreatic edema but worsened the development of the disease and inhibited apoptotic pathways that have been proposed to be more beneficial than the necrotic ones in the severity of AP. Thus, a complete inhibition of pancreatic protein synthesis does not seem a likely event to happen during AP; rather, a selective inhibition of the synthesis of specific proteins would most likely account for the reduction seen in total protein synthesis during AP. Since synthesizing digestive enzymes in the exocrine pancreas is the main purpose of the gland under physiological situations, inhibition of this synthesis could likely happen during adverse situations such as AP, when secretion is also blocked, in an attempt to prevent or reduce more acinar cell damage.

Additionally, despite the fact that numerous studies have shown the possible interaction between lysosomal hydrolases and digestive enzyme zymogens in acinar cells in the development of AP, whether lysosomal enzymes and pancreatic zymogens can be regulated at the translational level during AP has not been addressed.

Pancreatitis and the ER stress response

The events that regulate the severity of AP are, for the most part, unknown, and the exact mechanisms by which diverse etiological factors induce an attack are still unclear. The use of animal models of AP has permitted to advance in our understanding of the early cellular events that underlie the development of acute pancreatitis. However, there is...
still no understanding of a common triggering mechanism for the disease. These models share several common features including secretory blockade, \( ^{77,78,80,204} \) intracellular trypsin activation, \( ^{80,205,206} \) high levels of digestive enzymes in blood, cytoplasmic vacuolization, \( ^{78,205} \) and activation of NFkB with later induction of an inflammatory response. \( ^{207} \) Alterations in intracellular \( Ca^{2+} \) signaling, either by disturbances of calcium influx or by disturbances of calcium coming from the ER, \( ^{187,208-210} \) lead to the activation of multiple intracellular mechanisms that can involve the activation of ER stress, and it has been shown by us \( ^{86} \) and others \( ^{75-79} \) that total protein synthesis is inhibited in AP.

ER stress signals are already active in normal pancreas in conditions that are important for the development of the gland, \( ^{488} \) but these signals could likely be involved in the early stages of the development of the disease, to try to compensate any original imbalance. When intracellular calcium concentrations increase to supramaximal levels, \( ^{72} \) the compensatory mechanisms are overworked, and ER stress signals lead to the activation of programmed cell death mechanisms, \( ^{211} \) similar to the ones seen during acute pancreatitis. \( ^{212} \)

Kubisch et al. showed, for the first time, that all major ER stress sensing and signaling mechanisms are present in pancreatic acini and are activated early in the arginine model of experimental acute pancreatitis. \( ^{212} \) Arginine treatment caused an early activation of ER stress, as indicated by phosphorylation of PERK and its downstream target eIF2alpha, ATF6 translocation into the nucleus, and upregulation of BiP. XBP-1 splicing and CHOP expression were observed within 8 hours. After 24 hours, increased activation of the ER stress-related proapoptotic molecule caspase-12 was observed along with an increase in caspase-3 activity and TUNEL staining in exocrine acini. These results indicate that ER stress is an important early acinar cell event that could contribute to the development of acute pancreatitis and, with time, stimulate apoptosis in the arginine model.

Several studies directed at finding different physiologic and metabolic imbalances in the exocrine pancreas, such as oxidative stress, mitochondrial dysfunction and autophagy, deregulation of ATP generation and ER Acetyl-CoA (AT) availability, changes in lipid metabolism, and inflammatory and cell death responses, \( ^{213,214} \) have demonstrated that these mechanisms often first manifest by abnormal cytosolic \( Ca^{2+} \) signaling. After abnormal calcium cytosolic levels, there is a multifaceted set of organelles and cellular interactions that trigger ER stress in pancreatic acinar cells, leading the way to different degrees of cell damage \( ^{215} \) and, ultimately, to acute \( ^{216,217} \) and chronic \( ^{218} \) pancreatitis.

Other studies have demonstrated that mutations in some digestive enzymes, that can be hereditary, cause problems with their proper folding and trigger the ER stress response in the pancreas. This protein misfolding in the ER may also contribute to parenchymal damage by causing acinar cell death, and this may led to chronic pancreatitis. \( ^{219,220} \)

A proteomics study on the RER of pancreatic acinar cells, from rat pancreases of control and two experimental models of AP (arginine- and caerulein-induced), revealed an increase of many ER proteins during AP, compared to the control group, but showed differences in the amount of several others between the two models of AP, possibly due to the different degree of cell damage caused by the two models or to the different stages of the progression of cell damage. \( ^{221} \) For instance, there was a clear reduction of some chaperone proteins, like BiP, in the arginine model,

**Figure 16. Effects of caerulein-induced acute pancreatitis on eIF4F complex formation, measured as eIF4E and eIF4G coimmunoprecipitation.** (A) Data for coimmunoprecipitated eIF4G are expressed as percentage of pooled control levels and SE. Representative blots for eIF4G associated with eIF4E and total eIF4E are shown in the insets. #P < 0.01 vs. pooled control group. (B) Representative Western blotting showing the degradation of total pancreatic eIF4G 30 minutes after caerulein injection (from reference \( ^{86} \)).
possibly due to the activation of apoptotic processes, the latest steps of the UPR mechanisms, when chaperoning for unfolded proteins is no longer needed.

Along these lines, Lugea and coworkers suggest that the ER stress and UPR responses alone are not involved in the development of acute pancreatitis. They argue that to be considered as triggering factors of the development of AP and cause cell damage, these mechanisms need to be combined with other environmental or genetic stressors. These authors study the effects of more clinically relevant causes of pancreatic injury in experimental models. They show that alcohol abuse triggers a UPR response that attenuates the ER stress caused by alcohol to the pancreas and suggest that early UPR mechanisms are beneficial for the well-being and survival of pancreatic acinar cells, because they are directed to compensate the original ER stress. Lugea et al. demonstrate this hypothesis in another study where the UPR was inhibited and cell death mechanisms were activated after applying, to AR42J cells, two well-known risk factors for clinical AP and chronic pancreatitis: cigarette smoke and alcohol.

In summary, several cellular insults that cause damage to pancreatic acinar cells start with depleting Ca²⁺ from ER stores. This Ca²⁺ depletion triggers the initial ER stress response that inhibits digestive enzymes synthesis. The ER stress response tries to compensate for these original imbalances, through the UPR, and when the cell situation can no longer be counteracted, the UPR stimulates cell death mechanisms through apoptosis. Some of these early ER stress mechanisms could likely be involved in the triggering events of AP, but it is also very likely that several insults need to be present to develop disease. A balancing regulation in response to ER stress is essential for cell survival and may act as a protective mechanism in the pancreas, as it does in other tissues. Because of that, it has also been proposed that the ER protein folding machinery and the UPR responses could be potential therapeutic targets to prevent and treat pancreatic diseases.

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References


120. Cao YC, Yang XJ, Guo L, Zheng C, Wang DD, Cai CJ, Liu SM, Yao JH. Effects of dietary leucine and phenylalanine on pancreas development, enzyme activity, and relative gene...


Chapter 8
Calcium signaling in pancreatic acinar cells

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Introduction

Intracellular Ca\(^{2+}\) signaling and the primary function of the exocrine pancreas to secrete digestive enzymes are intimately linked. A secretagogue-induced rise in the level of intracellular Ca\(^{2+}\) signaling is the pivotal intracellular event triggering both the molecular machinery that results in exocytosis of zymogen granules and also the activation of ion channels that leads to the secretion of the primary fluid from acinar cells. Given the need to ensure vectoral secretion of a potentially hazardous cargo into the lumen of the gland, the Ca\(^{2+}\) signals and activation of effectors are elaborately orchestrated in time and space. The importance of the physiological Ca\(^{2+}\) signal is reinforced by increasing evidence that disordered Ca\(^{2+}\) signaling is causally associated with pancreatic pathology.\(^1\) The aim of this chapter is twofold—first to provide the reader with an overview of the spatiotemporal features of intracellular Ca\(^{2+}\) signals in acinar cells as measured by contemporary high-resolution fluorescence measurements. Second, a review will be presented of the current understanding of the molecular mechanisms underpinning Ca\(^{2+}\) release, Ca\(^{2+}\) influx, and clearance—processes that are fundamental for the specific properties of intracellular Ca\(^{2+}\) signals in acinar cells. The reader is referred to various “Method” submissions in The Pancreapedia regarding current imaging techniques used to monitor intracellular Ca\(^{2+}\) changes with high spatial and temporal resolution in acinar cells.

Signal transduction leading to secretagogue-induced cytosolic calcium signals in acinar cells

The primary pancreatic secretagogues, cholecystokinin (CCK), acetylcholine (ACh), and bombesin, share a common general mechanism to raise [Ca\(^{2+}\)]. It is well established that each agonist binds a specific, cognate, seven-transmembrane domain receptor in the plasma membrane (PM) that couples to the heterotrimeric G protein, G\(\alpha\)q. Activation of G\(\alpha\)q results in the increased activity of phosphoinositide (PI)-specific phospholipase C which cleaves phosphatidylinositol, 4,5-bisphosphate (PIP\(_2\)) in the PM and subsequently generates the second messengers inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol. Historically, studies in pancreatic acinar cells have played important roles in establishing the signal transduction pathway utilized by so-called “calcium-mobilizing” agents. A seminal paper by Streb and colleagues\(^2\) was the first to demonstrate that IP\(_3\) could induce Ca\(^{2+}\) release in studies using permeabilized pancreatic acini.\(^2\) Notably, release could only be evoked from rough ER vesicles but not mitochondria or vesicles prepared from plasma membrane.\(^3\) These data confirmed earlier studies that secretagogues stimulation resulted in substantial Ca\(^{2+}\) loss from ER but not zymogen granules or mitochondria.\(^4\) Finally, the causative “loop” was then closed by the demonstration that stimulation with pancreatic secretagogues resulted in the rapid formation of IP\(_3\).\(^5\) Notably, this pathway has subsequently been shown to be ubiquitous and accounts for the generation of Ca\(^{2+}\) signaling events important for a multitude of cellular events in all tissue systems.

The Characteristics of Secretagogue—Induced Cytosolic Calcium Signals in Acinar Cells

Temporal properties at high concentrations of secretagogues

In common with many nonelectrically excitable cell types, the spatiotemporal patterns of Ca\(^{2+}\) signals elicited in pancreatic acinar cells are dependent on the concentration of agonist. Regarding the temporal characteristics of signals in isolated pancreatic acinar cells, experiments monitoring the global intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) using fluorometric techniques in single rodent cells reported that maximum concentrations of CCK, ACh, or bombesin evoked a similar Ca\(^{2+}\) signal. Exposure to either agonist results in a rapid 5–12-fold increase in [Ca\(^{2+}\)]\(_i\), from a basal value of [Ca\(^{2+}\)]\(_i\) ~75–150 nM to reach a peak within seconds of approximately 1 μM (Figure 1A). This peak then declined over 2–5 minutes to reach a new plateau level.
Calcium signaling in pancreatic acinar cells

around 100 nM above basal, which is maintained as long as the agonist is present.\(^6\)\(^-\)\(^9\) The initial peak was shown to be the result of Ca\(^{2+}\) release from intracellular stores, since the early transient response was essentially unaffected by removal of extracellular Ca\(^{2+}\) while the later plateau phase was absent (Figure 1B).\(^8\)\(^-\)\(^11\) Removal of extracellular Ca\(^{2+}\) during the plateau phase resulted in the rapid attenuation of the signal (Figure 2B), thus indicating an absolute dependence on extracellular Ca\(^{2+}\) for this maintained phase and implying the presence of a mechanism for Ca\(^{2+}\) influx from the extracellular milieu. A study monitoring ACh-induced Ca\(^{2+}\) signals in human pancreatic acinar cells confirmed this general paradigm; the initial phase of the signal occurs independently of extracellular Ca\(^{2+}\), while the sustained plateau requires the presence of Ca\(^{2+}\) in the extracellular milieu.\(^12\)

Temporal properties at physiological concentrations of secretagogues

In contrast to the situation with maximal concentrations of agonists, similar experimental techniques demonstrated that lower, physiological concentrations of agonist resulted in the initiation of Ca\(^{2+}\) oscillations.\(^7\)\(^-\)\(^9\),\(^13\)\(^,\)\(^14\) These oscillations are characterized by repetitive, regular cycles of elevated and subsequently decreasing Ca\(^{2+}\) levels. In single
acinar cells or small acini, physiological concentrations of secretagogues (1–50 pM CCK; 50–300 nM ACh) induce after a latency of 30 seconds to 2 minutes fairly regular Ca$^{2+}$ oscillations at a frequency of between 1 and 6 cycles per minute. The maximal global [Ca$^{2+}$], reached during the release phase is generally between 200 nM and 1 μM. At least in mouse pancreatic acinar cells, the global temporal profile stimulated by ACh differs from that stimulated by CCK and bombesin. Oscillations stimulated by peptide secretagogues tend to be characterized by slow, relatively long-lived transients originating and returning to basal levels between Ca$^{2+}$ spikes while ACh-induced oscillations are characterized by faster short-lasting transients originating from an elevated plateau (Figure 2A and B).

**Mechanisms underlying oscillating Ca$^{2+}$ signals**

Consistent with a prominent role for receptor-activated, Gαq-stimulated, PIP$_2$ hydrolysis as the underlying mechanism, Ca$^{2+}$ oscillations in pancreatic acinar cells can be mimicked by agents, which activate heterotrimeric G proteins such as GTPγS, sodium fluoride, or mastoparan and by introduction of IP$_3$ into the cell, for example, via dialysis from a whole-cell patch clamp pipette. Oscillations are primarily the result of cycles of intracellular Ca$^{2+}$ release and ATP-dependent reuptake because the oscillations can be initiated in the absence of extracellular Ca$^{2+}$ and are inhibited by agents, which deplete ATP or inhibit the Ca$^{2+}$ pump on the endoplasmic reticulum.

Although Ca$^{2+}$ release during each cycle only minimally depletes the intracellular Ca$^{2+}$ store and reuptake is efficient, the maintenance of oscillatory behavior is dependent on extracellular Ca$^{2+}$. These observations again indicate an absolute requirement for Ca$^{2+}$ influx to sustain the Ca$^{2+}$ signal presumably primarily by maintaining the level of Ca$^{2+}$ in the intracellular store.

Oscillating levels of IP$_3$ are not necessary per se for oscillatory behavior, since nonmetabolizable IP$_3$ is capable of initiating Ca$^{2+}$ oscillations in mouse pancreatic acinar cells. These data indicate that the mechanism underlying Ca$^{2+}$ oscillations is most likely the result of an inherent property of the Ca$^{2+}$-release mechanism. Nevertheless, these data do not preclude the possibility that upon agonist stimulation the [IP$_3$] itself fluctuates and contributes to the kinetics of Ca$^{2+}$ release. Indeed mathematical predictions based on the experimental behavior of Ca$^{2+}$ oscillations when Ca$^{2+}$ or IP$_3$ is artificially raised during agonist exposure predict that the [IP$_3$] is itself oscillating during CCK stimulation. One mechanism whereby oscillating IP$_3$ could occur is through periodic activation cycles of RGS proteins. RGS proteins stimulate the GTPase activity of Gα subunits, thereby terminating the stimulus for activation of effectors such as PLC β. In rat pancreatic acinar cells, infusion of RGS proteins via the patch pipette results in dampening of Ca$^{2+}$ signals. Interestingly, the common catalytic core of RGS proteins, the so-called RGS box, is much less effective than infusion of full-length RGS proteins. In addition, specific RGS proteins appear to affect the Ca$^{2+}$ signals generated by different secretagogues differentially. These observations may indicate that individual RGS proteins are associated in a signaling complex with specific secretagogues receptors and other signaling proteins. This interaction may impact the kinetics of IP$_3$ production and contribute to the agonist-specific characteristics of secretagogue-stimulated Ca$^{2+}$ signals in pancreatic acinar cells.

**Spatial properties of Ca$^{2+}$ signals**

Measurements of global Ca$^{2+}$ signals have provided a wealth of data regarding the general temporal properties of Ca$^{2+}$ signals in pancreatic acinar cells. However, by the nature of the measurements, the experiments report the [Ca$^{2+}$], as a mean value integrated from throughout the cell. Modern imaging techniques, however, allow the monitoring of [Ca$^{2+}$] at the subcellular level and in multiple cells.
of a coupled acinus. Probes, both chemical and genetically encoded sensors, are available with a choice of spectral characteristics and affinities for Ca\(^{2+}\) and with the ability to be targeted to various cellular compartments.\(^{29}\) Importantly, transgenic animals have been generated expressing a variety of these probes.\(^{30,31}\) Highlighting the promise of these techniques to enable the study of Ca\(^{2+}\) signals in living animals and thus in their native environment, an initial study using intravital two-photon microscopy in YC3.60 expressing animals reported Ca\(^{2+}\) signals following the injection of the muscarinic agonist bethanechol with characteristics similar to that seen in isolated acini.\(^{32}\) The combination of the flexibility of the probes plus the imaginative use of digital imaging techniques, such as confocal, multiphoton, and total internal reflection fluorescence (TIRF) microscopy, has revealed that the Ca\(^{2+}\) signal displays remarkable spatial intricacy, which appears to be fundamental for the appropriate activation of downstream effectors. An early study by Kasai and Augustine utilizing digital imaging of small acinar clusters demonstrated a profound spatial heterogeneity in the Ca\(^{2+}\) signal following stimulation with a maximal concentration of ACh (Figure 3A). Stimulation resulted in the initiation of Ca\(^{2+}\) release in the apical region of the cell immediately below the luminal plasma membrane and the subsequent spread of the signal as a wave toward the basal aspects of the cell.\(^{33}\) This Ca\(^{2+}\) wave has generally been reported to travel across the cell at a speed of between 5 and 45 μm/s, consistent with the velocity observed in other cell types.\(^{33-38}\)

These data were somewhat counterintuitive since contemporary studies had demonstrated that secretagogue receptors were expressed on the basolateral face of the cell\(^{30}\) and it was known that the ER, the presumed site of Ca\(^{2+}\) release, was present throughout the cell. Nathanson and colleagues, using line-scanning confocal microscopy, later confirmed that a similar pattern of apical to basal Ca\(^{2+}\) wave was initiated by high concentrations of CCK.\(^{34}\)

At physiological concentrations of agonists using similar techniques, Ca\(^{2+}\) signals are also shown to initiate in the apical region of acinar cells.\(^{39,41}\) This initial site of Ca\(^{2+}\) release has been termed the “trigger zone”\(^{39}\) (Figure 3B). Ca\(^{2+}\) release invariably occurs at this specialized site even under conditions where stimulation of agonist is restricted to the basal region by focal application of agonist. This has been most elegantly demonstrated by focal flash photolysis of caged-carbachol contained in a whole-cell patch-clamp pipette isolated in the base of the cell.\(^{42}\) It should be noted, however, that the apical portion of the acinus, immediately proximal to the tight junctions, may express a relatively high number of secretagogue receptors since this area has been reported to be most sensitive to focal agonist stimulation.\(^{33,44}\)

At threshold concentrations of ACh, repetitive short-lasting Ca\(^{2+}\) transients are initiated, which strikingly are contained to the apical third of the cell and do not propagate to the basal region.\(^{39,41}\) These spikes, although short-lived, have been shown using low-affinity Ca\(^{2+}\) indicators to be of large amplitude in the order of 1–4 μM Ca\(^{2+}\).\(^{45}\)

At intermediate concentrations of ACh, apically initiated global Ca\(^{2+}\) transients dominate, often superimposed on a slight global elevation of [Ca\(^{2+}\)]. The frequency of these transients corresponds to the frequency of oscillations noted in microfluorimetry studies.

Low concentrations of CCK predominately result in apically initiated global Ca\(^{2+}\) signals, which are of longer duration.\(^{15,16}\) In studies of whole-cell patch-clamped acinar cells, where the Ca\(^{2+}\) buffering of the cell is set by dialysis from the patch pipette, the broad CCK-induced transients have been reported to be preceded by short-lasting apically localized transients.\(^{46}\) Studies indicate that the precise site of initiation of each transient by specific agonists is very similar but possibly not identical.\(^{44}\) The site of initiation of each Ca\(^{2+}\) transient in the trigger zone is nevertheless tightly coupled functionally to both the exocytosis of zymogen granules and the activation of Cl\(^{-}\) channels required for the process of fluid secretion from the pancreatic acinar cells.\(^{45,47}\) Notably, the entire spectrum of signals evoked by secretagogues, from localized apical release to propagation of global Ca\(^{2+}\) waves, can be mimicked by global, uniform application of IP\(_{3}\) either through the patch pipette or via flash photolysis from a caged precursor (Figure 3C).\(^{17,36,41,48}\) It is envisioned that future work utilizing intravital microscopy in animals expressing genetically encoded Ca\(^{2+}\) indicators will further our understanding of the spatial and temporal properties of Ca\(^{2+}\) signals evoked by physiological stimulation.

The impact of cell-cell communication on Ca\(^{2+}\) signaling

Individual cells in the pancreatic acinus are extensively coupled by the expression of gap junctional proteins.\(^{49}\) These channels effectively allow the passage of small molecules up to a molecular mass of 1–2 kDa between cells and furthermore provide electrical coupling of large numbers of cells in the acinus. While stimulation of small acini with maximal concentrations of agonist results in the Ca\(^{2+}\)-dependent closure of these junctions,\(^{50,51}\) at physiological levels of CCK and bombesin where global Ca\(^{2+}\) transients predominate the junctions remain open and Ca\(^{2+}\) signals appear to spread as waves between individual cells.\(^{31,52}\) In contrast, brief, apically confined transients initiated by threshold concentrations of ACh have been reported not to propagate between coupled cells.\(^{51}\) Each cycle of CCK-induced intercellular Ca\(^{2+}\) signaling has been reported to be initiated by a “pacemaker cell.”\(^{55}\) This pacemaker presumably represents the individual cell within the acinus most sensitive to agonist. The propagation of a Ca\(^{2+}\) wave between adjacent cells obviously requires relatively
Figure 3. Spatial characteristics of Ca$^{2+}$ signals in pancreatic acini. Digital imaging of Ca$^{2+}$ indicators reveals spatial homogeneity in agonist-stimulated Ca$^{2+}$ signals. (A) Stimulation of a triplet of mouse pancreatic acinar cells (shown in a) with a maximal concentration of ACh results in the initiation of the Ca$^{2+}$ signal in the extreme apical portion of the acinar cells (shown in b). The signal subsequently spread toward the basal aspects of each cell (from reference 33). The pseudocolor scale indicates the levels of [Ca$^{2+}$]. In (B), a single pancreatic acinar cell is stimulated with a threshold concentration of ACh. Ca$^{2+}$ signals are again initiated in the apical portion of the cell but remain in the apical third of the cell without spreading to the basal aspects of the cell (images (B) Ab-Ah). The kinetic recorded from an apical region of interest (yellow trace in (B)) and from the basal region (blue trace) demonstrates that [Ca$^{2+}$] elevations are only observed in the apical pole of the acinar cell under these conditions (from reference 39). (C) Shows the changes in Ca$^{2+}$ following photolytic liberation of 1,4,5 InsP$_3$ from a caged precursor induced into a single mouse acinar cell via a whole-cell patch-clamp pipette. Following global elevation of 1,4,5-InsP$_3$, Ca$^{2+}$ changes initially occur at the apical pole of the acinar cell and spread to the basal pole in a similar fashion to secretagogue stimulation. The kinetic shows the Ca$^{2+}$ changes in the apical (blue trace) vs. basal pole (red trace) of the cell together with the activation of a chloride conductance as measured by whole-cell patch clamp (from reference 17).
long-range messengers. It appears that IP₃ and small amounts of Ca²⁺ are capable of diffusing between coupled cells to act in concert in this manner providing a signal to synchronize the intercellular Ca²⁺ wave. The primary evidence for this contention is that a Ca²⁺ signal can be observed in neighboring cells when 1,4,5-IP₃ is injected into an unstimulated individual cell. In addition, while Ca²⁺ injected into a resting cell fails to measurably increase [Ca²⁺] in adjacent cells, microinjection of Ca²⁺ into cells previously stimulated with threshold concentrations of CCK leads to a measurable increase in [Ca²⁺], in neighboring cells. These data are consistent with Ca²⁺ acting to facilitate further Ca²⁺ release from intracellular stores as will be described in detail in the remainder of this chapter. The physiological function of propagating Ca²⁺ waves in pancreatic acinar cells is not at present firmly established. A reasonable proposal, however, is that gap-junctional communication represents a mechanism to increase the responsiveness of an acinus to threshold concentrations of agonist. In this scenario, the acinus is rendered as sensitive to secretagogues stimulation as the pacemaker cell. In support of this idea, isolated single cells are much less sensitive to secretagogues stimulation than isolated acini and in addition experimental maneuvers which increase gap-junctional permeability lead to increased secretagogue-induced amylase secretion.

Molecular Mechanisms Underlying Ca²⁺ Signaling in Pancreatic Acinar Cells

Intracellular Ca²⁺ release

IP₃-induced Ca²⁺ release

The invariable apical initiation of Ca²⁺ release dictates the view that this trigger zone must represent a specialized region of ER, highly sensitive to IP₃. Studies have shown that this exquisite sensitivity to IP₃ is a result of the abundant expression of IP₃ receptors (IP₃Rs) in the extreme apical region of pancreatic acini. IP₃Rs were first isolated and cloned from cerebellum and have subsequently been shown to represent a family of three proteins named the type-1 IP₃Rs (IP₃R1), type-2 IP₃Rs (IP₃R2), and type-3 IP₃Rs (IP₃R3), which are all related to the ryanodine receptor Ca²⁺ release channel. Initially, it was reported that IP₃R3 was expressed in the apical pole. Later studies, however, showed that all three subtypes had essentially identical expression; all IP₃Rs were excluded from areas containing zymogen granules and were apparent immediately below the apical and lateral plasma membrane (Figure 4A–D). This localization is essentially identical to the “terminal web” of actin-based cytoskeleton in this region (Figure 4E). The localization to this region may be dependent on lipid rafts because cholesterol depletion results in the redistribution of IP₃R and disruption of the apical to basal Ca²⁺ wave. By this technique, no other significant localization of IP₃R was noted except for moderate expression on perinuclear structures. The later distribution is consistent with a recent report of IP₃-induced Ca²⁺ release from isolated nuclei prepared from mouse pancreatic acinar cells. Studies where IP₃ was released from a caged precursor in various localized regions of mouse acinar cells have also functionally confirmed that the apical region of the cell is more sensitive to IP₃ than the basal area of the cell. These data were later confirmed by a study imaging permeabilized pancreatic acini. Quantitative Western analysis and PCR have indicated that there is approximately equal expression of IP₃R3 and IP₃R2 in pancreatic acinar cells making up ~ 90 percent of the total complement of IP₃R. The pivotal importance of IP₃R2/3 for Ca²⁺ signaling and subsequent exocrine secretion has been confirmed by studies of transgenic animals in which both subtypes have been knocked out. Individual knockdown of IP₃R2 or IP₃R3 resulted in a modest reduction in the magnitude of Ca²⁺ release but no obvious global phenotype. However, the compound IP₃R2/3 null animal dies soon after weaning. The lack of viability results from a failure to ingest and subsequently digest food as a direct consequence of a general failure of exocrine gland secretion. In both salivary and pancreatic acinar cells from IP₃R2/3 null animals, secretagogue-stimulated Ca²⁺ signals and secretion of fluid and protein are essentially absent. These data also reveal that the residual IP₁,R-1 in the IP₃R-2/3 null animal cannot compensate for the loss of the other subtypes in pancreatic acinar cells.

IP₃R structure and regulation

The functional IP₃R is formed cotranslationally by the tetrameric association of four individual receptor subunits. In pancreatic acinar cells, there is evidence that individual channel can form a heterotetramer since multiple types of IP₃R can be detected in immunoprecipitates of specific individual receptor types. Indeed, a recent study has shown by sequential immunodepletion of individual subtypes from pancreatic lysates that homotetrameric IP₃Rs likely constitute a minority of the functional channels. Each subunit has a binding site for InsP₃, which is formed by a cluster of positively charged amino acids thought to coordinate the negatively charged phosphate groups of IP₃. The Kᵩ for binding of IP₃ to pancreatic membranes has been reported to be between 1 and 7 nM, a figure similar to that reported for other peripheral tissues such as liver. The c-terminus of each subunit is postulated to span intracellular membranes six times and a single cation selective pore is formed from this region of the protein in the tetrameric receptor. When purified or expressed in a heterologous system and then reconstituted
in planar lipid bilayers, the protein can be demonstrated to function as an InsP$_3$-gated cation channel with many of the characteristics of the release channel. The fact that the outer nuclear membrane is continuous with the ER has been exploited in patch-clamp experiments to study IP$_3$R channel activity in isolated nuclei from Xenopus laevis oocytes, COS, and DT40–3KO cells. These experiments have provided insight into the activity and regulation of the channel in a native membrane; however, to date, no information is available regarding the activity of IP$_3$R in native pancreatic membranes.

While the IP$_3$ binding pocket and channel pore are highly conserved between IP$_3$R family members, the intervening sequence between the binding region and pore is more divergent and consists of the so-called “regulatory and coupling” or “modulatory” domain. This region consisting of ~1,600 amino acids is thought to be important in modulating the Ca$^{2+}$ release properties of the IP$_3$R. Indeed, Ca$^{2+}$ release through the IP$_3$R is markedly influenced by many factors, most importantly by Ca$^{2+}$ itself. The majority of studies have indicated that all forms of the IP$_3$R are biphasically regulated by Ca$^{2+}$ in the range of 0.5–1 μM increases the steady-state open probability of the channel while at higher concentrations the activity decreases. This property of the IP$_3$R is thought to be fundamentally important in the generation of the different spatial and temporal patterns of Ca$^{2+}$ signals observed in cells. In pancreatic acinar cells, IP$_3$-induced Ca$^{2+}$ release has been shown to be inhibited when Ca$^{2+}$ is elevated and enhanced when Ca$^{2+}$ is buffered with chelators. The [Ca$^{2+}$]$_i$, together with the range of action of Ca$^{2+}$, can be manipulated by dialyzing cells with buffers exhibiting differing on-rates for Ca$^{2+}$ binding. In pancreatic acinar cells, restriction of the range of action of Ca$^{2+}$ using the slow on-rate buffer EGTA resulted in spatially restricted IP$_3$-induced spikes and the attenuation of global waves consistent with EGTA inhibiting the positive effect of Ca$^{2+}$ to facilitate Ca$^{2+}$ release between spatially separated release

Figure 4. Localization of IP$_3$R in pancreatic slices. The localization of IP$_3$R1, (A) IP$_3$R2, (B) and IP$_3$R3 (C) was determined with specific antibodies to individual InsP$_3$R types and visualization by confocal microscopy. IP$_3$R of all types predominately localized to the extreme apical pole of acinar cells, immediately below the luminal plasma membrane (arrows in A–D; compare localization of zymogen granules visualized by staining for amylase in panel D). IP$_3$R1 and IP$_3$R3 also localized to perinuclear structures (arrowheads in A and C) (from reference 53). (E) IP$_3$R3 (stained in red) are colocalized with the terminal web of the actin cytoskeleton (stained in green).
sites. In contrast, the fast on-rate buffer BAPTA resulted in larger monotonic Ca$^{2+}$ release and this was interpreted to reflect the loss of local Ca$^{2+}$ inhibition of IP$_3$R.\(^{85}\)

IP$_3$R activity is also influenced through interaction with numerous factors such as proteins, adenine nucleotides, and phosphorylation in particular by cyclic nucleotide-dependent kinases (for review see references\(^{66,84}\)). The IP$_3$R1 represents one of the major substrates for phosphorylation by protein kinase A in brain and thus represents a potentially important locus for cross-talk between the cAMP and Ca$^{2+}$ signaling systems.\(^{85}\) In pancreatic acinar cells, PKA activation results in phosphorylation of IP$_3$R.\(^{18,19}\) Functionally, phosphorylation of IP$_3$R in pancreatic acinar cells correlates with IP$_3$-induced Ca$^{2+}$ release which is decreased in terms of the magnitude and kinetics of Ca$^{2+}$ release.\(^{19,86}\)

Physiologically relevant concentrations of CCK, but not Ach, also result in PKA-dependent phosphorylation of IP$_3$R.\(^{19}\) This observation is consistent with earlier reports that CCK stimulation leads to an increase in cAMP and PKA activation. CCK-induced phosphorylation of InsP$_3$R has characteristic distinguishing effects on individual IP$_3$R subtypes\(^{89}\) and despite an approximately equal complement of IP$_3$R2 and IP$_3$R3 in acinar cells, the modulation has essentially identical properties to IP$_3$R2 in wild-type animals and is indistinguishable from IP$_3$R3 in IP$_3$R2 null animals. These data indicate that the specific properties of the IP$_3$R2 appear to dominate the overall profile of IP$_3$-induced release in pancreatic acinar cells\(^{92}\) and are consistent with the effects of ATP occurring on heterotetrameric IP$_3$R in which IP$_3$R2 is present.\(^{69}\) This hypothesis was directly tested in a heterologous expression system where concatenated IP$_3$R constructs consisting of tandem cDNAs encoding defined heterotetramers were expressed in IP$_3$R null cells. The characteristics of Ca$^{2+}$ release from heterotetrameric IP$_3$R consisting of at least two subunits of IP$_3$R2 were indistinguishable from homotetrameric IP$_3$R.\(^{69,93}\)

Introduction of G-protein βγ subunits into pancreatic acinar cells has also been shown to induce Ca$^{2+}$ release. An initial report attributed this to the activation of PLC β2 by βγ and the subsequent production of IP$_3$.\(^{94}\) A later report however demonstrated that the Gβγ-induced Ca$^{2+}$ release was independent of InsP$_3$ production and the result of a direct interaction of Gβγ with IP$_3$R.\(^{95}\) This association was confirmed by co-immunoprecipitation and shown to increase the open probability of IP$_3$R in a manner independent of IP$_3$. This interaction of IP$_3$R and βγ may be important for the action of Gαi-linked agonists in pancreatic acinar cells.

Ryanodine receptor-induced Ca$^{2+}$ release

Evidence exists for Ca$^{2+}$ release initiated through activation of ryosynode receptors (RyRs) in pancreatic acinar cells. This family of channels, best studied as the Ca$^{2+}$ release channel in skeletal and cardiac muscle, is classified as belonging to the same gene superfamily as IP$_3$R. Indeed, RyRs are modulated by similar regulators and share some sequence homology with the IP$_3$R especially in the putative Ca$^{2+}$-conducting pore region of the c-terminus. However, while IP$_3$R have an absolute requirement for IP$_3$ with Ca$^{2+}$ as an important coagonist for gating, RyRs only require Ca$^{2+}$ to open through a process termed calcium-induced calcium release (CICR). The functional expression of RyRs in pancreatic acinar cells has been indicated by a number of studies; for example, microinjection of Ca$^{2+}$ in the presence of the IP$_3$R antagonist heparin results in Ca$^{2+}$ release in mouse pancreatic acinar cells.\(^{39}\) In addition, treatment of pancreatic acinar cells with high concentrations of ryosynode known to block RyR dampens secretagogue-induced Ca$^{2+}$ signals.\(^{34,36}\) and one report has shown that low concentrations of ryanodine, which permanently opens the RyRs in a subconductance state, results in Ca$^{2+}$ release.\(^{96}\) In muscle cells, caffeine activates RyRs and results in emptying of sarcoplasmic reticulum Ca$^{2+}$ stores. In contrast, the majority of reports from pancreatic acinar cells indicate that caffeine does not elicit Ca$^{2+}$ release and actually
inhibits secretagogue-induced Ca\(^{2+}\) signaling through an action to inhibit phospholipase C and IP\(_3\),R-mediated Ca\(^{2+}\) release.\(^{96,97}\) This later effect of caffeine, together with presumably much lower numbers of RyRs in pancreatic acinar cells, probably explains the absence of caffeine-induced Ca\(^{2+}\) release.

The physical presence of RyR has, however, been difficult to demonstrate in pancreatic acinar cells with conflicting positive and negative reports of expression. For example, in one study RyR could be detected using western analysis in rodent salivary gland acinar cells but not in pancreatic acinar cells.\(^{21}\) In contrast, an initial study reported the expression using PCR analysis of RyR type 2 (RyR2) but not RyR type 1 or 3 (RyR1 and RyR3, respectively) in mRNA extracted from rat pancreatic acini.\(^{98}\) A later report, however, using single-cell PCR and western analysis demonstrated that all three types of RyR were expressed in pancreatic acini.\(^{99}\) A study performed in human acini has reported mRNA for RyR1 but not RyR2/3. Notably, in pancreatic acini.

Subsequently, it has been shown in experiments performed in sea urchin eggs where the expression using PCR analysis of RyR type 2 (RyR2) but not RyR type 1 or 3 (RyR1 and RyR3, respectively) in mRNA extracted from rat pancreatic acini.\(^{98}\) A later report, however, using single-cell PCR and western analysis demonstrated that all three types of RyR were expressed in pancreatic acini.\(^{99}\) A study performed in human acini has reported mRNA for RyR1 but not RyR2/3. Notably, \(\left[^{3}H\right]\)-ryanodine binding to membrane preparations provides evidence that the RyR protein is indeed expressed in human pancreatic acini.\(^{100}\) In all probability, the lack of consensus in these studies relates to the relatively low expression of RyR in pancreas when compared to muscle cells. While IP\(_3\),R have a well-defined localization in pancreatic acinar cells, several studies have reported that ryanodine receptors have a more diffuse distribution. Immunohistochemistry and studies with fluorescently labeled ryanodine have indicated that RyR are distributed throughout acinar cells with perhaps the greatest concentration in the basal aspect of the cell.\(^{36,98,101}\) As a result of this localization to areas of the cell with low levels of IP\(_3\),R, it has been suggested that activation of RyR plays an important role in the propagation of Ca\(^{2+}\) signals from the initial release of Ca\(^{2+}\) in the trigger zone to the basal aspects of the cell. In support of this contention, high concentrations of ryanodine in some studies have been shown to slow or spatially limit the spread of Ca\(^{2+}\) waves in mouse pancreatic acinar cells (Figure 5A and B)\(^{34,36}\) and also to selectively attenuate the peak [Ca\(^{2+}\)]\(_i\) in the basal aspects of cells.\(^{101}\)

**Ca\(^{2+}\) release stimulated by cADPR**

In a number of cell types, there is evidence that in addition to activation by Ca\(^{2+}\), the RyR activity are modulated by cyclic adenosine diphosphoribose (cADPR). cADPR was first suggested to be a Ca\(^{2+}\)-releasing second messenger based on experiments performed in sea urchin eggs where it was shown to release Ca\(^{2+}\) and function as a messenger during fertilization.\(^{102}\) Subsequently, it has been shown to release Ca\(^{2+}\) and satisfy some of the criteria for a second messenger in mammalian systems including lymphocytes, pancreatic \(\beta\)-cells, and cardiac myocytes.\(^{103-105}\) Ca\(^{2+}\) release following cADPr exposure is inhibited by blocking concentrations of ryanodine and appears to reduce the threshold for CICR through RyR.\(^{106}\) Opinion is however divided as to whether it functions through a direct effect on the RyR or indirectly through interaction with an accessory protein such as FKBP 12.6 or calmodulin.\(^{107,108}\)

In pancreatic acinar cells, the intracellular application of cADPr results in Ca\(^{2+}\) release. This observation has been reported in both rat and mouse acini with either whole-cell pipette dialysis of cADPr, liberation of cADPr from a caged precursor by two-photon flash photolysis, and by direct application to permeabilized acini.\(^{61,109-112}\) In addition, cADPr has been reported to release Ca\(^{2+}\) from a rat pancreatic microsomal preparation.\(^{113}\) The spatial localization of cADPr-induced Ca\(^{2+}\) release remains to be conclusively resolved. In an initial report, cADPr introduced by dialysis from a patch-clamp pipette into mouse pancreatic acinar cells resulted in Ca\(^{2+}\) release from the apical pole of the cell.\(^{109}\) This Ca\(^{2+}\) release was blocked by both ryanodine and interestingly the IP\(_3\),R antagonist heparin. These data were interpreted as indicating that Ca\(^{2+}\) release mediated by cADPr was dependent on both IP\(_3\),R and RyR, presumably as highly localized Ca\(^{2+}\) release initially through RyR-sensitized neighboring IP\(_3\),R to basal levels of IP\(_3\).\(^{109}\) Consistent with this view, it was also shown that low concentrations of ryanodine also resulted in Ca\(^{2+}\) release from the apical pole in a manner apparently dependent on functional IP\(_3\),R.\(^{96}\) In contrast, in a study more consistent with the localization of the majority of RyR, selective local uncaging of cADPr in different regions of an acinar cell by two-photon photolysis reported that the basal aspect of the cell was more sensitive to cADPr.\(^{110}\) These data are consistent with a report that in permeabilized acini the apical pole exhibited a higher affinity for IP\(_3\) but cADPr released Ca\(^{2+}\) exclusively from the basal aspects of the cell.\(^{61}\)

Important questions to address, which are required to establish cADPr as a bona fide Ca\(^{2+}\)-releasing second messenger in pancreatic acinar cells, are to demonstrate that the molecule is produced following secretagogues stimulation and that it is necessary for Ca\(^{2+}\) release. To these ends, it has been reported that CCK and ACh but not bombesin stimulate the activity of a cytosolic ADP-ribosyl cyclase resulting in the production of cADPr.\(^{114}\) One such enzyme which possesses this activity is CD38. Ca\(^{2+}\) signaling events in acinar cells prepared from CD38 null mice are dampened and appear reminiscent of RyR blockade.\(^{115}\) In addition, 8-NH\(_2\),cADPr, a structural analog of cADPr which antagonizes the effect of cADPr, has been reported to block CCK and bombesin but not ACh or IP\(_3\),R-induced Ca\(^{2+}\) signals in mouse pancreatic acinar cells.\(^{116,117}\) This later result, while not internally consistent with the ability of ACh to induce the formation of cADPr, has been suggested to indicate that CCK stimulation preferentially couples to the generation of cADPr and thus may account for
some of the distinct characteristics of CCK-induced Ca\(^{2+}\) signals reported.

**Nicotinic acid dinucleotide phosphate-induced Ca\(^{2+}\) release**

An additional putative messenger that potently induces Ca\(^{2+}\) release in pancreatic acinar cells is nicotinic acid adenine dinucleotide phosphate (NAADP). Once again, the activity of this agent was first reported to play a role in invertebrate fertilization. Although it has been reported that NAADP-induced Ca\(^{2+}\) release from nuclei isolated from mouse pancreatic acini is dependent on RyR,\(^ {60}\) the majority of evidence suggests that the receptor is likely to represent a novel Ca\(^{2+}\) release channel since while the activity of IP\(_3\)R and RyR exhibits a bell-shaped dependence on Ca\(^{2+}\), the putative NAADP receptor does not support CICR.\(^ {118}\) While this property of NAADP is not well suited to play a role in the propagation of Ca\(^{2+}\) waves, it has been suggested that NAADP is required to initiate Ca\(^{2+}\) signals and this initial Ca\(^{2+}\) increase subsequently recruits IP\(_3\)R and RyR. This idea is supported by the observation that NAADP introduced via the patch pipette into mouse pancreatic acinar cells results in Ca\(^{2+}\) release in the apical pole but that this release is absolutely dependent on both InsP\(_3\)R and RyR.\(^ {111}\) These data suggest that Ca\(^{2+}\) release through

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**Figure 5. Contribution of RyR to global Ca\(^{2+}\) signals in pancreatic acinar cells.** Global Ca\(^{2+}\) signals were initiated by photolysis of caged-IP\(_3\). In (A) the images and kinetic traces show that global Ca\(^{2+}\) signals can be initiated multiple times following exposure to 1,4,5-IP\(_3\). However, as shown in (B), exposure to a high concentration of ryanodine, known to inhibit RyR, leads to a slowing in the progression of the Ca\(^{2+}\) wave and to the restriction of the signal to the apical pole of the cell following stimulation with 1,4,5-IP\(_3\) (compare (B) I; absence of ryanodine and (B) IV; exposure to ryanodine for 15 minutes) (from reference \(^ {36}\)).
NAADP is quantitatively very small but ideally localized to sensitize IP\(_3\)R and RyR. While IP\(_3\)R and RyR reside in the ER, it has been suggested that NAADP primarily acts on a distinct store probably an acidic lysosome-related organelle.\(^{119}\) This idea is primarily based on the observation that NAADP-induced Ca\(^{2+}\) signaling but not cADP-ribose or IP\(_3\)-induced Ca\(^{2+}\) release is inhibited by experimental maneuvers which either inhibit vacuolar-type H\(^+\) ATPase or result in osmotic disruption of lysosomes.\(^{119}\)

The concentration-response relationship for NAADP-induced Ca\(^{2+}\) release also displays unique properties. NAADP-induced Ca\(^{2+}\) release is biphasic; nanomolar levels induce Ca\(^{2+}\) release while micromolar concentrations fail to release Ca\(^{2+}\) but render the mechanism refractory to subsequent stimulation.\(^{120}\) Provocatively, exposure of mouse pancreatic acinar cells to inactivating concentrations of NAADP also renders cells refractory to stimulation with threshold concentrations of CCK but not ACh or bombesin, and disruption of the NAADP releasable store selectively disrupts CCK-induced Ca\(^{2+}\) signals.\(^{117,120}\) In addition, NED-19 (an inhibitor of NAADP action) attenuates CCK- but not ACh-induced Ca\(^{2+}\) responses.\(^{121}\) These data suggest that CCK and NAADP under these conditions may utilize a common mechanism to induce Ca\(^{2+}\) release.

In support of this contention, physiologically relevant concentrations of CCK can also be shown to result in the production of NAADP while ACh stimulation does not result in measurable accumulation.\(^{122}\) The molecular mechanism responsible for coupling CCK receptor occupation to NAADP production is at present not completely understood. Evidence suggests, however, that the enzyme CD38 may also be a candidate for the hormone-responsive NAADP synthase because CCK-stimulated NAADP formation is absent in CD38 null transgenic animals.\(^{115}\) This activity of CD38 occurs at acidic pH and this requirement appears to be fulfilled by the expression of some of the enzymes in an endosomal compartment.\(^{115}\)

The two-pore channel (TPC) family of proteins has recently been proposed as a candidate for the cognate receptor for NAADP.\(^{118,123}\) The TPC gene family is related to the superfamily of voltage-gated ion channels and consists of three distinct gene products which are expressed in both plants and animals.\(^{123}\) The primary supporting evidence for the idea that TPC represents a NAADP receptor is that the expression of TPCs appears to confer Ca\(^{2+}\) release activity in the presence of NAADP.\(^{118,123,124}\) Additionally, the expression of TPCs also leads to specific binding activity of radiolabeled NAADP, and TPCs are primarily expressed in an endolysosomal compartment, consistent with the prominent site of NAADP-induced Ca\(^{2+}\) release.\(^{118,123}\) Since this specific binding is much lower affinity than the functional effects of NAADP and specific binding can be observed to co-immunoprecipitated proteins,\(^{125,126}\) it is questionable whether the effects of NAADP are mediated through an accessory protein. In support of a role for TPC2 as a target of NAADP, Ca\(^{2+}\) release was reduced in acini prepared from TPC-2 knockout animals.\(^{121}\) Of note, NAADP-induced Ca\(^{2+}\) release was also reduced in RYR3 knockout animals and in permeabilized cells incubated with α-RYR1 antibodies. The latter observation is consistent with reports from T lymphocytes that suggest RYR are the target of NAADP.\(^{127}\) In total, these data led the authors to suggest that both RYRs and TPC channels might function in pancreatic acinar cells to provide the trigger for Ca\(^{2+}\) release following CCK stimulation.

**Cellular mechanisms underlying Ca\(^{2+}\) influx across the plasma membrane**

Ca\(^{2+}\) influx from the extracellular space is essential to sustain Ca\(^{2+}\) signaling in pancreatic acinar cells and in turn the long-term maintenance of secretion.\(^{11}\) This influx is not blocked by antagonists of voltage-gated Ca\(^{2+}\) channels but is attenuated by lanthanides.\(^{10,11}\) Ca\(^{2+}\) influx is also sensitive to changes in extracellular pH, being enhanced by alkaline and inhibited in acidic conditions.\(^{10,11}\)

**Store-operated Ca\(^{2+}\) entry and its molecular components**

Functionally, Ca\(^{2+}\) influx can be initiated by substantial depletion of intracellular Ca\(^{2+}\) pools, the so-called “store-operated” or “capacitative” Ca\(^{2+}\) entry (SOCE) pathway.\(^{128}\) This pathway can be readily demonstrated by inhibition of ER Ca\(^{2+}\) pumps with the plant sesquiterpene lactone thapsigargin, which results in Ca\(^{2+}\) influx independent of receptor activation and PI hydrolysis.\(^{11,128-130}\) Recently, significant progress has been made regarding the molecular candidates responsible for the SOCE pathway. Using siRNA screens, the first breakthrough was the identification of stromal interaction molecule 1 (STIM-1) as the ER Ca\(^{2+}\) sensor responsible for coupling intracellular Ca\(^{2+}\) store depletion to the opening of the Ca\(^{2+}\) permeable conductance in the plasma membrane.\(^{131-133}\) STIM-1 is an integral ER membrane protein, which harbors an EF hand-type Ca\(^{2+}\)-binding domain localized in the ER lumen. Following depletion of ER stores, Ca\(^{2+}\) dissociates from the ER hand and this results in the aggregation of STIM-1 molecules to areas of the ER close to the plasma membrane where it can physically gate the ion channel responsible for mediating Ca\(^{2+}\) influx.\(^{134,135}\) Strong candidates for the actual pore-forming components of the pathway have also been identified. Using siRNA screens and complimented by linkage analysis of patients with severe combined immunodeficiency, studies have identified members of the Orai family of proteins as the channel constituents of the archetypal SOCE current, the so-called, calcium-release activated current (I\(_{\text{CRAC}}\)). The channel has been extensively characterized in lymphocytes as a highly selective Ca\(^{2+}\) channel.\(^{136-138}\) In particular,
Calcium signaling in pancreatic acinar cells

homing multimers of Orai form channels with the biophysical characteristics of $I_{\text{CRAC}}$.\textsuperscript{138} Both STIM-1 and Orai-1 are expressed in mouse pancreatic acinar cells and thus represent good candidates for mediating SOCE.\textsuperscript{137,140,141} Lower levels of secretagogue stimulation result in only modest ER Ca\textsuperscript{2+} depletion, which may not be sufficient to activate STIM-1. While not directly demonstrated in pancreatic acinar cells, minor store depletion may be sensed by STIM-2, a family member which because of increased affinity for ER Ca\textsuperscript{2+}, can activate Orai channels following physiological stimulation.\textsuperscript{142} It is, however, clear that marked global depletion of ER Ca\textsuperscript{2+} occurs upon stimulation with maximal concentrations of secretagogues and upon exposure to pancreatic toxins, such as bile acids and fatty acid esters of ethanol.\textsuperscript{1,22,143} Thus, the SOCE pathway may be important during strong stimulation and during pathological Ca\textsuperscript{2+} depletion of ER stores. Indeed, it has been suggested that activation of this pathway is responsible for the inappropriate intracellular activation of trypsin, which occurs in models of acute pancreatitis.\textsuperscript{144–147} Consistent with this idea, inhibition of Ca\textsuperscript{2+} influx with pharmacology that targets SOCE has been shown to reduce the severity of pancreatitis in several experimental animal disease models\textsuperscript{148–150} as well as mitigating the detrimental cellular hallmarks of pancreatitis \textit{ex vivo}.\textsuperscript{151} Further, maneuvers which inhibit Ca\textsuperscript{2+} release, including blockade of IP$_R$ with caffeine, which prevents depletion of the ER and thus activation of SOCE, also are protective in experimental pancreatitis.\textsuperscript{152}

\textbf{Contribution of TRP channels}

In exocrine cells there is also evidence that members of the canonical transient receptor potential channel (TRPC) family contribute to Ca\textsuperscript{2+} influx following store depletion.\textsuperscript{137,153,154} This channel, while Ca\textsuperscript{2+} permeable, is a nonselective cation channel, exhibiting significant Na\textsuperscript{+} permeability. STIM-1 has been reported to also function as the ER calcium sensor for this class of proteins.\textsuperscript{155} Further, there is molecular and biochemical evidence to suggest that a tertiary complex of STIM-1/TRPC/Orai-1 may contribute to the native SOCE in acinar cells.\textsuperscript{137,156} TRPC 3 and 6 are expressed in pancreatic acinar cells.\textsuperscript{153} In TRPC 3 \textit{null} transgenic animals, the magnitude of secretagogue-stimulated Ca\textsuperscript{2+} influx is reduced and this is associated with reduced agonist-stimulated amylase secretion.\textsuperscript{153} In addition, the frequency of agonist-stimulated Ca\textsuperscript{2+} oscillations was reduced in TRPC 3 \textit{null} animals supporting the view that Ca\textsuperscript{2+} influx through this channel may also serve to maintain Ca\textsuperscript{2+} oscillations.\textsuperscript{155} Interestingly, the absence of TRPC 3 is also protective in animal models of pancreatitis indicating that SOCE may be deleterious under these conditions.\textsuperscript{153}

While the spatial properties of the Ca\textsuperscript{2+} signal can be largely attributed to the localization of the Ca\textsuperscript{2+} release machinery, recent data have also suggested that the distribution of the components of the SOCE Ca\textsuperscript{2+} influx pathway may also be spatially heterogeneous. The majority of Orai-1 and TRPC3 is reported to be localized to the apical and lateral plasma membrane.\textsuperscript{140,141,153,157} One study has shown that following store depletion some STIM-1 translocates to the apical domain and is colocalized with Orai-1. Further, this study demonstrated that Ca\textsuperscript{2+} influx following store depletion could be detected earlier in the apical region when compared to more basal aspects of the cell.\textsuperscript{137} Of note, a majority of STIM-1 does not colocalize with Orai-1 following store depletion.\textsuperscript{137,140} Indeed, a study monitoring the localization of adenovirally expressed fluorescently tagged STIM-1 reported little colocalization with Orai-1 and that the vast majority of STIM-1 translocated to the basolateral aspects of cells following stimulation.\textsuperscript{140} Further work will be necessary to unequivocally confirm the cellular localization of this influx pathway in acinar cells.

\textbf{Contribution of ARC channels}

As stated earlier, during secretagogue stimulation only relatively minor depletion of the ER pool as a whole occurs.\textsuperscript{22} This observation raises the question whether SOCE operates under physiological conditions. If global ER Ca\textsuperscript{2+} is not markedly reduced during physiological stimulation, it follows that for SOCE to be activated under these conditions, local depletion of the strands of ER that infiltrate the apical domain,\textsuperscript{158} which are replete with IP$_R$,\textsuperscript{53} must occur allowing the activation of STIM1/2. A contrary view is that a different mechanism largely controls Ca\textsuperscript{2+} influx during physiological stimulation. In support of this idea, an electrophysiological study of mouse pancreatic acinar cells failed to detect SOCE currents following stimulation with physiologically relevant concentrations of agonists. Under these conditions, it was reported that a channel activated by arachidonic acid was predominately responsible for Ca\textsuperscript{2+} influx.\textsuperscript{159} This conductance has been termed $I_{\text{ARC}}$, and is present in many nonexcitable cell types.\textsuperscript{160,161} Notably, this channel also requires STIM-1 for activation and the pore is formed from multimers of Orai1 and Orai3.\textsuperscript{162}

\textbf{Cellular mechanisms underlying Ca\textsuperscript{2+} clearance from the cytosol}

Following [Ca\textsuperscript{2+}]i elevation, mechanisms must be present to reduce [Ca\textsuperscript{2+}]i rapidly and efficiently during the falling phase of Ca\textsuperscript{2+} oscillations and ultimately to terminate Ca\textsuperscript{2+} signals following secretagogue removal. Moreover, the resting [Ca\textsuperscript{2+}]i of 50–200 nM must be maintained in the face of a large concentration gradient from the cell exterior, leak from the ER, and the negative intracellular potential, all of which would tend to drive Ca\textsuperscript{2+} into the
cytoplasm. Homeostasis is accomplished by a variety of pumps and transporters that have specific distribution on both the plasma membrane and on the membranes of various organelles.

**Ca\(^{2+}\) pumping across the PM**

Tepikin and colleagues employing a technique where the extracellular [Ca\(^{2+}\)] is monitored by an indicator in a small volume of extracellular fluid demonstrated that Ca\(^{2+}\) is extruded across the plasma membrane following agonist stimulation.\(^{163,164}\) This in all probability occurs by Ca\(^{2+}\) pumps of the plasma membrane Ca\(^{2+}\)-ATPase gene family (PMCA). Ca\(^{2+}\)-ATPase activity is present in plasma membrane preparations isolated from pancreatic acinar cells and immunoblotting demonstrates the expression of PMCA family members.\(^{21,165,166}\) Upon supramaximal stimulation with CCK or Ach, the amount of Ca\(^{2+}\) lost from the cell approximates the entire agonist-releasable pool.\(^{167}\) Ca\(^{2+}\) extrusion also occurs during more physiological stimulation with CCK and the activity follows the [Ca\(^{2+}\)]\(_i\).\(^{163}\) PMCA is also important for maintaining resting [Ca\(^{2+}\)]\(_i\), since its activity can be demonstrated at basal [Ca\(^{2+}\)]\(_i\). Indeed, the rate of pumping has been shown to have a steep dependence on [Ca\(^{2+}\)]\(_i\) (Hill coefficient of ~3) and is effectively saturated at [Ca\(^{2+}\)]\(_i\) above 400 nM.\(^{168}\) The activity of the PMCA can also be increased by agonist stimulation in a manner independent of Ca\(^{2+}\) and this may reflect modulation of the pumps activity by phosphorylation or association with regulatory proteins.\(^{169}\) PMCA is not homogeneously distributed over the entire plasma membrane and appears to be most abundant in the luminal and lateral plasma membrane.\(^{21}\) The localization of PMCA correlates with the site of most apparent Ca\(^{2+}\) pumping activity since Ca\(^{2+}\) extrusion occurs preferentially from the apical aspects of mouse pancreatic acinar cells when monitored using an indicator with limited diffusional mobility.\(^{170}\)

**Ca\(^{2+}\) uptake into the ER**

Ca\(^{2+}\) pumps are also expressed on ER membranes.\(^{171,172}\) Indeed, the ER in pancreatic acinar cells has been shown to effectively function as a single continuous Ca\(^{2+}\) store.\(^{22,173}\) This has been best illustrated by demonstrating that ER Ca\(^{2+}\) pump activity, exclusively at the basal pole, can recharge the ER following maximal agonist stimulation such that Ca\(^{2+}\) signals can be initiated at the apical pole.\(^{173}\) ER Ca\(^{2+}\)-ATPases belong to the sarcoplasmic and endoplasmic reticulum Ca\(^{2+}\) ATPase gene family (SERCA). Both SERCA 2A and SERCA 2B have been reported to be expressed in pancreatic acinar cells and specific subcellular distributions of specific SERCA pumps have been reported.\(^{21}\) SERCA 2A appears to be distributed very similar to InsP\(_3\)R exclusively in the apical pole while SERCA 2B predominately resides in the basal aspects of the cell. All SERCA pumps are inhibited by thapsigargin and treatment results in Ca\(^{2+}\) leak from the ER store. In pancreatic acinar cells, thapsigargin treatment results in a uniform rise in [Ca\(^{2+}\)]\(_i\), and abolishes the characteristic secretagogue-stimulated apically initiated Ca\(^{2+}\) wave even before the ER is fully depleted.\(^{21}\) The later observation indicates that the microenvironment created by SERCA pumps is crucial for the initiation of Ca\(^{2+}\) signals in the apical pole during Ca\(^{2+}\) oscillations. This could occur as a function of the SERCA pump controlling either the cytosolic [Ca\(^{2+}\)] or local luminal ER [Ca\(^{2+}\)] in the apical pole as both SERCA pumps and IP\(_3\)R are markedly influenced by the [Ca\(^{2+}\)] on both faces of the ER membrane.\(^{23,174}\)

**Ca\(^{2+}\) uptake by mitochondria**

A further Ca\(^{2+}\) uptake mechanism that plays a significant role in spatially shaping Ca\(^{2+}\) signals in pancreatic acinar cells is through Ca\(^{2+}\) sequestration into mitochondria. Mitochondrial Ca\(^{2+}\) uptake occurs as a function of the large electrical potential across the inner mitochondrial membrane via a ruthenium red-sensitive Ca\(^{2+}\) uniporter.\(^{175}\) Because the Ca\(^{2+}\) uniporter is a relatively low-affinity, high-capacity transporter, it was generally believed that mitochondrial Ca\(^{2+}\) uptake was only relevant under pathological conditions when the [Ca\(^{2+}\)] was significantly elevated for prolonged periods of time. Recently, however, with the advent of mitochondrially targeted indicators, this idea has been reevaluated given that it has become clear that mitochondria function during normal physiological Ca\(^{2+}\) signaling in many cell types.\(^{176}\) Moreover, it is now thought that mitochondrial Ca\(^{2+}\) uptake is important not only for shaping cytosolic Ca\(^{2+}\) signals but also for stimulating the production of ATP as key enzymes in the TCA cycle are Ca\(^{2+}\)-dependent.\(^{177}\) It appears that the privileged localization of mitochondria very close to Ca\(^{2+}\)-release sites, where Ca\(^{2+}\) is presumably very high, allows the Ca\(^{2+}\) uniporter to function effectively under these conditions.

The mitochondria play a key role in regulating calcium homeostasis. The mitochondrial calcium uniporter (MCU) is a highly Ca\(^{2+}\)-selective channel that takes up calcium from the mitochondrial matrix. The MCU is composed of several subunits, including MCU1, MCU2, and MCU3. When calcium levels in the mitochondrial matrix rise to a threshold level, the MCU opens, allowing calcium to be pumped into the matrix. This helps to maintain the low calcium concentration in the cytosol and prevents calcium overload in the mitochondrial matrix. The MCU is regulated by calcium, with an increase in intracellular calcium levels leading to its activation. The MCU is also regulated by other factors such as the calcium-sensing receptor (CaSR) and the mitochondrial calcium release-activated calcium channel (MIRC). The MCU is important for the regulation of calcium homeostasis in various physiological processes, including muscle contraction, neuronal transmission, and cell proliferation.
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...evidence for its role in acini. Interestingly, disruption of mitochondrial Ca\textsuperscript{2+} signaling did not alter the severity of experimental pancreatitis despite compromising mitochondrial bioenergetics.

In pancreatic acinar cells, mitochondria are indeed in close proximity to Ca\textsuperscript{2+}-release sites since stimulation with physiological concentrations of both CCK and Ach, which raise global [Ca\textsuperscript{2+}], only to submicromolar levels, leads to mitochondrial Ca\textsuperscript{2+} uptake. Furthermore, mitochondrial Ca\textsuperscript{2+} uptake in pancreatic acinar cells is coupled to the conversion of NAD to NADH. Further, stimulation of pancreatic acinar cells with Ca\textsuperscript{2+}-mobilizing secretagogues has been shown to result in the net generation of ATP as reported by adenovirally expressed mitochondrially targeted luciferase constructs. In common with other Ca\textsuperscript{2+} clearance mechanisms in acini, energized mitochondria also have specific subcellular localization (Figure 6A). The majority of studies have reported that mitochondria are concentrated in a perigranular “belt,” together with additional further subpopulations surrounding the nucleus and immediately below the basolateral plasma membrane. Strikingly, it appears that these perigranular mitochondria play a role in limiting the spread of Ca\textsuperscript{2+} signals upon stimulation with threshold concentrations of

Figure 6. Functional consequences of mitochondrial distribution in pancreatic acini. In (A), the localization of active mitochondria was visualized by confocal microscopy in living mouse pancreatic acini loaded with mitotracker red. (A) shows mitotracker red fluorescence merged with a phase image of a small mouse acini. The predominant localization of mitochondria is to a perigranular belt surrounding the zymogen granules. (B) Photolysis of threshold concentrations of 1,4,5-IP\textsubscript{3} results in apically limited Ca\textsuperscript{2+} signals as shown in the images and kinetic traces in (B) I (red trace apical ROI; black trace basal ROI). In the same cell, following disruption of the mitochondrial membrane potential, and thus mitochondrial Ca\textsuperscript{2+} uptake, an identical exposure to 1,4,5 IP\textsubscript{3} results in a global Ca\textsuperscript{2+} signal. These data suggest that functional mitochondria are required to constrain Ca\textsuperscript{2+} signals in the apical portion of the acinar cell (from reference 36).
Figure 7. Intracellular Ca\(^{2+}\) signaling in pancreatic acinar cells is initiated by the binding of acetylcholine to muscarinic M3 receptors (M3R) and by cholecystokinin (CCK) to CCK receptors, predominately the CCK1R in mice and rats. Both receptors are classical seven-transmembrane domain receptors coupled to guanine nucleotide-binding proteins (G proteins). Activation of both receptors leads to stimulation of Gαq and increased activity of phospholipase C-β (PLC), which cleaves the membrane phospholipid phosphatidylinositol,4,5,-bisphosphate (PIP2) into diacylglycerol and inositol 1,4,5-trisphosphate (IP\(_3\)). IP\(_3\) diffuses through the cytoplasm and interacts with InsP3 receptors (IP3Rs), largely type 2 and 3 present predominantly on the apical endoplasmic reticulum (ER) resulting in Ca\(^{2+}\) release into the cytoplasm. Ca\(^{2+}\) release from IP3R acts a coagonist to increase further IP3R activity and also acts on ryanodine receptors (RyRs) to induce Ca\(^{2+}\) release. Depletion of Ca\(^{2+}\) within the ER results in Ca\(^{2+}\) influx from the extracellular space. The ER Ca\(^{2+}\) sensor has been identified as stromal interaction molecule-1 (stim-1). Following ER depletion, Ca\(^{2+}\) is released from an EF hand present in a domain of stim-1 within the ER lumen and this results in aggregation of several stim-1 molecules. Aggregation of stim-1 is sufficient to gate plasma membrane Ca\(^{2+}\) channels and leads to Ca\(^{2+}\) influx. Good candidates for the channel pore are the proteins Orai-1 and TRPC3. CCK receptor stimulation also stimulates ADP-ribosyl cyclase activity resulting in the formation of two additional Ca\(^{2+}\)-mobilizing second messengers: nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic-ADP ribose (cADPr). The particular cyclase responsible has not been defined in pancreas but good candidates include CD38 and CD157. cADPr is generally thought to act on RyR, while the target of NAADP is currently the subject of intense research. Candidates include the RyR and two-pore channel (TPC). In addition to the ER, Ca\(^{2+}\) can also be released from a store, which accumulates Ca\(^{2+}\) in a manner dependent on a proton gradient—known as the “acidic store.” This pool likely represents the endolysosomal compartment. This pool has been reported to be responsive to IP3, cADPr, and NAADP and may represent the store initially released following receptor stimulation. Ca\(^{2+}\) is removed from the cytoplasm by the concerted action of SERCA (ER Ca\(^{2+}\)-ATPase), PMCA (PM Ca\(^{2+}\)-ATPase), and the action of mitochondrial uniporter (MCU).
agonist as disruption of mitochondrial Ca\(^{2+}\) uptake facilitates the spread of normally spatially confined Ca\(^{2+}\) transients (Figure 6B).

Summary

A cartoon summarizing the molecular components currently believed to be involved in the processes of Ca\(^{2+}\) release, Ca\(^{2+}\) influx, and Ca\(^{2+}\) clearance from the cytosol of pancreatic acinar cells following secretagogue stimulation is shown in Figure 7.

References


15. Park MK, Petersen OH, Tepikin AV. The endoplasmic reticulum as one continuous Ca\(^{2+}\) pool: visualization of rapid Ca\(^{2+}\) movements and equilibration. EMBO J 1999; 18:5729–5739.


62. Wojcikiewicz RJ. Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. J Biol Chem 1995; 270:11678–11683.


87. Camello PJ, Petersen OH, Toescu EC. Simultaneous pres-
effect on the agonist-evoked Ca$^{2+}$ signal in pancreatic acinar cells. Pflugers Arch 1996; 432:775–781.
111. Cancela JM, Gerasimenko OV, Gerasimenko JV, Tepikin AV, Petersen OH. Two different but converging messenger pathways to intracellular Ca$^{2+}$ release: the roles of nicotinic acid adenine dinucleotide phosphate, cyclic ADP-ribose and inositol trisphosphate. EMBO J 2000; 19:2549–2557.
117. Burdakov D, Cancela JM, Petersen OH. Bombesin-induced cytosolic Ca$^{2+}$ spiking in pancreatic acinar cells depends on...


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Chapter 9
Role of the actin cytoskeleton in acinar cell protein secretion
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Introduction

The actin cytoskeleton that is ubiquitous in nonmuscle cells is largely made up of β-actin and γ-actin, the products of the ACTB and ACTG1 genes, respectively. These two actins differ by only four amino acids but they appear to have different functions, as mutations in the two genes have very different effects. In the mouse acinar cell, Actb mRNA is expressed more than twofold greater than Actg1 (Table 1); presumably their protein levels match this expression, although this has not been measured in the pancreas. In gastric parietal cells, β-actin is more localized to the apical cortical cytoskeleton whereas γ-actin is more localized along the basolateral plasma membrane (BLPM). If the same is true in the acinar cell, then β-actin is more likely to be important in protein secretion, which is also localized to the apical surface of the cell. In the acinar cell, γ-actin may be involved in the BLPM blebs that form under supraphysiologic stimulation. From here on, actin will be referred to as if it were a single gene/protein.

Results from studies of the actin cytoskeleton in the exocrine pancreas are the primary source for this review. However, there are many gaps in our knowledge regarding exocrine pancreas function. When data from pancreas are lacking, results from other cell types that can reasonably be assumed to be true of the acinar cell are briefly discussed. It should be noted that even though there are commonalities shared by all cells with a regulated secretory pathway (e.g., regulation by small GTPases and use of SNAREs to mediate granule-vesicle—plasma membrane (PM) recognition and fusion), there can be important differences.

For example, the role of actin coating of granules-vesicles during the content release process may serve different functions related to the speed of product release required (see: Dynamics of Acinar Actin in the Stimulated State; below).

Actin Basics

Actin is a 42-kDa protein that exists in two distinct forms in the cell: monomeric (globular or G-actin) and polymeric (filamentous or F-actin). Actin monomers have the inherent behavior of self-assembly into filaments and prevention of inappropriate polymerization is controlled by several G-actin-binding proteins that stoichiometrically outnumber G-actin by more than threefold. However, in general, the majority of a cell’s actin is in the form of F-actin, estimated to comprise ~75 percent of the total. The structure of F-actin microfilaments (MFs) is a polarized double helix such that the barbed (+) end is where new monomers are primarily added and the pointed (-) end is where F-actin primarily releases actin monomers. Actin is an ATPase, and for G-actin the ATP-bound form predominates and is thus primed for addition to an actin filament. Once added to the filament, the ATPase is activated and ATP is hydrolyzed to ADP.

F-actin is highly dynamic and there are numerous proteins that regulate the assembly and disassembly of MF. Interrogation of a published microarray study done in the author’s lab for actin cytoskeleton elements was performed and is presented in Table 1. This yielded a number of genes, the most highly expressed of which not surprisingly were β- and γ-actins as well as several proteins known to bind to or regulate dynamics of actin. Many of these proteins directly interact with actin and are thus called actin-binding proteins (ABPs). The majority of genes on this list and their functions were discovered using other cell systems and have not been investigated in the pancreatic acinar cell. Microfilament (MF) structures in cells may appear to be highly stable structures, but they are constantly undergoing growth at the (+) end and disassembly at the (-) end, a process called treadmilling. Thus, the apparent stability of MFs is actually due to a dynamic equilibrium. It has been estimated that the ~1-μm-long MFs of the intestinal microvillus turns over every 20 minutes by treadmilling, and a similar rate is also believed to be true of the terminal web (TW) that underlies the apical plasma membrane (APM). In addition to proteins that induce disassembly of MFs, there are numerous actin-binding proteins that regulate nucleation of new fibers and growth of existing fibers. Actin can exist as higher-order bundles of multiple MFs in linear arrays as well as branched MF structures, with accessory proteins providing lateral cross-links.
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Actin Localization and Dynamics in the Acinar Cell

Methods to visualize F-actin

Most studies of actin in the acinar cell have been of MFs and usually after chemical fixation of the cells in either the unstimulated basal state, with a secretory stimulus, and/or after a specific experimental manipulation of the cells. The most commonly used technique is fluorescently tagged phalloidin, a fungal toxin with a high affinity for F-actin (see references from Williams et al., Jang et al., and Hou et al. on the Pancreapedia website). An example of FITC-phalloidin labeling of mouse acini from the author’s lab is

Table 1. Microarray analysis of mouse pancreas for actin and associated proteins. From microarray analysis of mouse pancreas (http://www.ncbi.nlm.nih.gov/geo/, accession GDS567). Data are means from three wild-type young adult mice (see reference for details). Value: “normalized signal count data, reflecting the relative measure of abundance of each transcript” as defined on the GEO website. This list is not comprehensive. Many actin cytoskeletal genes were expressed below detection limits or were not represented on the microarrays.

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Figure 1. FITC-phalloidin staining of F-actin in mouse acinar cells under basal and stimulated conditions. Mouse acini were incubated without (basal) or with 1 μM carbachol (stimulated). The cells were fixed in suspension with 2 percent formaldehyde and 0.1 percent saponin to permeabilize cell membranes and 1 mM MgCl₂ and 5 mM EGTA to stabilize F-actin. The cells were then stained with 0.6 μM FITC-phalloidin and imaged on a confocal microscope, collecting 0.5-μm optical sections. These images are single optical slices. The acinar lumen is bounded by the APM (left panel), under which is the strongly labeled TW. The BLP (left panel) is much weaker stained than the APM. After stimulus, there are actin-coated granules (ACGs) along the APM (right panel, arrowheads) and the TW is more weakly stained (these images were preliminary data for the work presented in reference 12).

shown in Figure 1, which shows the MFs of acinar cells under basal and stimulated conditions. Phalloidin is a stabilizer of F-actin MFs, so when used in living cells it perturbs the actin cytoskeleton. This property limits use of phalloidin to static imaging of F-actin after fixation and precludes its use to visualize dynamics of actin in live cells where other techniques are used (see below).

F-actin can also be recognized in standard transmission electron microscopy as electron-dense ~6-nm-diameter filaments (smaller than microtubules, ~24 nm; and intermediate filaments, ~10 nm).

Until recently, MFs have been difficult to visualize in living cells without perturbing the structure and function of the cytoskeleton. Adding tags such as green fluorescent protein (GFP) to β-actin perturbs the actin cytoskeleton into which it is incorporated (discussed in reference 13). A newer approach is to make chimeric fluorescent proteins from ABPs. Using such an approach the Lifeact reporter was made, which consists of a 17-amino-acid actin-binding domain from the ABP140 protein, fused to GFP. 14 This chimeric protein has a 30-fold greater affinity for F-actin than G-actin, was found to not interfere with known activities of the actin cytoskeleton, and it faithfully reproduces known F-actin staining patterns in a variety of cell types even under dynamic situations where the actin cytoskeleton is being remodeled. 13 This construct was later used to make a transgenic mouse expressing the fluorescent F-actin reporter in all tissues, called the Lifeact mouse. 15

G-actin can also be visualized using fluorescently tagged DNase I, which binds to both G- and F-actin, with higher affinity to the former, 16 although an analysis of DNase I inhibition by highly purified G- and F-actin concluded that both forms have similar affinity for DNase I. 17 An example of fluorescent DNase I staining of G-actin in the acinar cell shows a diffuse cytoplasmic distribution which is not very informative. 18 Visualization of G-actin has not been used to study the actin cytoskeleton of the acinar cell to any extent. Nor has there been systematic investigation of G-actin ABPs in the acinar cell.

Acinar cell actin in the resting state

The single largest pool of F-actin in the acinar cell is in the TW, 19 also called subapical actin or cortical actin (Figure 1, left panel).

The TW is a dense arrangement of MF that forms a continuous sheet-like network ~200 nm thick 21 under the APM. Among ABPs, members of the red blood cell spectrin family cross-link TW MFs and also link the TW to perpendicular MF that extend into the microvilli (MV), as shown in intestinal epithelium. 22 As compared to the villus epithelium of the small intestine where MV are best characterized, the acinar cell MV are relatively sparse. A second actin-based structure is a ring of MFs that are attached to the adherens junctions near the apical surface and encircle each acinar cell. Because this is a ring-shaped structure, it is less easily visualized than the sheet-like TW. There are also recently described MF bundles that project perpendicularly from the TW into the cytoplasm that are called actin cables. 23 Lesser amounts of MFs are found along the BLP of the acinar cell (Figure 2, left panel). Because of the large amount of F-actin in the TW, it can be difficult to visualize the other sites of MFs when using fluorescent labeling approaches. For example, the recently described actin cables that run perpendicular to the TW in acinar cells are not observed using phalloidin staining but are visualized using a chimeric ABP domain fused to GFP (Lifeact; see below). 23

Effects on secretion of pharmacological agents and molecular approaches that perturb the actin cytoskeleton

There is a large data set about the acinar cell actin cytoskeleton obtained using a variety of approaches. Because the actin cytoskeleton is so important to eukaryotic cells, there are numerous naturally occurring substances produced by a variety of organisms that interfere with the organization of actin, and many of these are available as research tools. Also, investigators have produced constitutively active (CA) and dominant negative (DN) constructs to stimulate or inhibit upstream pathways that control the actin cytoskeleton.
A summary of these toxins and reagents and their effects on the actin cytoskeleton and acinar cell amylase release is given in Table 2. Some of the reported effects of a particular agent are contradictory but these are likely attributable to differences in concentration of the agent (e.g., latrunculins\textsuperscript{24} have been used over the wide range of 0.3 μM to 100 μM), time of exposure, and temperature at which the experiment was performed (discussed in reference \textsuperscript{25}). The

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mechanism</th>
<th>Cytoskeleton Effects</th>
<th>Amylase Release / Other</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>LatA</td>
<td>Binds G-actin monomers, inhibiting addition to MF</td>
<td>no effect on ACG; loss of actin cables</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TW resistant; BPLM MFs lost</td>
<td>inhibits / 28% decrease in MF in 10 min; 44% decrease at 2 hr</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>inhibits / prevents BLM blebs by supramaximal CCK</td>
<td>29</td>
</tr>
<tr>
<td>LatB</td>
<td>Binds G-actin monomers, inhibiting addition to MF</td>
<td>loss of ACG</td>
<td>/ done at room temperature</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>loss of ACG; reduces TW</td>
<td>/ early pore closure</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>loss of ACG</td>
<td>no effect /</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>loss of TW</td>
<td>/ perturbs Ca\textsuperscript{2+}-signaling</td>
<td>24</td>
</tr>
<tr>
<td>Cytochalasin</td>
<td>Binds to barbed end of MF; disassembly of existing MFs</td>
<td>no effect /</td>
<td></td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reduces lifetime of ACG</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>loss of TW</td>
<td>reduces / dilation of lumen</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>loss of TW</td>
<td>reduces / rebound after washeout / dilates acinar lumen with stimulation; compound exocytosis increases</td>
<td>44</td>
</tr>
<tr>
<td>Jasplakinolide</td>
<td>Stabilizes existing MFs, enhances MF nucleation</td>
<td>reduces; prevents high-dose CCK inhibition / causes BLM blebs in absence of stimulation</td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>BDM</td>
<td>Inhibits myosin ATPase</td>
<td>ACG accumulate</td>
<td>totally blocks / prevents loss of MF by latrunculin or cytochalasin</td>
<td>31</td>
</tr>
<tr>
<td>BDM &amp; LatB</td>
<td></td>
<td>ACG accumulate</td>
<td>inhibits /</td>
<td>31</td>
</tr>
<tr>
<td>C3 (Rho-toxin)</td>
<td>ADP-ribosylates &amp; inactivates Rho</td>
<td>no effect /</td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>CA-Rho</td>
<td>Stimulates downstream effectors</td>
<td>increases total F-actin</td>
<td>enhances / prevents supramaximal CCK inhibition of amylase release; causes BLM blebs</td>
<td>29</td>
</tr>
<tr>
<td>CA-Rac</td>
<td>Stimulates downstream effectors</td>
<td>increases total F-actin</td>
<td>enhances / prevents supramaximal CCK inhibition of amylase release; causes BLM blebs</td>
<td>29</td>
</tr>
<tr>
<td>DN-Rho</td>
<td>Inhibits downstream effectors</td>
<td>reduces, both phases affected /</td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>DN-Rac</td>
<td>Inhibits downstream effectors</td>
<td>reduces, primarily late phase / partially blocks supramaximal CCK inhibition of amylase release</td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>DN-Rho &amp; DN-Rac</td>
<td>Inhibits downstream effectors</td>
<td>reduces, slightly greater than sum of individual DN effects /</td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>CA-mDia</td>
<td>Recruits G-actin for MF growth</td>
<td>no effect on ACG</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>DN-mDia</td>
<td>Blocks mDia-induced MF growth</td>
<td>no effect /</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>β-thymosin</td>
<td>Binds G-actin monomers, inhibiting addition to MF</td>
<td>no effect /</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>Gelosin fragment</td>
<td>Binds G-actin monomers, inhibiting addition to MF</td>
<td>no effect on TW</td>
<td></td>
<td>26</td>
</tr>
<tr>
<td>Phalloidin</td>
<td>Stabilizes existing MF</td>
<td>inhibits /</td>
<td></td>
<td>26</td>
</tr>
</tbody>
</table>
importance of a specific agent’s concentration is illustrated by the finding discussed below that a small perturbation of the actin cytoskeleton increases amylase release, whereas stronger perturbation blocks amylase release.

The data covered here attempt to give the consensus of the available data.

**Signals that affect the actin cytoskeleton**

The principal mechanism of acinar cell stimulation begins with ligand binding to membrane receptors coupled to G proteins that feed into several pathways. With respect to the acinar cell cytoskeleton, the known pathways are activation of the small GTPases RhoA (via Gα13) and Rac1 (via Ga13 and Gαq). These activated small GTPases in turn activate their downstream effectors, such as Diap1 and ROCK1 and WASP/WAVE and ARP2/3, respectively, that lead to actin cytoskeleton remodeling.

As discussed below, a major downstream effector of Rho activation is the formin family member Dia. In drosophila, the formin diaphanous is activated by the small GTPase Rho, resulting in nucleation and formation of linear F-actin filaments that comprise the TW of epithelial cells. Although not yet experimentally verified, Rho activation in the mammalian pancreas is expected to activate mDia1 and it has been shown in mouse acinar cells that constitutively active (CA)-mDia1 leads to F-actin cables perpendicular to the apical PM. It was suggested these F-actin cables serve to recruitzymogen granules (ZGs) through the TW to the apical membrane during secretory stimulation. Consistent with the requirement for apical MF in secretion, protein release into the tracheal lumen of Drosophila lacking Dia, which also lack TW F-actin, was absent. A notable difference between Drosophila and mammalian acinar cells is that Dia in drosophila is required for formation of the TW of epithelia, whereas in mouse acini, the only observed role of mDia was in formation of the F-actin cables perpendicular to the APM, and the TW persisted in the presence of a DN-mDia construct. Also in contrast to Drosophila, mouse acini expressing dominant negative (DN)-mDia1 have unchanged basal and stimulated amylase release. Clearly, the roles of Dia/mDia1 differ in Drosophila and mouse pancreatic acini.

**Dynamics of acinar actin in the stimulated state**

Actin undergoes a variety of changes during secretory stimulation. The TW in the resting acinar cell is a barrier to ZG exocytosis, and the F-actin here must be rearranged to allow ZG access to the APM when the cell is stimulated. Using streptolysin O-permeabilized acinar cells, it was demonstrated that mild perturbation of the actin cytoskeleton by adding low concentrations of G-actin sequestering proteins (β-thymosin or a gelsolin fragment; Table 2) resulted in amylase release, especially the early phase of release. Interestingly, even when Ca<sup>2+</sup> was chelated at low levels using EGTA, mild perturbation of the actin cytoskeleton resulted in amylase release. If high concentrations of the G-actin ABPs were used that resulted in extensive loss of F-actin, there was reduced amylase release. Similarly, if the F-actin-stabilizing drug phalloidin was introduced to permeabilized acini, amylase release was inhibited. Together, these data indicate that the TW is a barrier to ZG exocytosis and that, while some remodeling of the actin cytoskeleton is required for release, extensive disruption or stabilization of F-actin is detrimental to protein secretion.

The dynamics of G-actin per se have not been investigated in relation to acinar cell stimulation. However, there have been measurements of the relative changes in the total amount of F-actin in acinar cells, which show that an increase in polymerized actin is associated with amylase release. Furthermore, pharmacological reduction of F-actin (1.0 μM latrunculin, a G-actin sequestering agent; Table 2) inhibits CCK-stimulated amylase release from mouse acini without affecting Ca<sup>2+</sup> dynamics. Since an acute increase in F-actin means less G-actin, these data add to the concept that dynamic changes in actin are involved in protein secretion in the acinar cell.
A similar conclusion regarding actin remodeling during secretion can be drawn from experiments using supramaximal CCK which induces large alterations in the actin cytoskeleton and shows inhibition of amylase release. When DN-Rho and DN-Rac (Table 2) constructs were used, they prevented supramaximal CCK inhibition of amylase release and at the same time lessened reorganization of the actin cytoskeleton. In that study, a low dose of jasplakinolide (1 μM) also prevented supramaximal CCK inhibition of amylase release, but a higher dose (3 μM) inhibited amylase release at all concentrations of CCK, consistent with the effect of jasplakinolide reported by Valentijn et al. (Table 2). So again, these results show there is a “sweet spot” for amylase release with respect to organization of the acinar cell actin cytoskeleton.

Remarkably, during exocytosis the ZG membrane (ZGM) becomes “coated” with F-actin (actin-coated granules, ACGs) (Figure 1; right panel). In the acinar cell, the role of the F-actin coat has been proposed to stabilize the fused ZG at the PM. This may be related to the duration of acinar protein secretion (seconds, which is long relative to neuronal exocytosis which occurs on a millisecond timescale), which may be required for the highly concentrated digestive enzymes to solubilize and diffuse out of the granule lumen into the beginning of the pancreatic ductal system. In other cells with the regulated pathway whose contents are more rapidly solubilized, the F-actin coat appears to flatten the fused vesicle, thus quickly forcing out the secretory product. The potential differences in the roles of F-actin granule/vesicle coats in various secretory cells are discussed in some excellent reviews. Interestingly, the G-actin sequestering drug latrunculin blocks coating of ZG with actin but does not prevent ZGM-PM fusion.

Using the C3 Rho toxin from Clostridium botulinum, it was shown that ACG formation is Rho-dependent. When acini were pretreated with the C3 toxin and then stimulated to secrete, ACGs were not observed but large vacuoles formed that were filled with the fluorescent extracellular tracer, showing that granule fusion had still occurred but that the individual granules in the absence of actin coating appear to fuse into a large vacuole. Thus, the rapid formation of ACG in stimulated cells appears to be Rho-dependent, and ACG formation may prevent vacuole formation. Amylase release was not measured in these experiments.

The timing of F-actin coating of ZGs relative to ZGM-PM fusion has evolved since its discovery. Early descriptions of ACGs suggested that the coat developed before ZGM-PM fusion, based on failure of entry of a fluorescent lectin (WGA) from the medium into ACGs, indicating their lumens were not yet accessible to the medium. Later experiments concluded that the F-actin coat forms after membrane fusion, while the exocytic pore is still open (see also the review from Jang and Thorn). This conclusion was based on the observation that only ZGs accessible to the acinar lumen (able to take up lysine-fixable fluorescent dextran from the medium) were actin-coated and no ZGs without fluorescent dextran were actin-coated. An example of acini stimulated in the presence of a lysine-fixable dextran followed by fixation and fluorescent phalloidin staining is shown in Figure 2. This clearly shows dextran in the lumens of ACGs. Subsequent work using a soluble tracer (sulforhodamine B) with living cells convincingly showed that access to the ZG lumen occurred on average 6.7 seconds before appearance of the actin coat, as visualized in acini from a transgenic mouse expressing Lifeact-GFP (Figure 3). This study also showed that latrunculin blocked formation of ACG while ZG fusion was not inhibited (Figure 4). Hence, ZGM-PM fusion occurs rapidly followed by actin coating of the fused ZG while it is still in the “Ω” shape, and that fusion per se is independent of ACG formation.

A different interpretation of the timing of ZG fusion and ACG formation has been made recently by Geron et al., using Lifeact-GFP, expressed in acini by adenoviral infection. This study was of the role of the formin family member diaphanous (DIAP1, called mDia in mouse), which is a Rho effector whose activation leads to linear MFs in the pancreatic acinar cell that they called actin cables. They state in the introduction to their paper that “secretory vesicles undergo actin coating before fusing to the apical membrane . . .” (emphasis added) and they cite these other references.

One can observe in their videos accompanying the paper ACGs moving apparently along F-actin cables that run perpendicular to the apical PM. Since these ACGs appear deeper inside the cells than the PM, it was concluded that coating preceded ZGM-PM fusion. Even though they had included FM4–64, a fluorescent membrane tag that should enter fused ZGs and label their membranes, in none of the data shown is FM4–64 fluorescence ever observed in ZGs. Therefore, it is not clear that the experiments actually could reveal when ZGM-PM fusion occurred. A more consistent interpretation of the role of mDia-induced actin cables is that fused ZGs are moving along these cables near the apical PM.

Another issue with their interpretation is that it does not concur with what was observed when the cables were interfered with. They showed that expression of a CA-mDia1 resulted in an increase in actin cables, and that a DN-mDia1 mutant caused a strong decrease in the number of cables. Neither of these affected basal or stimulated amylase release. If the cables are an important mechanism to move ZGs in the cytoplasm to the PM, so they can then fuse, one would expect a decrease in stimulated amylase release when the cables are decreased, which was not observed. The DN-mDia1, which reduced cable formation, caused an increase in ACG fused to one
Figure 3. Actin coating of ZGs occurs shortly after ZGM-PM fusion. (A) Low-magnification images show a lumen lying diagonally between two acinar cells identified with SRB and Lifeact-EGFP (green) in the subapical region. (Ai) is an image taken before the appearance of exocytic events at the time point indicated “i” on the graph of fluorescence intensity over time in panel (C). (Av) is an image at a time point after induction of a number of exocytic events which can be seen as bright spots of SRB fluorescence along the lumen; the time point v” is indicated on the lower graph (C). (B) Shows an image sequence from an enlarged region (box shown in A) of Lifeact-EGFP and SRB and the overlay, for two exocytic events. The images were taken at the time points i, ii, iii, iv, and v as indicated on the graph in panel (C). (C) is a graph of fluorescence changes over time taken from a region of interest placed over the lower exocytic event (indicated by an arrowhead). SRB fluorescence is plotted normalized to the peak and rises rapidly to a peak and then decays to a plateau. The simultaneously recorded Lifeact-EGFP signal, plotted in arbitrary fluorescence units, rises slowly and nearly reaches a maximum by the end of the record. The starting points of the SRB and Lifeact-EGFP signals, as determined by a positive deflection of the signal by more than five times the standard deviation of the signal noise, are shown by the color-coded triangles on the X axis. The black dotted lines were mono-exponential fits to the data with τ values of 6.9 s (SRB) and 29.4 s (Lifeact-EGFP) (from reference 37, Figure 4).
Figure 4. Latrunculin A abolishes actin coating of ZGs but does not inhibit ZGM-PM fusion. (A) Low-magnification images show complex lumens, identified by SRB²⁰ and Lifeact-EGFP fluorescence (green), lying between the cells within a pancreatic fragment. (Ai) is an image taken before the appearance of exocytic events, (Av) is taken after, at the time points “i” and “v” as indicated on the graph in panel (C). (B) shows an image sequence from an enlarged region (box shown in A) of Lifeact-EGFP and SRB and the overlay, for a single exocytic event. The images were taken at the time points i, ii, iii, iv, and v as indicated on the graph in panel (C). (C) is a graph of fluorescence changes over time taken from a region of interest placed over the exocytic event (indicated by an arrow). SRB fluorescence is plotted normalized to the first, rapid peak and shows a rapid rise followed by a slower increase. The simultaneously recorded Lifeact-EGFP signal, plotted as arbitrary fluorescence units, shows only very small changes over time (from reference 3⁷, Figure 5).
another, which was interpreted as compound exocytosis. So, it would appear that the role of these mDia1-induced actin cables is to keep ZGs separated such that individual granule fusion predominates and minimizes compound exocytosis.

It is likely an issue in these disparate results regarding the timing of ACG formation whether the acinar preparation was optimal for allowing access from the medium to the acinar lumen. This is discussed in references 38 and 39, where it is shown that the vigor of the tissue dissociation technique can yield groups of cells ranging from multicellular clusters to single acini or even smaller than acinar-sized groups that retain normal acinar secretory function. To allow access of the bathing solution to the acinar lumen, groups consisting of as few as three to six cells with a short unhindered connection between the acinar lumen and the medium are optimal. (In my lab, we could not get lysine-fixable dextran into fused ZGs as achieved by other labs, likely because my “acini” were actually larger multicellular clusters.)

Although mDia is a downstream effector of activated Rho, introduction of DN-mDia and DN-Rho A in acinar cells does not produce the same effects (Table 2). DN-mDia does not affect amylase release whereas DN-Rho inhibits amylase release. These different effects on amylase release indicate that mDia is not the sole target of activated Rho in the acinar cell, and perhaps another of the Rho effectors is involved.

The conclusion that the F-actin coat develops rapidly after ZGM-PM fusion contributes to the idea that this coat functions to stabilize the pore in the open state, providing sufficient time for the stored zymogens to become soluble and diffuse into the acinar lumen. An actin coat attached along the cytosolic surface of the fused ZGs would stiffen this membrane, preventing closure of the energetically unfavorable pore. The presence of the nonclassical myosin Vc (Myo5c) on ZGs, possibly tethered to the membrane by the small GTPase Rab27B, is believed to provide a motive force to move ZGs along the TW. Another myosin, Myo2A, is localized to the apical pole of acinar cells, is phosphorylated when the cell is stimulated, and when its activity is inhibited access of the ZG lumen to the soluble markers in the medium is reduced. This was interpreted as more rapid fusion pore closure when this myosin activity is inhibited. Also, when the TW was disassembled using cytochalasin B treatment, the acinar lumen enlarged under stimulation but amylase was not released; when the cytochalasin and secretory stimulus was washed out, the TW recovered and amylase was released from the already fused ZGs. It was suggested that contractile forces on the fused ZGM by actin were needed to force the ZG content out. These results can be reinterpreted in light of the newer data indicating that MFs are required to maintain an open pore for protein release.

In addition to providing sufficient time for zymogens to dissociate and diffuse into the lumen, the actin coat of fused ZGs has been proposed to help counteract the expected hydrostatic pressure of these dissolving contents from the fused ZGs in salivary glands. Also, there is compelling evidence that the fused ZGM transports electrolytes and fluid from the acinar cytosol into the lumen during protein secretion (see review by Frank Thévenod), which will add to hydrostatic pressure within the fused ZG lumen.

While it has been shown that mDia1 is involved in the formation of actin cables in the acinar cell, and is regulated by the small GTPase Rho, it remains to be determined what controls localized disassembly of the TW required to allow passage of ZG to the PM for exocytosis. The F-actin structure that coats granules is likely to consist of a network of short interconnected linear filaments or, alternatively, of branching actin filaments. This is because linear F-actin filaments <10 μm in length are rigid rods and such long linear filaments could not be accommodated on the highly curved surface of granules which are ~1 μm in diameter. Consistent with this idea, a subunit of the ARP2/3 complex and N-WASP, factors involved in nucleation of branches on existing MFs, were immunolocalized to the region of ACGs in stimulated acini. Based on this, Rac1 may be involved, because Rac1 has been shown to regulate WASP/WAVE ABPs that control nucleation/elongation of branched MF in other systems. However, WASP nucleates a new branch on existing F-actin, and proteomic analysis of highly purified ZGMs revealed the presence of small GTPases and molecular motors (dynein, myosins) but not actin or other cytoskeletal elements. On the other hand, ultrastructural immunogold localization showed actin in the region of ZGs. Together these results suggest that actin in the ZG region of the cell is either loosely bound or not bound at all to the ZGM. In any case, it appears F-actin is not present on ZG before fusion, so there needs to be a process to nucleate new MF on the fused ZGM and at this time, how this occurs remains to be identified.

**After the stimulus ends**

Once the fused ZG has released its content and exocytosis can be considered complete, the actin cytoskeleton has the further role of helping drive compensatory endocytosis. In order to maintain the balance of membranes between apical and basolateral domains, the ZGM added to the apical surface during protein secretion must be taken back into the cell. The endocytic process is believed to retrieve the ZGM in a clathrin-mediated fashion, and this also requires the actin cytoskeleton. It was observed in cytochalasin D-treated acinar cells that the APM became dilated and pits on this surface were immunoreactive for...
the clathrin adaptor AP-2, clathrin, dynamin, and caveolin, all of which are components of various endocytic mechanisms. When cytochalasin was washed out, these proteins rapidly disappeared from the APM and the lumenal membrane recovered to its prestimulus size. From this it was suggested that the actin cytoskeleton is also involved in the compensatory endocytosis process that follows protein secretion. The roles of actin in endocytosis are well known.\(^{52}\)

Since this chapter was written in 2015, a PubMed search was carried out in October 2020 and showed that recent work concerning the actin cytoskeleton in acinar cells was primarily focused on endocytosis and autophagy and not digestive enzyme secretion. By contrast, recent work exists on actin in islet beta cells and its relationship to insulin secretion. This addendum will review this recent work on the acinar cell actin cytoskeleton in endocytosis linked to autophagy during models of pancreatitis and in insulin release from islet β-cells.

Role of Actin in Acinar cell autophagy and pancreatitis

Autophagy is a process whereby cells breakdown and recycle their own components.\(^{53}\) Autophagy can be induced by starvation, thus providing the cell with energy and substrates to survive this stress. Additionally, and relevant to this discussion, cytotoxic events in the cell can also induce autophagy in an attempt to clear malformed, cytotoxic organelles and materials, again to save the cell and/or protect the wider organism. In the canonical autophagy pathway, this occurs by formation of an autophagosome, a double-membrane structure, to engulf the damaged organelle/material which then fuses with a lysosome to degrade it. In the noncanonical form of autophagy, the machinery that drives phagosome formation (a marker of which is LC3, microtubule-associated protein 1A/1B-light chain 3), is recruited to a single-membrane organelle which promotes their fusion with lysosomes for degradation of the organelle contents.

During pancreatitis, there is widespread disruption of ongoing protein synthesis and the packaging of zymogens into functional secretory granules (for review see reference \(^{54}\)). For example, during alcohol-induced pancreatitis, zymogen synthesis is increased at the transcriptional level and, at the same time, exocytosis is inhibited resulting in an overabundance of ZGs in the cell. Because of the high synthetic rate, the capacity of the protein folding machinery of the endoplasmic reticulum (ER) is exceeded leading to an unfolded protein response by the cell. These conditions promote activation of zymogens, especially important of which is activation of trypsin from trypsinogen. Active trypsin in turn can lead to cell/organ damage and is accompanied by inflammation, which together enhance the severity of pancreatitis.

One of the cell responses to stress, as mentioned above, is to activate autophagy, in this case to get rid of the excess of ZGs. In pancreatitis, there is a strong body of evidence that lysosomal biogenesis fails to keep up and that there is insufficient flux into the autophagy pathway such that trypsin activation occurs in the cell with its damaging sequelae.

As with all membrane trafficking, formation and movement in the autophagy pathway involves cytoskeletal elements, including actin (see reference \(^{55}\) for review). Evidence for this includes the observation that actin-depolymerizing agents (e.g., cytochalasin D, latrunculin B) block autophagosome formation. The actin-regulating Rho family GTPases are also involved. RhoA is needed during starvation-induced autophagy, while Rac is inhibitory to induction and itself is inactivated during autophagy. The GTPase Rab1 is also involved, stimulating actin assembly and tubulation of the ER membrane to produce autophagosomes.

Recent studies investigated the role of endocytosis, which is well known to involve actin, in experimental pancreatitis. Chyanov et al. used LifeAct to follow actin dynamics during pancreatitis along with extracellular fluorescent tracers to monitor fluid-phase endocytosis.\(^{56}\) They found that endocytic structures that form during pancreatitis are much larger than during physiological stimulation, forming endocytic vacuoles (EVs) which are positive for active trypsin. These EVs can be as large as 12 μm in diameter and appear to form from the enlarged apical plasma membrane that result from compound exocytosis during acinar protein release. These EVs are actin-coated, often not continuously. The small “fenestrae” in the actin coat that were sometimes observed appear to be weak spots where rupture of EVs can occur (as revealed by movement of the fluid-phase fluorescent tracer from the EV to the cytosol; see Figure 5, reproduced from this paper) and deliver active trypsin to the cytosol. Once rupture occurs, it is quickly followed by plasma membrane blebbing in most of their observations and cell death about 30 minutes later. Importantly, the presence of the F-actin-stabilizing drug jasplakinolide decreased the rupture of EVs from about 30 percent of observed cells down to about 15 percent. This led the authors to suggest that the actin coat on the EVs is a stabilizing force against rupture and is cell protective.

Another interesting observation was that EVs can sometimes fuse with the plasma membrane, as revealed by mixing of preloaded fluorescent tracer of one color with a differently colored tracer in the medium.\(^{56}\) This exocytosis of EVs occurred at both the apical and basolateral surfaces of the cells (see Figure 6; reproduced from reference \(^{56}\)). Because of the technical setup for these high-resolution confocal studies, not many individual events could be recorded, so it is not known how frequently this occurs or whether the propensity is for apical or basolateral
exocytosis. The basolateral exocytosis is consistent with that investigated by Gaisano and colleagues, which is proposed as mechanism to deliver harmful active trypsin to the interstitial space during pancreatitis. The process of exocytosis from the autophagic pathway has been termed secretory autophagy (see reference for review).

Subsequent work from this group looked in greater detail at the formation of EVs and presented evidence that they form by a noncanonical pathway, LC3-associated phagocytosis (LAP). This process involves an EV with a single membrane which is initially actin-coated and as it loses actin it becomes LC3-positive and is converted into an autophagic organelle. Importantly, most LC3-positive EVs also exhibited activation of trypsinogen.

A different study implicating the actin cytoskeleton in autophagy and pancreatitis studied β1 syntrophin, which binds and regulates dystrophin and is in turn an important linker between the actin cytoskeleton and transmembrane glycoproteins. In this case, actin remodeling driven by β1 syntrophin is involved in curvature of the membrane at the ER to form tubules which become autophagosomes. Using a β1 syntrophin knockout mouse, they observed dilation of the acinar lumen under resting conditions, and the knockout showed an unaltered cerulein dose-response amylase release. However, during cerulein supramaximal stimulation to induce pancreatitis, the β1 syntrophin mice had less F-actin and reduced autophagy: a marked reduction in ER nucleations, autophagosomes, and autolysosomes. The β1 syntrophin knockouts exhibited greater severity of pancreatitis (higher serum amylase and trypsin; more necrosis; more neutrophil infiltration; and greater edema), showing the protective role of autophagy during pancreatitis. At this time, there does not appear to be enough information to know the relative importance of canonical (ER-derived) versus noncanonical (endocytic organelle) autophagic pathways in pancreatic function and in pancreatitis.

Figure 5. Cytosolic presence of membrane-impermeant fluorescence probe in the cell located in undissociated pancreatic fragment. Small (~1-mm) section of mouse pancreas was stimulated by 100 nM CCK for 2 hours at 35°C in the presence of diS-Cy5 (shown in magenta), washed, and imaged in the presence of FITCD (shown in green). The lower gallery of images depicts the fragment containing two cells within the section: one with a large intact EV (white arrow) and the adjacent cell with increased cytosolic fluorescence of diS-Cy5. The FITCD image indicates that the plasma membrane of this cell is intact, suggesting that the increase of the cytosolic fluorescence occurred as a result of EV rupture. Representative of six similar experiments (from reference , with permission).
Figure 6. Exocytosis of endocytic vacuoles (EVs). (A) Fusion of LY-filled EV (green; the EV undergoing fusion is highlighted by an arrow) with the basal plasma membrane of a CCK-stimulated acinar cell. The extracellular medium contains TRD and a fusion event is characterized by mixing of the probes (yellow). Following fusion, the EV loses LY and gains TRD. The region containing the fusing EV is shown in the bottom row of images. The graph shows the time course of fluorescence of the two dyes recorded in the EV (highlighted by the dashed line). (B) LY-filled EV (green) fuses with TRD-filled post-exocytic structure in the apical region of the CCK-stimulated cell. The region containing the fusing EV (white arrow) is shown in the bottom row of images. Following fusion, the composite structure contains both probes (yellow) (representative of n = 7 observations). Scale bars = 10 μm (from reference 56, with permission).
**The role of actin in β-cell exocytosis**

Although there are known differences in the cell biology of endocrine and exocrine pancreatic cells, there are more recent investigations of the actin cytoskeleton in the β-cell than in the acinar cell. The Pancreapedia: Exocrine Pancreas Knowledge Base includes a review of the physiology and cell biology of insulin release and the intracellular pathways controlling it. The final signaling step in β-cell exocytosis of insulin granules, similar to most secretory cells, is an influx of Ca\(^{2+}\) resulting in activation of the SNARE complex (soluble N-ethylmaleimide sensitive factor attachment protein receptor). The complex consists of SNAREs on the granule and plasma membranes whose conformational changes provide the force to fuse these two membranes and release the granule content to the extracellular space. The major physiological stimulus for β-cell exocytosis is elevated concentration of blood nutrients, especially glucose. Glucose-stimulated insulin release proceeds in two phases. The initial phase results in a large spike of insulin release and lasts about 10 minutes. If elevated glucose is maintained, the second phase occurs, and this is a lower rate of release but is capable of being sustained for hours, ensuring the circulating insulin remains sufficient throughout nutrient uptake from the intestines to assimilate those nutrients by target organs throughout the body.

As in the acinar cell, glucose-stimulated insulin release from the β-cell involves remodeling of the actin cytoskeleton, which in the case of the β-cell has roles in both phases of exocytosis (see Kalwat and Thurmond for review). As in other secretory cells, the β-cell has a cortical F-actin cytoskeleton which presents a barrier to granule exocytosis, as revealed by actin depolymerizing agents that increase insulin secretion. Actin remodeling is also involved in recruitment of new granules to the plasma membrane during the second, sustained phase of insulin release.

If we look at upstream events, the influx of glucose has two pathways that lead to insulin release. Glucose is taken up from the circulation via GLUT glucose channels in the plasma membrane into the cytosol. Metabolism of glucose in the cell increases the ATP/ADP ratio, which inactivates the ATP-sensitive K\(^+\) channel resulting in membrane depolarization and an influx of Ca\(^{2+}\), stimulating SNARE-mediated membrane fusion. The other pathway is that elevated glucose leads to glucosylation of Cdc42, a Rho GTPase, and its glycosylation decreases the level of Cdc42-GTP, which is the active form. Activated Cdc42 interacts with two proteins that affect actin remodeling. One is Wiskott-Aldrich syndrome protein (WASP), which through actin-related protein complex 2/3 (ARP2/3) stimulates branching of F-actin. The other is mDia, which promotes actin polymerization. Thus, inhibition of Cdc42 results in actin remodeling and increases insulin release in a similar fashion as do actin depolymerizing drugs. Further evidence of the importance of Cdc42 is that a constitutively active form of the protein (Q61L) inhibits glucose-stimulated insulin release; and a constitutively inactive dominant negative form (T17N) is without effect. The authors also used jasplakinolide (nucleates and stabilizes F-actin) which unexpectedly potentiated glucose-stimulated insulin release, without effect on unstimulated (basal) release. Jasplakinolide did not enhance release with KCl depolarization even though the abundance of granules near the plasma membrane was increased. Importantly, glucose stimulation dramatically diminished cortical F-actin whereas KCl depolarization did not. These results indicate that cortical actin is decreased at the same time deeper F-actin is increased during glucose stimulation, thus promoting the initial phase of release and recruiting new granules for the sustained second phase.

They next investigated whether the SNARE syntaxin 1 (plasma membrane localized), which is known to interact with actin, is involved. They immunoprecipitated (IP) syntaxin 1 and measured co-IP actin. Glucose stimulation decreased actin associated with syntaxin 1 approximately twofold. The additional presence of jasplakinolide during glucose stimulation did not affect this decrease in co-IPed actin. Depolarization with KCl had no effect on co-IP of actin, alone or in the presence of jasplakinolide. Therefore, glucose-stimulated actin remodeling occurs even in the presence of the actin nucleating and polymerization effects of this drug. The authors proposed that glucose initiates the transient reduction of filamentous cortical actin to coordinate granule mobilization and pool refilling and thereby promotes granule exocytosis.

Another recent study has investigated F-actin coating of insulin granules with a focus on ARP2/3, a nucleator that leads to branched F-actin filaments. Using endocrine pancreas cells from LifeAct transgenic mice and a fluorescent fluid-phase endocytic marker, with multiphoton microscopy, they provide high-resolution data showing that insulin granules become actin-coated following membrane fusion (labeling with the fluid-phase marker preceded actin coating) and that this is ARP2/3 dependent. Using drugs that block ARP2/3 activity and short hairpin RNA (shRNA) to deplete the cells of the protein, they observed loss of F-actin coating. Interestingly, with this high-resolution technique they found that there was not a global change in the cortical F-actin cytoskeleton but only localized changes to individual fusing granules. When ARP2/3 was inhibited or absent, fused granules accumulated at the plasma membrane and insulin release was decreased even though fusion had occurred. They then examined whether linear F-actin has a role by interfering with formin and find that while actin coating was disrupted, insulin release was unaffected. The authors concluded that ARP2/3-mediated nucleation of branched F-actin plays an active role post-fusion in events involved in insulin release.
Because of the complexity, especially delineating the two phases of insulin release and their regulatory mechanisms, a recent study presented the initial development of a statistically based computer model along the lines of Cellular Automaton, to integrate what is known and provide a platform for evaluating new data and hypotheses regarding the role of the actin cytoskeleton in insulin granule transport in the β-cell.\(^{66}\) Since the model they develop needs to incorporate what is known about β-cell insulin release, this paper also serves as an up-to-date review of granule movement and the various roles of the actin cytoskeleton in the β-cell.

The authors interpret recent total internal-reflection fluorescence (TIRF: a very narrowly focused signal with high signal to noise, in close proximity to the plasma membrane that reveals only granules docked/primed and can reveal exocytic events) and confocal data that insulin granules can be docked at the plasma membrane (responsible for first rapid phase of insulin release); can be freely diffusible toward the plasma membrane and back again deeper into the cytosol; and can be anchored via various accessory proteins to F-actin filaments such that these are being transported and will be released during the prolonged second phase.

They set up their initial model such that “the following processes and hypotheses are considered:

- Granules are created inside the cell.
- Granules move either diffusely or are directed, with stimuli promoting a directed movement.
- Granules can move along actin fibers, and are prevented from moving perpendicular to the strand.
- With a certain probability, granules can pass through the actin network, in cases of actin remodeling.
- Granules must exist for a certain time before they can fuse at the membrane.
- Under constant stimuli, a steady state exists for insulin secretion.”

In the simulation, they reduce a β-cell to a cube 10 μm on each edge with 343,000 cubic elements approximating the size of a single insulin granule. Focusing on just granules and actin, they define 11 possible states for each element denoting granules in various associations with actin, its movement, and the granule’s age (new or in the immediately releasable pool). With this model they can investigate the actin cytoskeleton and how it relates to granule movements and insulin release. To set up initial conditions, they used fluorescence microscopy data from LifeAct expressing cultured mouse islets and MIN6 cells. The simulation is populated with actin filaments and granules, randomly assigned to fit the known data. For changes in granule behavior (movement, actin association, fusion, etc.), the stimulus (glucose, KCl depolarization) and intracellular Ca\(^{2+}\) levels are used to define the probabilities of such changes. They start the simulation by randomly placing 13,000 granules within the actin mesh and assigning the majority to diffusing and a small fraction to different motions, to match data in the literature regarding these parameters.

The simulations were run and compared visually to see how well the simulations approximate actual cells and then quantitatively to see how stimuli affect insulin release. They also used latrunculin to disassemble F-actin and compared results from real cells with the simulations.

In summary, the authors conclude that “three major results were achieved:

1. The simulated pattern of insulin secretion in response to high glucose shows the typical biphasic pattern of cultured islets and cell lines
2. The simulated effect of the modifier of the actin cytoskeleton, Latrunculin B, namely the increase of both phases of glucose-induced secretion, concurs with experimental observations, provided the simulated network fulfills a certain set of criteria.
3. This network is also compatible with a simulated interaction between glucose- and potassium-stimulated secretion, which mirrors earlier experimental observations.”

“Thus, on the one hand, extensions can be implemented informatically in an uncomplicated way, and on the other hand, computing capacities are not yet a problem. Rather, the addition of further findings and hypotheses is an important component in the further development of the model, so that quantitative predictions should also be possible in the future.”

In conclusion, although there has been little recent progress on investigating the roles of the actin cytoskeleton in acinar cell protein secretion under normal physiological conditions, there are new data in the context of pancreatitis as well as in other systems such as the β-cell. The phasic release of insulin from the β-cell shows several roles for actin and its remodeling. It should be emphasized that much of this progress relied on use of high-resolution live cell imaging. The TIRF signal is limited to ~100 nm from the coverslip surface into the attached cell. Similarly, multiphoton microscopy is able to focus into approximately 600-nm-thick focal plane of interest. These techniques exclude signals outside of these thin focal planes, thus considerably reducing background that would obscure the region of interest. Interestingly, there are also kinetic differences for zymogen secretion from granules that are older or newly synthesized (e.g., see reference \(^{6}\)). The issue of release of old versus new ZGs has not been explored with respect to actin, but it would seem likely to be important to this process.
References


33. Turvey MR, Thorn P. Lysine-fixable dye tracing of exocytosis shows F-actin coating is a step that follows granule fusion in pancreatic acinar cells. Pflugers Arch 2004; 448:552–555.
Pancreatic Acinar Cell, an Important Model for Secretion

The pancreatic acinar cell possesses a robust protein synthetic machinery that synthesizes a large spectrum of enzymes that digest all components of ingested food. Proteinases and some lipases are synthesized as inactive proenzymes. All are sorted through the trans-Golgi and then vectorially transferred via a still incompletely defined maturation process to the zymogen granules (ZGs). These mature ZGs, among the largest secretory vesicles (~1 micron), accumulate at the apical pole of the highly polarized acinar cell and occupy ~30 percent of total cell volume. Ca\(^{2+}\)-mobilizing neural (acetylcholine) and endocrine (cholecystokinin: CCK) stimulation, and to lesser extent cAMP-acting agonists (vasoactive intestinal polypeptide: VIP, secretin), acts on their respective plasma membrane G-protein-coupled receptors to trigger two signaling cascades. The first is the inositol triphosphate (IP\(_3\))-mediated intracellular Ca\(^{2+}\) release partnered with diacylglycerol (DAG) formation and protein kinase C (PKC) pathways; the second is elevation of cAMP levels partnered with protein kinase A (PKA)-activated pathways; extensive cross talk exists between these two cascades. Other chapters in this book deal with these topics. These cell signals act on the complex exocytotic fusion machinery discussed below that is comprised of a large number of molecules on the ZGs and the plasma membrane (PM) to culminate in fusion of ZGs with apical PM, releasing the ZG cargo of nutrient hydrolases (e.g., proteases, lipase, amylase) into the ducal lumen, which then transit to the duodenum for a timely meeting with a food bolus whose entry into the duodenum from the stomach is metered by the pylorus.

The pancreatic acinar cell, historically from the work of George Palade (Nobel Prize for Medicine or Physiology in 1999) and coworkers, had long served as the basic model to elucidate the complex processes of secretion. This was however largely overtaken by the neuron and endocrine fields that have exponentially accelerated as a result of the rapid identification of the exocytotic machinery (originally known as the SNARE hypothesis)\(^3\) and membrane ion channel machinery that orchestrates plasma membrane Ca\(^{2+}\) channel opening with the ensuing Ca\(^{2+}\) influx as the major source of Ca\(^{2+}\) for secretory granule fusion. The rapid progress in the neural and endocrine fields was further supported by the rapid development of new technology, including genetic models, electrophysiology, and microscopy imaging. The pancreatic acinar cell eventually followed in hot pursuit to initially show that it too has the fundamental components that support the SNARE hypothesis.\(^5\) To the delight of neuroscientists, probably paralleled by the undeclared dismay of earlier workers in pancreatic acinar biology, was that the levels of the SNARE and associated proteins were at much higher levels in neurons than in acinar cells; this made the neuron a better model to isolate and characterize the large number of proteins of the exocytotic machinery.\(^6\) The acinar cell exocytotic machinery however does not simply mimic the neuron and still lends itself to be an outstanding model for nonexcitable cell secretory biology. For instance, the pancreatic acinar apical PM constitutes less than 10 percent of the total cell surface area even when maximally stimulated, therefore exocytosis of the few ZGs abutting the apical PM would be insufficient in exporting enough proteases for delivery into the duodenal lumen to efficiently digest the huge amount of food continually being emptied from the stomach during a meal. Instead, the pancreatic acinar cell has a different secretory granule architecture from the neuronal synapse, which enables an orderly and rapid (within 1 minute) fusion of majority (>70%) of the ZGs within the apical pole, termed sequential exocytosis,\(^7\) whereby fusion pores between homotypically fused ZGs (also called compound exocytosis) remained open for very long periods (>20 minutes)\(^7\) to ensure efficient emptying of zymogen cargo from the deepest ZG layers within the apical pole. This provides an exquisitely regulated and metered machinery that can match hydrolase outputs to the varying amounts of food throughout the entire duration of the meal. Similar
exocytotic machineries can be greatly accelerated in mast cells and neutrophils during an allergic reaction or to mount an effective kill against invading bacteria.\textsuperscript{5,10} Furthermore, the exocytotic machinery of the acinar cell can be misdirected (i.e., ectopic exocytosis at the basolateral PM) by disease-causing stimulation such as alcohol, as a major contributing mechanism to alcoholic pancreatitis.\textsuperscript{11,12}

SNARE Hypothesis for Membrane Fusion and Exocytosis

The 2013 Nobel Prize in Physiology or Medicine was awarded to James Rothman, Randy Schekman, and Thomas Südhof for their discoveries of the machinery regulating vesicle traffic. This was actually the convergence of four fields (reviewed in reference \textsuperscript{13}). James Rothman and coworkers were reconstituting membrane traffic events to show fusion of donor vesicles with target membranes using cell-free assays. Randy Schekman and coworkers were studying the secretory pathway in yeast, which led to the identifying SEC genes and their mutants that accounted for accumulation of various intermediated vesicles along the ER to the Golgi. Thomas Südhof cloned a large number of synaptic vesicle proteins found to mediate neuroexocytosis, the latter actually was pivotally shown by a fourth field of coworkers who found that the synaptic proteins Südhof cloned were actually the substrates for proteolytic cleavage by tetanus and botulinum neurotoxins known to block neurotransmission.

The SNARE hypothesis as originally proposed by abovementioned major contributors stated that cytoplasmic N-ethylmaleimide-sensitive fusion (NSF) proteins and soluble NSF attachment proteins (SNAPs) bind SNAP receptors (SNAREs) on the donor vesicle (v-SNARE) and target membrane (t-SNARE) to form a multicomponent complex capable of mediating fusion between the two membranes.\textsuperscript{3,4} The v-SNAREs are vesicle-associated membrane proteins (VAMPs) and t-SNAREs are syntaxins (STX) and SNAP25 (synaptosomal associated protein of 25kDa), which form trans-SNARE complexes to constitute the minimal machinery for membrane fusion. The original neuronal SNARE proteins\textsuperscript{5} were found to belong to large families that included numerous nonneuronal isoforms which have very broad tissue distribution\textsuperscript{14} and with each cell type containing multiple isoforms. Compartmental specificity of membrane fusion was initially thought to be encoded by the specific pairing of these SNARE proteins.\textsuperscript{15} This thinking turned out to be inaccurate as promiscuity was increasingly found (i.e., VAMP8 discussed below); and that compartmental specificity actually required many accessory proteins that serve to spatially target and bind distinct SNARE complexes, including Sec1/Munc18 (SM), Munc13, and RIM proteins, which further act to prime the SNARE complex to become fusion-ready.\textsuperscript{4,6} For more exquisite regulation of fusion that achieves a higher level of precision and speed, these primed SNARE complexes would pull the cognate-fusing membranes together only up a hemi-fused state, whereby complete fusion is prevented by fusion clamps (i.e., complexins, synaptotagmins) that also act as Ca\textsuperscript{2+} sensors that are ready to be activated by Ca\textsuperscript{2+} to trigger fusion.\textsuperscript{4,6} Many of these proteins turned out to be the precise substrates for specific cell signals, including DAG (Munc13), PKC (SM proteins), Ca\textsuperscript{2+} (SNARE complex, synaptotagmins), and even cAMP/PKA (RIMs and EPACs).\textsuperscript{6,16} These fusion clamps also act to super-prime the SNARE complex, which is required to ensure speed and precision for neuroexocytosis,\textsuperscript{5,16} but probably the full complement of these proteins is not required for the less efficient and less precise secretory cells, such as acinar cells.\textsuperscript{16} Nevertheless, the pancreatic acinar cell has been an ideal epithelial cell model to examine these tenets of the SNARE hypothesis, wherein many isoforms of these proteins (i.e., SNARE and SM proteins, complexins, synaptotagmins, EPAC) were indeed reported (discussed below), whereas others that were found in neurons (i.e., Munc13, RIM) have not been yet found in the acinar cell.

Physiologic Apical Exocytosis in the Pancreatic Acinar Cell

SNARE proteins

The pancreatic acinar cell was one of the first nonneuronal cells to show the multiple isoforms of each SNARE protein, along with the accessory proteins described above for the neuron.\textsuperscript{5} In fact, the investigation of SNARE-mediated exocytosis in the pancreatic acinar cell somewhat followed the developments reported in neurons as depicted below and shown in Figure 1. First, we showed the cellular locations of v-SNARE (VAMPs) and t-SNAREs (syntaxins (STX) and SNAP-25) that mapped out to different exocytotic membrane compartments. The three syntaxin isoforms for each of the three exocytotic compartments include syntaxin 2 (STX-2) for the apical PM, syntaxin 3 (STX-3) for the ZG membrane, and syntaxin 4 (STX-4) for the basolateral PM.\textsuperscript{17} SNAP-23 was the ubiquitous nonneuronal SNAP-25 isoform that is present in ZGs, apical, and basolateral PM.\textsuperscript{18} Of the first two VAMPs reported, VAMP-2, was first thought to be the main one for regulated exocytosis, whereas VAMP-3 might have a role in constitutive secretion.\textsuperscript{19}

To assess the function of these SNARE proteins, we first used the tetanus and botulinum neurotoxins. However, unlike neurons, pancreatic acinar cells do not have PM receptors to internalize these neurotoxins, prompting us and others to employ cell permeabilization strategies (i.e., streptolysin O) to enable internalization of the neurotoxins, showing that tetanus toxin and botulinum neurotoxin C1
selectively cleaved pancreatic acinar VAMP-2 and syntaxins (STX-2 and STX-3 only), respectively, which blocked Ca\textsuperscript{2+}-evoked enzyme release.\textsuperscript{19,20} However, tetanus toxin, while completely cleaving VAMP-2, resulted in only ~30 percent inhibition of acinar secretion.\textsuperscript{19}

It was much later that we discovered a third VAMP, VAMP-8, which when genetically deleted blocked most of the apical exocytosis.\textsuperscript{21} This finding was unexpected as VAMP8 is prominent in endosomal vesicles in most cells and was originally known as endobrevin.\textsuperscript{22} Here, VAMP-8 accounted for the multiple layers of compound ZG-ZG fusions within the apical pole, whereas exocytosis of the few ZGs with the apical PM is mediated by VAMP-2.\textsuperscript{21,23,24}

Using a perifusion assay to measure amylase release from acini of VAMP8-KO mice and adenovirus-tetanus toxin-infected acini that acutely cleaved VAMP2, VAMP-2 was shown to mediate an early immediate phase of secretion, whereas VAMP8 regulates a later prolonged phase of secretion.\textsuperscript{25} These findings matched the exocytosis imaging results wherein VAMP-2-mediated first-phase secretion corresponds to the fusion of the fewer ZGs abutting the apical PM, and VAMP8-mediated later-phase secretion corresponds to the continued emptying of the much larger number of sequentially fused ZGs that lie behind then fusing with the apical PM-fuse ZGs that would provide a more sustained second-phase secretion.\textsuperscript{21,23,24} STX-2 was initially thought to mediate apical exocytosis,\textsuperscript{26} which was misled by its location on the apical PM.\textsuperscript{17,26} It turned out that STX-2 actually acts as an inhibitory SNARE capable of competing and blocking STX-3 and STX-4 from forming their SNARE complexes, thus blocking apical and basolateral exocytosis (see below), respectively.\textsuperscript{27} We had reasoned that this inhibitory SNARE protein STX-2 may be a deliberate mechanism to reduce the efficiency of membrane fusion to a much slower secretory rate (than neurons) of digestive enzyme release over a period of hours during a meal to ensure optimal nutrient digestion.\textsuperscript{27} Some SNARE proteins were distinguished by their resistance to botulinum neurotoxin cleavage, of which VAMP8 and also SNAP-23 are two examples. We therefore initially assessed SNAP-23 function...
with a dominant-negative carboxyl-terminal deleted SNAP-23, which would form nonfunctional SNARE complexes, and indeed this SNAP-23 mutant inhibited acinar secretion.28 A recent report unequivocally confirmed that SNAP-23 deletion in mice indeed blocked apical exocytosis.29

**Accessory proteins involved in the apical exocytotic machinery**

Although neuronal Munc18a is not present in acinar cells, Munc18b and Munc18c are 21 and were found to bind and prime the predicted SNARE complexes shown to mediate apical exocytosis, ZG-ZG fusion, and basolateral exocytosis. Specifically, Munc18b binds STX-3/SNAP-23 with VAMP2 to mediate exocytosis of ZGs with apical PM, and with VAMP8 to mediate sequential ZG-ZG fusion21, whereas Munc18c binds STX-4/VAMP8/SNAP-23 to mediate basolateral exocytosis (discussed below). Of the several isoforms of synaptotagmins (Syt 1, 3, 6, and 7) found in pancreatic acinar cells, Syt-1 found on ZGs and apical PM was the only one involved in enzyme secretion.31 Pancreatic acinar cells also expressed complexin 2 in its apical pole,32 and apical VAMP2 could bind both Syt-1 and complexin 2, and their truncated mutants introduced into permeabilized acini inhibited enzyme secretion.31,32 It is therefore plausible that Syt-1 and complexin 2 may be the fusion clamps and calcium sensors for physiologic exocytoses.33 However, gene deletion or depletion of Syt-1 or complexin 2 in pancreatic acinar cells will be required to definitively show this. Cab45 (the 45-kDa calcium-binding protein) was found to specifically bind the Munc18b/STX-3 complex and also acts as a Ca$^{2+}$ sensor for exocytosis as shown by Cab45 antibody blockade of enzyme secretion by a remarkable 90 percent.34 cAMP-binding protein, EPAC (exchange protein directly activated by cyclic AMP) partially reversed supramaximal carbachol and CCK-induced inhibition of secretion.35 While this suggests a role for EPAC in either enhancing apical exocytosis or reversing the apical blockade, further work will be needed to elucidate how EPAC interacts with the apical exocytotic machinery to accomplish these effects.

**Other proteins involved in other steps of apical exocytosis**

Coordinated interactions of the Rho and Rab family of small G proteins (i.e., Rab3D, Rab 27A/B)36–40 and synaptotagmin-like proteins,41 and their subsequent concerted actions on primary exocytotic fusion proteins, including SNARE and accessory proteins (i.e., Munc18b),42 appear to assist in ZG exocytosis perhaps by their potential ZG-tethering actions. Of these, the stronger evidence seems to building up for Rab27, localized to ZGs, wherein dominant-negative Rab27 constructs inhibited secretion in pancreatic and parotid acini.37,39 Whereas Rab27A was shown to be involved only on a minor secretory (lysosomal/endosomal) pathway,38 Rab27B was found to be more important for ZG exocytosis in pancreatic and parotid acini.37,39,43 Synaptotagmin-like proteins (SLPs) include various isoforms (Slp1 to Slp5, and rabphilin), of which Slp1 and Slp4 were shown to regulate amylase release in pancreatic and parotid acini in part by their binding to Rab27B.41,42

The actin cytoskeleton also plays multiple roles in apical exocytosis, including actin coating of ZGs during acinar stimulation44 that serves in part to support ZG-ZG fusion45 and also fusion pore opening46—and the actin-myosin complex that facilitates the expulsion of ZG contents.47 Interestingly, small G-proteins RhoA and Rac1 were shown to also regulate actin rearrangement in pancreatic acinar cells to support secretion.48

**Pathologic Basolateral Exocytosis Contributing to Pancreatitis**

The basolateral PM accounts for 90 percent of the pancreatic acinar cell surface area where little or no exocytosis normally occurs. Nevertheless, the acinar basolateral PM possesses an intact SNARE fusion machinery consisting of t-SNARE proteins (STX-4, SNAP-23) and SM protein Munc18c, which would indicate that exocytosis can potentially occur,40 as shown in Figure 1. In fact, early studies had shown that supramaximal CCK or cholinergic stimulation, capable of inducing pancreatitis in vivo, caused apical exocytotic blockade and redirected exocytosis to the lateral PM,49 releasing enzymes into the interstitial space to cause interstitial pancreatitis.50 This contribution to pancreatitis that was largely ignored was shown to be of importance when we elucidated the exocytotic machinery mediating this pathologic fusion event (reviewed in reference 11). Using various fluorescence imaging tools (FM143, syncollin-pHluorin), we were able to observe basolateral exocytosis in rat pancreatic acini in response to supramaximal CCK or carbachol stimulation—and after clinical alcohol sensitization by physiologic CCK or carbachol stimulation51–53 that mimicked alcoholic pancreatitis. We further showed that not only clinical concentrations of ethanol but also clinical concentrations of ethanol metabolites, acetaldehyde, ethyl palmitate, and ethyl oleate reduced the formation of apical exocytotic complexes with consequent blockade of CCK-8-stimulated apical exocytosis in rat pancreatic acini; and furthermore, acetaldehyde and ethyl oleate redirected VAMP8-containing ZGs toward and fusing with the basolateral PM, along with an increased formation of basolateral PM fusion complexes (Munc18c/STX-4, SNAP-23, VAMP8).54 It is remarkable that despite only a few ZGs normally close to the basolateral PM,
supramaximal stimulation could induce VAMP-8-labeled ZGs to move from the apical pole toward the lateral and basal PM. Mechanistically, these pancreatitis stimuli caused Munc18c on the basolateral PM to be phosphorylated by PKC-α, which induced its binding and activation of the STX-4/VAMP-8/SNAP-23 complex to become receptive to binding VAMP-8 of the approaching ZGs, whereby this complete SM/SNARE complex would then mediate basolateral exocytosis. Eventually, when Munc18c-depleted mice (Munc18c−/−) became available, and along with Munc18c knockdown in human pancreas by lentiviral delivery, we confirmed that depletion of Munc18c in pancreatic acini still exhibited normal apical exocytosis during physiologic CCK-8 stimulation, but upon supraphysiologic CCK-8 stimulation, there was reduction in basolateral exocytosis resulting from a decrease in STX4 complexes, with consequent alleviation of caerulein pancreatitis. Very recently, we reported that SNAP-23 depletion in rat and human pancreatic acini blocked not only apical exocytosis but also basolateral exocytosis; the latter also led to reduction in the severity of pancreatitis. The Munc18c phosphorylation also caused Munc18c displacement from the basolateral PM into the cytosol where it is rapidly depleted by the cytosolic proteolytic cleavage. This Munc18c displacement from the PM may be of clinical relevance, as in a human case of quiescent chronic alcoholic pancreatitis, Munc18c was similarly displaced into the cytosol of residual intact acinar cells, suggesting that this might be a contributing mechanism predisposing to the clinically observed recurrent pancreatitis. Finally, we showed that VAMP-8 depletion in mice completely blocked basolateral exocytosis, which resulted in reducing not only caerulein pancreatitis but also alcoholic pancreatitis, the latter shown in alcohol diet-fed mice stimulated with postprandial carbachol stimulation that mimicked clinical alcoholic pancreatitis. Whereas the mechanism for pathological basolateral exocytosis of ZGs is now clear, how thezymogens particularly trypsinogen that are emptied into the interstitial space are activated into damaging trypsin is not and requires further investigation. In a number of our more recent reports, we have performed these studies directly on the human exocrine pancreas using a slice technique that allowed preservation of secretory function over at least four days, enabling us to not only overexpress candidate proteins but also knockdown protein expression with shRNA adenviruses, thereby providing direct translational value over the conventional use of genetic mouse models.

Dysregulated SNARE Protein-Mediated Fusion Underlying Abnormal Autophagy in Pancreatitis

Autophagy SNARE proteins

Autophagy is the major cellular process that removes damaged organelles and other cellular debris. Autophagy is controlled by a series of autophagy-related proteins (ATGs) but also involves membrane fusion events which are intrinsically regulated by SNARE proteins that also interact with ATGs. Autophagy comprises four key stages. The first is phagophore nucleation induced by AMP kinase and PI3K-III complex-1. Second is phagophore expansion to engulf damaged organelles (i.e., mitochondria by mitophagy, ER by ER-phagy, ZG by zymophagy). Phagophore expansion is by an aggressive recruitment of membranes from various sources (PM, ER, endosomes), which requires ATG16L1 complexes and fusion events mediated by a distinct SNARE complex (STX7/STX8/VAMP7)—and eventual closure of the phagophore ends involving LC3 to form the autophagosome. The third stage is autophagosomal fusion with lysosome to form the autolysosome, which is mediated by two other SNARE complexes (STX7/SNAP29/VAMP8/ATG14 and STX7/SNAP29/YKT6). STX17 translocates from the cytosol to the autophagosome to initiate fusion with lysosome by first recruiting SNAP29 and then to form a complete complex with lysosomal VAMP8; this SNARE complex is stabilized by ATG14. Lysosomal STX7 can also bind SNAP29, which then forms the complete SNARE complex with autophagosome v-SNARE YKT6. The fourth and last stage is autolysosome maturation whereby its cargo is degraded for reuse. Defects in autophagy, particularly in autolysosome formation and maturation, the latter resulting in inadvertent trypsinogen activation, are now well known to occur and has become the current dogma for the cellular mechanism of pancreatitis. However, there is no work that directly addresses the role of the STX17 SNARE complex (STX17/SNAP29/VAMP8/ATG14) in autophagy, whose dysregulation could possibly contribute to the defective autolysosome formation or maturation in pancreatitis; and possibly some defects might also arise in phagophore formation and expansion that could be contributed by dysregulation of the STX7/STX8/VAMP7 SNARE complex.

Exocytic SNARE proteins affecting autophagy

While we were investigating the role of the exocytotic SNARE proteins, we inadvertently ventured into their unexpected roles in autophagy that contributed to pancreatic acinar injury and pancreatitis. Likely as a result of the basolateral exocytotic blockade, Munc18c-depleted acini unexpectedly activated a component of the ER stress response which contributed to autophagy induction, resulting in accumulation of autophagic vacuoles and autolysosomes. We found that STX-2 deletion also led to an accumulation of autophagic vacuoles as a result of increased autophagic induction, but by a different mechanism. ATG16L1 translocates to the PM during intense autophagy to recruit more membranes to expand the phagophore, which is mediated
by PM-bound clathrin heavy chain-1 (CHC-1) that forms a complex with pre-autophagosome-linked ATG16L1.71 As depicted in Figure 1, we discovered that STX-2 also binds ATG16L1, which blocked ATG16L1 binding to clathrin in the PM; thus STX-2 deletion resulted in the increased binding of ATG16L1 to clathrin, which leads to increased formation of pre-autophagosomes that drives autophagosome biogenesis.27 This increased autophagy induction at least in part accounted for the CCK-8-induced increase in autolysosome formation and activation of trypsinogen.27 As depicted in Figure 1, we also discovered a novel link between SNAP-23, autolysosome formation, and their connection to IKK (inhibitor of NF-κB kinase),56 the latter well-known to mediate NF-κB-mediated production of proinflammatory chemokines and cytokines that initiates and perpetuates the inflammatory response of pancreatitis.22 Pancreatitis stimuli of acini resulted in the translocation of SNAP-23 from its native location at the PM to the autophagosomes where SNAP-23 was able to perturb the STX-17 SNARE complex-mediated autophagosome-lysosome fusion.56 This SNAP-23 translocation was mediated by IKKβ-induced phosphorylation of SNAP-23, which was blocked by IKKβ inhibitors and IKKβ-insensitive mutants of SNAP-23.56 Interestingly, these pathologic SNAP-23-mediated events would not occur during physiologic starvation,56 which in part explains why this was not previously discovered. SNAP-23 depletion in rats therefore resulted in reduced autolysosome formation with consequent reduction in trypsinogen activation that takes place in autolysosomes, which together with SNAP-23 depletion-induced blockade of basolateral exocytosis contributed to a major reduction in pancreatitis severity.56 Of note, this is the first report that a single protein could serve as a mechanistic link of the three major cellular pathways for pancreatitis, including inflammatory signaling and the pathologic fusion events of dysregulated autolysosome formation and basolateral exocytosis. This therefore has major therapeutic implication in the targeting of SNAP23 for the treatment of pancreatitis.

Future Directions in the Investigation of Pathologic Fusion Underlying Pancreatitis

While of interest, the above work on exocytotic SNAREs affecting autophagy are nevertheless not the primary SNARE proteins that mediate autophagy. Of note, STX-17 expression was recently reported to be reduced after cerulein pancreatitis.73 This reduction of STX-17 levels can conceivably perturb autolysosome formation or maturation, which could contribute to the acinar injury. However, there is no reported work on autophagy SNAREs or their role in pancreatitis. Another major pathologic fusion contributing to pancreatitis is the very large (>2.5 micron) trypsin-containing endocytic vacuoles enveloped by F-actin whose rupture inside the pancreatic acinar cell releases damaging trypsin.74,75 The membrane fusion machinery that mediates this pathologic endocytosis has not been elucidated. VAMP8, originally discovered as an endosomal VAMP,22 was shown to play an additional and important role in endosomal trafficking by its interactions with early endosomal proteins D52 and Rab5, whereby VAMP8 depletion resulted in depletion of these endosomal proteins with consequent accumulation of activated trypsin in the autolysosomes.25,76,77 Remarkably, replenishment of D52 or Rab5 prevented the accumulation of activated trypsin.25 Finally, there is pathologic endocytosis of zymogens by the macrophages invading the acini during pancreatitis, which led to activation of macrophage NF-κB that in turn induced the production of inflammatory cytokines to perpetuate and accentuate pancreatitis injury.78 Much more work is needed to pursue these lines of investigation in elucidating the fusion machinery of each of these pathologic fusions that contribute to pancreatic acinar injury in pancreatitis; and from those insights, novel therapeutic strategies could then be devised to treat pancreatitis.

Acknowledgments

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References

Physiologic exocytosis in pancreatic acinar cells and pathologic fusion underlying pancreatitis


Introduction

The exocrine pancreas produces and secretes multiple digestive enzymes and has been the model in which the structure and functional organization of the mammalian secretory pathway was originally discovered and intensively studied.\textsuperscript{1,2} The pancreatic acinar cells exhibit one of the highest protein synthesis rates among mammalian cells. More than 90 percent of the newly synthesized proteins are targeted to the secretory pathway\textsuperscript{3} and packaged into large secretory granules, called zymogen granules (ZGs). In contrast to the smaller neuroendocrine and endocrine granules, ZGs have an averaged diameter of around 1 µm. They are responsible for the transport, storage, and secretion of digestive enzymes and have long been a model for studying the general mechanisms of secretory granule biogenesis and regulated exocytosis. Stimulation of the acinar cells by secretagogues such as acetylcholine and cholecystokinin triggers fusion of ZG membrane with the apical plasma membrane and the release of digestive enzymes into the pancreatic ductal system. In the duodenum, trypsinogen is converted to trypsin by proteolytic cleavage via enterokinase and activated trypsin then proteolytically activates the other zymogen enzymes.\textsuperscript{4,5}

Physiological stimulation of acinar cells by secretagogues such as acetylcholine and cholecystokinin triggers fusion of ZG membrane with the apical plasma membrane and the release of digestive enzymes into the pancreatic ductal system. In the duodenum, trypsinogen is converted to trypsin by proteolytic cleavage via enterokinase and activated trypsin then proteolytically activates the other zymogen enzymes.\textsuperscript{4,5}

Protein Composition of Zymogen Granules

Zymogens and digestive enzymes

The major secretory products of the acinar cells, namely the content of ZGs, are digestive enzymes which belong to five functional groups of hydrolytic enzymes including endo and exoproteases, lipases, glycosidases, and nucleases. In contrast to endocrine cells, which often produce a predominant peptide or protein product such as insulin, acinar cells synthesize, package, and secrete a mixture of nearly 20 different enzymes and isoenzymes including amylase, trypsinogens, chymotrypsinogens, carboxypeptidases, esterases, lipases, and ribonucleases. Most pancreatic proteases are synthesized as inactive precursors or zymogens, which only become activated by a cascade of limited proteolysis within the intestinal lumen. Because of the importance of ZGs to digestive enzyme storage and regulated secretion and as a general model for secretory vesicles, the identification and characterization of both the soluble and membrane proteins of ZGs have been of great interest in the field. In an early pioneering study, the secreted ZG contents from the guinea pig exocrine pancreas were analyzed by two-dimensional gel electrophoresis, which resolved 19 distinct high-molecular-weight proteins. Thirteen of the nineteen proteins were identified by actual or potential enzymatic activity.\textsuperscript{13} In more recent studies using mass spectrometry-based proteomics analyses, the identities of these enzymes have been confirmed and additional isoforms were found.\textsuperscript{14,15} The ZG content and ZG membrane components exposed to the lumen of ZGs. The cytoplasmic surface of the ZG membrane must contain vesicular trafficking proteins including Rabs, SNARE proteins, as well as molecular motors to interact with the cytoskeleton. Defective ZG biogenesis and trafficking can result in various pancreatic diseases such as acute and chronic pancreatitis.\textsuperscript{10–12} A comprehensive understanding of the ZG content and membrane protein composition is necessary to provide critical insights in the biogenesis and regulated secretion of pancreatic ZGs.
make up the major protein components of the pancreatic juice secreted into the duodenum. Therefore, the identification of ZG content proteins also significantly impacts biomarker studies in the pancreatic juice.16

**Components and topology of the ZG membrane proteins**

It is believed that the integral and peripheral ZG membrane proteins serve critical functions for zymogen sorting/packaging, vesicular trafficking, and regulated exocytosis. Therefore, comprehensive identification of ZG membrane proteins is expected to shed new light on our understanding of ZG biogenesis and secretion. In early studies, characterization of rat ZG membranes by SDS-PAGE indicated a relatively simple protein composition of about 10 components with GP2 (glycoprotein 2) accounting for 40 percent of the proteins.17–19 In the past decade, studies have been carried out to characterize ZG membrane proteins using two-dimensional gel electrophoresis. These efforts led to the identifications of two additional major ZG membrane components, GP3 (glycoprotein 3)20 and membrane dipeptidase,21 by N-terminal amino acid sequence analysis. However, due to the lack of sensitive tools for protein identification, the identities of many spots resolved on the 2D gels remained unknown. In another study, 14 spots corresponding to small GTP-binding proteins were resolved on a 2D gel of ZG membrane proteins by [35S]GTPγS blotting analysis.22 However, the identities of the spots were not determined. Different from the above abundant ZG membrane proteins, a number of low-abundance regulatory proteins have been identified on the ZG membrane by immunoblotting and immunocytochemistry. Examples of these proteins included the small GTPase, Rab3D23,24 and the SNARE proteins, vesicle-associated membrane protein 2 (VAMP2),25 and syntaxin 3.26,27 More recently, VAMP 8 was found on ZG membrane and to play a major physiological role in regulated exocytosis.28 Despite the significant amount of knowledge of ZG membrane proteins accumulated in the past decades on an individual basis, a comprehensive characterization of the membrane protein components of this organelle was not achieved until the application of modern mass spectrometry revealed a much more complex makeup of the ZG membrane.14,15,29

Organellar proteomics represents an analytical strategy that combines biochemical fractionation and comprehensive protein identification. Initial purification of organelles leads to reduced sample complexity and links proteomics data to functional analysis.30–32 In the past decade, organellar proteomic analysis has been carried out for virtually every subcellular compartment in the mammalian secretory pathway, including Golgi, ER, and secretory granules.30 The first comprehensive analysis of rat ZG membrane was carried out by combining mass spectrometry-based proteomics technologies and a well-established protocol of ZG purification.14 Using this protocol (outlined in Figure 1, left), a crude granule pellet (P2) was prepared by two consecutive low-speed centrifugations and then further purified by ultracentrifugation in a self-forming Percoll gradient. A heavy white band, containing highly purified ZGs, was observed and collected just above the bottom of the tube. The ZG membrane and content proteins were then separated by osmotic lysis of ZGs with the ionophore, nigericin, followed by ultracentrifugation. The membrane pellet was washed first with 0.25M KBr and then with 0.1M Na2CO3 (pH 11.0) to remove soluble content proteins and loosely associated proteins. The known ZG membrane marker, such as Rab3D was highly enriched in the purified membrane fractions (Figure 1, right).

These results indicated a much more complex protein composition of the ZG membrane. By combining multiple separation strategies including one-, two-dimensional gel electrophoresis and two-dimensional HPLC with tandem mass spectrometry, over 100 proteins were identified from purified ZG membrane.14 Most of the known ZG membrane proteins were identified, including high-abundance matrix proteins such as GP2, GP3, ZG16, and syncoUlin, which are likely involved in ZG sorting and packaging low-abundance proteins such as dynactin23 and VAMP2, which are involved in ZG trafficking and exocytosis. Many novel ZG membrane proteins were also identified, including the SNARE protein, SNAP 29, the small G proteins Rab27B, Rab11A, and Rap1, and the molecular motor protein, myosin Vc. Indicative of the interest in understanding the ZG membrane proteome, a later study15 using 1D SDS-PAGE coupled with 1D LC-MS/MS identified, in a more redundant database, 371 proteins from both ZG membrane and content. A large degree of overlap between proteins was found in these two independent studies. The overlap is higher (nearly 100%) for high-score protein identifications and major new observations, including all the new vesicular trafficking proteins, whereas the disagreement increases in low-score identifications, some of which may be contaminants. Representative ZG proteins found in multiple proteomic analyses14,15,29 and in some individual studies are summarized in Table 1.

As a second step toward a comprehensive architectural model of ZG membrane, a systematic topology analysis of ZG membrane proteins was performed by combining a global protease protection assay with iTRAQ-based quantitative proteomic technology.29 In this study, isolated ZGs were incubated with or without protease K. The control and protease K-treated ZG membrane proteins were digested with trypsin and the resulting peptides were labeled with iTRAQ reagents to compare the relative abundance of each peptide in the two samples. Because protease K treatment removed cytoplasm-sided ZGM proteins or domains, the iTRAQ ratios (protease K treated vs. control) from all peptides fell into two
clusters. The peptides from cytoplasm-sided proteins or domains showed reduced iTRAQ ratios (<<1.0), whereas those luminal ZG proteins showed little change (ratios around 1.0) (Figure 2). A threshold was determined using a training set of ZG membrane proteins with well-characterized membrane topology. The category with iTRAQ ratios below the threshold included the cytoplasm-sided peripheral membrane proteins, such as synaptotagmin-like protein 1 and Myosin Vc, and membrane proteins with single transmembrane structure or posttranslational lipid modification, such as VAMP 2, 8, Syntaxin 7, and all the Rab proteins. The second category with the ratios above the threshold included the lumen-sided peripheral membrane proteins, such as GP2, GP3, syncollin, and all of the digestive enzymes. Altogether, this analysis was able to successfully assign the membrane topology for 199 identified ZG proteins. The advantage of this technique was to analyze a large number of endogenous proteins simultaneously without the necessity to exogenously express fusion proteins. In addition, this technique was also able to map both cytoplasm- and lumen-sided domains from the same transmembrane proteins.

The overall goal of these recent proteomics studies is to build a quantitative, architectural model of the pancreatic ZG, which will lead to new hypotheses for subsequent functional analysis of this prototypic secretory granule. The ZG localization of a number of novel proteins, including Rap1, Rab6, Rab11A, Rab27B, SNAP29, and myosin Vc, were confirmed by immunocytochemistry (Figure 3). Several such novel observations have already led to hypothesis-driven functional studies. One example was Rab27B, subsequently demonstrated as an essential regulator of acinar exocytosis. Other examples included Rap1 and SNAP 29. The fact that Myosin Vc was also identified on ZG membrane in this study led us to hypothesize that Myosin Vc and Rab27B form a complex to tether ZGs at the apical actin web in an analogy to the Rab27A/melanophilin/Myosin Va complex on melanosomes.
Representative pancreatic ZG proteins identified from proteomics analysis and their major functional categories. Representative ZG proteins from major functional categories are listed. These proteins were identified on purified rat pancreatic ZGs from multiple proteomics studies. \(^{14,15,29}\) Proteins potentially copurified from other subcellular organelles are not included. References for their original discovery or with independent functional characterization or immunostaining are also included. Note: (a) SCAMP2, 3, 4, and (b) syntaxin 3 were also identified on ZG membrane.

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hypothesis is further supported by the more recent findings of two potential Rab27 effector proteins, synaptotagmin-like protein (Slp) 1 and 4.\textsuperscript{15,29}

A comprehensive model of the ZG requires the absolute quantity of each individual ZG protein as well as the stoichiometry among different ZG proteins. However, to date, this information has not yet been determined for any ZG protein. Recently, an absolute quantification (AQUA) proteomics strategy\textsuperscript{58,59} using LC-SRM and isotope-labeled synthetic peptides was used to obtain absolute molar abundances for selected mouse ZG proteins, Rab3D and VAMP8.\textsuperscript{60} The absolute quantities of mouse Rab3D and VAMP8 were determined as 1242 ± 218 and 2039 ± 151 (mean ± SEM) copies per ZG. The size distribution and the averaged diameter of ZGs (~750 nm) were determined by atomic force microscopy.\textsuperscript{60} For an average-sized ZG, the densities of these two proteins, if evenly distributed on the ZG membrane, were 702 molecules/µm\textsuperscript{2} for Rab3D and 1152 molecules/µm\textsuperscript{2} for VAMP8, respectively. As a comparison, it was estimated that Rab3A had 10 copies and VAMP2 had 70 copies on a synaptic vesicle (an average diameter of 45.18 nm) with their corresponding membrane densities being 1,572 and 11,003 molecules/µm\textsuperscript{2}, respectively.\textsuperscript{61} Notably, some ZG proteins can be present on a subpopulation of ZGs or concentrated on specific domains of the ZG membrane. The average copy numbers and membrane densities determined here can serve as a starting point to further examine the relative distributions of specific ZG proteins. In addition to mouse and rat ZGs, ZGs were purified from human acini obtained from pancreatic islet transplantation center, and the constituents of human ZGs were comprehensively characterized for the first time.\textsuperscript{60} A total of 180 human ZG proteins were identified, including membrane and the content proteins. The identification of human ZG-specific content and membrane proteins could significantly impact translational studies of biomarkers in pancreatic juice from cancer and pancreatitis patients. From these proteomics analyses in past decades, a comprehensive molecular model of ZG has emerged to include the protein components, membrane topologies, copy numbers per ZG, and protein-protein interactions. A diagram of such a ZG molecular model with selective ZG membrane proteins is shown in Figure 4.

**Functional categories of ZG proteins**

The secretory granules in neuroendocrine, endocrine, and exocrine cells share fundamental molecular mechanisms in granule formation, intracellular trafficking, and regulated exocytosis.\textsuperscript{62–64} For ZGs, this multistep process includes budding of immature granules (IGs) off the trans-Golgi network, granule maturation, and granule transport toward the apical pole in the vicinity of plasma membrane, tethering/docking at the plasma membrane, and regulated

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**Figure 2.** Workflow of iTRAQ-based topology analysis of pancreatic ZG membrane proteins. Left: Isolated ZGs were treated with or without proteinase K and then lysed. ZG membrane proteins were digested with trypsin, and resulting peptides were labeled with iTRAQ reagents. The peptides were mixed and analyzed by 2D LC-MALDI-MS/MS. Right: iTRAQ ratio distributions of tryptic peptides from ZG membrane proteins with known topology. A histogram of iTRAQ ratios (proteinase K versus control) from identified peptides illustrates the presence of two distinct clusters of tryptic peptides from cytoplasm-orientated and lumen-orientated ZG proteins, respectively (modified from reference\textsuperscript{29}).
exocytosis of ZG contents triggered by the rise of local Ca\(^{2+}\) concentration upon hormonal and neuronal stimulations (Figure 5). Despite the general model outlined above, the detailed molecular mechanisms are not yet completely understood. The ZG membrane is believed to carry at least part of the molecular machinery responsible for each step. Therefore, the recent comprehensive identification of the ZG protein components has shed new light
Figure 4. Molecular architecture of pancreatic zymogen granule proteins. A number of identified ZGM proteins and their topology assignments are shown on a single ZG.

on ZG biogenesis, trafficking, and exocytosis.\textsuperscript{14,15,29,65} The identified ZG proteins fall into several broad functional categories which link their identities to potential functional importance. These categories include proton pumps and ion channels, enzymes, vesicular trafficking proteins, matrix and glycoproteins, and small GTP-binding proteins (Table 1).

The ZG matrix protein group includes the highly abundant ZG proteins such as GP2, GP3, ZG16, and syncollin, previously known to be present on the inner surface of ZG membrane and likely involved in zymogen sorting and packaging (more detailed discussion in the next section).\textsuperscript{44,64,67,68} In addition to the high-abundance structural proteins and enzymes, many of the known functional and regulatory proteins were also identified on the outer surface of the ZGs. These included vesicular trafficking proteins such as SNARE proteins VAMP 2 and VAMP 8, molecular motors myosin Vc, and dynactin 2, a subunit of dynactin adaptor complex for the minus end-driven microtubular transport motor, dynein. A significant number of small GTPases were identified, including multiple Rabs and Rap1. Only Rab3D was reported on ZGs.\textsuperscript{23,24,69,70} These newly identified ZG-localized small GTPases represented one of the major novel findings from the extensive proteomics analyses of ZG membrane. Ion channels and transporters are usually low-abundant proteins and multi-transmembrane domains, making them hard to detect by proteomics approaches. Within this category, various subunits of the vacuolar H\textsuperscript{+}-ATPase (V-ATPase) have been consistently identified in multiple proteomics studies.\textsuperscript{14,15,29}

Figure 5. A working model of ZG biogenesis and regulated exocytosis. For ZGs, this multistep process includes the budding of immature granules from the trans-Golgi network (TGN), granule maturation through condensation and transport toward the apical pole in the vicinity of plasma membrane, tethering/docking at the plasma membrane, and regulated exocytosis of ZG contents through membrane fusion (modified from reference 66).
A few other ion channels and transporters have also been identified in individual proteomics studies (Table 1). In addition, functional evidence indicates the presence of several ion channel and transporter proteins in ZG membranes (aquaporins, vacuolar-type H⁺-ATPase, zinc influx transporter SLC30A2). The evidence for the K⁺ channels, Kv7.1 and Kir6.1, for CIC Cl⁻ channels and the vesicular nucleotide transporter SLC17A9 in ZG is less strong. A detailed discussion can be found in reference 71.

**Zymogen Granules Biogenesis—Sorting and Maturation**

Protein sorting at the trans-Golgi network (TGN) is essential for professional secretory cells, such as pancreatic acinar cells. In contrast to most eukaryotic cells that secrete proteins constitutively using TGN-derived vesicles, acinar cells store their secretory product in granules that undergo regulated exocytosis in a stimulus-dependent manner. In acinar cells, a mixture of different zymogens are packaged within the TGN; some zymogens form protein complexes and progressively aggregate in a Ca²⁺ and pH-dependent manner. Parts of the Golgi cisternae become dilated and these condensing vacuoles (CVs) pinch off to become IGs and then mature into ZGs. The selective aggregation of pancreatic secretory proteins has been documented, but the underlying molecular mechanism by which the secretory proteins are sorted into the regulated secretory pathway is still not well understood. Two hypotheses, not necessarily mutually exclusive, have been developed to explain the selection of content proteins for storage in the secretory granules.

The sorting-for-entry hypothesis proposes that the TGN acts as the primary protein sorting station in the biosynthetic transport pathway. This is based on the paradigm for newly synthesized lysosomal hydrolases sorted into clathrin-coated vesicles by mannose-6-phosphate receptor (MPR) and then targeted to endosomal membranes. For the regulated secretory proteins, the sorting-for-entry model postulated the presence of TGN membrane-associated sorting receptors to facilitate cargo entry into IG. Through this mechanism, only selected secretory proteins can enter IGs, whereas other proteins, such as those targeting the constitutive secretory pathway, are efficiently excluded. Immunelectron microscopy observations have demonstrated the segregation of regulated and constitutive cargo at the level of the TGN, supporting an active sorting mechanism.

In the sorting-by-retention hypothesis, the IGs serve as an important post-TGN sorting station. In this model, protein entrance into IGs is largely unselctive, and high-order intermolecular associations allow regulated secretory proteins for efficient retention within maturing granules. Concurrently, a subset of protein components is removed by receptor-mediated sorting or bulk flow. The driving force underlying this subtractive retention involves assembly of granule core proteins within IGs by aggregation/condensation, the progressive protein insolubility within the luminal environment of maturing granules in a Ca²⁺- and pH-dependent manner. Condensation of regulated secretory proteins allows them to remain in the maturing granules while the lysosomal proteins are removed by constitutive-like vesicle budding.

In pancreatic acinar cells, membrane proteins involved in the sorting and packaging of zymogens are predicted to have their functional domains exposed on the luminal side of the ZG membrane. Studies have been focused on several abundant luminal ZG membrane proteins for their potential roles in zymogen sorting and ZG formation. GP2 represents up to 40 percent of the total ZG membrane proteins in rat ZG. The membrane association of GP2 is via a glycosylphosphatidylinositol (GPI) anchor. Since GP2 can form stable complexes with zymogens at mildly acidic pH but not at alkaline pH, it was suggested that GP2 may act as a sortase for aggregated secretory proteins. However, the findings that ZGs in mouse pancreas can form in the absence of GP2 indicated it is not required for ZG biogenesis. Another abundant luminal ZG membrane protein, syncollin, is a component of lipid rafts. While the rates of synthesis and intracellular transport of secretory proteins were reduced in syncollin-deficient mice, these mice are viable and showed no detectable changes in pancreatic morphology, regulated exocytosis, or zymogen content. Thus, syncollin does not seem to be required for ZG biogenesis either. Though several other luminal membrane-associated ZG proteins have also been studied in ZG biogenesis, it is still unclear if any of these proteins alone is indispensable for ZG formation. However, it has been shown that the assembly of a proteoglycan scaffold at the ZG membrane promotes the efficient packaging of zymogens and the proper formation of stimulus-competent storage granules in pancreatic acinar cells. Other proteins identified as associated with the inner surface of the ZGM include chymase and peptidyl-prolyl isomerase B.

**Zymogen Granules Trafficking and Exocytosis**

Upon nervous and/or hormonal stimulation, ZGs move toward the apical plasma membrane in a microtubule- and actin-dependent manner. Secretagogue stimulation of the cells causes an elevation of the intracellular Ca²⁺ concentration, which in turn triggers granule fusion. Detailed discussion of ZG exocytosis can be found in Chapter 6 by Cooley and Gaisano et al.

Major new findings from recent studies came from the vesicular trafficking and small GTP-binding proteins groups for ZG membrane proteins. Among the small GTPase identified on ZG membrane, only Rab3D...
was previously reported on ZGs. In addition, punctate subapical labeling of Rab11 just below the apical plasma membrane was observed in pancreatic acinar cells. The ZG localization of the majority of newly identified small G proteins including Rab27B, Rab11A, Rap1, and Rab6 were confirmed by immunocytochemistry at the isolated acini and ZG level (Figure 3). In subsequent functional studies, it was shown that Rab27B localizes on ZGs and plays an important role in regulating acinar exocytosis. Furthermore, Rap1 was localized on pancreatic ZGs, although it had previously been localized on parotid secretory granules and it was found that Rap1 activation regulates pancreatic amylase secretion. Though Rab3D and Rab27B were present on all the ZGs, Rab6 and Rab11A localized to only a fraction of ZGs (Figure 3). This could indicate the existence of different subpopulations of ZGs. However, because corresponding GDIs can extract rabs from the membranes and cycles between membrane and cytosol, an alternative interpretation is that this represents ZGs at different stages in the secretory pathway. Notably, Rab27A was later shown to be present in mouse acinar cells and partially co-localized with ZGs and was required for digestive enzyme secretion. In addition to the small G proteins, we also found a novel SNARE protein, SNAP29, on ZGs. The ZG membrane localization of SNAP29 was confirmed by immunocytochemistry and it was also found that SNAP29 and VAMP2 formed a complex on ZG membrane.

Another important category of molecules critical to vesicular trafficking and exocytosis is molecular motors and corresponding adaptors. It was reported previously that myosin Vc localized to the exocrine pancreas and largely overlapped with apical F-actin. Proteomics analysis identified the presence of myosin Vc on ZG membrane. Rab27a forms a complex with myosin Va on melanosome through a synaptotagmin-like linker protein, melanophilin; the complex is required to tether melanosomes to the actin cytoskeleton. Rab27B, myosin Vc, and at least two synaptotagmin-like proteins, slp1 and slp4, are present on the ZG membrane in acinar cells. By analogy, Rab27B, slps, and myosin Vc could form a complex on ZGs to regulate the tethering of ZGs at the apical membrane. In exocrine pancreas, targeting of ZGs to the apical cell surface first requires an intact microtubule system and is then transferred to actin. The minus end-driven microtubular transport is thought mediated by molecular motor, dynein rather than kinesin. Purified ZGs are associated with cytoplasmic dynein intermediate and heavy chain and contained the major components of the dynein activator complex, dynactin. Consistent with this report, proteomics analysis identified a component of the dynactin complex, dynactin2/dynamitin, from highly purified ZG membrane. Interestingly, Rab6 functioned as a tethering factor controlling the recruitment of dynactin to membranes.

Conclusions and Future Directions

The pancreatic ZG has been a prototypic model for all secretory granules in the regulated secretory cells. Since its discovery and initial morphological characterization by electron microscopy, its protein compositions and the related molecular mechanisms that govern its biogenesis, intracellular transport, and regulated exocytosis have been described. Through modern proteomics technologies, a very detailed molecular model of ZG content and membrane is being established. A comprehensive molecular architecture of ZG will be developed in the foreseeable future, including its protein components and protein complexes, their membrane topologies, and protein copy numbers per ZG. In addition, the lipid composition of the ZG membrane will be described using state-of-the-art lipidomic techniques. This comprehensive molecular view will facilitate a thorough mechanistic understanding of how the IGS are formed from TGN, condense into mature ZGs and subsequently released with physiological stimulation.

References


47. Braun JE, Scheller RH. Cysteine string protein, a DnaJ family member, is present on diverse secretory vesicles. Neoporpharmacology 1995; 34:1361–1369.


Chapter 12
Chains and transporters in zymogen granule membranes and their role in granule function: A critical assessment

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Introduction

An update of this review on pancreatic zymogen granule (ZG) channels and transporters and their function is timely since its first publication in 2015. The advent of the post-genomic era has led – similar to other areas of cell biology – to the discovery of a number of ZG transport proteins and the characterization of their function in pancreatic acinar secretion. Recent studies have combined functional and molecular approaches to identify ZG channels and transporters and their contribution to pancreas physiology and pathology. A better understanding of the physiology of pancreatic acinar cell secretion and the role played by ZG in this process is prerequisite to comprehend the pathogenesis of major pancreatic disorders, such as pancreatitis, cystic fibrosis or cancer. Yet the fact that only a very limited number of studies have been published in this area of research is surprising, despite tremendous progress of knowledge and methodologies to investigate the molecular and cellular biology and physiology of the pancreas.

In the following paragraphs, I review significant advances in the characterization of ZG channels and transporters in the last two decades and discuss their putative role in pancreatic acinar secretion (Figure 1). The review ends with an outlook on unsolved issues that require clarification to move the field forward.

Proteomics

In the past, ion pathways in ZG were either characterized in permeabilized acini in which the effect of the ionic composition of the “extended cytosol” on stimulated secretion was investigated, and/or by recording macroscopic ion fluxes using an osmotic swelling assay (and lysis as an end-point) in suspensions of isolated ZG, and has been previously exhaustively reviewed. That piece of work has provided important ‘background’ knowledge for the interpretation of the post-genomic work (see below).

A key advance in the last decade has been the widespread availability of proteomics that allows the simultaneous identification of large numbers of expressed proteins. The use of proteomics for analysis of ZG and their membranes has propelled the identification of channels and transporters of pancreatic ZG although various caveats must be contemplated. Several proteomic studies have been published that used highly purified ZG membranes (ZGM) to identify cloned transporters and channel proteins. In most of these studies, granules were disrupted by different techniques, and membranes were subjected to carbonate and/or bromide extraction, which is a standardized and reliable procedure to obtain pure membranes without peripheral proteins. A critical step in this isolation procedure represents the initial “purification” of ZG by either differential or continuous Percoll gradient centrifugation. These methods yield fractions that are enriched 4–8-times in α-amylase compared to homogenate, which is close to the theoretical limit of purity. Yet, it is practically impossible to avoid contaminations by lysosomes, plasma membranes or membranes of other (disrupted) organelles. These contaminants are, however, a major drawback for proteomic analyses because even minimal contaminations by membranes originating from other organelles or plasma membranes may be prominent in a proteomic analysis of ZGM because of proteins that are highly expressed in these contaminating membranes. This issue needs to be considered, particularly for “mitochondrial” proteins such as voltage-dependent anion channels (VDACs) and subunits of ATP-synthase that have been detected in ZGM in several independent studies, or for “plasma membrane” proteins, such as the α-subunit of Na+/K+-ATPase. Hence, these particular observations need to be taken with caution. In addition, detection of a particular channel or transporter in ZGM may not infer any physiological/functional significance of that identified protein in mature ZG (see the paragraph on Vacuolar-type H+-ATPase). Finally, it should be born in
mind that several cloned channel proteins and transporters that have been identified by functional or immunological approaches have not been detected by proteomic analyses. Some studies even claim that no transmembrane proteins or classical membrane-anchored proteins are expressed in ZGM. But this conclusion may be due to the relative insensitivity of the proteomic approaches used because of low expression levels of transport proteins in ZGM.

Interestingly, among a variety of putative novel transporters and channels identified in ZGM by Rindler and coworkers, the ATP-gated receptor P2X4 deserves special attention. Indeed, P2X4 has been recently identified in lysosomes and lamellar bodies where it operates as a nonselective cation channel that is inhibited by luminal acidity, while increasing luminal pH in the presence of ATP elicits channel activation. These functional properties of a P2X4 ATP receptor located in ZGM - if not due to lysosomal contamination - are attractive for ZG exocytosis, considering the fact that maturing ZG lose their acidity (see the paragraph on Vacuolar-type H^+-ATPase). Moreover, the P2X4 cation channel is activated by ATP concentrations that have been measured in ZG (see the paragraph on Vesicular nucleotide transporter SLC17A9 for a discussion of this concept). Nonetheless, the detection of the P2X4 ATP receptor offers an example of a potentially novel aspect of ZG physiology that is derived from proteomic approaches. Hence, P2X4 is a promising candidate for future studies on ZG exocytosis.

Figure 1. The model summarizes current evidence (strong or weak) for the presence of ion channels and transporters in zymogen granules (ZG) of pancreatic acinar cells. The Figure also describes relevant ion concentrations in mature ZG and emphasizes the decreasing lumen acidity during maturation along the compartments of the secretory pathway that participate in ZG maturation (for further details, see text).

Channels

H_2O Channels

Digestive macromolecules are packaged and condensed together with various osmotically active ions and small organic molecules in the lumen of ZG during maturation, where granular Cl^- and K^+ concentrations display lower concentrations than in the cytosol. It is a long-standing observation that isolated ZG and other secretory granules suspended in isotonic sugar or salt solutions, such as KCl, remain stable for hours. Consequently, the stability of secretory granules in isotonic solutions indicates that the intragranular osmolarity does not exceed cytosolic osmolarity and/or that granules exhibit a low basal permeability for H_2O, K^+ and Cl^- in situ and in vitro. Reduction of extragranular osmolarity below 245 mOsmol/l, however, or addition of cation or anion ionophores to isolated ZG suspended in iso-osmotic KCl buffers elicits granular swelling and lysis. In the latter case, ionophores generate an electrochemical membrane potential that activates
endogenous conductive pathways for counterions, thus allowing entry of osmotically active KCl into the granular lumen, which attract H2O and induce swelling (and lysis) of secretory granules. Thus, these observations imply that H2O permeable pathways, e.g. aquaporins, are present in ZGM.

The expression and function of aquaporins in pancreas has been recently reviewed. Aquaporin 1 (AQP1) has been identified in rat pancreatic ZGM by a full range of immunodetection methods, namely immunoblotting, immunofluorescence confocal microscopy and immunogold labeling. Furthermore, swelling of ZG suspended in hypo-osmotic KCl solution was detected by atomic force microscopy (AFM) when 40 µM GTP was added and blocked by Hg2+, an inhibitor of AQP1, as well as by a functional AQP1 antibody that was raised against the carboxyl-terminal domain of AQP1. These changes of ZG swelling were paralleled by corresponding effects on fluxes of H2O. The effect of GTP on AQP1 activation is assumed to be mediated by interaction of GTP with a complex of several proteins that were co-immunoprecipitated with an antibody against AQP1 and identified by immunoblotting. They included the GTP-binding protein subunit Gαi3, the inwardly rectifying K+ channel IRK8 (Kir6.1/KCNJ8), the voltage-gated Cl channel CIC-2 and phospholipase A2 (PLA2). Furthermore, exposure of ZG to either the K+ channel blocker glyburide, or the PLA2 inhibitor ONO-082, blocked GTP-induced ZG swelling measured by AFM, whereas the non-specific Cl channel inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) had no effect. Interestingly, proteomic analyses have identified AQP8, but not other AQP isoforms, in rat ZGM, thus confirming previous immunohistochemical studies in rat pancreas tissues. AQP8 may be a genuine ZG membrane protein, however a contamination by mitochondria cannot be ruled out, which express AQP8 as well.

An intriguing observation has been made by Ohta et al., who found AQP12 expressed in the rough endoplasmic reticulum (rER) of rat pancreatic acinar cells and extends a previous study demonstrating expression of AQP12 in rat pancreatic acinar cells by in situ hybridization and reverse transcription-polymerase chain reaction (RT-PCR). In rats treated with the secretagogue cholecystokinin octapeptide (CCK-8), Otah et al. detected AQP12 in the rER and also on the membranes of ZG near the rER. Furthermore, AQP12 knockout mice were more prone to caerulein-induced pancreatitis. The authors suggested that AQP12 may be involved in the mechanisms underlying the proper generation, maturation, and trafficking of ZG in the secretory pathway and that adequate H2O flux may be necessary for this process to function adequately.

Altogether, it seems highly likely that AQP H2O channels are expressed in pancreatic ZGM because ZG swell and lyse in hypotonic solutions, although the AQP isoform(s) involved remain(s) uncertain. Moreover, the physiological role of AQPs in the complex process of secretory granule maturation, trafficking and exocytosis required for secretagogue-induced pancreatic acinar enzyme and fluid secretion are poorly understood.

K+ Channels

In pancreatic acinar cells, ZG are about 4–8-fold lower in K+ compared to the cytosol, as measured by X-ray microanalysis or using a potassium-sensitive fluorescence dye. Hence, any K+ conductive pathway should allow flux of K+ into the ZG lumen and increase its osmotic load. Based on the work by Hopfer and coworkers, I proposed in 1992 that (ATP- and glibenclamide-sensitive) K+ channels are expressed in ZGM where they contribute to osmotic swelling of isolated pancreatic ZG. This observation was soon confirmed by others. Subsequent studies have suggested that glibenclamide may modulate ZG K+ permeability indirectly, possibly by binding to a 65-kDa multidrug resistance P-glycoprotein (ABC1)-like regulatory protein, and/or the ZG membrane-associated protein ZG-16p. Additional support for the presence of K+ channels in secretory granules came from studies with mucin granules from goblet cells and suggested a Ca2+/K+ ion-exchange mechanism via parallel operation of a Ca2+ channel and a Ca2+-activated K+ channel. Ca2+/K+ ion-exchange has been subsequently proposed to play a critical role in the premature activation of trypsin in ZG and development of acute pancreatitis.

Jena and coworkers have identified an inwardly rectifying K+ channel IRK8 (Kir6.1/KCNJ8) in ZGM by immunoblotting and measured whole vesicle currents of patched single ZG with KCl as permeating ions that were reduced by ~15% by either 40 µM quinidine or 20 µM glibenclamide. Proteomic analyses of purified rat ZGM have detected the K+ channel TWIK-2/KCNK6, a member of a two-pore-domain K+ channel family that produces constitutive inward rectifying K+ currents of weak amplitude and that is highly expressed in the pancreas. However, their functional properties and their wide distribution suggest that these channels are mainly involved in the control of background K+ conductive pathways in the plasma membranes of many cell types. Hence, it cannot be excluded that plasma membranes may have contaminated ZGM in the proteomic study reporting TWIK-2/KCNK6 expression in ZGM (see also the paragraph on Proteomics).

Kv7.1 (KvLQT1/KCNQ1) is a very low-conductance, voltage-gated six-membrane-spanning K+ channel distributed widely in epithelial and non-epithelial tissues, including pancreatic acinar cells. KCNQ1 channels associate with all five members of the KCNE β-subunit family, resulting in a β-subunit-specific change of the current characteristics. Kv7.1 K+ channels are blocked by
the chromanol 293B\textsuperscript{45} and by the more potent and selective derivative HMR1556\textsuperscript{46}. Indeed, we showed that 293B selectively blocks osmotic swelling of ZG induced by activation of the \(K^+\) permeability (IC\(_{50}\) \(\sim\) 10 \(\mu M\))\textsuperscript{47}. Upon incorporation of ZG into planar bilayer membranes, \(K^+\) selective channels were detected with linear current-voltage relationships. Single channel analysis identified several \(K^+\) channel groups with distinct channel behaviors. \(K^+\) channels were inhibited by 100\(\mu M\) 293B or HMR1556, but not by the \(max\) \(K^+_\text{cs}\) channel inhibitor, charybotoxin (5\(nM\)). Kv7.1 protein was demonstrated by immunoperoxidase labelling of rat pancreatic tissue, immunogold labelling of ZG and immunoblotting of ZGM. Moreover, 293B also inhibited cholecystokinin (CCK)-induced amylase secretion of permeabilized acini (IC\(_{50}\) \(\sim\) 10\(\mu M\)) when applied together with the ZG nonselective cation conductance blocker, flufenamate. Thus these data indicated that Kv7.1 accounts at least partially for ZG \(K^+\) conductance and contributes to pancreatic secretagogue-stimulated enzyme and fluid secretion\textsuperscript{47}.

A question of some significance for the discussion of ZG \(K^+\) channels is whether the presence of several \(K^+\) channels with distinct biophysical properties in our bilayer experiments\textsuperscript{47} argues against the purity of the ZGM used for single channel studies with the planar bilayer technique. However, the observation by others that single patched ZG also display a mixture of channel types\textsuperscript{40} supports our model that ZG carry several different cation and anion conductive pathways (reviewed in\textsuperscript{4}). To further exemplify the concept that several conductive pathways are localized in ZGM, a study should be cited that investigated the effect of 293B and HMR-1556 on fluid and enzyme secretion induced by acetylcholine in the perfused rat pancreas\textsuperscript{48}. Interestingly, the authors were unable to observe any inhibitory effect of Kv7.1 channel blockers on secretion and concluded that Kv7.1 is not essential for secretagogue-mediated secretion of pancreatic acini. These results appear to question a role of Kv7.1 in enzyme secretion of rat pancreatic acini, as described in our study\textsuperscript{47}. However, we showed that CCK-stimulated enzyme secretion in permeabilized rat pancreatic acini was only abolished if flufenamate was applied together with 293B (see above), which is a strong indication that several ZG cation channels with functional redundancy are involved in enzyme secretion\textsuperscript{47}.

In a more recent study, we followed up the role of the ZG \(K^+\) conductance in CCK-induced enzyme secretion and showed that the immunosuppressive drug cyclosporin A (CsA) (but not FK506, another commonly used immuno-suppressant) activates ZG \(K^+\) conductance and selectively increases the open probability of ZG \(K^+\) channels incorporated into planar bilayers\textsuperscript{49}. The electrophysiological (and other) data let us conclude that CsA has a direct effect on the underlying \(K^+\) channel\textsuperscript{49}. CsA also increased basal enzyme release of permeabilized rat pancreatic acini, but did not enhance CCK-induced enzyme secretion\textsuperscript{49}. These results are concordant with previous studies that had investigated the role of CsA on exocrine pancreas function\textsuperscript{50, 51}, although these authors suggested a different mode of action of CsA. We speculated that selective activation of ZG \(K^+\) channels by the immunosuppressant CsA may cause an increased intracellular release of digestive enzymes due to osmotic swelling and lysis of ZG \textit{in situ}, and that this pathological process may account for the increased incidence of post-transplant allograft pancreatitis following hypoxia-reperfusion injury in patients treated with CsA (but not with FK506)\textsuperscript{49}.

In summary, although contamination by other organelles and membranes cannot be completely excluded, it seems likely that several \(K^+\) channels with different biophysical properties and pharmacological profiles are expressed in ZGM. This conclusion is supported by different electrophysiological techniques (incorporation of ZG in planar bilayers and single ZG patch techniques), as well as by the detection of several different \(K^+\) channel proteins by immunological or proteomic approaches. However, as for AQPs, the significance of ZG \(K^+\) channels for the physiology of secretion by pancreatic acinar cells remains unclear.

\textbf{Cl\textsuperscript{\textdagger} Channels}

\textit{CIC Cl\textsuperscript{\textdagger} channels and transporters}

X-ray microanalysis studies of pancreatic acinar cells have demonstrated that the ZG \(Cl\textsuperscript{\textdagger}\) concentration is about half the cytosolic \(Cl\textsuperscript{\textdagger}\) concentration\textsuperscript{20}. When measured with a \(Cl\textsuperscript{\textdagger}\) sensitive fluorescent dye, cytosolic \(Cl\textsuperscript{\textdagger}\) concentrations vary between 63 and 83 mM in resting cells\textsuperscript{48, 52}, suggesting that ZG \(Cl\textsuperscript{\textdagger}\) concentrations are well below those values. Early osmotic swelling studies with isolated ZG postulated the presence of regulated \(Cl\textsuperscript{\textdagger}\) channels (reviewed in reference\textsuperscript{6}). Subsequently, several candidates for CIC ion channels\textsuperscript{53} have been identified in ZGM that have been linked to ZG \(Cl\textsuperscript{\textdagger}\) conductive pathways. The earliest report suggested the presence of the \(Cl\textsuperscript{\textdagger}\) channel CIC-2 in ZGM\textsuperscript{54}. Using the whole-cell patch-clamp technique, the authors identified a hyperpolarization-activated \(Cl\textsuperscript{\textdagger}\) current in isolated pig pancreatic acinar cells. This current had the characteristic biophysical properties of CIC-2\textsuperscript{55} and was activated by extracellular hypotonicity, similar to CIC-2\textsuperscript{56}, suggesting a role in volume regulation. An antiserum raised against the C-terminus of CIC-2 localized the channel to secretory granules containing amylase by immunofluorescence microscopy of acinar cells, and the authors suggested that the channel protein incorporates into the apical plasma membrane following granule exocytosis\textsuperscript{54}. By measuring whole vesicle currents of patched single ZG and using KCl as permeating ions, Jena and coworkers\textsuperscript{40} detected...
a conductance that could be reduced by the non-specific anion transport blocker DIDS (40 µM) by ~50% and concluded that Cl- channels/transporters were present in ZGM. Immunoblots of ZG with CIC-2 and CIC-3 antibodies showed several bands, including immunoreactive bands of ~100 kDa, as expected for CIC-2 and CIC-3. However, CIC-2 (as well as CIC-1) is a plasma membrane Cl- channel, whereas CIC-3 through CIC-7 Cl-/H+ exchangers are predominantly localized in intracellular vesicles of the endosomal/lysosomal system, further arguing against a significant role of CIC proteins in the secretory pathway. Yet proteomic analyses of rat ZGM identified CIC-3 and CIC-5, thus supporting the notion that CIC channel proteins are expressed in ZGM. Although the lack of pancreas or salivary phenotype of CIC-2 and CIC-3 knockout mice does not exclude that ZG express these channel proteins - also because of the assumed functional redundancy of ZG ion channel proteins - the evidence provided for CIC-2 and CIC-3 expression in secretory granules largely depends on immunological studies. However, these studies have to be taken with caution as long as negative control experiments with ZG from knockout tissues have not been performed, thus proving specificity of the antibodies (see below for a critical discussion). Hence, the decisive experiments proving expression of CIC proteins in ZG are still missing.

**Other candidate Cl- channels**

Another possible candidate for ZG anion channels may be AQP6 that is unique among the AQPs because it is activated by HgCl2 rather than being inhibited as for other AQPs; and it does not operate as a water channel, but as an anion permeable channel with the halide permeability sequence NO3- > I- > Br- > Cl- > F-. Analysis of sequence alignments led to the identification of a critical amino acid residue responsible for anion permeability: An asparagine residue (Asn-60) at the position corresponding to Gly-57 in other AQP H+ channels is responsible for anion permeation. Interestingly, AQP6 is almost exclusively localized in acidic intracellular organelles where it co-localizes with vacuolar-type H+-ATPases (V-ATPases) and is activated by acidic pH. Although no evidence is available for AQP6 localization in pancreatic ZGM, AQP6 has been detected in secretory granules of parotid acinar cells by immunological methods. Furthermore, osmotic swelling and lysis of secretory granules suspended in iso-osmotic KCl buffer displayed the same conductive properties as the native AQP6 channel when induced by the AQP6 activator HgCl2. Interestingly, osmotic swelling showed a HgCl2-independent component (about 33%) and a HgCl2-dependent swelling was about 50% DIDS-sensitive, again suggesting at least three different Cl- conductive pathways (however without functional redundancy) in these granules.

A family of putative anion channel proteins predominantly expressed in epithelial tissues that are gated by Ca2+ were named CLCA (chloride channel, Ca2+-activated), but rather represent chloride channel accessory modulatory proteins (see below and ). Mouse CLCA1/2 (mCLCA1/2) was detected in ZGM with a variety of immunological techniques using several antibodies that were thoroughly characterized. Nevertheless, the gold standard of antibody characterization (namely to test the specificity of antibodies in knockout tissues used as negative controls) was not performed, because mCLCA1/2 knockout mice were not available at the time of those studies. In one study, an anion permeability was demonstrated using a ZG osmotic swelling assay whose permeability sequence, Ca2+-dependence and inhibitor sensitivity was reminiscent of CLCA associated anion currents. It is now established that hCLCA1, the human homolog of mCLCA3 that is expressed in mucous cells of the airway epithelium is a secreted protein rather than being a Cl- channel although it seems to modulate the conductance of endogenous Ca2+-activated Cl- channels. It is likely that all the CLCA proteins cannot act in solo as ion channels but instead interact with other proteins in a possible signaling or regulatory capacity (reviewed in ). Their role in ZG function is unclear and awaits further elucidation.

Whether a member of the TMEM16/anoctamin (ANO) protein family of Ca2+-activated Cl- channels and phospholipid scramblases contributes to ZG anion permeability remains an interesting question, especially because the Ca2+-activated Cl- channel TMEM16A/ANO1 is expressed in the area of pancreatic acinar ZG. Interestingly, TMEM16A/ANO1 is permeated by HCO3-, a selectivity process which is thought to be regulated by the Ca2+/calmodulin complex. Hence, TMEM16A/ANO1-dependent HCO3- secretion through ZGM fused with the apical plasma membrane may also contribute to solubilization of exocytosed secretory products during secretion of pancreatic acinar cells stimulated by Ca2+-dependent secretagogues.

In summary, although there is some suggestive evidence for the expression of intracellular CIC Cl- channel proteins, AQP6 and TMEM16A/ANO1 in ZGM, no strong candidate for a ZG Cl- channel has been identified.

**Ca2+ Channels**

Pancreatic ZG are a significant Ca2+ store, although the majority of ZG Ca2+ appears to be bound to proteins. Depending on the Ca2+ sensor used, the free Ca2+ concentration in the ZG lumen varies between 9 µM and 55 µM. How Ca2+ accumulates in ZG is unclear, but Ca2+ uptake could occur in compartments of the secretory pathway upstream of secretory granules, e.g. the rER or the Golgi apparatus, and involve active transport.
mediated by different Ca\textsuperscript{2+}-ATPases, such as SERCA (Sarcoplasmic/endoplasmic Reticulum Calcium ATPase) and SPCA (Secretory Pathway Calcium ATPase) pumps\textsuperscript{84,85}. Ca\textsuperscript{2+} released from ZG into the pancreatic juice may have a paracrine function on pancreatic duct cells that express Ca\textsuperscript{2+}-sensing receptors on their apical surface, as proposed by Bruce et al.\textsuperscript{85}.

Secretagogues have been shown to evoke local increases of cytosolic Ca\textsuperscript{2+} in the secretory pole region of pancreatic acinar cells\textsuperscript{86}. Finding studies demonstrating IP\textsubscript{3}-induced Ca\textsuperscript{2+} release from acidic neuroendocrine chromaffin granules induced by the second messenger inositol 1,4,5-trisphosphate (IP\textsubscript{3})\textsuperscript{87}, experimental evidence has suggested that secretory granules from both endocrine and exocrine cells, including pancreatic acinar and airway goblet cells, are IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} stores that play a significant role in Ca\textsuperscript{2+}-dependent secretion (reviewed in\textsuperscript{88,89}). In particular, using ZG isolated by differential centrifugation or intact pancreatic acinar cells, Petersen and colleagues have demonstrated release of Ca\textsuperscript{2+} from an acidic store in the apical pole of acinar cells induced by IP\textsubscript{3}, cyclic ADP ribose (cADPR) or nicotinic acid adenine dinucleotide phosphate (NAADP) (i.e. suggestive of functional IP\textsubscript{3} and ryanodine receptors) that they associated with ZG\textsuperscript{83,90,91}. Moreover, Gerasimenko and coworkers\textsuperscript{82,83} (reviewed in\textsuperscript{85}) hinted at the importance of this acidic ZG store for the pathogenesis of alcohol-induced pancreatitis. However, two independent studies have challenged these observations in pancreatic\textsuperscript{17} and parotid acinar cells\textsuperscript{94}. These authors provided functional and biochemical evidence indicating that ZGM do not express IP\textsubscript{3} or ryanodine receptors, provided that ZG are not contaminated by other organellar structures, especially ER or mitochondria. Although Gerasimenko et al.\textsuperscript{83} had shown a three-fold enrichment of the ZG marker α-amylase in the ZG preparation, the assay used to exclude ER contamination relied on the fluorescent dye DiOC6(3) that is generally applied to assess ATP of energized mitochondria [sic!]\textsuperscript{95}. Using the ZG isolation procedure of Gerasimenko et al.\textsuperscript{83}, Yule et al.\textsuperscript{17} showed disrupted mitochondria and ER membranes by electron microscopy that contaminated the ZG preparation. Interestingly, Gerasimenko et al.\textsuperscript{96} revisited this issue and proposed that the main apical Ca\textsuperscript{2+} pools that are responsible for release of Ca\textsuperscript{2+} by all three intracellular Ca\textsuperscript{2+} messengers IP\textsubscript{3}, cADPR and NAADP are the ER and ill-defined “acidic stores”, where these second messengers elicit Ca\textsuperscript{2+} release by activation of the Ca\textsuperscript{2+} channels IP\textsubscript{3} and ryanodine receptors. The two stores interact with each other via Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release and can be separated using pharmacological tools. The ER relies on SERCA pumps that can be blocked by the specific inhibitor, thapsigargin; Ca\textsuperscript{2+} uptake in the acidic store relies on a bafilomycin-sensitive V-ATPase and probably a Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger, as originally proposed by Thévenod & coworkers for an IP\textsubscript{3}-sensitive ER Ca\textsuperscript{2+} pool\textsuperscript{97,98}. Gerasimenko et al. suggested that a large part of the acidic store is “co-localized” with ZG, however, other acidic organelles like endosomes and lysosomes may also participate in intracellular Ca\textsuperscript{2+} signaling\textsuperscript{96}. This conclusion is more cautious and accurate than the earlier proposal of ZG as acidic Ca\textsuperscript{2+} stores but is still not satisfying. Three lines of evidence argue against ZG being an acidic Ca\textsuperscript{2+} store involved in secretagogue-induced apical Ca\textsuperscript{2+} release. Firstly, the majority of ZG are not acidic\textsuperscript{89} (see also the paragraph on Vacular-type H\textsuperscript{+}-ATPase); secondly, ER also co-localizes with ZG\textsuperscript{100,101}; thirdly, an acidic IP\textsubscript{3}-sensitive ER Ca\textsuperscript{2+} pool has also been described that takes up Ca\textsuperscript{2+} via parallel activity of a V-type H\textsuperscript{+} pump and a Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger, both in pancreatic\textsuperscript{93,98} and parotid acinar cells\textsuperscript{102}. It also appears unlikely that ZG are an IP\textsubscript{3} sensitive Ca\textsuperscript{2+} pool because all proteomic studies of ZGM so far did not detect any IP\textsubscript{3} receptors\textsuperscript{82-13}. Hence, it remains an open question whether other mechanisms of Ca\textsuperscript{2+} release are operative in ZG.

The ATP-gated cation channel P2X4 is Ca\textsuperscript{2+} permeable\textsuperscript{103} and has been identified in ZGM\textsuperscript{9} (see the paragraphs on Proteomics and Vesicular nucleotide transporter SLC17A9). This receptor could play a role in Ca\textsuperscript{2+} release from weakly acidic Ca\textsuperscript{2+} stores\textsuperscript{104}, such as ZG, because P2X4 receptors are normally inhibited at acidic pH and are activated when intraluminal pH increases\textsuperscript{105-107}. Finally, Hille and coworkers\textsuperscript{108} have shown a release of Ca\textsuperscript{2+} via “store-operated” Orai channels in the membrane of secretory granules from neuroendocrine PC12 cells (interestingly, they did not detect IP\textsubscript{3} receptors in these secretory granules, in contrast to previous studies\textsuperscript{89}). The granule Orai channels are activated by regulator stromal interaction molecule 1 (STIM1) on the ER and may raise local cytoplasmic Ca\textsuperscript{2+} concentrations for refilling of Ca\textsuperscript{2+} stores of the ER and promotion of exocytosis\textsuperscript{105}. A subsequent study in RBL-2H3 mast cells identified Orai-2 on secretory granules\textsuperscript{106}. Ca\textsuperscript{2+} release from a Ca\textsuperscript{2+} store induced by antigen stimulation was attenuated by Orai-2 silencing, while that induced by thapsigargin was not affected. Furthermore, exocytotic release induced by antigen stimulation was inhibited in Orai-2 silenced cells\textsuperscript{106}.

**Transporters**

**Vacular-type H\textsuperscript{+}-ATPase (V-ATPase)**

One of the hallmarks of many vesicular compartments is the expression of a V-ATPase that operates to acidify the vesicular lumen\textsuperscript{107}. Subunits of the V-ATPase have been clearly identified in the membrane of rat pancreas ZG by immunological methods\textsuperscript{108} and by proteomic analyses\textsuperscript{85,86,109}. Nevertheless, conflicting results were obtained in several early studies when measuring luminal pH of secretory granules in freshly isolated acini or purified granules.
Using fluorescence microscopy and the weak base acridine orange (AO) that accumulates in acidic organelles and changes its fluorescence from green to orange when concentrated, ZG were found to be acidic whereas parotid secretory granules showed a neutral pH\(^{23,109-111}\). To resolve this issue, in a landmark study Orci et al.\(^9\) used the probe 3-(2,4-dinitroanilino)-3-amino-N-methyldipro-pylamine (DAMP), a weak base that accumulates in the lumen of acidic organelles, where it can be fixed\(^{112}\). Quantitative immunostaining of DAMP in conjunction with electron microscopy was used to measure pH in the secretory pathway of fixed isolated pancreatic acini. The data revealed that condensing vacuoles are acidic but lose their acidity during maturation\(^9\). To account for these results, the authors speculated that the loss of acidity of maturing ZG may be the consequence of either removal of H\(^+\)-pump subunits, inactivation of the V-ATPase, or closing of an anion conductance. It is noteworthy that it took more than 20 years to confirm the observation that the acidity of ZG decreases during maturation, using freshly isolated pancreas acini with AO and live-cell two-photon imaging\(^{113}\), thus indicating that earlier AO measurements with fluorescence microscopy techniques were not accurate. Of note, the study by Thorn and coworkers\(^{113}\) also showed that the H\(^+\) concentration of mature granules is not affected by pre-incubation with a blocker of the V-ATPase. However, in contrast to the data by Orci et al.\(^9\), labeling of the cells with the ratiometric pH indicator, Lysosensor Yellow/Blue, suggested that mature secretory granules have a pH of less than 6.0 (yet without proof that the labelled acidic structures are indeed mature ZG!)\(^{113}\). Furthermore, upon secretagogue-stimulated secretion, the pH in the acinar lumen never dropped below 7.0, which suggests that ZG pH is not or weakly acidic. To account for this discrepancy, Thorn and coworkers have subsequently described a luminal secretagogue-stimulated HCO\(_3^-\) conductance, mediated by TMEM16A/ANO1 that is activated coincidently with ZG fusion and exocytosis\(^8\).

An increase of pH in the secretory pathway during maturation is anomalous because along the secretory pathway of pancreatic (and parotid) acinar cells could also result in a dissipation of the pH gradient during maturation.

**Vesicular nucleotide transporter SLC17A9**

SLC17A9 (also named VNUT for Vesicular Nucleotide Transporter) is a vesicular ATP transporter widely expressed in various organs, but predominantly in the adrenal gland, brain, and thyroid gland (the pancreas was not tested)\(^{113,122}\) (reviewed in references\(^{123,124}\)). SLC17A9 mediates concentative accumulation of ATP in secretory vesicles, such as chromaffin or synaptic vesicles, to a luminal concentration of 100 mM or more\(^{125-127}\) (reviewed in references\(^{125,126}\)). SLC17A9 protein shows a relative mobility varying between 61 and 68 kDa in SDS-PAGE, depending on the origin of the protein (native or recombinant). When reconstituted into proteoliposomes, SLC17A9 transports ATP (as well as ADP, GTP and UTP as shown by \(c\)is inhibition experiments) and uses a \(\Delta\Psi\) (positive inside) but not a \(\Delta\phi\) (the vesicle lumen being acidic) as driving force for uptake. ATP transport depends on external Cl\(^-\) and saturates at ~4 mM external Cl\(^-\). ATP uptake is inhibited by DIDS with an IC\(_{50}\) of ~1.5 \(\mu\)M. Evans blue is even more potent (IC\(_{50}\) ~40 \(\mu\)M) whereas 200 \(\mu\)M atractyloside inhibits SLC17A9 only in the presence of Mg\(^{2+}\). All these properties are similar to those that have been reported for ATP uptake in chromaffin granules and granule membrane ghosts\(^{127}\). Although ATP transport rate appeared to be independent of Ca\(^{2+}\) or Mg\(^{2+}\), subsequent studies showed that SLC17A9 also transports both divalent cations in an ATP- and Cl\(^-\)- dependent manner, which is driven by an inside-positive \(\Delta\phi\), but not by an inside-positive \(\Delta\phi\), and with a similar inhibitor sensitivity as ATP transport\(^{129}\). Hence, divalent cation transport probably occurs by complexation with ATP and may contribute to vesicular accumulation of divalent cations in secretory vesicles.

It has been suggested that during pancreas secretion evoked by secretagogues, ATP is released from ZG into the primary fluid to mediate paracrine signaling between acinar and duct cells and thereby coordinates ductal and acinar fluid secretion\(^{129}\). This may occur via purinergic receptors expressed on the apical membrane of duct cells\(^{130}\). Indeed, pancreatic ZG do contain ATP. From measurements with freshly isolated ZG, it has been estimated that the \(\textit{in vivo}\) concentration of ATP in ZG is 0.5–1 \(\text{mM}\) ATP\(^{117}\), a more than 100-times lower concentration than in neuroendocrine secretory vesicles expressing SLC17A9 and a concentration that is also below the average cytosolic concentration of 2–5 \(\text{mM}\) of a differential activity or expression of V-ATPases and/or differences in H\(^+\) permeability, as suggested by Orci et al.\(^9\). Moreover, activity of exchange pathways for counterions, e.g. Na\(^+\)/H\(^+\), Ca\(^{2+}\)/H\(^+\) or Cl\(^-\)/H\(^+\)-exchangers\(^{118-121}\) along the secretory pathway of pancreatic (and parotid) acinar cells would also result in a dissipation of the pH gradient during maturation.
ATP$^{126}$, thus questioning concentrative ATP transport in ZG. Nevertheless, Novak and coworkers have suggested that SLC17A9 is expressed in the membrane of ZG where it mediates ATP uptake$^{117, 131}$. Their experimental evidence supports expression of SLC17A9 in ZGM, e.g. by detection of a ca. 65-kDa protein with a SLC17A9-specific antiserum, co-localization of immunolabeling for SLC17A9 with the ZG membrane marker Rab3D, or inhibition of ATP uptake into ZG by 100 µM DIDS and Evans blue$^{117, 131}$. Furthermore, induction of rat AR42J acinar cells into a secretory phenotype by dexamethasone administration increases carbachol-induced ATP release and SLC17A9 expression. In Haanes’ and Novak’s work on isolated ZG, ATP uptake depends on external Mg$^{2+}$ and high Cl$^{-}$$^{117, 131}$. Moreover, ATP uptake is driven by an inside-positive ΔpH (either generated by an artificial pH gradient or as demonstrated by bafilomycin A1-sensitive uptake) and by an inside-positive K$^{+}$ diffusion potential. Furthermore, ATP uptake shows no cis inhibition by ADP or UTP$^{117}$. As a matter of fact, most of these properties differ from the transport properties of SLC17A9$^{122}$ (reviewed in$^{123, 124}$). It may be argued that results obtained in intact ZG may differ from transport of a protein reconstituted into proteoliposomes$^{117}$. However, transport of reconstituted SLC17A9 matches transport in intact chromaffin granules$^{122, 127}$. Hence, ATP transport in ZG may occur independently from SLC17A9 expression.

On the other hand, these observations in ZG have gained recent support from studies in lysosomes$^{18, 132}$ and lamellar bodies (LB)$^{19}$. SLC17A9 is expressed in these acidic intracellular organelles, which contain ATP concentrations between <0.5 mM (in lysosomes) and ca. 2 mM (in LB). Interestingly, SLC17A9 expression was functionally linked to the ATP-gated cation channel P2X4$^{19, 133}$ (reviewed in$^{134}$), which is also expressed in ZGM$^{6}$ (see the paragraphs on Proteomics and Ca$^{2+}$ channels). The study by Fois et al.$^{19}$ demonstrates a physiological role of lysosomal P2X4 receptors during the secretion of surfactant from alveolar type II epithelial cells. Apparently, LB fusion triggers P2X4 receptors within the LB membrane to generate a highly localized, cytosolic Ca$^{2+}$ signal in the vicinity of the fused LB, which drives expansion of the fusion pore and facilitates surfactant release (reviewed in$^{135}$). LB are likely lysosome-derived but less acidic than juxtanuclear lysosomes by virtue of reduced V-ATPase activity and an increase in proton leak$^{135}$, once more emphasizing the importance of electrogenic conductive pathways for ions, in particular H$^{+}$, for concentrative ATP uptake in intracellular vesicles.

In conclusion, experiments providing evidence for a causal relationship between ZG ATP accumulation and SLC17A9 are needed to confirm the postulated role of SLC17A9 in (concentrative?) ATP uptake into ZG. This requires, for instance, silencing of the transporter and measurements of ZG ATP concentration and/or secretagogue-induced ATP release from acinar cells, as well as measurements of the driving force for ATP accumulation, namely ΔΨ, in ZG. Moreover, it remains to be tested whether SLC17A9 - if it is indeed expressed in ZGM - may also contribute to the accumulation of Ca$^{2+}$ as nucleotide complex in ZG that, apart from the endoplasmic reticulum represent a significant intracellular Ca$^{2+}$ pool$^{21, 82, 136}$ (see also the paragraph on Ca$^{2+}$ channels). In summary, the available evidence that SLC17A9 is responsible for concentrative ATP uptake into pancreatic ZG awaits further experimental confirmation.

**Zinc transporter SLC30A2**

The SLC30A2 gene encodes a zinc (Zn$^{2+}$) transporter (also named ZnT2 for Zinc Transporter 2) that was cloned from a rat kidney cDNA expression library by complementation of a Zn$^{2+}$-sensitive Baby Hamster Kidney cell line$^{137}$ and is highly expressed in pancreas (reviewed in$^{138–146}$). Using ZnT2-GFP fusion proteins and immunofluorescence microscopy, the transporter was localized in Zn$^{2+}$ accumulating acidic intracellular vesicles (as opposed to the Zn$^{2+}$ efflux transporter SLC30A1/ZnT1 that is expressed in the plasma membrane$^{141}$). The authors suggested that ZnT2 contributes to protection against Zn$^{2+}$ toxicity by facilitating Zn$^{2+}$ influx into an endosomal/lysosomal compartment thereby lowering cytosolic Zn$^{2+}$ jointly with plasma membrane ZnT1$^{137}$. Subsequent studies have revealed that, in addition to the pancreas, SLC30A2 is also expressed in mammary gland, prostate, retina, small intestine and kidney. In these organs, it is localized on the membrane of intracellular organelles (e.g. endosomal/lysosomal and secretory vesicles) and sequesters cytoplasmic Zn$^{2+}$ for secretion, storage, or for use in proteins that require Zn$^{2+}$ for their activities (reviewed in$^{138–146}$). Interestingly, a loss-of-function mutation or silencing of SLC30A2 results in reduced Zn$^{2+}$ secretion from mammary gland epithelial cells in vivo and in vitro$^{142}$. Subsequently, transient neonatal Zn$^{2+}$ deficiency (TNZD) in infants was found to be caused by a variety of loss-of-function mutations in the SLC30A2 gene in women, resulting in poor secretion of Zn$^{2+}$ into the breast milk. Consequently, infants exclusively breastfed with Zn$^{2+}$-deficient breast milk develop severe Zn$^{2+}$ deficiency (reviewed in$^{143}$). The transport mechanism of SLC30A2 has been recently elucidated$^{144}$: Similarly to vesicular Zn$^{2+}$ transport mediated by other mammalian SLC30 transporters (SLC30A1 and SLC30A5)$^{145, 146}$, SLC30A2 is an electroneutral proton-coupled vesicular antipporter displaying an apparent stoichiometry of two H$^{+}$ per Zn$^{2+}$ ion and is driven by the vesicular H$^{+}$ gradient generated by V-ATPases$^{144}$.

In pancreatic endocrine β-cells, secretory granules accumulate Zn$^{2+}$ almost 40-fold above cytosolic levels,
whereas no difference between cytosolic and acinar ZG Zn²⁺ concentration has been found, as measured by X-ray microanalysis²⁰. In β-cell granules (and other islet secretory granules), SLC30A8 is the Zn²⁺ transporter that provides Zn²⁺ for maturation of stored macromolecules, such as insulin, and their crystallization before secretion¹⁴⁷, and its possible importance in the etiology of diabetes has been highlighted¹⁴⁸. Because insulin containing granules are acidic⁶⁹–¹⁵¹, SLC30A8-mediated Zn²⁺ accumulation against a concentration gradient is likely driven by the vesicular H⁺ gradient generated by V-ATPases and could occur via H⁺/Zn²⁺ exchange, as demonstrated for SLC30A1, SLC30A2 and SLC30A5¹⁴⁴–¹⁴⁶.

The situation in ZG is much less clear. In rats, Zn²⁺ deficiency caused Zn²⁺ depletion in acinar but not in β-cells¹⁵², which is in line with the ability of β-cells to store Zn²⁺ in insulin granules in a slowly-exchanging intra-granule compartment and also with the apparent inability of pancreatic ZG to accumulate Zn²⁺. Liuzzi et al.¹⁵³ showed that Zn²⁺ depletion in mice is associated with a reduction of pancreatic acinar SLC30A1 and SLC30A2, and the authors speculated that SLC30A1/2 may mediate lowering of cytosolic Zn²⁺ in pancreatic acinar cells of Zn²⁺ replete animals. Furthermore, De Lisle and coworkers showed secretion of the cytosolic Zn²⁺-binding protein metallothionein into pancreatic juice¹⁵⁴. All these studies led to the hypothesis by Cousins and colleagues that exocytotic secretion of Zn²⁺ by pancreatic acinar cells may be modulated by Zn²⁺ transporter expression, which would thus affect enteropancreatic Zn²⁺ trafficking and also regulate physiological Zn²⁺ body homeostasis by controlling Zn²⁺ losses into the pancreatic fluid and intestinal excretion of Zn²⁺. More recently, Cousins and coworkers demonstrated SLC30A2 expression in ZG, and reduction of the Zn²⁺ diet of mice was paralleled by decreases of Zn²⁺ concentrations and SLC30A2 expression in ZG. Rat AR42J acinar cells, when induced into a secretory phenotype with dexamethasone, exhibited increased SLC30A2 expression that was associated with a reduction of cytosolic⁶⁵ Zn²⁺ content and an increase of the ᵅ⁶⁵Zn²⁺ content of ZG. SLC30A2 silencing of AR42J cells by RNAi (~70% mRNA reduction) increased cytoplasmic⁶⁵ Zn²⁺ by 36% and decreased ZG⁶⁵Zn²⁺ by 15%, suggesting that SLC30A2 mediates the sequestration of Zn²⁺ into ZG. The relatively low impact of SLC30A2 silencing on ZG Zn²⁺ content may be explained by low ZG Zn²⁺ below cytosolic concentrations, which is likely caused by the loss of acidity of ZG during maturation (see the paragraph on Vacular-type H⁻/ATPase). Alternatively, other Zn²⁺ transporters may also contribute to Zn²⁺ fluxes across the ZG membrane.

What could be the role of Zn²⁺ in ZG? For instance, it is known that ZG contain Zn²⁺-metalloenzymes, such as carboxypeptidases that require Zn²⁺ for their function¹⁵⁷,¹⁵⁸, but also for inhibition of their function at higher (micromolar) Zn²⁺ concentrations¹⁵⁹. Although SLC30A2 is expressed in ZG, Zn²⁺ uptake via SLC30A2 (or other members of the SLC30 protein family) is more likely to be operative in precursor acidic organelles of the secretory pathway, e.g. the Golgi apparatus or immature granules after protein synthesis¹⁶⁰. Whether secretagogue-evoked exocytotic secretion of Zn²⁺ from ZG and/or secretion of Zn²⁺-metallothionein¹⁵⁴ are effective means to regulate Zn²⁺ body homeostasis remains to be shown. In fact, regulation of the expression of transporters for Zn²⁺ uptake (from dietary sources and/or the pancreatic juice) in the intestinal mucosa may be a more effective and sensitive means to control Zn²⁺ body homeostasis¹⁶¹–¹⁶³. However, a divergent view, based on zebrafish studies, concluded that the responses of epithelial tissues to Zn²⁺ deficiency and excess are best explained by local epithelial homeostasis, with no evidence of systemic control¹⁶⁴.

Conclusion and Perspectives

From a scientific perspective, the major unresolved issue is not so much the presence/absence of specific pancreatic ZG channels and transporters - there is no doubt that channels and transporters are expressed in the membrane of ZG - but rather a clarification of their physiological role in pancreatic acinar cells during the process of maturation, fusion and exocytosis of ZG. Here the development of organ-specific channel/transporter knockout animal models is mandatory to move the field forward. In this context the anomalous property of the pancreatic acinar cell, which displays a decreasing ZG acidity during maturation (see Figure 1 and the paragraph on Vacular-type H⁻/ATPase) remains an enigma because other secretory cells, in particular neuroendocrine cells, rather exhibit increasing acidity along the secretory pathway¹¹⁴,¹¹⁵. This is particularly puzzling because studies in neuroendocrine cells have highlighted the important role of the V0 domain of the V-ATPase as a sensor of intragranular pH that identifies and regulates the ability of granules to undergo exocytosis¹⁶⁵,¹⁶⁶. Hence, the functional implication of a decreasing acidity of maturing ZG for exocytosis needs to be addressed. Moreover, more studies investigating differences in H⁻ permeability¹¹⁴ and/or activity of exchange pathways for counter-ions, e.g. Na⁺/H⁺-, Ca²⁺/H⁺- or Cl⁻/H⁺-exchangers¹¹⁸–¹²¹ along the secretory pathway of pancreatic acinar cells and their contribution to dissipation of the pH gradient during maturation are required, to better understand their significance in the process of regulated exocytotic secretion.

As this review has sought to demonstrate, many unsolved – and neglected – problems remain in ZG physiology. They need to be addressed and require clarification before we can frame accurate concepts of efficient
preventive and therapeutic strategies for diseases of the pancreas, such as acute and chronic pancreatitis.

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References


57. Stobrawa SM, Breiderhoff T, Takamori S, Engel D, Mukhopadhyay AK, Davidoff MS, Holstein AF, Jentsch TJ. Male germ cells and photoreceptors, both dependent on ClC-2 Cl− channel disruption. EMBO J 2001;20:1289–1299.


trypsinogen activation elicited via Ca\textsuperscript{2+} release through IP\textsubscript{3} receptors. Proc Natl Acad Sci U S A 2011;108:5873–5878.
100. van de Put FH, Elliott AC. The endoplasmic reticulum can act as a functional Ca\textsuperscript{2+} store in all subcellular regions of the pancreatic acinar cell. J Biol Chem 1997;272:27764–27770.
108. Roussa E, Alper SL, Thévenod F. Immunolocalization of anion exchanger AE2, Na\textsuperscript{+}/H\textsuperscript{+} exchangers NHE1 and NHE4, and vacuolar type H\textsuperscript{+}-ATPase in rat pancreas. J Histochem Cytochem 2001;49:463–474.


Chapter 13
Secretory portal—porosome in pancreatic acinar cells

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text

Introduction

Secretion is a fundamental process through which cells communicate with their environment and exchange information in a multicellular context to reach homeostasis and sustain life. For decades, the prevailing view was that secretion operates as an all-or-none “complete fusion” event, where vesicles are trafficked to the cell surface to fuse and completely incorporate into the plasma membrane. The vesicle contents then diffuse out of the cell. This hypothesis, although attractive at first glance, had several key setbacks. First, it predicted a quantization of secretory products packaged into each secretory vesicle, when in fact, secretory vesicle size greatly varies even within the same cell, sometimes as much as sixfold. Second, the level of additional regulation necessary to rapidly and precisely internalize and sequester vesicle-associated lipids and proteins following incorporation into the cell plasma membrane seems extraordinarily complex, given the tens of thousands of different membrane lipids and their differential distribution even between the same bilayer leaflets. Third, following a secretory episode, partially empty secretory vesicles accumulate within cells as observed in electron micrographs (Figure 1), demonstrating that secretory vesicles are capable of transient fusion at the cell plasma membrane and incomplete or partial content release.

We therefore hypothesized some 25 years ago the presence of a tunable dial at the cell plasma membrane for secretory vesicle docking and fusion without full collapse and the generation of hydrostatic pressure within vesicles to drive vesicular contents to the cell exterior. Through the use of atomic force microscopy, a force spectroscopy, we identified nanoscale transmembrane cup-shaped lipoprotein structures at the cell plasma membrane first in acinar cells of the exocrine pancreas and later in all cells examined including neurons. We named the structures “porosomes” that have since been implicated in a wide range of secretory events. The family of proteins that make up the porosome has been biochemically identified and the mesoscale structure of the complex has been well characterized through electron microscopy and solution X-ray methods. Defects in one or more porosome components have measurable, often highly potent effects on the regulation of secretion, establishing links between point mutations and secretion-defective disease states such as neurological disorders and cystic fibrosis that were previously correlative and are now causative. The discovery of the porosome solved the conundrum of fractional discharge of intravesicular contents from cells by providing an explanation for regulated graded secretion. Porosomes are cup-shaped unidirectional (inside cells to the outside) transport machineries, which are supramolecular lipoprotein structures at the cell plasma membrane ranging in size from 15 nm in neurons to 100–180 nm in endocrine and exocrine cells and composed of around 30–40 proteins. In comparison, the 120-nm bidirectional transport machinery, the nuclear pore complex, is composed of nearly 1,000 protein molecules.

Porosomes in the Exocrine Pancreas

In 1973, the “transient” or “kiss-and-run” mechanism of secretory vesicle fusion at the cell plasma membrane enabling fractional release of intravesicular contents during secretion was proposed.2 Nearly two decades later in 1990, it was hypothesized that the fusion pore, a continuity established between the vesicle membrane and the cell plasma membrane, results from a “preassembled ion channel-like structure that could open and close.”3 A later 1992 review4 opined that the principal difficulty in observing these hypothetical pore structures was the lack of ultrahigh resolution imaging tools to directly monitor their presence and study their activity in live cells.

In the mid-1990s, employing the then newly developed technique of atomic force microscopy (AFM), nanometerscale cup-shaped pore structures and their dynamics were discovered at the apical plasma membrane in live pancreatic acinar cells.5 Circular pit-like structures containing 100–180 nm cup-shaped depressions or pores were observed at the apical plasma membrane of pancreatic acinar cells

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where secretion is known to occur. During secretion, the depression or pore openings grew larger, returning to their resting size following completion of cell secretion.

Studies next established the observed depressions to be the secretory portals at the cell plasma membrane. Following stimulation of secretion, gold-conjugated amylase antibodies (amylase being one of the major intravesicular enzymes secreted by pancreatic acinar cells) accumulate at depressions. These results established depressions to be the long sought-after secretory portals in cells. The study further reported the presence of t-SNAREs at the pore base facing the cytosol, firmly establishing depression structures to be secretory portals where zymogen granules (ZGs) transiently dock and fuse for intravesicular content release during secretion. Subsequently, porosomes and their dynamics at the cell plasma membrane in growth hormone (GH)-secreting cells of the pituitary gland and in rat chromaffin cells were reported. In 2003, following immunosolation of the porosome structures from acinar cells of the exocrine pancreas, its composition was determined, and isolated porosomes were functionally reconstituted into artificial lipid membranes. Morphological details of porosome associated with docked secretory vesicles were revealed using high-resolution electron microscopy. In the past decade, employing a combination of approaches such as AFM, biochemistry, electrophysiology, conventional EM, mass spectrometry, and small-angle X-Ray solution scattering analysis, this specialized secretory portal has been extensively characterized and documented and found to be present in all cells examined, including neurons. Studies establish porosomes to be secretory portals that perform the specialized task of fractional discharge of intravesicular contents from cells during secretion. The porosome structure and the associated transient fusion mechanism accompanied by fractional discharge of vesicular contents from cells has greatly progressed in the past two decades. It has been demonstrated in exocrine, endocrine, and neuronal cells that “secretory granules are recaptured largely intact following stimulated exocytosis in cultured endocrine cells” that “single synaptic vesicles fuse transiently and successively without loss of identity”, and that “zymogen granule exocytosis is characterized by long fusion pore openings and preservation of vesicle lipid identity.” The past two decades have additionally witnessed much progress in our understanding of Ca^2+ and SNARE-mediated membrane fusion and on secretory vesicle volume regulation required for precise fractional release of intravesicular contents from cells during secretion. These findings have greatly contributed to the progress in our understanding of porosome-mediated fractional release of intravesicular contents from cells during secretion.

**Porosome Discovery: AFM Trained on Pancreatic Acini**

As noted earlier, the porosome was first discovered in acinar cells of the rat exocrine pancreas nearly 25 years ago. This discovery was made possible by the use of a then new microscope, the AFM. In AFM, a probe tip microfabricated from silicon or silicon nitride and mounted on a cantilever spring is used to scan the surface of the sample at a constant force. Either the probe or the sample can be precisely moved in a raster pattern using a xyz piezo to scan the surface of the sample. The deflection of the cantilever measured optically is used to generate an isoforce relief of the sample. Force is thus used by the AFM to image surface profiles of objects at nanometer resolution and in real time, objects such as live cells, subcellular organelles, and even biomolecules submerged in physiological buffer solutions.

Compared to neurons and neuroendocrine cells, exocrine pancreas are slow secretory cells, secreting digestive...
enzymes over a period of minutes following a meal, as opposed to milliseconds or seconds in a fast secretor such as neurons and endocrine cells. Hence, pancreatic acinar cells were ideal for the discovery of possible plasma membrane structures that could be involved in the fractional release of intravesicular contents from cells during secretion. Similar to neurons, exocrine pancreatic acinar cells are polarized secretory cells possessing an apical and a basolateral end. Digestive enzymes in pancreatic acinar cells are stored within 0.2–1.2 μm in diameter apically located membrane-bound secretory vesicles, called zymogen granules (ZGs). It had been well established that following a secretory stimulus, ZGs dock and fuse at the apical plasma membrane to release their contents. The AFM was therefore trained at the apical plasma membrane of live acinar cells in buffer, before and following stimulation of secretion. These studies demonstrate the presence of pore structures only at the apical plasma membrane of the cell where secretion is known to occur. A group of circular “pits” measuring 0.4–1.2 μm in diameter, contain smaller 100–180 nm in diameter “depressions” structures are identified (Figure 3). Typically, three to four depressions are found within each pit structure. Basolateral membrane of acinar cells is devoid of such pit and depression structures. High-resolution AFM images of depressions in live acinar cells further reveal a cup-shaped basket-like morphology, each cup measuring 35 nm in depth and 100–180 nm in diameter. View of the porosome structure at the cytosolic compartment of the plasma membrane in pancreatic acinar cells has also been determined at near nm resolution (Figure 4).

When isolated plasma membrane preparations of pancreatic acinar cells in near-physiological buffered solution are imaged at ultrahigh resolution using the AFM, inverted cup-shaped structures, some with docked ZGs, are demonstrated (Figure 4). AFM surface topology maps of inside-out acinar cell plasma membrane preparations reveal scattered circular disks (pits) measuring 0.5–1 μm in diameter, with inverted cup-shaped structures (depressions or porosomes) within. On a number of occasions, ZGs ranging in size from 0.4–1 μm in diameter are observed to be docked at the base of one or more porosomes (Figure 4). These results further demonstrated that a single secretory vesicle may dock at more than one porosome at the cell plasma membrane. Porosomes in acinar cells of the exocrine pancreas have also been examined using high-resolution transmission electron microscopy (TEM) (Figures 5 and 6), both in isolated cells and tissues (Figure 5), and in association with ZGs prepared from stimulated acinar cells (Figure 6). In Figure 5, the electron micrograph of a porosome sectioned at a certain angle reveals its distinct and separate bilayer and the bilayer membrane

![Figure 2](image-url)
attachment of the associated ZG. A cross-section through three lateral knob-like structures that circle around the porosome cup is clearly delineated. The apical knob-like density at the lip of the porosome appears most prominent and is hypothesized to be composed of actin and myosin, involved in the dilation of the porosome opening during cell secretion. The TEM micrograph further suggests that the lower knob, likely representing the t-/v-SNARE ring or rosette complexes, formed as a consequence of ZG docking and fusion at the base of the porosome complex. Elegant TEM studies in the exocrine pancreas by Craciun and Barbu-Tudoran report docked and post-docked or dis-associated ZGs. Similarly, secretory vesicles at the porosome base have been reported at the nerve terminal and the arrangement of the SNARE complex in a ring or rosette conformation at the porosome base demonstrated. As previously outlined, in vitro studies further reveal the Ca\(^{2+}\) and SNARE-mediated membrane fusion to establish the fusion pore and the molecular process of secretory vesicle volume regulation required for the precise fractional release of intravesicular contents from cells during secretion.

Exposure of pancreatic acinar cells to a secretory stimulus results in a time-dependent increase (20–45%) in both the diameter and relative depth of porosomes (Figure 7). Porosomes return to their resting size on completion of cell secretion. No demonstrable change in pit size is detected following stimulation of secretion. 

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**Figure 3.** To the left is an AFM micrograph of the apical plasma membrane of a live pancreatic acinar cell demonstrating the presence of a pit (yellow arrow) with depressions or porosomes within (blue arrow). To the right is a schematic drawing demonstrating pits and cup-shaped porosomes where zymogen granules (ZGs), the secretory vesicles in exocrine pancreas, dock and transiently fuse to release intravesicular digestive enzymes from the cell (from reference 9).

**Figure 4.** AFM micrograph showing cup-shaped depression or porosome structures at the pancreatic acinar cell plasma membrane. (A) Several circular “pits” (yellow arrowheads) with depressions or porosomes (red arrowheads) are seen in this AFM micrograph of the apical plasma membrane in live pancreatic acinar cell. (B) AFM micrograph of the cytosolic compartment of isolated pancreatic plasma membrane preparation depicting a “pit” (yellow arrowheads) containing several inverted cup-shaped porosomes (red arrowhead) within. ZG (blue arrowhead) is found associated with porosomes in figures c and d. (C) The “pit” and inverted fusion pores in b is shown at higher magnification. (D) AFM micrograph of another “pit” with inverted fusion pores within, and associated with a ZG, is shown. Bar = 200 nm (from reference 9).
of porosome diameter and an increase in its relative depth, following exposure to a secretory stimulus, correlates with secretion (Figures 7 and 8). Additionally, exposure to cytochalasin B, a fungal toxin that inhibits actin polymerization and secretion, results in a 15–20 percent decrease in porosome size and a consequent 50–60 percent loss in cell secretion. These results suggested depressions or porosomes to be the secretory portals in pancreatic acinar cells. Results from these studies further demonstrate actin to compose the porosome in addition to implicating actin in regulation of both the structure and function of porosomes.

To further confirm if porosomes serve as cellular secretory portals, the direct observation of the release of secretory products through the structure was required. This was accomplished using immuno-AFM studies in acinar cells of the exocrine pancreas (Figures 9 and 10) where gold-conjugated amylase-specific antibody was demonstrated to selectively decorate the porosome opening following a secretory stimulus. Since
ZGs dock and fuse at the plasma membrane to release vesicular contents, it was hypothesized that if the inverted cups or porosomes are indeed the secretory portals, then plasma membrane-associated t-SNAREs should be present at the base of the porosome cup facing the cytosol. Since the t-SNARE protein SNAP-23 is present in pancreatic acinar cells, a polyclonal monospecific SNAP-23-specific antibody recognizing a single 23-kDa protein in Western blots of pancreatic acinar cell plasma membrane fraction was used in the study. Immuno-AFM studies demonstrate the selective binding of the SNAP-23 antibody to the base of the porosome complex (Figure 11). These results establish that the inverted cup-shaped structures in the inside-out pancreatic plasma membrane preparations are indeed secretory portals or porosomes, where secretory vesicles transiently dock and fuse to release their contents during cell secretion.

Immunooisolation studies using SNAP-23-specific antibody on solubilized pancreatic plasma membrane fractions demonstrate the isolation of the porosome complex as assessed both structurally (Figures 12 and 13) and functionally (Figure 14). Furthermore, immunochemical characterization of the pancreatic porosome complex demonstrates the presence of SNAP-23, syntaxin 2, actin, fodrin, vimentin, chloride channels CLC2 and CLC3, calcium channels β3 and α1c, and the SNARE regulatory protein NSF, among other proteins. Transmission electron micrographs of pancreatic porosomes reconstituted into liposomes exhibit a 150–200-nm cup-shaped basket-like morphology (Figure 13), similar to its native structure observed in cells and when coisolated with ZG preparation. To test the functionality of the immunooisolated porosome complex, purified porosomes obtained from exocrine pancreas have been reconstituted in the lipid membrane of the EPC9 electrophysiological bilayer apparatus and exposed to isolated ZGs (Figure 14). Electrical activity of the porosome-reconstituted membrane as well as the transport of vesicular contents from the cis to the trans compartments of the bilayer chambers demonstrate that the lipid membrane-reconstituted porosomes are indeed functional. Thus, in the presence of calcium, isolated secretory vesicles dock and fuse to transfer intravesicular contents from the cis to the trans compartment of the bilayer chamber (Figure 14). ZG fusion and content release through the reconstituted porosome is demonstrated by the increase in capacitance and conductance and a time-dependent transport of the ZG enzyme amylase from cis to the trans compartment of the bilayer chamber measured using Western blot assays. Amylase is detected using immunoblot analysis of the buffer in the cis and trans compartments of the bilayer chambers. Chloride channel activity present in the porosome complex has been demonstrated to be critical to porosome function, since the chloride channel blocker DIDS is inhibitory to the function of the reconstituted porosome (Figure 14). Interestingly, recent studies demonstrate the interaction between the cystic fibrosis transmembrane conductance regulator (CFTR) and the porosome complex in human airways epithelia. This study implies that the CFTR chloride selective ion channel affects mucus composition and secretion through the porosome complex at the cell plasma membrane of the airways epithelia. Similarly, in cystic fibrosis, CFTR dysfunction is known to reduce secretory activity of pancreatic duct cells, which leads to blockage of the ducal system and eventual fibrosis of the entire gland. This is just one examples of diseases resulting from altered porosome-associated protein.

**Porosome Proteins in Secretory Defects and Disease**

Porosomes are composed of 30–40 proteins. The composition of proteins in porosomes from different tissue has been demonstrated to be similar, if not identical. Among porosome proteins of the exocrine pancreas are the t-SNAREs (SNAP-23 and syntaxin), actin, vimentin, α-fodrin, Ca²⁺- α1c, and Ca²⁺-β1c. Similarly, in the well-characterized neuronal porosomes, actin, SNAREs, tubulin, and calcium
and potassium transporters have also been reported.\textsuperscript{7,10,24} It has long been established that actin is involved in neurotransmission,\textsuperscript{71} and our own studies demonstrate its role in secretion of amylase from the exocrine pancreas.\textsuperscript{5} Similar to the exocrine pancreas demonstrating the collapse of the porosome opening and loss of amylase secretion following exposure to the actin depolymerizing cytocholasin D,\textsuperscript{35} studies performed on neurons in presence of latrinculin A, also an actin depolymerizing agent, partially blocked neurotransmitter release at the presynaptic terminal of motor neurons.\textsuperscript{11} Actin b protein, a posttranslational product of actb mRNA, is important in formation of excitatory synapses, which is promoted by interaction of actb mRNA with Src associated in mitosis (Sam68 protein). Loss in Sam68 diminishes its interaction with actb mRNA leading to lower levels of synaptic actin b, which in turn leads to neurological disorders involved with abnormal synaptic transmissions.\textsuperscript{72} Although tubulin involvement in
Figure 8. Changes are observed only in depressions or porosomes following stimulation of secretion. Analysis of the dimensions a–d, schematically represented at the top and graphically presented below, demonstrates a significant increase in the porosome diameter at 5 minutes and a return toward prestimulatory levels after 30 minutes. No changes (100%) in a–c are seen throughout the times examined. Pit and porosome diameters were estimated using section analysis software from Digital Instruments. Each single pit and porosome was measured twice, once in the scan direction and once at 90° to the first (from reference 6).

neurotransmission is not fully understood, its association with several synaptosomal proteins at the presynaptic membrane suggests an important role in neurotransmission. Besides actin and tubulin, other important classes of proteins identified in the neuronal porosome are the membrane-integrated ion-channel proteins that are important in maintaining cellular homeostasis. A good example is the alpha subunit 3 of Na⁺/K⁺ATPase present in the porosome and plays a critical role in neurotransmission. Na⁺/K⁺ATPase activity is known to be blocked by dihydrouobain (DHO). Activation of Na⁺/K⁺ATPase inhibits synaptic transmission, which results from the secretion of a presynaptic protein “follistatin-like 1” which activates the alpha 1 subunit of the ATPase. Transient blocking of Na⁺/K⁺ATPase pump using DHO results in an increase in both the amplitude and number of action potentials at the nerve terminal. Interestingly, Na⁺/K⁺ATPase inhibition is

Figure 9. Intravesicular contents are expelled from within cells to the outside through the porosome during cell secretion. (A) and (B) AFM micrograph and section analysis of a pit and two of the four porosomes, demonstrating enlargement of porosomes following stimulation of cell secretion in the acinar cell of the exocrine pancreas. (C) Exposure of live cells to gold-conjugated amylase antibody (Ab) results in selective localization of gold particles to these secretory sites. Note the localization of amylase-specific 30-nm immunogold particles at the edge of porosomes. (D) AFM micrograph of pits and porosomes with immunogold localization demonstrated in cells immunolabeled and then fixed. Blue arrowheads point to immunogold clusters and the yellow arrowhead points to a porosome opening (from reference 6).
calcium dependent, and it has been demonstrated that increased intracellular calcium levels inhibit Na⁺/K⁺ATPase, which increases excitability of neurons. Similarly, the porosome protein plasma membrane calcium ATPases (PMCA) is an important class of proteins known to be involved in maintaining calcium homeostasis within cells including neurons. PMCA2 has been shown to colocalize in synaptosomes with synaptophysin. At the presynaptic membrane, syntaxin-1, also a porosome protein, has been demonstrated to colocalize with PMCA2 and the glycine transporter 2 (GlyT2) that is found coupled to the Na⁺/K⁺ pump, suggesting the presence of a protein complex involved in neurotransmission. Similarly, deletion of PMCA2 generates an ataxic phenotype in mice, where the neurons possess prolonged hyperpolarized membrane, resulting from an increase in the basal levels of calcium within these neurons. Mutation in the PMCA2 encoding gene is known to result in homozygous deafwaddler mice (dfw/dfw) and they show high levels of calcium accumulation within their synaptic terminals. NAP-22, also known as BASP-1, is a protein found in the neuronal porosome complex. Due to its localization at the pre- and postsynaptic membranes and also in synaptic vesicles, it has been long speculated to be involved in synaptic transmission. NAP-22 is known to bind to the inner leaflet of lipid rafts suggesting interaction with cholesterol. Adenylyl cyclase associated protein-1 (CAP-1) is known to regulate actin polymerization and both actin and CAP-1 are present in the porosomal complex. In cells depleted of CAP-1, using RNAi results in lamellipodia growth and F-actin accumulation along with other cytoskeletal abnormalities. In Alzheimer’s, the levels of CNPase (2,3-cyclic nucleotide phosphodiesterase) and the heat shock protein 70 (HSP70) are found to increase while the levels of

Figure 10. AFM and immune-AFM micrographs of the pancreatic acinar cell porosome demonstrating pore morphology and the release of secretory products at the site. (A) A pit with four porosomes within, found at the apical surface in a live pancreatic acinar cell. (B) After stimulation of secretion, amylase-specific immunogold localize at the pit and porosome openings, demonstrating porosomes to represent the secretory release sites in cells. (C) Some porosomes demonstrate greater immunogold localization, suggesting greater secretory product release through them. (D) AFM micrograph of a single porosome in a live acinar cell (from reference 7).
Dihydropyrimidinase-related protein-2 (DRP-2) decrease. Decreased levels of CNPase have been detected in the frontal and temporal cortex of patients with Alzheimer’s disease and/or Down’s syndrome. Low CNPase levels have also been detected in postmortem anterior frontal cortex in schizophrenic patients. Additionally, an allele that is associated with low levels of CNPase is also reported to be linked to schizophrenia. CNPase is also detected as a
marker for oligodendroglia and myelin and several diseases associated with low levels of CNPase indicating low myelin on neurons.\textsuperscript{23} CNPase-positive cells have been shown to increase in corpus callosum of rats exposed to an enriched environment, meaning given to perform a task. Similarly, alterations in the levels of several of the SNARE proteins are associated with various neurological disorders. For instance, SNAP-25 and synaptophysin are significantly reduced in neurons of patients with Alzheimer’s disease. Mice that are SNAP-25 (+/-) show disabled learning and memory and exhibit epileptic-like seizures.\textsuperscript{23}

Overexpression of SNAP-25 also results in defects in cognitive function. Loss of SNAP-25 is also associated with Huntington’s disease and a reduction in rabphilin 3a, another protein involved in vesicle docking and fusion at the presynaptic membrane. SNARE knockout mice are neonatally lethal and mice with a dominant mutation in SNAREs are known to develop ataxia and show impairment in vesicle recycling capability. Increase in synaptophysin levels along with SNAP-25 are also observed in Brodman’s area in the postmortem brain of patients with bipolar disorder I. Protein levels of synaptotagmin and
syntaxin 1 are highly upregulated in areas of cerebral ischemia, which are known to exhibit highly active levels of neurotransmission. Mutations in certain regions of the protein syntaxin 1A, such as Ca\(^{2+}\) channel-binding region, are known to increase neurotransmitter release, which suggests that syntaxin 1A is involved in regulating Ca\(^{2+}\) channel function. A point mutation in syntaxin 1A is known to result in augmented release of neurotransmitters in models of the fruit fly *Drosophila melanogaster*. Synaptotagmin similar to syntaxin has Ca\(^{2+}\)-binding domains and is known to form dimers and interact with syntaxin to form complexes in the presence of calcium. Binding of synaptotagmin to SNAP 25 is also calcium-dependent. Reticulons are proteins which contribute to lipid membrane curvature and are found in the neuronal porosome complex. The involvement of reticulons with the porosome and diseases associated with their deregulation lend credence to the role of membrane curvature in regulation of synaptic vesicle fusion at the porosome complex. These studies reflect on the critical role of various neuronal porosome proteins in protein–protein and protein–lipid interactions within the porosome complex and in their participation in normal neurotransmission at the nerve terminal and in disease. In addition to the above studies discussed on the involvement of porosome proteins in neurotransmission-related disease, recent morphological studies on the neuronal porosome complex have also shed light on both the health and disease status of the neuronal porosome complex as examined using high-resolution EM. Ultrastructure of the neuronal porosome complex in rats subjected to continuous white noise that is relevant to the increasing random noise encountered by humans in
today’s environment is known to provoke diverse effects on different brain regions such as the documented alteration in the length of the porosome complex. Constant exposure to such noise is further known to sabotage the development and normal function of audition, impair hearing, language acquisition, memory performance, and other cognitive functions.

Summary

In summary, these studies demonstrate how the power and scope of the AFM enabled the discovery of the porosome, first in acinar cells of the exocrine pancreas and later in other cell types including neurons. Porosomes are permanent supramolecular lipoprotein structures at the cell plasma membrane in pancreatic acinar cells, where membrane-bound secretory vesicles called ZGs transiently dock and fuse to release intravesicular contents from within cells to the outside. A schematic drawing of porosome-mediated fractional release of intravesicular contents during cell secretion is presented in Figure 15. As opposed to the complete merger of secretory vesicles at the cell plasma membrane (via an all or none mechanism), the porosome complex prevents secretory vesicle collapse at the cell plasma membrane during the secretory process and provides precise regulation of intravesicular content release during cell secretion. Whether secretion involves the docking and fusion of a single vesicle or compound exocytosis where a docked vesicle may involve a number of vesicles fused, the porosome would provide specificity and regulation of content release without compromising the integrity of the secretory vesicle membrane and the cell plasma membrane. In this porosome-mediated secretion, following fractional discharge of intravesicular contents, the partially empty vesicle may undergo several docking-fusion-release cycles until empty, prior to being recycled. However, in neurons, synaptic vesicles could be rapidly refilled via neurotransmitter transporters present at the vesicle membrane. A number of secretory defects and disease states are now recognized as a consequence of dysfunction of one or more porosome proteins. Further understanding of the molecular structure and regulation of the porosome complex will provide new and novel therapeutic applications. The recent functional reconstitution of the insulin-secreting porosome...
complex in live beta cells of the endocrine pancreas opens a window in the treatment of diabetes. To determine the distribution of constituent proteins within the complex will require further studies using small-angle X-ray solution scattering, chemical cross-linking followed by mass spectrometry, immuno-EM, immuno-AFM, and single-particle cryo-electron tomography.

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References

32. Taraska JW, Perrais D, Ohara-Imaizumi M, Nagamatsu S, Almers W. Secretory granules are recaptured largely intact


Exocrine Pancreas Integrated Responses
Chapter 14

Regulation of pancreatic secretion

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Introduction
The exocrine pancreas secretes digestive enzymes, fluid, and bicarbonate in response to food ingestion. This is a critical digestive process that is regulated by neural reflexes, gastrointestinal hormones, and absorbed nutrients. Secretion is highly regulated by both stimulatory and inhibitory influences that coordinate the delivery of digestive enzymes with food emptying into the intestine to assure adequate digestion of a meal. In the absence of proper pancreatic secretion, maldigestion and malabsorption of nutrients may cause malnutrition and associated complications. This review will describe the physiological processes that regulate pancreatic exocrine secretion.

Phases of Meal Response
Pancreatic secretion in response to a meal occurs in four distinct but overlapping phases which are named based on the location of ingested food. The four phases of pancreatic secretion are cephalic, gastric, intestinal, and absorbed nutrient. Considerable crosstalk and inter-regulation is associated within the phases, thereby ensuring adequate, but not excessive, enzyme and bicarbonate secretion. Each phase is regulated by a complex network of neural, humoral, and paracrine feedback mechanisms which help to maintain an optimal environment for food digestion and absorption.

Cephalic phase
Sensory inputs such as sight, smell, taste, and mastication (prior to swallowing) lead to the anticipation of food. These sensations initiate the first phase of pancreatic secretion known as the cephalic phase. In addition to sensory input, interaction of certain food molecules such as long-chain fatty acids (but not triglycerides or medium-chain fatty acids) with receptors in the oral cavity also induce the cephalic phase. Furthermore, studies in animals have implicated a gustatory vagopancreatic reflex in mediating the cephalic phase of pancreatic secretion. Approximately 20–25 percent of the total pancreatic exocrine secretion occurs during the cephalic phase. This estimate is based on data obtained by sham feeding, a process by which food is anticipated by sight, smell, and taste, but not ingested. Sham feeding in animals, such as dogs, has been evaluated by inserting a surgically prepared gastric fistula that diverts food from the esophagus, allowing swallowing but not entry of food into the stomach. In humans, sham feeding involves chewing but not swallowing. The pancreatic response to sham feeding in humans lasts approximately 60 minutes while in dogs it can last for more than 4 hours. Sham feeding stimulates pancreatic secretion which is low in bicarbonate but rich in enzymes, suggesting that pancreatic acinar, rather than ductal, cells are stimulated in this phase.

The cephalic phase of exocrine secretion is under the control of the vagus nerve. Sensory inputs arising from anticipation of food are integrated in the dorsal vagal complex (located in the brainstem) and transmitted to the exocrine pancreas via the vagus nerve. Cholinergic agonists produce secretory responses similar to cephalic stimulation while vagotomy blocks the cephalic responses, suggesting that acetylcholine released by vagal efferents is the primary mechanism by which sensory inputs lead to exocrine secretion. Secretion of the islet hormone, pancreatic polypeptide (PP), increases with sham feeding and serves as an indicator of vagal innervation of the pancreas, as its secretion is inhibited by cholinergic blockers. When sham feeding is accompanied by swallowing, the pancreatic secretory and PP responses are much greater, implying that chewing and swallowing stimulate PP secretion by cholinergic mechanisms.
The exocrine pancreas also contains peptidergic nerve terminals and there is some evidence to suggest that neuropeptides such as vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP) may influence the cephalic phase. In addition, thyrotropin-releasing hormone stimulates pancreatic exocrine secretion of protein and bicarbonate through vagal efferents and this process involves both muscarinic and VIP receptors. In contrast, the effects of inhibitory cerebral calcitonin gene-related peptide (CGRP) are mediated by sympathetic noradrenergic efferents acting upon α-adrenergic receptors. Sham feeding and electrical vagus nerve stimulation in dogs triggers the release of cholecystokinin (CCK), although this response may be absent in humans. Endogenous CCK was shown to enhance PP release in humans during sham feeding. Therefore, although peptidergic neurotransmitters are released during vagal stimulation, acetylcholine is believed to be the main neurotransmitter which regulates the cephalic phase.

**Gastric phase**

Entry of food into the stomach initiates the gastric phase of pancreatic secretion. This phase has been difficult to study in anaesthetized animals because presence of food in the stomach initiates neural reflexes and release of hormones. Therefore, physiological data regarding this phase have been collected by gastric distention induced either by balloon dilation or instillation of inert substances in the antrum. Experiments in which gastric contents were prevented from emptying into the duodenum demonstrated that the gastric phase accounted for approximately 10 percent of pancreatic secretion. Secretions induced during this phase consist mainly of enzymes with minimal release of bicarbonate suggesting that acinar cells are primarily involved in the induction of this phase.

The role of gastrin in this phase of pancreatic secretion remains unclear. Stepwise alkaline distension of the antrum induced graded release of gastrin and pancreatic enzymes. However, when exogenous gastrin was administered to dogs the amount required to stimulate exocrine secretion was much greater than normal postprandial gastrin levels, suggesting that gastrin did not have a physiological role. These findings are supported by other studies demonstrating that gastrin release is not required for pancreatic enzyme secretion during this phase.

The vagus nerve plays an important role in the gastric phase of pancreatic secretion. Early experiments in anaesthetized cats demonstrated that stimulation of the antrum resulted in vagal stimulation of pancreatic amylase release. Antral distension in dogs also increased pancreatic secretion by long-route vagal pathways. An antropancreatic short reflex pathway that is blocked by hexamethonium and atropine also mediates this phase.

In addition, atropine and vagotomy block the gastric phase providing further evidence that gastric contributions to pancreatic secretion are mediated by vagovagal cholinergic reflexes that originate in the stomach and terminate in the pancreas. CCK release plays an important role in antral motility and gastrin release in humans as suggested by sham feeding experiments.

In the stomach, pepsin and gastric lipases catabolize proteins and fats into peptides and triglycerides plus fatty acids, respectively, while salivary amylase contributes to the continued digestion of carbohydrates. Peptic digests of proteins are effective in stimulating the intestinal phase. Thus when gastric chyme enters the duodenum, it stimulates the intestinal phase of pancreatic secretion. In a clinical setting, surgical procedures that slow the rate of gastric emptying reduce pancreatic secretion. Therefore, the rate of gastric emptying regulates the discharge of nutrients into the intestine and consequently the activation of the intestinal phase through neural and hormonal pathways.

**Intestinal phase**

Digestion of food in the stomach is followed by release of acidic chyme into the duodenum, which initiates the intestinal phase of pancreatic secretion. The pancreas has already been primed by cephalic and gastric influences, which enhance blood flow and initiate exocrine secretion. A majority of the pancreatic secretory response (50–80%) occurs during the intestinal phase and is regulated by hormonal and neural mechanisms.

The intestinal phase is more easily studied than the gastric phase as food can be instilled directly into the intestinal lumen without concern for gastric emptying. Stimulation of both acinar and ductal cells results in the production of enzyme and bicarbonate secretion. Pancreatic digestive enzyme secretion is stimulated by food molecules such as sodium oleate, monoglycerides, peptides, and amino acids (particularly tryptophan and phenylalanine). In the duodenum the high volume of bicarbonate released neutralizes the acidity of gastric chyme, while pancreatic enzymes catabolize partially digested food into molecules that are easily absorbed by intestinal enterocytes.

In the intestinal phase, pancreatic response is regulated primarily by the hormones secretin and CCK and by neural influences including the enteropancreatic reflex which is mediated by the enteric nervous system and amplifies the pancreatic secretory response. Entry of low pH gastric chyme into the intestine stimulates release of secretin from S cells into the blood. The main action of secretin is to stimulate bicarbonate release from pancreatic duct cells, but it also has a direct effect on acinar cells and potentiates enzyme secretion. CCK is released by proteins and fats and their partial digestion products: peptides and fatty acids.
Experiments in dogs with chronic pancreatic fistulae have shown that CCK antagonism diminishes pancreatic protein response to a meal and duodenal perfusion suggesting that CCK plays an important role in this phase. Similar results were also obtained in humans, where CCK receptor antagonism reduced pancreatic enzyme secretion during the intestinal phase.\(^{(40,41)}\)

Cholinergic regulation plays a critical role during this phase of pancreatic secretion. In the absence of secretin, atropine partially inhibits pancreatic bicarbonate secretion stimulated by low pH due to acidic chyme in the duodenum.\(^{(42,43)}\) In addition, the amount of bicarbonate produced by infusion of secretin is lower than that released by entry of food into the duodenum suggesting that other factors contribute to meal-stimulated pancreatic bicarbonate secretion.\(^{(44)}\) Atropine inhibited pancreatic enzyme secretion from 30 to 120 minutes following meal ingestion, implicating cholinergic mechanisms.\(^{(44)}\) Vagovagal enteropancreatic reflexes mediated by M1 and M3 muscarinic receptors and CCK receptors play an important role in the intestinal phase of secretion.\(^{(7,45)}\) These vagovagal enteropancreatic reflexes are modulated by input from the dorsal motor nucleus of the vagus projecting into the pancreas. Vagal stimulation activates pancreatic bicarbonate secretion through both cholinergic muscarinic and noncholinergic transmission.

**Role of gastric acid**

The physiological effects of acid on pancreatic secretion were evaluated by various methods such as diversion of gastric and pancreatic contents with fistulae and instillation of acidic solutions into the duodenum. Both gastric acid and exogenous HCl are powerful regulators of postprandial pancreatic enzyme secretion in humans and this effect was similar in potency to that observed with intravenous CCK injection.\(^{(56)}\) In contrast, triglycerides administered directly into the duodenum (in the absence of endogenous lipase) were unable to induce pancreatic secretion. However, following lipase digestion of fatty acids, monoglycerides stimulated pancreatic secretion but glycerol was ineffective indicating that fatty acids are the major component of ingested fats that stimulate pancreatic secretion.\(^{(56,57)}\)

Intraduodenal infusion of hydrochloric acid elicited a concentration-dependent increase in both the amount of bicarbonate and volume of pancreatic secretion. Secretion was similar to that attained with intravenous infusion of exogenous secretin suggesting that pH changes resulting from entry of acidic contents into the duodenum are important in inducing pancreatic secretion. Administration of a peptone meal of varying pH (pH 1–5) produced a maximal secretory response at pH 3.0, which was comparable in magnitude to that obtained with exogenous secretin.\(^{(47)}\) Acid infusion in both the duodenum and upper jejunum elicited pancreatic secretion suggesting that the proximal small intestine responds to this stimulus.\(^{(38)}\)

Entry of gastric contents into the duodenum creates an acidic environment with a pH of 2.0–3.0 in the initial segment of the duodenum, while the pH of the distal segment remains alkaline.\(^{(48,46)}\) This difference in pH is largely due to pancreatic bicarbonate release, which is augmented in large part by gastric acid-induced secretin release from the intestinal mucosa. In conscious rats with gastric and pancreatic fistulae, diversion through a gastric fistula produced a small increase in pancreatic secretion. However, instilling hydrochloric acid into the duodenum with an open gastric fistula augmented pancreatic secretion.\(^{(50,51)}\) In addition, pancreatic bicarbonate secretion was much greater when pancreatic juice was diverted from the intestine signifying a correlation between intestinal pH and quantity of pancreatic bicarbonate release.\(^{(52,53)}\)

The pancreatic bicarbonate response is dependent on the concentration of free unbuffered hydrogen ions and not on the total load of buffered acid entering the duodenum. Inhibition of gastric acid production by cimetidine (an histamine H2 receptor blocker) or omeprazole (an H+/K+ ATPase inhibitor) substantially reduced the pancreatic bicarbonate response to a meal.\(^{(50,54)}\) The pH of a liquid gastric meal also plays a significant role in pancreatic bicarbonate secretion; in cats and dogs, pH > 4.5 resulted in little pancreatic bicarbonate secretion, while at pH < 4.0 secretion increased substantially suggesting that a pH threshold of <4.5 is critical for stimulation of pancreatic secretion.\(^{(47,55)}\) This evidence implies that gastric acid is an important regulator of pancreatic bicarbonate secretion, which neutralizes the acid to create an alkaline environment optimal for the action of pancreatic enzymes and continued digestion of food.

**Role of dietary fat in pancreatic secretion**

Dietary fats stimulate pancreatic enzyme and bicarbonate secretion. Intestinal perfusion of monolein stimulated pancreatic enzyme secretion in humans and this effect was similar in potency to that observed with intravenous CCK injection.\(^{(56)}\) In contrast, triglycerides administered directly into the duodenum (in the absence of endogenous lipase) were unable to induce pancreatic secretion. However, following lipase digestion of fatty acids, monoglycerides stimulated pancreatic secretion but glycerol was ineffective indicating that fatty acids are the major component of ingested fats that stimulate pancreatic secretion.\(^{(56,57)}\)

There is some evidence to suggest that both free and saponified fatty acids induce pancreatic secretion, while other experiments suggest effectiveness only in a micellar form. Secretion has been shown to be dependent on fatty acid chain length, with C4 being least effective and C18 being most effective.\(^{(58)}\) Although the reason for this difference in potency is not entirely clear, it is not believed to be related to the efficiency of absorption.\(^{(59)}\) Other studies have demonstrated that intraduodenal administration of propionate (C3) was more effective than oleate (C18) in stimulating acinar cell secretion.\(^{(60)}\) The reason for the differences between the two studies is not entirely clear but could be species related as these experiments have been performed.
in humans, rats, and rabbits. Both oleate and neutral fats stimulate bicarbonate and fluid secretion, whereas only neutral fats stimulate pancreatic enzyme secretion. In dogs, oleic acid was shown to potentiate acidified protein-mediated pancreatic enzyme and bicarbonate secretion.\textsuperscript{61} Fat emulsions given to conscious rats produced a threefold increase in pancreatic protein secretion. The route of fat administration also has an impact on pancreatic secretion. Intravenous administration of fat did not produce pancreatic secretion, whereas intraduodenal administration led to elevated protein, bicarbonate, and fluid secretion.\textsuperscript{62,63}

Administration of fat emulsions increases plasma CCK and secretin levels. Fat-mediated pancreatic secretion was blocked by proglumide, a CCK receptor antagonist, implicating the importance of CCK in stimulating pancreatic secretion.\textsuperscript{64} Both C12 and C18 fatty acids augment the effects of secretin-induced bicarbonate secretion.\textsuperscript{65} In humans, introduction of different concentrations of oleic acid into the duodenum induces pancreatic secretion, although the threshold for CCK stimulation is much lower than for secretin.\textsuperscript{66} Secretin release is physiologically important since injection of anti-secretin antibodies in conscious rats greatly reduces fat-mediated protein and bicarbonate secretion.\textsuperscript{67}

A critical fatty acid chain length of C12 was required for CCK release from STC-1 cells, a neuroendocrine tumor cell line. Fatty acids with less than 10 carbon atoms did not augment secretion. This dependence on fatty acid chain length is similar to that observed previously for \textit{in vivo} CCK release in humans. In addition to the fatty acid carbon chain length, a free carboxyl terminus is also important as esterification of the carboxylic terminus abolished CCK secretion, while modification of the methyl terminus had no effect.\textsuperscript{68–70} Two cell surface receptors have been identified and demonstrated to promote fat-mediated CCK release. Mice with global deletion of GPR40 show partial reduction in CCK secretion following fatty acid administration.\textsuperscript{71} The recently discovered immunoglobulin-like domain-containing receptor (ILDR) is expressed in the duodenum in a subtype of EECs known as I cells that secrete CCK. ILDR appears to play an essential role in fat-stimulated CCK release as deletion of ILDR in mice completely eliminates fatty acid-stimulated CCK secretion.\textsuperscript{72}

Thus, fats and fatty acids are important regulators of pancreatic secretion. Experimental evidence suggests that the degree and extent of acinar and ductal cell activation may vary depending on the animal species and the route of fat administration.

\textit{Contributions of proteins, peptides, and amino acids to pancreatic secretion}

Studies performed in dogs, rats, and humans have shown that proteins, peptides, and amino acids stimulate pancreatic secretion.\textsuperscript{73} In rats, proteins such as casein stimulate pancreatic secretion in proportion to their trypsin inhibitor capacity.\textsuperscript{74} However, in dogs, intact, undigested proteins such as casein, albumin, and gelatin did not stimulate pancreatic secretion, whereas protease digests of these proteins were very effective.\textsuperscript{34} In contrast, studies in rats suggested that intestinal administration of hydrolyzed casein produced a smaller response than some of the other proteins which potently stimulated pancreatic enzyme secretion, suggesting that the amino acid composition of a protein is relevant in determining the extent of stimulation.\textsuperscript{74,75}

Although intravenous infusion of amino acids in humans stimulated pancreatic enzyme and bicarbonate secretion, a mixture of L-amino acids when infused intravenously in dogs was not effective. In contrast to intravenous infusion, intraduodenal delivery of amino acids in dogs induced pancreatic fluid, bicarbonate, and protein secretion which was comparable to an elemental diet suggesting the importance of the route of administration on pancreatic secretion.\textsuperscript{62,76} Only L-amino acids stimulate pancreatic secretion which is consistent with the overall physiological importance of these stereoisomers. Of all the amino acids, aromatic amino acids such as phenylalanine and tryptophan have the greatest potency.\textsuperscript{36,77,78}

Acidification of amino acid\textsuperscript{77,79} and peptide\textsuperscript{78} preparations with hydrochloric acid potentiates the bicarbonate response but pancreatic enzyme secretion is not influenced beyond that observed in the absence of acid. Aromatic amino acids are capable of inducing maximal secretory response as potentiation of pancreatic enzyme secretion is not observed when amino acids or peptides are administered concomitantly with lipid molecules such as oleate or monoolein.\textsuperscript{56,61}

The pancreatic secretory response to intraduodenal administration of amino acids appears to be concentration dependent. A minimal concentration of 8 mM is necessary for stimulation by most amino acids,\textsuperscript{76} although the more potent aromatic amino acids such as tryptophan stimulate secretion at concentrations as low as 3 mM.\textsuperscript{80} The length of the intestine exposed to amino acids also plays a critical role in pancreatic secretion. In dogs, exposure of the first 10 cm was least effective, while perfusion of the whole intestine produced significant enzyme output,\textsuperscript{36} suggesting that the pancreatic response was dependent upon the entire load of nutrients, not just their concentration. The majority of stimuli responsible for pancreatic stimulation originate in the proximal small intestine. In humans, amino acids stimulated pancreatic secretion only when perfused into the duodenum and no response was observed upon perfusion in the ileum.\textsuperscript{81} Therefore, similar to fats, the primary mechanisms that stimulate pancreatic secretion are limited to the proximal regions of the small intestine.

The amount of bicarbonate released by intraluminal administration of tryptophan is similar to that produced
Role of bile and bile acids in pancreatic secretion

Bile is produced by hepatocytes as a complex mixture of bile acids, cholesterol, and organic molecules. It is stored and concentrated in the gall bladder and released into the duodenum upon entry of chyme. Bile acids such as cholate, deoxycholate, and chenodeoxycholate are conjugated with glycine or taurine amino acids, which increase their solubility. In the intestine, bile acids assist in the emulsification and absorption of fatty acids, monoacylglycerols, and lipids and stimulate lipolysis by facilitating binding of pancreatic lipase with its colipase.

Under basal conditions, intraduodenal administration of physiological concentrations of bile or the bile salt sodium taurocholate elevated plasma secretin and stimulated pancreatic fluid secretion in cats. Secretin was released only in response to perfusion of sodium taurocholate in the duodenum. Perfusion in the upper jejunum produced a significantly diminished pancreatic response, while no response was observed upon ileal perfusion. Pancreatic fluid secretion was stimulated by the free ionized form of taurocholate and was not dependent on its detergent properties. In humans, infusion of bovine bile augmented secretin release along with pancreatic exocrine secretions of fluid, bicarbonate, and enzymes.

In addition to secretin, infusion of bovine bile and bile acids in humans and dogs was shown to stimulate the release of several hormones and neuropeptides such as CCK, neurotensin, VIP, gastric inhibitory peptide (GIP), PP, and somatostatin. Fluid and bicarbonate release was enhanced when elevated levels of VIP were present in the plasma, suggesting that bile activates peptidergic nerves resulting in pancreatic secretion. Additionally, cholinergic mechanisms are also important as atropine blocked bile- and taurocholate-stimulated exocrine secretion. The composition of bile is important in mechanisms regulating this secretory response as some differences in hydrokinetic and ecbolic responses were observed with administration of bile versus various bile acids.

However, a stimulatory effect of bile acids on pancreatic fluid secretion was not observed in the presence of digestive intraluminal contents. In some studies, where bile acids were administered concomitantly with amino acids or fat, an inhibition of pancreatic enzyme secretion was observed. The mechanism underlying this observation is not completely understood, although it is possible that bile acids inhibit CCK release by a negative feedback mechanism, which helps to relax and refill the gallbladder. Chemical sequestration of bile acids in dogs augmented the release of CCK and pancreatic enzyme secretion in response to amino acids and addition of taurocholate reversed this effect. Long-term diversion of bile in dogs also augmented basal and oleate-stimulated pancreatic fluid, bicarbonate, and enzyme secretion along with plasma CCK levels, further supporting the role of bile acids in inhibiting CCK release.

Other studies have shown that the bile salt chenodeoxycholate when infused in humans inhibited bombesin- and CCK-stimulated gallbladder emptying along with elevation of plasma CCK levels. These results led the authors to hypothesize that chenodeoxycholate, by a yet unknown mechanism, reduced the sensitivity of the gallbladder to stimulation by bombesin and CCK.

In contrast to many species including mice and humans, rats do not possess a gallbladder and multiple pancreatic ducts join the lower end of the common bile duct. In rats, diversion of bile and pancreatic juice stimulates the release of pancreatic enzymes. This augmentation of enzyme secretion has been suggested to compensate for the increased degradation of proteolytic enzymes in the absence of bile. Thus, exocrine secretion in rats is regulated by a luminal feedback mechanism. Additional experiments have shown that certain bile salts stimulate bicarbonate secretion via CCK release, whereas other bile salts inhibit exocrine secretion. Two inhibitory mechanisms have been proposed—one dependent on the stabilization of luminal proteases and the other independent of protease activity. Stimulation of pancreatic fluid secretion in anesthetized rats has been demonstrated to be mediated by taurocholate-induced transcription of Na+/K+/2Cl− cotransporter, which plays a key role in regulating the entry of Cl− from the basolateral surface of acinar cells.

The physiological role of bile and bile salts in regulating pancreatic secretions is not completely understood and appears to be dependent on multiple factors, including the chemical properties of bile salts, the animal model...
being evaluated, and prandial status of the animal being studied.\textsuperscript{112}

\textbf{Absorbed nutrient phase}

Once nutrients are absorbed from the intestinal lumen, they can directly stimulate pancreatic secretion as part of the absorbed nutrient phase. Nutrients can either directly stimulate pancreatic acinar cells or they may indirectly activate hormonal and neural pathways to further regulate exocrine secretion. In contrast to intraduodenal administration of amino acids, which produces large increases in pancreatic secretion,\textsuperscript{113–115} intravenous administration of amino acids stimulates trypsin and chymotrypsin secretion but not lipase or amylase. Less is known about lipids and glucose, although they appear to have little if any direct effect on pancreatic secretion.\textsuperscript{63,116}

\textbf{Feedback regulation of pancreatic secretion}

The concept of feedback regulation of pancreatic secretion emanated from a series of studies demonstrating that\textsuperscript{117} instillation of trypsin inhibitor into the upper small intestine or\textsuperscript{118} surgical diversion of the bile-pancreatic duct removing bile and pancreatic juice from the duodenum of rats stimulated pancreatic enzyme secretion.\textsuperscript{119} Conversely, infusion of trypsin into the duodenum during bile-pancreatic juice diversion suppressed pancreatic enzyme release. Thus, the protease concentration in the upper small intestine appears to be intimately linked to pancreatic secretion through a negative feedback system in which active proteases within the duodenum limit pancreatic secretion but reduced protease activity stimulates pancreatic secretion. When assays for CCK became available, it was shown that CCK mediated the effects of proteases on pancreatic secretion\textsuperscript{120} through protease-sensitive CCK-releasing factors\textsuperscript{121,122} (see Figure 1). In the absence of proteases, CCK-releasing factor can stimulate CCK cells, but in the presence of proteases, the releasing factors are inactivated and CCK secretion is low. Negative feedback regulation of pancreatic secretion has been shown to exist in many species, although other proteases such as elastase may be more important in regulating pancreatic secretion in humans.

Pancreatic exocrine secretion is also influenced through a positive feedback mechanism. Monitor peptide is a 61-amino acid peptide produced by pancreatic acinar cells and possessing CCK-releasing activity. Although monitor peptide has modest trypsin inhibitor capability, its ability to stimulate CCK is independent of this action because monitor peptide can directly stimulate CCK secretion from isolated CCK cells in vitro.\textsuperscript{123,124} Monitor peptide is secreted in pancreatic juice, therefore, it does not stimulate CCK secretion unless pancreatic secretion is underway. Thus, monitor peptide cannot account for the increase in CCK during bile-pancreatic juice diversion, but it may serve to reinforce pancreatic secretion once the process has been initiated.

\textbf{Pancreatic Exocrine Secretion}

The exocrine pancreas delivers its secretions of digestive enzymes, fluid, and bicarbonate ions to the duodenum
following ingestion of food. The pancreas is composed of both endocrine and exocrine components. The endocrine pancreas is comprised of $\alpha$, $\beta$, $\delta$, $\epsilon$, and PP (F) cells, which are located in the islets of Langerhans. These specialized cells secrete the hormones glucagon, insulin, somatostatin, ghrelin, amylin, and pancreatic polypeptide into the blood, which exert endocrine and paracrine actions within the pancreas. Ninety percent of the pancreas is composed of acinar cells which secrete digestive enzymes such as trypsin, chymotrypsin, and amylase for digestion of food in the small intestine. The acinar cells are triangular in shape and arranged in clusters with the apex of the cell opening into a centrally located terminal duct. The terminal or intercalated ducts merge to form interlobular ducts, which in turn congregate to form the main pancreatic duct. The pancreatic duct delivers exocrine secretions into the duodenum. The ductal cells secrete fluid and bicarbonate ions, which neutralize acinar cell secretions, as well as the acidic gastric contents entering the duodenum. The pancreas is heavily innervated by sympathetic and parasympathetic peripheral nerves and contains a dense network of blood vessels which regulate blood flow and modulate pancreatic secretion. Pancreatic exocrine secretion is a highly integrated process mediated by neural and hormonal signals arising from the gut as well as by factors secreted by other tissues and hormones released from pancreatic islets. The secretory pathways can be stimulatory or inhibitory in nature and represent a highly regulated system that responds to ingestive signals. The agents that modulate pancreatic exocrine secretion are discussed in Table 1.

**Neural Innervation**

The pancreas is innervated by parasympathetic nerve fibers, postganglionic sympathetic neurons, as well as a network of intrapancreatic nerves. Together these nerves regulate pancreatic exocrine function by releasing neurotransmitters such as acetylcholine, serotonin, and neuropeptides such as VIP, GRP, and neuropeptide Y (NPY). The pancreatic ganglia receive input from pre- and postganglionic nerve fibers and regulate exocrine and endocrine secretion. Intrapancreatic postganglionic neurons are activated by central input during the cephalic phase and by vagovagal responses initiated during the gastric and intestinal phases of stimulation. They stimulate enzyme and bicarbonate secretion primarily by releasing acetylcholine, which activates muscarinic receptors located on acinar and duct cells.

**Vagal innervation**

The dorsal vagal complex in the brainstem is comprised of the nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus (DMV) and exerts parasympathetic control on pancreatic secretion. Information relayed by sensory vagal afferent nerves innervating the pancreas is first processed in the NTS, which then projects onto the preganglionic motor neurons of the DMV. The DMV receives inputs from other regions of the brain such as the hypothalamus and from numerous hormones and neuropeptides through the afferent limb of the vagus nerve.

Parasympathetic preganglionic efferent vagal nerves innervating the pancreas originate primarily from the DMV and terminate in the pancreatic ganglion. Electrical and chemical stimulation of the DMV induces rapid pancreatic secretion, and this response is inhibited by vagotomy or blockade of muscarinic receptors by atropine. It has been suggested that vagal cholinergic neurons mediate pancreatic secretion during low loads of intestinal stimulants whereas hormones mediate the response during high loads of intestinal stimuli.

CCK affects pancreatic secretion through both a direct effect on pancreatic acinar cells and an indirect effect on the vagus nerve (Figure 2). However, the effects on the vagus nerve are complex and the firing response of neurons in the DMV appears to be dictated by their spatial location. In one study, neurons in the caudal region were activated; those in the rostral region were unaffected, while neurons in the intermediate region were inhibited by a direct action of CCK. Although not fully understood, it appears that CCK’s effects on the vagus nerve influence the overall pancreatic secretory response.

The exocrine pancreas is regulated directly by the vagus. Studies with muscarinic receptor knockout mice demonstrated that both M1 and M3 receptors mediate amylase release from dispersed acini. It is likely that M3 receptors are more relevant physiologically since the level of M3 receptor expression was significantly higher in acinar cells and M1 receptors were found to have only a minor effect on bicarbonate secretion in conscious dogs. The vagus nerve also possesses group II metabotropic glutamate receptors that couple primarily to $G_{i/o}$. These receptors are located on excitatory and inhibitory presynaptic terminals of pancreas-projecting DMV neurons that are also activated by CCK and pancreatic polypeptide. Thus, in addition to $\gamma$-amino butyric acid, glutamate also modulates pancreatic exocrine secretion through distinct vagal neurons.

**Vasoactive intestinal peptide**

Vasoactive intestinal peptide (VIP) is a 28-amino acid neuropeptide that is found throughout the body. Immunocytochemical evidence suggests that VIP is localized in pancreatic nerve fibers and functions as a vagal neurotransmitter. In the chick, VIP immunoreactive nerve endings are found in close proximity to acinar cells and
Table 1. Regulators of pancreatic exocrine secretion

<table>
<thead>
<tr>
<th>AGENT</th>
<th>TYPE OF SECRETION</th>
<th>SITE OF ACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>STIMULATE</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Neurotransmitter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>Protein</td>
<td>Acinar cell</td>
</tr>
<tr>
<td>Vasoactive intestinal peptide</td>
<td>Bicarbonate</td>
<td>Acinar cell</td>
</tr>
<tr>
<td>Gastrin-releasing peptide</td>
<td>Protein</td>
<td>Acinar cells</td>
</tr>
<tr>
<td>Neuromedin C</td>
<td>Protein</td>
<td>Acinar cell</td>
</tr>
<tr>
<td>PACAP</td>
<td>Bicarbonate, protein</td>
<td>Acinar cell</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>Bicarbonate, protein</td>
<td>Direct effect on acinar cells; indirect effect via dopamine and bile acids</td>
</tr>
<tr>
<td>Substance P</td>
<td>Protein, fluid, bicarbonate</td>
<td>Acinar cells, duct cells</td>
</tr>
<tr>
<td>Calcitonin gene-related peptide</td>
<td>Protein</td>
<td>Perivascular nerves</td>
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<tr>
<td>Cholecystokinin</td>
<td>Protein</td>
<td>Acinar cell</td>
</tr>
<tr>
<td>Peptide histidine isoleucine</td>
<td>Bicarbonate, fluid</td>
<td>Pancreatic nerves, ganglia, blood vessels, acinar cells</td>
</tr>
<tr>
<td>Catecholamine</td>
<td>Protein</td>
<td>Acinar cell</td>
</tr>
<tr>
<td>Dopamine</td>
<td>Protein, fluid, bicarbonate</td>
<td>Acinar cell</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Protein</td>
<td>Paracrine effect via vagal afferent fibers in duodenal mucosa</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>Fluid, protein</td>
<td>Acinar cell, nerve terminals</td>
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<tr>
<td><strong>Hormone</strong></td>
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<td>Cholecystokinin</td>
<td>Protein</td>
<td>Acinar cell</td>
</tr>
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<td>Secretin</td>
<td>Bicarbonate. fluid, protein</td>
<td>Acinar cell, duct cell</td>
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<td>A-natriuretic peptide</td>
<td>Fluid, protein</td>
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<td>C-natriuretic peptide</td>
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<td>Insulin</td>
<td>Protein</td>
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<tr>
<td>Bombesin</td>
<td>Protein or Bicarbonate</td>
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<td>Melatonin</td>
<td>Protein</td>
<td>Acinar cell</td>
</tr>
<tr>
<td>Amylin</td>
<td>Amylase release from AR42J cells</td>
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</tr>
<tr>
<td>Histamine</td>
<td>Fluid, protein</td>
<td>Presynaptic parasympathetic nerve terminals; acinar cell</td>
</tr>
<tr>
<td><strong>INHIBIT</strong></td>
<td></td>
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<tr>
<td><strong>Neurotransmitter</strong></td>
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<tr>
<td>Calcitonin gene-related peptide</td>
<td>Protein</td>
<td>Neurons</td>
</tr>
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<td>Neuropeptide Y</td>
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<td>Neurons</td>
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<tr>
<td><strong>Hormone</strong></td>
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<tr>
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<td>Fluid, protein</td>
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<td>Pancreatic polypeptide</td>
<td>Protein</td>
<td>?</td>
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<tr>
<td>Somatostatin</td>
<td>Protein</td>
<td>Acinar cells, ganglia, cholinergic neurons</td>
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<td>Acinar cell</td>
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<tr>
<td>Pancreastatin</td>
<td>Fluid, protein</td>
<td>Blood vessels?</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Bicarbonate, protein</td>
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<tr>
<td>Ghrelin</td>
<td>Protein</td>
<td>Intrapancreatic neurons</td>
</tr>
<tr>
<td>Leptin</td>
<td>Protein</td>
<td>Neurons</td>
</tr>
<tr>
<td>Adrenomedullin</td>
<td>Protein</td>
<td>Acinar cell</td>
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</table>
Figure 2. CCK stimulates pancreatic secretion through hormonal and neuronal pathways. CCK is released from I cells of the small intestine and diffuses into the bloodstream where it is carried to the pancreas. CCK binds to receptors on acinar cells to stimulate pancreatic enzyme secretion. Secreted CCK also diffuses through the paracellular space and binds to CCK1-bearing nerves in the submucosa. Vagal afferent signals are integrated in the dorsal vagal complex which also receives signals from other regions of the brain (e.g., hypothalamus). Vagal efferent fibers transmit cholinergic signals to the pancreas to stimulate pancreatic secretion.

epithelial cells of arterioles. Small clear vesicles were present in VIP-positive nerves indicating that these neurons are were cholinergic in nature. In normal human pancreas, autonomic ganglia receive an abundant supply of VIP-positive fiber plexi and VIP-positive nerves and appear to innervate acinar cells, ducts, and blood vessels. After atropine treatment, electrical stimulation of the vagus still increased bicarbonate secretion concurrent with detection of VIP in pancreatic venous effluent suggesting that VIP release is coupled with bicarbonate secretion. The effects of VIP are especially prominent in the pig as perfusion of the pancreas with VIP antibodies inhibited fluid and bicarbonate secretion, and treatment of rats with a VIP antagonist reduced bicarbonate secretion concomitant with vasodepression further supporting a direct relationship. High- and low-affinity VIP receptors have been identified on pancreatic acinar membranes. The high-affinity receptors are coupled to cAMP-mediated amylase release, while activation of low-affinity receptors did not cause cAMP elevation or amylase release, suggesting that only high-affinity receptors are important in protein secretion. These effects of VIP were attenuated by somatostatin and galanin, which reduced VIP-mediated fluid and protein output. One of the main functions of VIP appears
to be increasing blood flow by vasodilation, and as a result its effects on pancreatic secretion independent of blood flow in the pancreas are difficult to evaluate.\textsuperscript{140,141}

**Gastrin-releasing peptide**

Gastrin-releasing peptide (GRP) is a 27-amino acid neuropeptide that is present in postganglionic vagal afferents and has been detected in neurons innervating the feline, porcine, rodent, and human pancreas.\textsuperscript{142} Receptors responsive to GRP have been identified in rat pancreatic membranes and cancer cells where they mediate enzyme secretion.\textsuperscript{143,144} In the cat, GRP is present in intrapancreatic ganglia, acinar and stromal regions, and occasionally on the vasculature and ducts.\textsuperscript{145} In humans, the pattern of GRP expression was similar to that of VIP; GRP was localized on nerve fibers in proximity to pancreatic acini, capillaries, ductules, and arterial walls.\textsuperscript{146} Several studies in different species have demonstrated that GRP modulates exocrine secretion. Vagal stimulation of porcine pancreas resulted in GRP release, which enhanced pancreatic exocrine secretion.\textsuperscript{147} In isolated perfused rat pancreatic preparations, electrical field-stimulated GRP release potentiated secretin-mediated fluid and amylase secretion through a noncholinergic pathway.\textsuperscript{148} The effects of GRP on rat pancreatic exocrine secretion were enhanced by \(\gamma\)-amino butyric acid.\textsuperscript{149} Neuromedin C, a decapeptide of GRP, also enhanced pancreatic secretion by direct action on canine acinar cells as well as indirectly by stimulating CCK release.\textsuperscript{150,151} However, since bombesin (GRP analog) does not stimulate pancreatic secretion in dogs, it appears that its effects may be dependent on the species being evaluated.\textsuperscript{152}

**Other peptide neurotransmitters**

Immunohistochemical staining has revealed that the neuropeptides listed below are present in pancreatic nerves and their functional significance and ability to regulate pancreatic secretion has been demonstrated by \textit{in vitro} and/or \textit{in vivo} studies.

\textbf{PACAP:} Pituitary adenylate cyclase-activating polypeptide (PACAP) has been identified in nerve fibers and intrapancreatic ganglion in rodents.\textsuperscript{153,154} PACAP has been shown to evoke bicarbonate and enzyme secretion from the pancreas albeit with a slower time course than VIP.\textsuperscript{157,155} In the acinar cell line AR42J, PACAP activated phospholipase C, which led to elevation of intracellular \(Ca^{2+}\) and amylase release.\textsuperscript{156}

\textbf{Neurotensin:} Neurotensin is a 13-amino acid neuropeptide that is widely expressed in the central nervous system and is also present in pancreatic nerves. It stimulates amylase secretion and its effects are potentiated by carbachol (a cholinergic agonist), secretin, and caerulein (a CCK analog).\textsuperscript{157,158} Other studies demonstrated that neurotensin stimulates bicarbonate but not protein secretion in dogs and may act indirectly by stimulating dopamine release.\textsuperscript{159}

\textbf{Substance P:} Substance P is expressed in periportal nerves in the guinea pig pancreas and inhibits ductal bicarbonate secretion by modulating neurokinin 2 and 3 receptors.\textsuperscript{160–162} It enhanced caerulein-stimulated enzyme secretion in isolated perfused pancreas as well as in anesthetized rodents.\textsuperscript{163}

\textbf{CGRP:} CGRP is a 37-amino acid peptide that is present in central and peripheral neurons. The effect of CGRP on exocrine secretion is not clear and may be species-specific. Interaction of CGRP with receptors on guinea pig acinar cells led to amylase release, although its effect was not as potent as VIP.\textsuperscript{164} In rat acinar cell preparations, CGRP inhibited amylase release by a mechanism involving cholinergic (muscarinic) neural pathways.\textsuperscript{165}

\textbf{NPY:} NPY, a 36-residue peptide, is expressed in intrapancreatic ganglia and nerve fibers that surround exocrine pancreatic tissue.\textsuperscript{166} NPY inhibited CCK- and vagally mediated amylase secretion from intact pancreas and lobules, but not from dispersed acini, suggesting that its actions were mediated by neurons innervating the exocrine pancreas.\textsuperscript{167} Other evidence suggests that NPY plays at best only a modest role in pancreatic exocrine secretion.\textsuperscript{168}

\textbf{CCK:} The presence of CCK in intrapancreatic nerves has led to the suggestion that it may serve a dual role as neurotransmitter and hormone in pancreatic secretion. However, CCK could not be detected in the venous effluent of isolated perfused porcine pancreas after vagal stimulation following a meal\textsuperscript{136} suggesting that synaptic release of CCK does not occur within the pancreas. Therefore, the role of CCK as a neurotransmitter in pancreatic secretion merits further investigation.

\textbf{Peptide histidine isoleucine:} Peptide histidine isoleucine (PHI) is a 27-amino acid peptide with an N-terminal histidine and C-terminal isoleucine and is derived from the same precursor molecule as VIP. It has been reported in pancreatic nerves and ganglia and stimulates fluid and bicarbonate secretion in a cAMP-dependent fashion.\textsuperscript{166,169}

**Adrenergic nerves**

Compared to cholinergic stimulation, adrenergic nerves play a relatively minor role in pancreatic exocrine secretion. Catecholamine-containing nerves are found in the celiac ganglion and extend to intrapancreatic ganglia, blood vessels, ducts, and islets.\textsuperscript{170} Epinephrine and norepinephrine (NE) evoked amylase release from superfused rat pancreatic preparations, similar to that induced by electrical stimulation in the presence of cholinergic blockade.\textsuperscript{171} This process is dependent on elevated intracellular \(Ca^{2+}\) and inhibited by propranolol, suggesting that \(\beta\)-adrenergic receptors are involved.\textsuperscript{172} Catecholamines also interact with \(\alpha\)-adrenergic receptors expressed in pancreatic acini.
and inhibit amylase secretion.\textsuperscript{173} NPY is coexpressed with NE in some nerve fibers, and stimulation of splanchnic nerves leads to the release of NPY and NE.\textsuperscript{166,174} Infusion of PACAP into the pancreatoduodenal artery enhanced release of NE after electrical stimulation of nerves. However, the physiological relevance of this observation is not clear.\textsuperscript{175}

Celiac denervation reduces pancreatic secretion by \textasciitilde70 percent while increasing blood flow by 350 percent. This dissonance presumably occurs by the disruption of stimulatory fibers and sympathetic fibers that maintain tonic constriction of pancreatic vessels.\textsuperscript{176} The effect of adrenergic transmitters on pancreatic secretion has been difficult to discern due to the wide-ranging effects of norepinephrine on multiple processes including blood pressure, blood flow, neural reflexes, and release of hormones. Even though high concentrations of norepinephrine have been found in rabbit pancreatic ganglia, ducts, and blood vessels, its effects are controversial.\textsuperscript{177} Norepinephrine has been reported to stimulate, inhibit, or have no effect on pancreatic secretion.\textsuperscript{170,178–180} Unfortunately \textalpha- and \textbeta- adrenergic receptor agonists and antagonists have not provided information that could be used to delineate mechanisms important in adrenergic regulation of pancreatic secretion.\textsuperscript{184,185}

**Dopamine**

Dopamine was detected in pancreatic ducts and ampullae and dopamine \textbeta-hydroxylase (DBH)positive fibers were identified along the vasculature, ducts, and ganglia suggesting it may play some role in pancreatic secretion.\textsuperscript{177,186}

There is conflicting evidence regarding the role of dopamine in pancreatic secretion. Dopamine stimulates pancreatic secretion in anesthetized dogs and rats, although the effect is negligible in conscious animals.\textsuperscript{180,182,187–190} Other data suggest that the secretory response to dopamine differs between dogs, cats, rabbits, and rats, and species-specific effects must be taken into consideration when evaluating its effects.\textsuperscript{191}

**Serotonin**

Like dopamine, serotonin is present in pancreatic ducts and ampullae. Autoradiography of tissue sections after tritiated serotonin uptake demonstrated the presence of serotonergic innervation of blood vessels, but not exocrine parenchyma in rats, suggesting a limited role in pancreatic secretion.\textsuperscript{177,192} However, phenylbiguanide, a 5-HT3 receptor agonist, activated preganglionic neurons located in the caudal DMV, inhibited those in the intermediate DMV, and had no effect on rostral DMV neurons, suggesting complex spatial regulation of pancreatic vagal neurons by serotonin.\textsuperscript{20} Intraduodenal infusion of melatonin (a serotonin derivative) increased pancreatic amylase secretion, while pretreatment with 5-HT2 serotonin antagonist ketanserin or the 5-HT3 antagonist MDL72222 decreased amylase release. Serotonin-induced amylase release was blocked by bilateral vagotomy supporting a role for serotonergic mechanisms on exocrine secretion.\textsuperscript{193}

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**Nitric oxide**

Nitric oxide (NO) is a gaseous signaling molecule that is synthesized by NO synthase from L-arginine in the presence of nicotinamide adenine dinucleotide hydrogen phosphate (NADPH). It is a potent vasodilator and modulates secretory activity as well as pancreatic blood flow in the pancreas.\textsuperscript{194} Because it is not practical to directly measure NO in biological tissues, the presence of NO has been identified by expression of NOS or histochemistry of NADPH diaphorase (NADPH-d), since NOS and NADPH-d colocalize in neurons of the peripheral and central nervous systems. The actions of NO in tissues have been identified by the use of NO donors, NOS inhibitors, and agents that inactivate (e.g., superoxide-generating compounds) or stabilize NO (e.g., superoxide dismutase). Unlike ligands that signal through cell surface receptors, NO penetrates cells and activates guanylate cyclase to generate the second messenger cGMP.\textsuperscript{195}

Immunostaining of pancreas from a wide range of mammals (mouse, rat, hamster, guinea pig, cat, and man) indicates that NOS is expressed in the cell bodies of intra-pancreatic ganglia, interlobular nerve fibers, and along blood vessels. VIP is sometimes coexpressed with NOS in ganglia and nerve fibers. These studies suggest that NO is important in pancreatic exocrine secretion.\textsuperscript{196} In newborn guinea pig, nitrergic neurons were present primarily in the head and body of the pancreas, along blood vessels, the main pancreatic duct, and in association with pancreatic acini.\textsuperscript{197} These nerves also immunostained with antibodies against NPY, VIP, and DBH indicating complex coregulation of pancreatic secretion by various neurotransmitters.

In rat pancreas, the NO donor sodium nitroprusside and cGMP analog 8-bromo cGMP inhibited basal and vagal amylase secretion through a \textca^{2+}-dependent mechanism.\textsuperscript{198,199} The G protein-coupled receptor, protease-activated receptor-2 (PAR-2), modulates NO-mediated amylase release in mice, and inhibition of NOS abolished PAR-2-mediated amylase release suggesting that the effects of NO may be mediated by neuronal release of a PAR-2 agonist. Ablation of sensory nerves by capsaicin did not affect PAR-2-mediated amylase release, suggesting that TRPV1-expressing sensory vagal fibers are not involved in this pathway.\textsuperscript{200} Analysis of the effects of NO on pancreatic secretions in pigs supports the findings that NO is essential for pancreatic fluid and amylase secretion mediated by the vagus nerve.\textsuperscript{201} Thus, in addition to maintaining the vascular tone, nitrergic nerves play an important role in pancreatic fluid and amylase release.
Stimulatory hormones

Cholecystokinin

Cholecystokinin (CCK) is released from specialized enteroendocrine cells (I cells) located mainly in the upper small intestine. Using confocal microscopy to examine the intestine of transgenic CCK-enhanced green fluorescent protein mice (Cck-eGFP), it was noted that enteroendocrine cells (EECs) possessed basal processes that extended to other cells\(^2\) (Figure 3). In addition to hormones like CCK, it was found that these cells possessed many neuron-like properties including neurotransmitters and synaptic proteins. Their basal extensions, called neuropods, were filled with neurofilaments\(^3\) and connected to nerves.\(^4\) Thus, synaptic connections between EECs and nerves, including the vagus nerve,\(^5\) provide a way for EECs to sense gut contents and directly regulate neural function.

The major stimulants of CCK release are dietary fats and proteins. In the rat, intraluminal proteases via an active feedback system participate in the release of a putative intestinal CCK-releasing factor (e.g., LCRF), which in turn causes CCK secretion.\(^1\) Various molecular forms of CCK, ranging in size from CCK-8 \([\text{CCK-(26–33)}-\text{NH}_2]\) to CCK-58, have been described in dogs, rats, and humans.\(^6\)–\(^8\) CCK-58 was determined to be the predominant peptide in dogs and humans and the only form detected in rats after employing CCK isolation techniques that prevented degradation of CCK in blood.\(^9\) The actions of CCK-8 and CCK-58 appear to be functionally identical suggesting that CCK-8 retains the biological activity ascribed to this hormone.\(^10\) CCK is posttranslationally modified and has an amidated C-terminus. A sulfated tyrosine residue in CCK-8 is important for its biological actions including exocrine secretion.\(^11\),\(^12\) C-terminal amidation is critical for binding of CCK to its receptors and removal of the amide group decreases CCK activity. Other studies have reported that deamidation and desulfation do not significantly impair the ability of CCK to stimulate amylase release and these discrepancies may arise from differences between species. Shorter forms of CCK such as the tetrapeptide CCK-4 are generally much less effective in mediating exocrine secretion than CCK-8, while longer forms of CCK, such as CCK-33, are equally effective.\(^13\)–\(^16\) CCK mediates its hormonal effects through two G-coupled protein receptors, CCK-1 and CCK-2, previously known as CCK-A and CCK-B, respectively. The contribution of these receptors to pancreatic secretion has been evaluated in order to delineate the molecular mechanisms of CCK action. CCK receptors have been proposed to exist in two states, a high-affinity (picomolar) but low-capacity state and a low-affinity (nanomolar) but high-capacity state.\(^17\),\(^18\) Autoradiography of pancreatic membranes incubated with radiiodinated CCK-8 demonstrated that CCK-1 receptors are highly expressed in rat pancreas, while CCK-2 receptors are less abundant. CCK-1 receptors appear to modulate pancreatic secretion.

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**Figure 3.** Model of an enteroendocrine cell (EEC) with a neuropod. EECs (green) are sensory cells that reside in the intestinal mucosa. Some EECs possess neuropods that contain neurofilaments (light green), synaptic proteins, mitochondria (red), and neurotransmitters (yellow) and synapse with neurons in the submucosa. In this manner, EECs may sense intraluminal contents such as nutrients or bacteria and signal directly to nerves, including the vagus nerve.
as oral administration of loxiglumide, a potent CCK-1 receptor antagonist, reduces protein and fluid output in rats.\textsuperscript{219} Similarly, caerulein-induced pancreatic amylase release was blocked by CCK-1 receptor antagonists.\textsuperscript{220} CCK-8 did not induce amylase release in CCK-1 receptor knockout mice confirming that CCK-1 receptors are critical for CCK-mediated protein secretion.\textsuperscript{221} Since bicarbonate secretion was not observed from dispersed acinar cells, this effect is not believed to be regulated by CCK receptors.\textsuperscript{222} Pancreatic responses in CCK-2 receptor knockout mice were similar to wild-type mice suggesting that functional CCK receptors are expressed on human pancreas.\textsuperscript{223} In pigs, where a majority of receptors are of the CCK-2 subtype, acinar cells demonstrated a low responsivity to CCK and did not secrete amylase in response to caerulein or a CCK-1 agonist.\textsuperscript{224} In humans, the actions of CCK on pancreatic secretion have been variously reported. Infusion of CCK, caerulein, and secretin in the presence of amino acids substantially increased output of fluid, bicarbonate, and enzyme.\textsuperscript{225,226} Similar to porcine pancreas, CCK-2 is the major CCK receptor subtype expressed in human pancreas, although interestingly CCK-1 receptor antagonists are able to inhibit amylase secretion.\textsuperscript{227–229} In dispersed human acini, which responded to carbacholcholine and neumedin C, CCK did not stimulate amylase release presumably because of a paucity of cellular membrane receptors. It has been proposed that the effects of CCK on human pancreatic secretion are mediated through CCK-1 receptors on nerves which innervate the pancreas.\textsuperscript{230,231} However, recent data demonstrated that application of physiologic concentrations of CCK-8 and CCK-58 to human acinar cells produced intracellular Ca\textsuperscript{2+} oscillations and normal exocytosis of pancreatic enzyme, suggesting that functional CCK receptors are expressed on human pancreas.\textsuperscript{232} By visualizing Ca\textsuperscript{2+} oscillations in a unique slice preparation of human pancreas obtained from cancer resections, it has been unequivocally demonstrated that CCK-8 stimulates pancreatic secretions through direct action on CCK-1 receptors expressed on acinar cells and does not require a neuronal component.\textsuperscript{233} Thus, it appears that CCK receptors are expressed on acinar cells of both human and rodent pancreas, and the differences between the two may not be as great as previously predicted.

Recently, it was shown that rat and human pancreatic stellate cells express CCK receptors and secrete acetylcholine in response to CCK stimulation. This source of acetylcholine was sufficient to stimulate amylase release from acinar cells. Pancreatic stellate cells may represent a previously unrecognized intrapancreatic pathway regulating CCK-induced pancreatic exocrine secretion.\textsuperscript{234} Several hormones and neuropeptides regulate CCK-mediated exocrine secretion. Locally, insulin has been shown to influence exocrine secretion. Intra-arterial infusion of canine pancreas with anti-insulin antibodies prevented CCK release as well as secretin-mediated protein and fluid secretion from canine pancreas.\textsuperscript{235} Secretin potentiated, as well as attenuated, CCK-mediated amylase secretion by the inositol signaling pathway while VIP enhanced CCK-mediated enzyme secretion.\textsuperscript{236,237} Peptide YY (PYY), PP, and somatostatin also inhibited CCK-mediated protein secretion and their effects are discussed later in this review.

The mechanism of CCK-induced amylase secretion involves transient elevation in intracellular Ca\textsuperscript{2+}.\textsuperscript{238} It also requires phospholipase C activation and generation of second messengers inositol trisphosphate and diacylglycerol.\textsuperscript{239} In some instances, CCK activates secretion by elevation of cAMP, as 8-bromo-cAMP and a phosphodiesterase inhibitor augmented CCK-mediated amylase release.\textsuperscript{236} Heterotrimeric G proteins Go\textsubscript{13} and Go\textsubscript{q} through downstream interactions with small GTP-binding proteins RhoA and Rac1 regulate actin cytoskeleton reorganization which is required for exocytosis.\textsuperscript{240,241} Secretin

Secretin is a 27-amino acid hormone released by S cells of the small intestine.\textsuperscript{242} Secretin release is stimulated during the intestinal phase upon entry of gastric acid and ingested fatty acids into the duodenum.\textsuperscript{243} It augments fluid and bicarbonate secretion and is one of the most potent stimulators of pancreatic secretion.\textsuperscript{244} Examination of pancreatic ultrastructure shortly after secretin injection revealed that fluid is secreted by duct as well as acinar cells.\textsuperscript{245} Large increases in pancreatic fluid and bicarbonate secretion have been demonstrated with secretin infusions as low as 1–2.8 pmol/kg/hr.\textsuperscript{246,249} In humans, bolus injections of secretin as low as 0.125 pmol/kg stimulated fluid and bicarbonate secretion.\textsuperscript{250,251} Although secretin is believed to be the single most powerful stimulator of pancreatic bicarbonate secretion, infusion of exogenous secretin equivalent to postprandial blood levels only produced 10 percent of the maximal pancreatic bicarbonate secretory response suggesting that other hormones and neurotransmitters play important roles in postprandial pancreatic bicarbonate secretion in humans.\textsuperscript{247,250} Secretin receptors have been localized in acinar and duct cells in the rat pancreas.\textsuperscript{118} Secretin stimulates the release of fluid and bicarbonate and, to a lesser extent, protein from acinar cells by a cholinergic mechanism. Perfusion of acetic and lactic acids in the duodenum of anesthetized rats increased fluid and protein output from the pancreas concomitant with elevation of plasma secretin levels. In addition, treatment of rats with atropine decreased plasma secretin levels and inhibited fluid (but not protein) secretion, indicating that only fluid
secretion is dependent on cholinergic input. Electrical field stimulation of isolated perfused rat pancreas demonstrated that secretin-mediated exocrine secretion was sensitive to tetrodotoxin and atropine blockade, further suggesting cholinergic regulation. Nicotinic acetylcholine receptors are not involved in this mechanism as hexamethonium did not exert an inhibitory effect on pancreatic secretion.

Both cAMP-dependent and independent pathways contribute to secretin-mediated exocrine secretion. Interaction of secretin with its receptor induced a three- to fourfold increase in adenylyl cyclase activity, which was abolished in the presence of secretin antagonists. Secretin did not stimulate pancreatic fluid release or elevate acinar cell cAMP levels in secretin receptor knockout mice. Exocrine protein secretion by secretin was associated with phospholipase C activation in one report. At secretin concentrations >10⁻⁸ M, inositol trisphosphate and diacylglycerol were generated in acinar cells, which caused release of Ca²⁺ from intracellular stores and activated protein kinase C. However, not all investigators have observed this effect.

Several hormones and peptides modulate the effects of secretin on pancreatic secretion. CCK augmented secretin-induced pancreatic fluid and protein output by stimulating acetylcholine release and this effect was blocked by atropine or by dispersion of acini. Venous drainage from pancreatic islets bathes the exocrine pancreas with high concentrations of islet hormones. Several of these hormones have potent effects on pancreatic secretion. Insulin enhances secretin-mediated fluid and protein secretion through an ouabain-sensitive Na⁺,K⁺-ATPase while glucagon inhibits secretin-stimulated release of fluid and protein. Addition of anti-somatostatin antibodies increased secretion from perfused rat pancreas implying that somatostatin inhibits secretin-induced fluid and enzyme secretion.

Glucagon-like peptide 1

Glucagon-like peptide 1 (GLP-1) is a 29-amino acid peptide that is produced by enteroendocrine cells in the distal small intestine. Until recently the role of GLP-1 in pancreatic exocrine secretion was not clear. Using real-time PCR it has now been shown that GLP-1 receptor (GLP-1R) is expressed in both pancreatic acinar cells and islets. GLP-1 stimulated amylase release from wild-type but not GLP-1R knockout mice through a cAMP-dependent mechanism. These results have been supported by Satoh et al., who showed that when isolated rat pancreatic acini were stimulated by GLP, amylase was released in conjunction with phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) protein. Inhibition of MARCKS phosphorylation prevented GLP-1-mediated amylase secretion, suggesting it was a critical step in this signaling pathway.

Atrial natriuretic factor and C-natriuretic peptide

Atrial natriuretic factor (ANF) is a peptide hormone that is secreted by atrial stretch and regulates blood pressure and volume by inhibiting reabsorption of sodium by the kidney. Immunochemical studies showed that ANF is present in acinar cells. Early studies suggested that ANF did not influence protein or fluid secretion. However, incubation of rat acini with ANF caused a dose-dependent elevation of cellular cGMP showing that guanylate cyclase receptors transduce ANF signaling. Injection of human ANF in dogs induced bicarbonate but not sodium or protein secretion. ANF also stimulates pancreatic natriuretic peptide receptor-C (NPR-C)-mediated phosphoinositide-dependent pathway in rats, causing the release of fluid and protein. NPR-C is a nonguanylyl cyclase receptor and is coupled to adenylyl cyclase inhibition or phospholipase C activation through G₁ proteins. ANF attenuated secretin- and VIP-induced elevation of intracellular cAMP and this effect was blocked by inhibitors of protein kinase C and phospholipase C. Along with elevating intracellular cAMP, secretin mediates the efflux of cAMP from intact pancreas and acinar cells. ANF augmented secretin-induced cAMP efflux and caused the rapid elimination of cAMP from cells. The multidrug resistance protein 4 (MRP4) has been reported to play a role in the extrusion of cAMP in many cellular systems. MRP4 is also expressed in the pancreas and genetic knockdown of MRP4 expression reduced intracellular cAMP levels in acinar cells by ANF and an NPR-C-dependent mechanism.

C-natriuretic peptide (CNP) is structurally similar to ANF and is expressed in the CNS and gastrointestinal tract. CNP increases pancreatic protein, chloride, and fluid secretion (without influencing bicarbonate output) suggesting that it acts on acinar rather than duct cells. Truncal vagotomy or perivaginal application of capsaicin or hexamethonium attenuated chloride secretion, demonstrating that the effect of CNP is modulated by the parasympathetic nervous system. At low concentrations, CNP induced protein secretion by activation of NPR-C. Similar to ANF, CNP-induced amylase release was inhibited by PLC and PKC inhibitors. CNP also elevated intracellular cGMP and reduced cAMP concentrations suggesting that CNP can interact directly with receptors located on pancreatic acini.

Insulin

Insulin modulates pancreatic exocrine function and insulin receptors are present in high density on the basolateral...
surfaces of acinar cells. Insulin increases pancreatic enzyme synthesis and secretion and its effects are enhanced by CCK and secretin. Since CCK induces insulin release in the presence of glucose and amino acids, it is possible that these two hormones act in conjunction on exocrine stimulation following food intake.

Limited data suggest that insulin regulates exocrine secretion by potentiation of ouabain-sensitive Na\(^+\),K\(^+\)-ATPase and by vagal cholinergic input. The action of insulin on exocrine secretion is modulated by PP that exerts an inhibitory effect on pancreatic secretion.

Anderson and colleagues showed that melatonin induced pancreatic amylase release, which was mediated by CCK, vagal sensory nerves, and galanin, pancreastatin, and somatostatin are known to inhibit insulin secretion, it is possible that these peptides also regulate insulin-mediated amylase release.

**Bombesin**

Bombesin is a 14-amino acid peptide homolog of GRP and neuromedin B and has the ability to suppress appetite. The effects of bombesin on pancreatic exocrine secretion appear to vary based on the species. In pigs, administration of bombesin alone or in combination with secretin induced protein but not fluid secretion. In guinea pigs, bombesin was very effective in inducing bicarbonate release from interlobular ducts, and this effect was blocked by a GRP receptor antagonist. Administration of bombesin to rats resulted in pancreatic hypertrophy with increased pancreatic weight, protein, RNA, and enzyme content and this effect was not regulated by CCK or secretin.

**Melatonin**

Melatonin is a lipophilic hormone produced by the pineal gland as well as by certain neuroendocrine cells located in the gastrointestinal tract. Melatonin receptors are present on acinar cells and melatonin protects the pancreas against caerulein-induced acute pancreatitis. Initial studies showed that melatonin induced pancreatic amylase release, which was mediated by CCK, vagal sensory nerves, and melatonin type 2 receptors. However, melatonin did not appear to have a direct effect on pancreatic acinar cells. The extent and importance of melatonin-induced pancreatic secretion is not well understood and has recently been proposed to involve activation of the enteropancreatic reflex and CCK release from the duodenum.

**Amylin**

Amylin is a 37-amino acid hormone that is cosecreted along with insulin from pancreatic β-cells in response to nutrients. Amylin stimulates CCK-independent pancreatic secretion in rats, and this effect is blocked by proton pump inhibitors and atropine, perhaps due to inhibition of somatostatin release. Amylin was also shown to stimulate protein secretion from pancreatic AR42J cells by a mechanism involving activation of GPCRs and release of Ca\(^{2+}\) from intracellular stores. Others investigations have suggested that amylin has no effect on pancreatic exocrine secretion from isolated perfused pancreas, acinar preparations, or AR42J cells. Hence, the effects of amylin on exocrine secretion remain unresolved.

**Fibroblast growth factor 21**

Fibroblast growth factor 21 (FGF21) was initially discovered in the liver and thymus of mouse embryos and was later found to be expressed at high levels in pancreatic acinar cells. FGF21 overexpression protected mice against caerulein and alcohol-induced pancreatitis. By evaluating the function of FGF21 in transgenic and knockout mice, it was discovered that FGF21 regulates pancreatic enzyme secretion from acinar cells in a paracrine/autocrine manner. FGF21 binds its heteromeric receptor (comprised of FGFR1c and β-klotho) to elevate intracellular calcium levels by signaling through phospholipase C and inositol trisphosphate. Zymogen granule accumulation was observed in both FGF21 knockout and β-klotho knockout mice. FGF21 is secreted in acinar cells in response to ER stress. Unlike CCK, FGF21’s role in acinar cells is limited to augmentation of amylase secretion, as it does not increase synthesis of pancreatic enzymes.

**Histamine**

The amino acid histamine is a potential mediator of pancreatic exocrine secretion, although it may have a gender-dependent role. Activation of H1 receptors and inhibition of H2 receptors in the rabbit pancreas led to an increase in fluid and protein secretion suggesting differential action based on regulation and coupling of the two receptors. The effect of histamine on pancreatic secretion is considered to be minor at best under normal physiological conditions.

**Inhibitory hormones**

**Peptide YY and pancreatic peptide**

The NPY family of peptides consists of three hormones: NPY, PYY, and PP. All three peptides contain 36 residues several of which are tyrosines and share a tertiary structural motif known as the PP fold. The N-terminal amino acids of PYY and NPY can be cleaved by peptidases to generate truncated forms, PYY\(_{3–36}\) and NPY\(_{3–36}\), which are biologically active. NPY has been localized to sympathetic pancreatic nerves and its role has been discussed previously in this review. In islets, PYY is coexpressed with glucagon in α-cells, whereas PP is secreted postprandially.
by F cells of the islets of Langerhans. In certain species, PP immunopositive cells are also present in the exocrine pancreas and some of these PP cells also express PYY. These three hormones exert their effects through a family of five GPCRs denoted Y1–5. NPY and PYY possess similar affinity for Y1, Y2, and Y5, PYY3–36 interacts preferentially with Y2, whereas PP is the preferred ligand for Y4.

PYY levels in blood are elevated postprandially and following instillation of fatty acids into the distal small intestine. Physiologically relevant concentrations of PYY in the circulation inhibit both meal- and hormone-stimulated pancreatic secretion. Intravenous administration of PYY significantly diminishes secretin- and fluid secretion concomitant with a reduction in pancreatic blood flow. However, PYY does not inhibit 2-diacylglycerol-stimulated pancreatic secretion, suggesting that suppression of CCK-stimulated exocrine secretion occurs prior to activation by 2-diacylglycerol or does not involve protein kinase C-activated signaling. In denervated pancreas, PYY1–36 but not PYY3–36 reduced CCK-stimulated amylase release suggesting that hormonal effects of PYY are mediated by Y1 receptors. Autoradiographic analysis of rat pancreas with radio-iodinated PYY ligand demonstrated that Y1 receptors are present primarily on smooth muscle cells of blood vessels. Specific staining was not observed in acinar cells indicating that decreased protein and fluid secretion could be due to reduced blood flow.

PP secretion is also stimulated by ingesting a meal and can be reproduced by intraduodenal infusion of acid, aromatic amino acids, or fatty acids. Like PYY, PP attenuated secretin- and CCK-mediated exocrine secretion in dogs independent of cholinergic blockade. In one study, PP decreased secretin- and secretin plus CCK-mediated amylase release in dispersed acini, suggesting that PP can act directly on acinar cells. However, although bovine and rat PP inhibited CCK-stimulated protein secretion in vivo, both peptides were ineffective in vivo, and binding of bovine PP to rat acinar cells or lobules was not observed. Other studies have also shown direct effects of PP. In humans, unlike dogs, infusion of PP decreased pancreatic protein output but did not influence bicarbonate secretion suggesting species-specific differences in PP action. However, unlike PYY, PP does not affect pancreatic blood flow and therefore inhibits exocrine secretion at the cellular level.

**Somatostatin**

Somatostatin is composed of 14 or 28 amino acids and is produced by δ-cells of pancreatic islets. It is also secreted by certain intestinal cells and by neurons in the hypothalamus. It is released into the blood after a meal but functions primarily through a paracrine mechanism. It has broad inhibitory actions on the release of several hormones and their target organs.

Somatostatin and its analogs inhibited secretin- and CCK-induced protein secretion in a dose-dependent fashion. Low doses of somatostatin exerted a greater inhibitory effect on CCK-stimulated pancreatic secretion compared to secretin-stimulated secretion. Secretin-mediated activation of slowly activating voltage-dependent K+ channels (present on the basolateral surface of pancreatic acini) resulted in cAMP generation and secretion of chloride ions. Addition of somatostatin to acini decreased intracellular cAMP production as well as secretin-mediated enhancement of K+ current suggesting that somatostatin regulates exocrine secretion through this pathway. Additionally, somatostatin inhibited Ca2+-activated and cAMP-stimulated amylase release by inhibiting exocytosis through a Gi-protein-dependent mechanism.

Somatostatin also inhibits exocrine secretion via a neural mechanism. Based on atropine, hexamethonium, and tetrodotoxin sensitivities, it appears that peptidergic but not cholinergic and nicotinic acetylcholine receptors present on sympathetic and parasympathetic ganglia mediate somatostatin action. Somatostatin-mediated inhibition of secretin-stimulated fluid and protein secretion was not influenced by denervation, suggesting that extrapancreatic nerves are not involved. Bethanechol, a muscarinic receptor agonist, reversed the inhibitory effects of somatostatin, indicating that its actions are mediated primarily by intrapancreatic cholinergic neurons. The mechanisms by which somatostatin inhibits pancreatic secretion are not completely understood. However, it is believed that somatostatin has an inhibitory effect on the release of hormones and neurotransmitters that normally stimulate pancreatic secretion.

**Galanin**

Galanin is a 29-amino acid peptide that plays diverse roles including inhibition of insulin, somatostatin, and PP secretion from the pancreas. It is found in the secretory granules of central and peripheral neurons suggesting that it functions as a neurotransmitter. Galanin immunoreactivity was present in nerve fibers surrounding acini, ductules, and blood vessels, with 73 percent of fibers being dual positive for galanin and VIP. Galanin receptor 3 mRNA is present in acinar cells indicating that galanin may act directly on acini. Consistent with this finding, galanin inhibited CCK- and carbachol-stimulated amylase release from acinar cells. Galanin inhibited the sustained phase of amylase release stimulated by carbachol, suggesting that it attenuates cholinergic action possibly by a mechanism that involves pertussis toxin-sensitive Gi proteins.

Extrapancreatic nerves are not involved in its action since...
Galanin inhibited food-, secretin- and CCK-mediated fluid release, as well as food- and CCK-mediated protein release in both innervated and denervated dogs.\textsuperscript{346}

**Pancreastatin**

Pancreastatin is derived from the cleavage of chromogranin A and is expressed in many neuroendocrine tissues. It has been localized to duct cells of the exocrine pancreas and its numerous roles include inhibition of pancreatic exocrine secretion.\textsuperscript{117} Initial studies showed that pancreastatin inhibited postprandial fluid and protein secretion in rats with bile-pancreatic juice diversion. No effect was observed on basal secretion, secretin-stimulated secretion in conscious rats, or CCK-stimulated secretion from dispersed acini. However, pancreastatin inhibited CCK-stimulated pancreatic secretion in conscious rats, although it did not influence plasma CCK levels. These results suggest that pancreastatin does not have a direct effect on acinar cells but may regulate the intestinal phase of pancreatic secretion.\textsuperscript{283,347–349} Pancreastatin inhibited caerulein-induced blood flow in the exocrine pancreas raising the possibility that its inhibitory effects are derived from its role in regulating pancreatic blood flow.\textsuperscript{350}

**Glucagon**

Glucagon is released from the endocrine pancreas after ingestion of a meal.\textsuperscript{351} Early investigations suggested that glucagon inhibited secretin- or secretin- and CCK-stimulated pancreatic but not bicarbonate secretion.\textsuperscript{352,353} However, other studies have demonstrated that glucagon inhibits postprandial protein and bicarbonate secretion.\textsuperscript{354–356} The effect of glucagon on isolated pancreatic lobules and acini appears to be direct and stimulatory, instead of inhibitory, suggesting complex action at the cellular versus physiological levels.\textsuperscript{357–360} The experimentally observed effects of glucagon on exocrine secretion are inconclusive and merit further investigation. Another proglucagon-derived peptide known as oxyntomodulin is a dual agonist of GLP-1R and glucagon receptors. Although this peptide did not influence plasma amylase concentration on its own, it attenuated CCK-mediated plasma amylase release. The mechanism of action of oxyntomodulin has not been identified.\textsuperscript{361}

**Ghrelin**

Ghrelin is a 28-amino acid orexigenic hormone released by gastric endocrine cells under fasting conditions. Ghrelin stimulates acid secretion by oxyntic cells in the stomach, and plasma levels of ghrelin rise immediately before a meal suggesting a role in modulating digestive behavior. In the pancreas, both ghrelin and its receptor have been identified in acini by evaluation of protein and mRNA expression.\textsuperscript{362} Ghrelin expression was not altered by gastric acid inhibition, acute pancreatitis, or food deprivation, although its receptor was upregulated by gastric acid inhibition and downregulated during acute pancreatitis.\textsuperscript{362} Experimentally, ghrelin did not affect basal or CCK-stimulated amylase release from dispersed acini. However, ghrelin inhibited CCK-stimulated protein secretion in normal and vagotomized rats and amylase secretion from lobules exposed to depolarizing potassium concentrations, suggesting that it modulates intrapancreatic neurons.\textsuperscript{363}

**Leptin**

Leptin is a 16-kDa orexigenic peptide that is secreted by adipocytes and regulates energy homeostasis by reducing food intake while increasing energy expenditure. In the pancreas, intravenous or intraperitoneal administration of leptin reduced basal and CCK-stimulated protein output \textit{in vivo}. This effect was attenuated by CCK-1 receptor blockade, vagotomy, and capsaicin pretreatment suggesting that it inhibited pancreatic exocrine secretion through a CCK-dependent vagal pathway. Leptin had no effect on dispersed acini \textit{in vitro}, further supporting a neural mechanism of action.\textsuperscript{364,365} In contrast, intraduodenal infusion of leptin in fasted rats augmented pancreatic protein output possibly by elevating plasma CCK levels leading to activation of sensory neurons.\textsuperscript{366}

**Adrenomedullin**

Adrenomedullin is colocalized with PP in F cells of pancreatic islets and inhibits insulin.\textsuperscript{367} In one study, it interacted directly with acinar cells and inhibited CCK-stimulated pancreatic amylase release.\textsuperscript{368} The mechanism of adrenomedullin action is not well understood.

**Conclusion**

Pancreatic secretion is a complex process that is initiated by the sight and smell of food and progresses until food enters the duodenum. At each level of food digestion, this process is regulated by various stimuli which affect neuronal and hormonal pathways. These pathways are both stimulatory and inhibitory and optimize the release of enzymes, bicarbonate, and fluid.

**References**


75. Liddle RA, Green GM, Conrad CK, Williams JA. Proteins but not amino acids, carbohydrates, or fats stimulate
86. Wang Y, Chandra R, Samsa LA, Gooch B, Fee BE, Cook JM, Vigna SR, Grant AO, Liddle RA. Amino acids stimulate cholecystokinin release through the Ca2+-sensing receptor. Am J Physiol Gastrointest Liver Physiol 2011; 300:G528–G537.
105. Thimister PW, Hopman WP, Tangerman A, Rosenbusch G, Willems HL, Jansen JB. Effect of intraduodenal bile salt on...


231. Miyasaka K, Shinozaki H, Jimi A, Funakoshi A. Amylase secretion from dispersed human pancreatic acini: neither


Regulation of pancreatic secretion


Regulation of pancreatic secretion


Introduction

Secretion from the human exocrine pancreas is highly regulated and essential for nutrient digestion. Pancreatic secretions are coordinated with responses occurring in both digestive and interdigestive periods and vary with meal composition and patient-related factors. In diseases affecting the exocrine pancreas, the amount and/or composition of pancreatic secretion can change and lead to maldigestion. Although our understanding of pancreatic secretion in the healthy state has been well-characterized, knowledge of the changes that occur during disease states remains limited. Management of exocrine pancreatic insufficiency is primarily focused on replacement of digestive enzymes, particularly lipase, with exogenous pancreatic enzymes. There is renewed interest in the study of pancreatic secretion for studies of both pancreatic physiology and disease due to advances in its collection and analysis and the growing recognition that pancreatic insufficiency often follows acute pancreatitis, particularly when it’s severe. In this chapter, we review various aspects of human pancreatic secretion in health and disease.

Anatomy of the Exocrine Pancreas

The anatomy of the exocrine pancreas has been comprehensively reviewed. In brief, the functional exocrine unit of the pancreas consists of groups of acinar cells and a network of pancreatic ductules and ducts. The production of the vast majority of pancreatic juice proteins occurs in the acinar cells. Of the pancreatic enzymes, proteases (e.g., trypsin, carboxypeptidases, chymotrypsin, and elastase) are the most abundant (according to mass) and comprise around 90 percent of the enzymes found in human pancreas fluid; amylase, lipase, and nucleases are relatively less abundant. Following production and storage of inactive proenzymes (termedzymogens) and active enzymes in acinar cellzymogen granules, they are secreted into a network of pancreatic ductules and ducts in response to a meal. These conduits are lined with cuboidal ductal epithelial cells, which secrete a bicarbonate-rich fluid needed to solubilize proteins secreted from the acinar cell and carry them to the duodenum. Secretion from acinar and ductal cells is highly regulated, primarily in response to nutrient ingestion and neural stimulation. Diseases that interfere with either the function of the ductal cells or the continuity of the ductal network will lead to exocrine pancreatic insufficiency, as discussed below.

Normal Secretion of the Exocrine Pancreas

During the interdigestive (i.e., fasting) state, exocrine secretion is somewhat cyclic and associated with the three phases of gastrointestinal motility. Pancreatic secretion in the digestive period is much greater than the interdigestive period. This secretion is regulated by neurohormonal responses and occurs in three primary phases—cephalic, gastric, and intestinal. The initial cephalic phase (stimulated by the sight, smell, or taste of food) is primarily controlled by the vagal nerve and results in levels of enzyme secretion that account for 20–25 percent of the total elicited by a meal. Next, the gastric phase is activated by gastric distention, which only produces a small increase in pancreatic enzyme secretion. Finally, the intestinal phase is activated by chyme in the duodenum, resulting in the majority (50–80%) of the pancreatic stimulus associated with a meal. Together these responses cause a rapid increase in enzyme secretion to a maximal output about 1 hour after meal ingestion. The enzyme output generally remains elevated for approximately 3–4 hours but can vary depending on multiple factors. The cessation of pancreatic enzyme secretion is believed to be primarily the result of nutrient exposure to the distal small intestine based on experiments demonstrating the inhibition of endogenously stimulated secretion following ileal perfusion of carbohydrates or lipids. Hormones believed to be involved in this feedback inhibition include peptide YY and glucagon-like peptide-1.

Several meal-related factors influence exocrine pancreatic secretion, including caloric content, nutrient...
composition, and physical properties of the meal. In regards to caloric content, there appears to be a minimal and maximal threshold of pancreas stimulation, with the maximal enzyme response occurring after consumption of approximately 500 kcal. The meal composition of fats, carbohydrates, and proteins can also influence the relative and absolute levels of enzymes in pancreatic secretion. For example, healthy subjects exposed to a high-fat diet for 2 weeks had a total enzyme output that was two to four times greater than those on a high-carbohydrate diet in the immediate postprandial and interdigestive periods, respectively. Although temporary exposure to a high-fat diet for 24 hours also increased enzyme output in this study, the observation was not replicated with intraduodenal infusion of different nutrient compositions. Another key observation is that ingestion of a solid meal results in a more sustained enzyme response than an identical homogenized meal.

Pancreatic secretion of a bicarbonate-rich fluid has a distinct mechanism and has important consequences. The transport of bicarbonate by duct cells into the pancreatic duct lumen is mediated by the cystic fibrosis transmembrane conductance regulator (CFTR). This transporter can either secrete chloride to drive a chloride-bicarbonate exchanger on duct cells or directly transport bicarbonate into the duct lumen. The relative levels of ion secretion depend on the amount of stimulation; at low rates, chloride predominates but at high secretory rates, bicarbonate is dominant. Reduced bicarbonate and fluid secretion are seen in chronic pancreatitis, cystic fibrosis, and in some individuals with alcohol abuse possibly due to inhibition of CFTR function (discussed below). Duodenal pH is the primary driving force for ductal fluid and electrolyte secretion though nutrient digestive products can also enhance bicarbonate secretion. After ingestion of a meal in a healthy subject, the duodenal pH changes from a baseline near 7.0 to a nadir of 5.0–4.5 in the early postprandial phase. When a threshold of about pH 4.5 is reached in the duodenum, secretin is released and secretion of bicarbonate-rich fluids into the duodenum follows. The alkaline fluid in the duodenum has several functions including inactivating pepsin, increasing the solubility of fatty acids and bile acids, maintaining an optimal pH (>4.0) for pancreatic and brush border enzymes, and preventing intestinal mucosal damage.

Alteration of Exocrine Secretion in Disease

Pancreatic enzyme secretion is a highly controlled process in health, so it is not surprising that its dysfunction is often observed with disorders of the exocrine pancreas. Although enzyme response to different nutrients has been well characterized in a healthy state, much less is known regarding the extent of change in disease. Rather, most studies have focused on the clinical ramifications of inadequate enzyme production and/or secretion.

Mechanisms of abnormal exocrine function

The multiple mechanisms whereby pancreatic disorders can lead to decreased pancreatic enzyme activity can be categorized as:

1. Decreased enzyme production,
2. Impaired enzyme secretion, and/or
3. Impaired enzyme mixing with food.

Reduced acinar cell mass leads to decreased enzyme production and arises from disorders of primary and secondary exocrine pancreatic insufficiency (EPI). Primary disorders are illustrated by Shwachman-Diamond syndrome and Johanson-Blizzard syndrome; secondary causes include chronic pancreatitis, severe acute pancreatitis, or pancreatic surgical resection. Patients with impaired enzyme secretion can have impairment at the cellular (e.g., impaired duct cell function in cystic fibrosis) or macroscopic level (e.g., obstruction of the main pancreatic duct secondary to a stricture, intraductal stone, or mass). Impaired mixing of pancreatic enzymes with food often occurs in the postoperative setting, such as following a gastrojejunostomy. Additionally, impaired bicarbonate secretion by ductal cells can lead to impaired mixing, a suboptimal pH for enzyme activities, and reduced formation of micelles.

Chronic pancreatitis

Chronic pancreatitis is one of the most common causes of exocrine pancreatic insufficiency (EPI), with insufficiency observed in 30 to 90 percent depending on the clinical severity of disease. In a study examining the natural history of chronic pancreatitis, the onset of EPI was often delayed 10–15 years after symptom onset. This delay is attributable to the dramatic functional reserve of the exocrine pancreas, requiring loss of more than 90 percent of exocrine pancreatic function before developing malabsorption. Whether or not the reduction of the various pancreatic enzymes occurs in parallel remains unclear. However, the clinical abnormalities are predominantly related to fat mal-digestion. This is primarily a consequence of limited levels of lipase in the normal pancreas and minimal redundancy of lipase production from nonpancreatic sources. Further, other luminal conditions for fat digestion and absorption, especially the formation of micelles needed for both optimal lipid hydrolysis and lipid delivery to mucosal cells, are disordered in chronic pancreatitis. In contrast, only 5 percent of normal amylase content is needed to maintain starch digestion; this amount can typically be reached by a combination of salivary gland and brush border oligosaccharidase.
production of amylase. Similar levels of pancreatic proteases are likely needed for efficient protein digestion. In a landmark study, DiMagno and colleagues demonstrated that steatorrhea (>7 gram fat/24 hours) develops when the lipase output is <10 percent of normal. The reported lipase output in chronic pancreatitis may range from 1 to 60 percent of levels found in healthy controls. The wide variability is in part due to methodologic differences in the studies (e.g., method of pancreas stimulation and collection methods) but also likely reflects subjects studied at various stages of disease.

Chronic pancreatitis can alter all aspects of pancreatic enzyme synthesis and secretion, so EPI is typically multifactorial. First, parenchymal fibrosis can lead to acinar cell injury and loss of exocrine mass with a resultant loss of enzyme production. Second, duct cell function is impaired in chronic pancreatitis leading to decreased bicarbonate secretion. As a result, postprandial duodenal pH is lower in chronic pancreatitis than in healthy subjects and can exceed the pH threshold for irreversible lipase inactivation (pH 4.5). Obstruction of the main pancreatic duct can also occur due to fibrotic strictures and intraductal calculi and likely contributes to reduced duct pancreatic secretion. However, neither endoscopic nor surgical correction of main pancreatic duct obstruction has been convincingly demonstrated to restore pancreatic enzyme secretion. Since the functional and tissue loss found in chronic pancreatitis is currently not reversible with the possible exception of cystic fibrosis, clinical treatment of exocrine insufficiency is limited to oral supplementation with pancreatic enzyme replacement therapy.

Cystic fibrosis

The CFTR gene codes for a chloride channel, which is central to the production of fluid and electrolyte secretion in various exocrine glands. In the pancreas, the primary pathophysiologic result of CFTR mutations is decreased electrolyte and fluid secretion into the pancreatic duct lumen. The consequence of these changes is pancreas fluid that is more viscous and acidic than normal secretion. Although acinar cells may not be directly affected in early or mild disease, both pancreatic enzyme secretion and function are impaired. In addition to impaired flow, the acidic pancreatic duct lumen can result in precipitation of mucins, secretory protein aggregation, and premature activation of enzymes. Furthermore, the decreased bicarbonate secretion leads to a more acidic intraluminal pH in the duodenum. It has been observed that the pH is lower by 1–2 units in both the interdigestive and digestive periods in cystic fibrosis (CF) compared to controls. Collectively, EPI is prevalent in classic CF with estimates that 80 percent of patients with CF will develop EPI by 2 years of life. Importantly, EPI appears to be mostly limited to those subjects with two severe deleterious mutations, though pancreatic disease can rarely occur in those with less severe mutations. Recent studies reported that correction of EPI can occur with treatment using CFTR modulators. How often this response is seen in CF patients and whether this class of drugs might impact EPI in those having other pancreatic diseases remains to be studied. There is also evidence that prominent levels of alcohol ingestion can rapidly reduce ductal CFTR function.

As in other diseases, the EPI in CF and impaired lipase secretion may at least partially be compensated by increased activity of gastric lipase. Studies of pancreatic enzyme secretion in CF are limited. This is likely due to the multiple methodologic challenges to completing these studies, the greatest of which is that EPI manifests at a young age. This poses technical and ethical issues for research study design. In these patients with EPI, lifelong pancreatic enzyme replacement therapy is recommended and improvement in nutritional status has been associated with improvements in pulmonary function and overall survival.

Acute pancreatitis

In contrast to chronic pancreatitis and cystic fibrosis, which are chronic, progressive diseases, acute pancreatitis is a multiphasic disease. Thus, impairment of pancreas enzyme secretion is variable and depends on the clinical severity of acute pancreatitis and chronologic proximity to the inciting insult. Knowledge of pancreatic enzyme secretion during the early phase of acute pancreatitis is limited in part because of the belief that pancreatic stimulation in this setting may be harmful. Epidemiological studies have characterized EPI prevalence following acute pancreatitis. It is estimated that 62 percent of these patients have EPI during admission with persistent EPI in 35 percent of patients during follow-up. This prevalence trend suggests that transient exocrine pancreatic dysfunction is common after acute pancreatitis and that a smaller number will develop long-term EPI. Persistent EPI appears to be more common in patients with pancreatic necrosis or an alcohol etiology. This is a commonly overlooked sequela of acute pancreatitis, and additional understanding of the pathophysiology is needed.

Pancreatic cancer

The changes in pancreas enzyme secretion are not well characterized in pancreatic cancer. Although the prevalence of EPI in pancreatic cancer has not been characterized in a large clinical cohort, the pooled prevalence in a recent meta-analysis was over 70 percent. In addition
to tumor-mediated parenchymal destruction, abnormal secretion can develop as a consequence of pancreatic duct obstruction and following surgical resection.\textsuperscript{29} The location of the tumor is an important factor in determining whether pancreatic secretion remains adequate. In one study involving direct PFTs, a shorter length of unaffected pancreatic duct (i.e., a tumor located closer to the pancreatic head) was associated with decreased bicarbonate and enzyme output in response to CCK stimulation.\textsuperscript{29}

\textit{Pancreatic surgery}
Alterations in exocrine secretion following pancreatic surgery are influenced by the type of surgery, extent of resection, and health of the remnant pancreatic parenchyma. The exocrine function remains largely unchanged, and occasionally improves, in those undergoing a drainage procedure with or without minor resection of the parenchyma.\textsuperscript{30} Conversely, clinically diagnosed EPI is observed in approximately 25–50 percent of those following a pancreaticoduodenectomy.\textsuperscript{30–34} In patients undergoing surgery for a diffuse disease, such as chronic pancreatitis, the risk of developing EPI following surgery is increased. For example, in a clinical series limited to subjects with chronic pancreatitis, over 50 percent developed clinically overt steatorrhea following pancreaticoduodenectomy and up to 40 percent following distal pancreatectomy.\textsuperscript{33,35,36}

\textit{Nonpancreatic etiologies of impaired exocrine pancreatic function}
In addition to the preceding pancreatic etiologies, there are other nonpancreatic diseases that can indirectly impair exocrine pancreatic function. For example, in those undergoing a partial or total gastrectomy there are multiple factors that decrease intra-luminal enzyme activity. The greatest contributing factor is post-cibal asynchrony, which describes disordered mixing of meal contents and pancreatic enzymes in the small intestine.\textsuperscript{5} The asynchrony is further compounded by pancreatic denervation during surgical dissection and the loss of compensatory gastric lipase production. The dilution of pancreatic enzymes in the duodenal lumen that results from rapid delivery of an osmotic load also reduces nutrient digestion. Asynchrony is also a contributing factor to the EPI observed following pancreaticoduodenectomy. Although steatorrhea is not universally present in patients with Zollinger-Ellison syndrome (ZES), it is important to consider the effect on exocrine pancreatic function due to its unique pathophysiologic mechanism.\textsuperscript{37} In ZES there is excessive gastrin production, which leads to inappropriate gastric acid secretion. This lowers the duodenal pH beyond the threshold at which normally secreted pancreatic enzymes are active and leads to inactivation of some, most importantly lipase.\textsuperscript{38} The relatively acidic duodenal pH in ZES also leads to bile salt precipitation. This reduces the effectiveness of lipase-dependent triglyceride hydrolysis as well as formation of micelles that are needed to carry lipids to enterocytes. Last, impaired exocrine function has been shown in patients without clinically evident pancreatic disease. For example, patients with type 2 diabetes mellitus can develop fibrosis that is histologically similar to chronic pancreatitis.\textsuperscript{39} This condition, currently referred to as diabetic exocrine pancreatopathy, differs from chronic pancreatitis in that chronic inflammatory cells are absent and overt EPI is rare. Exocrine pancreatic insufficiency may also occur in celiac disease (when measured using fecal elastase levels) despite the absence of structural changes in the pancreas.\textsuperscript{40,41} The EPI in celiac disease is generally attributed with mucosal injury, which may disrupt an enteric-mediated cholecystokinin stimulation of the pancreas and gallbladder emptying. In children, short-term support with oral pancreatic enzymes is often considered. Pancreatic secretion usually resolves with mucosal healing on a gluten-free diet.\textsuperscript{40} EPI has also been reported to occur in patients with inflammatory bowel disease and following gastrointestinal surgeries, but additional studies are needed to further characterize the potential mechanisms of exocrine dysfunction in these scenarios.\textsuperscript{52}

\textbf{Measurement of Human Pancreatic Secretion (Direct Pancreatic Function Testing)}
Our knowledge of normal human pancreatic secretion primarily comes from human studies using luminal tube(s) to collect intestinal fluids following pancreatic stimulation with either a standardized meal or an intravenous secretagogue such as secretin or cholecystokinin. The use of exogenous stimulation to measure pancreatic function is referred to as direct pancreatic function testing (PFT) (Table 1). Although different types of tubes have been used, one of the most recognized is the Dreiling tube, which permitted simultaneous aspiration from the gastric and duodenal lumens. One drawback of this approach was that the enzyme output could not be determined because the flow rate could not be calculated. To resolve this issue, a method was developed using a second tube placed with ports in both proximal and distal duodenum. A nonabsorbable marker (typically polyethylene glycol) was infused through the proximal port and aspirated with pancreatic secretions through the distal port.\textsuperscript{43} Using this marker-perfusion method, permitted determination of correction coefficient for the amount of fluid not collected distally and accurate calculation of pancreatic enzyme output. A variety of endogenous and exogenous stimuli were administered, and the changes in pancreatic secretions could be characterized. Significant drawbacks to these techniques included
Table 1. Comparison of direct and indirect pancreatic function tests. Estimates of sensitivity and specificity are assigned semiquantitatively due to extreme heterogeneity in study designs, which precludes accurate pooling.44, 45

<table>
<thead>
<tr>
<th>Name</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCK-stimulated pancreatic function test</td>
<td>High</td>
<td>High</td>
<td>• Provides the most direct measure of pancreatic enzyme output</td>
<td>• Tubes are no longer available to measure enzyme output • Invasive</td>
<td>$$$</td>
</tr>
<tr>
<td>Secretin-stimulated pancreatic function test</td>
<td>High</td>
<td>Moderate</td>
<td>• Highly sensitive in early stages of disease • High negative predictive value to rule out chronic pancreatitis</td>
<td>• Invasive • False positives: CFTR mutations and cigarette smoking • Does not directly assess acinar cell function</td>
<td>$$$</td>
</tr>
<tr>
<td>Coefficient of fat absorption (CFA)</td>
<td>High</td>
<td>Moderate</td>
<td>• Highly accurate for fat malabsorption • Can be used to monitor PERT • Noninvasive</td>
<td>• Requires 3 day stool collection • False positives: any cause of fat malabsorption (which must be excluded before diagnosing EPI)</td>
<td>$</td>
</tr>
<tr>
<td>Fecal elastase (FE-1)</td>
<td>Mild-Moderate</td>
<td>Moderate-High</td>
<td>• Convenient collection • Noninvasive</td>
<td>• Lower accuracy in those with mild EPI or history of pancreatic resection • Cannot be used to monitor PERT • False positives: bacterial overgrowth, watery stool</td>
<td>$</td>
</tr>
<tr>
<td>$^{13}$C-mixed triglyceride breath test (MTBT)</td>
<td>Moderate</td>
<td>Moderate</td>
<td>• Can be used to monitor PERT • Noninvasive</td>
<td>• Limited availability • Time consuming test</td>
<td>$$</td>
</tr>
</tbody>
</table>

EPI, exocrine pancreatic insufficiency; PERT, pancreatic enzyme replacement therapy.

patient discomfort and prolonged fluoroscopy time to maintain proper tube location. Despite these challenges, the majority of the data related to human pancreatic secretory physiology were acquired using this technique.

Further studies regarding the mechanisms of human pancreatic secretion became possible following adaptation of early work demonstrating the ability to isolate functioning pancreatic acini from rodents.46 The ability to isolate acini from human pancreases and study them in a controlled environment has led to further understanding of the cellular mechanisms of pancreatic secretion in health and disease, which are described elsewhere.47

More than a decade ago, an endoscopic-based pancreatic function test (ePFT) was developed that greatly improved patient tolerance and eliminated the need for fluoroscopy.17 Although the stimuli for pancreatic secretion remained similar (i.e., cholecystokinin (CCK) and/or secretin), the method of fluid collection was through the suction channel of the endoscope rather than an enteric tube. Since there is only one “port” for collecting samples through the endoscope, this technique permits determination of enzyme and analyte concentration but not enzyme output (or flow rate). The most common use of the ePFT is for evaluation of suspected chronic pancreatitis. However, there is emerging use of this methodology for translational science, including the study of disease biomarkers.48

Currently, the most commonly used measurement made using ePFT is the peak bicarbonate concentration following secretin stimulation. Although this measurement is more directly an assay of pancreatic duct-cell function than acinar-cell function, previous studies have demonstrated that peak bicarbonate corresponds to peak lipase concentrations in the pancreatic fluid of healthy subjects as well as chronic pancreatitis.49 For the evaluation of chronic pancreatitis, measurement of peak bicarbonate concentration (following secretin stimulation) has improved discrimination compared to peak lipase or amylase concentrations (following CCK stimulation).50 Importantly, the negative predictive value (97%) of a normal bicarbonate response to secretin stimulation to exclude chronic pancreatitis is very high; however, the positive predictive value (45%) is much lower.51 Thus, the primary clinical use for endoscopic function tests is to “rule out” a diagnosis of chronic pancreatitis.
In addition to the previously mentioned electrolytes and pancreatic enzymes, there is a large number of other potential analytes in pancreatic fluid. During an ePFT, there is typically an abundant volume of fluid collected, which provides the opportunity for other studies. Investigators have begun to explore various molecular targets in pancreas fluid, including protein expression, cytokines, DNA methylation markers, microRNAs, and genetic mutations. Various markers are being examined for the purposes of identifying diagnostic or disease biomarkers, as well as observations that may lead to novel therapeutic approaches. Although early studies were primarily limited to proteomics, there has been a recent resurgence in other areas with exciting preliminary findings. For example, Abu Dayeh et al. demonstrated that prostaglandin E2 (PGE2) is a promising disease biomarker for the various stages of chronic pancreatitis. Levels are different in early and advanced chronic pancreatitis compared to healthy controls, with areas under the curve (AUCs) of 0.62 and 0.9, respectively. When used in combination with the pancreatic fluid bicarbonate, the AUCs for diagnosis of early and advanced chronic pancreatitis were 0.94 and 1.0, respectively. Another example of biomarker discovery includes the identification of DNA hypermethylation markers that identify patients with pancreatic cancer compared to controls with AUCs ranging from 0.62 to 0.92.

Indirect Pancreatic Function Testing

Tests measuring pancreatic function without the use of hormonal stimulation are referred to as indirect PFTs. Since the indirect tests are typically less accurate for detecting early stages of exocrine dysfunction, they are more helpful for quantifying the degree of insufficiency in those with known pancreatic disease, rather than diagnosis. Indirect PFTs are noninvasive and typically less expensive, so the selection of the pancreatic function test to be employed in the clinical setting requires considering the tradeoff between diagnostic test performance, invasiveness, and costs (Table 1). For example, indirect PFTs are generally adequate to identify EPI in patients with overt morphological changes (e.g., calcifications and/or main pancreatic duct dilation). In contrast, a direct PFT would be preferred to identify EPI in a patient with clinical suspicion of chronic pancreatitis and normal or equivocal imaging tests. Poorly studied indirect tests (including serum trypsin, fecal chymotrypsin, and qualitative fecal fat analysis) are not discussed here.

Coefficient of fat absorption

Among indirect testing methods, the coefficient of fat absorption (CFA) is considered the gold standard to diagnose fat malabsorption from any cause and to document and quantify EPI in those with pancreatic disease. This test involves consumption of a high-fat diet (100 grams/day) for at least 5 days with stool collection during the final 3 days of the diet. The daily dietary fat intake is recorded and factored into the final CFA calculation, using the following equation:

\[ \text{CFA} \% = 100 \times \frac{\text{mean daily fat intake} - \text{mean daily stool fat}}{\text{mean daily fat intake}} \]

A CFA of <0.93 (which corresponds to >7 grams of stool fat per 24-hour period while on a 100 g fat diet) is considered abnormal. Although this test is accurate when carefully performed, the three-day collection period can be inconvenient for patients and errors may occur when performed in a noncontrolled environment with either collection or processing. Since fat excretion is directly related to fat ingestion over a broad range of values (excretion ~7% of intake over a broad range of intakes), subjects must have an exact record of their intakes. Though this test is also useful to assess adequacy of PERT dosing, follow-up testing is rarely performed outside of the research setting.

Fecal elastase-1

In contrast to the CFA, a fecal elastase-1 (FE-1) level can be determined from a single formed stool sample. The test’s convenience makes it the most widely used indirect PFT in clinical practice. Pancreatic elastase is resistant to degradation as it passes through the gut, so it can be measured in the stool. Although early studies demonstrated strong correlation with pancreatic enzyme output during direct PFT, more recent studies have demonstrated only fair accuracy in mild EPI. False positives occur in about 10 percent of patients with low pretest probability for EPI. Similarly, levels are falsely low any time a liquid stool specimen is analyzed. Also, low levels are commonly observed in diabetes mellitus (both type 1 and type 2 diabetes), but it is uncertain if this truly represents EPI. Last, since FE-1 levels detected by monoclonal (but not polyclonal) assays are unaffected by PERT, this test is not useful for monitoring the adequacy of therapy.

\(^{13}\text{C-mixed triglyceride breath test}\)

The \(^{13}\text{C}-\text{mixed triglyceride breath test (MTBT)}\) is another indirect test that measures the intraluminal lipolytic activity as an estimate of exocrine pancreatic function. The test involves ingesting a standardized meal (including triglycerides with radiolabeled carbon tracers). The triglycerides are hydrolyzed by pancreatic lipase releasing \(^{13}\text{C}\)-fatty acid, which is absorbed, then transported to the liver. Lipolysis and beta-oxidation occur in the liver leading to the formation of \(^{13}\text{CO}_2\). These molecules are exhaled by the lungs and...
measured in serial breath samples. A decrease in the recovery of $^{13}$CO$_2$ is associated with decreased pancreatic lipase secretion and fat malabsorption as measured by CFA.\textsuperscript{53–65} Although this is a noninvasive test, it lasts approximately 6 hours and the radiotracers are not universally available, so the clinical utility of this test worldwide is limited.

**Summary**

Pancreas exocrine secretion represents a complex response to a meal which involves the coordinated release and transport of enzymes from acinar cells and fluid and electrolytes from duct cells into the pancreatic ductal system and then into the duodenal lumen where they are required for normal digestion. Essentially all pancreatic disorders may alter this process to different degrees and do so through a variety of mechanisms. There are various methods for determining enzyme output and bicarbonate secretion in response to endogenous and exogenous pancreatic stimulation. These tools have shaped our current understanding of pancreas physiology and hold significant potential for biomarker discovery and identification of novel therapeutic targets.

**Acknowledgments**

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**References**


53. Abu Dayyeh BK, Conwell D, Buttar NS, Kadiyala V, Hart PA, Baumann NA, Bick BL, Chari ST, Chowdhary S, Clain


58. Wollaeger EE, Comfort MW, Osterberg AE. Total solids, fat and nitrogen in the feces; a study of normal persons taking a test diet containing a moderate amount of fat: comparison with results obtained with normal persons taking a test diet containing a large amount of fat. Gastroenterology 1947; 9:272–283.


Chapter 16
Neural control of the pancreas
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Introduction
The pancreas plays a critical role in the control of nutritional homeostasis. It consists of two major parts: the exocrine pancreas, which secretes digestive enzymes, and the endocrine pancreas, which releases hormones such as insulin, glucagon, pancreatic polypeptide, and somatostatin and maintains glucose homeostasis. Cells in the endocrine pancreas are organized in pancreatic clusters of cells, the islets of Langerhans. Within the islets, the β-cells, which secrete insulin, are the predominant cell type and comprise approximately 70 percent of the cells within the islets. The remaining cells consist of α-cells that secrete glucagon, δ-cells that secrete somatostatin, and cells that secrete pancreatic polypeptide. The main function of the exocrine pancreas is to aid in digestion by secreting digestive enzymes and bicarbonate into the duodenum. The exocrine pancreas consists of only two major cell types, namely acinar cells that synthesize, store, and secrete digestive enzymes and ductal cells that secrete chloride and bicarbonate.

Both parts of the pancreas are innervated by the sympathetic and parasympathetic nervous system, with separate pathways regulating the exocrine and the endocrine pancreas. In this chapter, we provide an overview of the central neural pathways that control the pancreas and the main neurotransmitters expressed in these pathways.

Sensory Innervation of the Pancreas
Sensory information from the pancreas is transmitted to the central nervous system (CNS) via both vagal and spinal pathways. Cell bodies of the spinal afferent pancreatic neurons are located in the T6-L2 dorsal root ganglia (DRG) and their axons traverse the splanchnic nerves and celiac plexus, before they enter the pancreas. These fibers comprise small myelinated (Aδ) and unmyelinated (C) fibers that transmit both mechanoreceptive and nociceptive information to the preganglionic sympathetic neurons in the intermediolateral cell column (IML) via interneurons in the spinal cord laminae I and IV. Most DRG neurons are capsaicin-sensitive and contain substance P, calcitonin gene-related peptide (CGRP), or both. Mechanosensitive fibers are primarily associated with blood vessels and although their axons are located within the pancreatic parenchyma, they do not appear to innervate the ductal system. SP and CGRP may be involved in pain associated with chronic pancreatitis, as intrathecal administration of their antagonists attenuated behavioral pain responses in a rat model of chronic pancreatitis.

Pancreatic vagal afferent neurons originate in the nodose ganglia and are relatively sparse compared to spinal afferents. Most of these neurons are capsaicin-sensitive and contain substance P and calcitonin gene-related peptide or both. Anterograde tracing studies have shown that axons originating in the nodose ganglion supply large blood vessels, pancreatic ducts, acini, and islets and are only sparsely distributed in the pancreatic ganglia. Interestingly, injections of an anterograde tracer into the right nodose ganglion resulted in labeling primarily in the duodenal pancreatic lobe, whereas injections into the left ganglion predominantly labeled the splenic lobe, indicating that sensory innervation of the pancreas is distributed in a regionally specific manner.

The role of sensory nerves on pancreatic functions in control conditions is not completely understood, however. Chemical ablation of pancreatic sensory nerves has been shown to increase or have no effect on glucose-stimulated insulin secretion, suggesting that sensory nerves may exert tonic inhibition of insulin secretion. Similarly, substance P has been shown to either stimulate or inhibit glucagon secretion. Effects on glucagon secretion are equally contradictory. Calcitonin gene-related peptide has been reported to either stimulate or inhibit glucagon release. Furthermore, ablation of the sensory nerves with capsaicin has been reported to either reduce or have no effect on stimulated glucagon secretion. Although the role of sensory
afferents in the regulation of exocrine secretion has not been fully established, it has been shown that calcitonin gene-related peptide and substance P inhibit pancreatic exocrine secretion indirectly via actions on ganglionic transmission.\(^1\)

**Sympathetic Nervous System Control of the Pancreas**

**Anatomy of the sympathetic pathways regulating pancreatic functions**

Sympathetic innervation of the pancreas originates from the sympathetic preganglionic neurons in the lower thoracic and upper lumbar segments of the spinal cord. Axons from these neurons exit the spinal cord through the ventral roots and supply either the paravertebral ganglia of the sympathetic chain via communicating rami of the thoracic and lumbar nerves or the celiac and mesenteric ganglia via the splanchnic nerves. The catecholaminergic neurons of these ganglia innervate the intrapancreatic ganglia, islets, and blood vessels and, to a lesser extent, the ducts and acini. These differences in the innervation of various portions of the pancreas are evident following sympathetic nerve activation, as sympatho-activation decreases insulin secretion and results in vasoconstriction, while it has little or no effect on ductal and acinar cell secretions. The principal neurotransmitters released by the postganglionic sympathetic neurons that innervate the pancreas are noradrenaline, galanin, and neuropeptide Y (NPY).

Retrograde tracing studies using transsynaptic tracers such as the Bartha strain of pseudorabies virus have revealed the distribution of neurons that supply the sympathetic innervation to the pancreas. Unlike traditional retrograde tracers, transsynaptic tracers can cross synapses and therefore enable identification of higher-order neurons in the neurocircuits that innervate the locus of injection.\(^2\) Injections of the virus into the pancreas of vagotomized rats have demonstrated that second-order neurons in the sympathetic circuits to the pancreas are located in the brainstem, specifically in the A5 cell group, locus coeruleus, ventrolateral medulla, and the caudal raphe, as well as in the paraventricular, lateral, and retrochiasmatic nuclei of the hypothalamus and the prefrontal cortex. Third-order neurons are located in the bed nucleus of the stria terminalis, medial preoptic area, and subformical organ, in the dorsomedial, ventromedial, and arcuate nuclei of the hypothalamus and the central nucleus of the amygdala.\(^3\) A schematic representation of the sympathetic innervation of the pancreas is shown in Figure 1.

**Effects of sympathetic nervous system activation on pancreatic functions**

The role of the sympathetic nervous system in the regulation of pancreatic functions still remains somewhat controversial. Stimulation of the sympathetic nerves elicits diverse effects, including effects on blood pressure, blood flow, and hormone release and therefore direct effects of sympathetic nervous system stimulation are difficult to discern from effects secondary to changes in blood flow or hormone release. Nonetheless, the sympathetic nervous system has been shown to affect the function of the endocrine and, to a lesser extent, exocrine pancreas.

Stimulation of the splanchnic nerve, which supplies the sympathetic innervation to the pancreas has been shown to decrease plasma insulin levels, possibly via direct actions of noradrenaline on pancreatic β-cells.\(^22-26\) Splanchnic nerve stimulation also increases catecholamine levels, which have been shown to decrease insulin secretion via α2 adrenoreceptors on pancreatic β-cells.\(^25,27\) Furthermore, both splanchnic nerve stimulation and adrenaline administration/release increase glucagon secretion.\(^25,26,28\) In contrast, disruption of the splanchnic nerve increases insulin levels, suggesting that the sympathetic nervous system exerts a tonic inhibition of the endocrine pancreas. Taken together, these findings indicate that the overall effect of sympathetic nervous system stimulation is to maintain glycemic levels during stressful conditions by decreasing insulin and increasing glucagon secretion.

The effects of sympathetic nerve stimulation on pancreatic exocrine secretions (PES) are not as clear. Although the sparse innervation of acinar and ductal by the sympathetic nervous system would suggest that the sympathetic nervous system does not play a major role in the regulation of the exocrine pancreas, some studies have reported that the sympathetic nervous system may exert profound effects on exocrine secretions.\(^1\) Electrical stimulation of the splanchnic nerves inhibits, whereas cutting the splanchnic nerves in pigs increases PES, suggesting a tonic inhibition of pancreatic exocrine secretion by the sympathetic nervous system.\(^29\) However, studies using more selective stimulation of the sympathetic nervous system have reported conflicting results. Noradrenaline as well as selective α- and β-adrenoreceptor agonists or antagonists have been shown to decrease, increase, or have no effect on pancreatic exocrine secretion.\(^1\) These conflicting findings may be due to the fact that these agents influence blood flow, which exerts secondary effects on PES. For example, vasoconstriction induced by activation of α-adrenoreceptors would result in reduced blood flow to the exocrine pancreas, thus causing a decrease in the amount of fluid secreted by the exocrine pancreas. In support of this suggestion, noradrenergic vasoconstriction has been shown to decrease pancreatic exocrine secretion.\(^30\) In addition, denervation of the celiac ganglion in the dog reduced pancreatic secretions by approximately 70 percent but increased blood flow by approximately 350 percent, suggesting that the sympathetic nervous system exerts a tonic effect on both pancreatic exocrine secretion and vasoconstriction.\(^31\) Considering...
these constraints of studying pancreatic exocrine secretion independently of vasoconstriction, it is not clear how much influence the sympathetic nervous system has in the regulation of PES.

Parasympathetic Innervation of the Pancreas

Anatomy of parasympathetic pathways innervating the pancreas

The parasympathetic nervous system provides the major excitatory input to the pancreas. Preganglionic parasympathetic neurons that innervate the pancreas originate in the dorsal motor nucleus of the vagus (DMV) and activate parasympathetic postganglionic neurons in the pancreatic ganglia, primarily via activation of nicotinic acetylcholine receptors. Vagal motor output from DMV neurons is conveyed to the GI tract via two pathways, which can be distinguished based on their postganglionic neurotransmitters. The excitatory cholinergic pathway releases acetylcholine, which acts on muscarinic M3 and M1 receptors and provides a tonic input to the gastrointestinal viscera. The inhibitory nonadrenergic, noncholinergic pathway uses nitric oxide, vasointestinal peptide, gastrin-releasing peptide, or pituitary adenylate cyclase-activating polypeptide. Nicotinic transmission between pre- and postganglionic neurons can be modulated by various neurotransmitters and neuromodulators. It should also be kept in mind that species differences in the parasympathetic innervation of the pancreas have been reported. In the mouse, parasympathetic axons provide input to both alpha and beta cells, while parasympathetic axons are rare in the human islets.

The DMV, which contains preganglionic parasympathetic neurons that supply various regions of the GI tract, shows viscerotopic organization, with neurons that project to different parts of the GI tract distributed in anatomically distinct mediolateral columns. Neurons in the medial part of the DMV project to the proximal GI tract, whereas neurons in the lateral DMV project to the more distal parts of the GI tract. Vagal preganglionic DMV neurons that innervate the pancreas are usually located in the left DMV in the area that comprises the hepatic and anterior gastric branches of the vagus, although a few scattered neurons innervating the splenic end of the pancreas are located in the areas corresponding to the celiac branches. Pancreas-projecting DMV neurons can be distinguished from gastric- and intestinal-projecting DMV neurons based on their morphological and electrophysiological properties, further

Figure 1. Sympathetic innervation of the pancreas. Abbreviations: arcuate nucleus (ARC), dorsal root ganglion (DRG), dorsomedial hypothalamic nucleus (DMH), lateral hypothalamic area (LHA), medial preoptic area (MPO), nucleus of the tractus solitaries (NTS), organum vasculosum of the lamina terminalis (OVLT), prefrontal cortex (PFC), retrociasmatic area (RCA), suprachiasmatic nucleus (SCN), subfornical organ (SFO), ventromedial hypothalamus (VMH).
reinforcing the observation that DMV neurons display a highly specialized organization with respect to regulation of various GI functions. Some pancreas-projecting DMV neurons display a slowly developing apamin-insensitive afterhyperpolarization, which is not present in other DMV neurons. Compared to gastric-projecting neurons, pancreas-projecting neurons have a longer action potential duration and longer afterhyperpolarization decay time. Pancreas-projecting neurons also have higher input resistance, smaller afterhyperpolarization amplitude, and a higher firing rate in response to current injections compared to intestinal-projecting neurons. Furthermore, pancreas-projecting neurons have a smaller soma area and a larger diameter than gastric-projecting neurons and fewer segments than gastric- or intestine-projecting DMV neurons.

The major input to DMV neurons originates in the adjacent nucleus tractus solitarius (NTS) (Figure 2). Although NTS neurons express a wide variety of neurotransmitters and neuromodulators, NTS projects to the DMV primarily via glutamatergic, GABAergic, and catecholaminergic inputs. Despite this relatively simple neurochemistry, NTS-DMV synapses display a great deal of plasticity and can be modulated by numerous neurotransmitters, neuromodulators, hormones, and physiological conditions.

Studies using injections of transsynaptic retrograde tracers into the pancreas of sympathectomized rats have demonstrated the distribution of higher-order neurons that innervate the pancreas. These studies have revealed that neurons that comprise the parasympathetic circuitry to the pancreas show a wider distribution compared to the neurons involved in the sympathetic innervation to the pancreas, with some regions overlapping those that comprise sympathetic inputs to the pancreas. In addition to the NTS, second-order neurons that innervate the pancreas are located in the area postrema, accessory nucleus of the spinal trigeminal nerve, raphe pallidus, raphe obscurus, substantia reticulata, ventrolateral medulla, and the A5 area (Figure 2). Parasympathetic second-order neurons are also located in the hypothalamic areas, namely the para-ventricular, lateral, dorsomedial, and arcuate nuclei; medial preoptic area, retrochiasmatic area, subfornical organ, bed nucleus of stria terminalis. Furthermore, higher-order neurons have been detected in the prefrontal, piriform, and gustatory cortices, and these neurons provide anatomical basis for the cephalic phase of exocrine secretion.

Figure 2. Parasympathetic pathways innervating the pancreas. Abbreviations: area postrema (AP), arcuate nucleus (ARC), bed nucleus of the stria terminalis (BNST), dorsomedial nucleus of the hypothalamus (DMH), dorsal motor nucleus of the vagus (DMV), lateral hypothalamic area (LHA), medial preoptic area (MPO), nucleus of the tractus solitarius (NTS), organum vasculosum of the lamina terminalis (OVLT), prefrontal cortex (PFC), paraventricular nucleus (PVN), retrochiasmatic area (RCA), suprachiasmatic nucleus (SCN), subfornical organ (SFO), ventromedial hypothalamus (VMH).
Effects of parasympathetic stimulation on pancreatic functions

Parasympathetic innervation plays a major role in the regulation of pancreatic functions. Activation of the vagus nerve directly affects pancreatic exocrine and endocrine secretion. Electrical stimulation of the DMV or the NTS increases insulin secretion, as do microinjections of the GABA<sub>A</sub> receptor antagonist bicuculline. In addition, the vagus nerve modulates the intrinsic pacemaker activity of the pancreas, which is responsible for pulsatile insulin secretion, indeed patients with complete resection of the subdiaphragmatic vagus display a longer periodicity of plasma insulin oscillations.

The vagus nerve also plays a crucial role in the regulation of PES. Effects of peptides that modulate pancreatic secretions, such as cholecystokinin (CCK), somatostatin, calcitonin gene-related peptide (CGRP), and pancreatic polypeptide (PP), are vagally mediated. Furthermore, vagotomy has been shown to almost completely abolish pancreatic exocrine secretion induced by feeding or by pharmacological or electrical stimulation, whereas disinhibition of the DMV by microinjections of the GABA<sub>A</sub> receptor antagonist bicuculline increases pancreatic exocrine secretion.

The cephalic phase response, which refers to the release of gut hormones and digestive enzymes before the ingested nutrients have induced a systemic hormonal response, is also dependent on the vagus nerve and its inputs from the gustatory, piriform, and prefrontal cortices. In fact, vagally mediated exocrine secretion in the cephalic phase accounts for a significant portion of total postprandial enzyme secretion, suggesting that inputs from higher CNS centers to pancreas-projecting DMV neurons play an important role in regulation of PES.

Neurotransmitters in Central Pathways Regulating the Pancreas

The brainstem plays an important role in the regulation of autonomic outflow to the pancreas. The NTS and the DMV have reciprocal connections with higher CNS regions, and these connections contain many neurotransmitters and neuromodulators that influence efferent outflow to the pancreas. In addition to receiving inputs from other CNS regions, the dorsal vagal complex is a circumventricular organ with fenestrated capillaries, which expose it to the influence of circulating hormones. While autonomic output to the pancreas can be regulated by numerous substances, we will focus on the neurotransmitters that have been most extensively studied.

GABA and Glutamate

GABA and glutamate provide major inhibitory and excitatory synaptic inputs to pancreas projecting DMV neurons, respectively. GABA is the main inhibitory neurotransmitter in the CNS and is the principal neurotransmitter regulating vagal outflow to the pancreas. Microinjections of the GABA<sub>A</sub> receptor antagonist bicuculline into the dorsal vagal complex increase pancreatic exocrine secretion and glucose-stimulated insulin secretion, suggesting that GABA exerts a tonic inhibition on both pancreatic exocrine secretion and insulin release. In addition to modulating pancreatic functions directly, GABAergic synapses in the DMV are subject to modulation by various other neurotransmitters and hormones. Studies from our laboratory have shown that GABAergic synapses impinging on pancreas-projecting DMV neurons can be modulated by PP, GLP-1, CCK, as well as metabotropic glutamate receptor agonists.

Although glutamate is one of the principal neurotransmitters in synapses impinging onto pancreas-projecting DMV neurons, it does not appear to exert a major role on pancreatic functions under control conditions. In fact, microinjections of ionotropic glutamate receptor antagonist kynurenic acid into the DMV do not affect pancreatic exocrine secretion in control rats. However, glutamatergic synapses impinging on pancreas-projecting DMV neurons are subject to modulation by various neurotransmitters and hormones. Similar to GABAergic synapses, glutamatergic synapses are modulated by PP, GLP-1, and CCK as well as by metabotropic glutamate receptors.

Both GABAergic and glutamatergic synapses impinging on pancreas-projecting DMV neurons express metabotropic glutamate receptors (mGluR), which have also been shown to affect pancreatic functions. Unlike the ionotropic glutamate receptors, which couple to ion channels and mediate fast synaptic transmission, mGluRs are members of G-protein coupled receptor (GPCR) family of receptors and couple to different second messenger systems. There are eight known subtypes of mGluRs, which belong to three different groups (group I, II, and III mGluRs), each of which has unique pharmacological characteristics. Both GABAergic and glutamatergic synapses impinging on pancreas-projecting DMV neurons express group II and group III mGluRs and activation of either receptor type decreases inhibitory and excitatory synaptic transmission. These observations suggest that glutamate released from the synaptic terminals in the DMV not only activates pancreas-projecting neurons postsynaptically but also modulates synaptic transmission onto these neurons. Microinjections of the group II mGluR agonist into the dorsal vagal complex increases pancreatic exocrine secretion and decreases plasma insulin levels, whereas microinjections of the group III mGluR agonist decreases plasma insulin, without affecting pancreatic exocrine secretion. These findings further support the suggestion that mGluRs modulate pancreatic functions via actions on pancreas-projecting DMV neurons. Our laboratory has shown that
the responsiveness of DMV neurons to the group II mGluR agonist is altered in a rat model of acute pancreatitis, suggesting that these receptors may play a role in the development of pathological conditions of the exocrine pancreas.53

**Pancreatic polypeptide**

Pancreatic polypeptide (PP) is released by the cells of the pancreatic islets of Langerhans after ingestion of a meal. The release of PP is vagally mediated and involves activation of postganglionic muscarinic acetylcholine receptors.59 Circulating PP inhibits PES, not via direct actions on the pancreatic acini but rather via actions on the dorsal vagal complex.60 PP receptors are not expressed by acinar or ductal cells, and isolated acini or ducts are not inhibited by PP.54,66 Instead, PP receptors are expressed in the dorsal vagal complex, in the area postrema, NTS, and DMV.43,60,64 Microinjections of PP in the dorsal vagal complex inhibit pancreatic exocrine secretion by modulating vagal cholinergic output but does not affect basal plasma insulin, somatostatin, or glucagon secretion,41,43 suggesting that PP modulates PES but not endocrine pancreatic secretions. Electrophysiological studies from our laboratory have demonstrated that approximately half of the identified pancreas-projecting DMV neurons respond to PP. In these experiments, PP inhibited both excitatory and inhibitory postsynaptic currents elicited by the stimulation of the NTS and reduced the amplitude of currents stimulated by chemical activation of the area postrema.34 Interestingly, pancreas-projecting DMV neurons that responded to PP did not respond to GLP-1, suggesting that these two peptides affect separate populations of pancreas-projecting neurons.

**Cholecystokinin**

Cholecystokinin (CCK) is released from enteroendocrine cells in the small intestine in response to ingestion of a meal and exerts various effects along the GI tract, including increased PES, gastric relaxation, decreased gastric acid secretion, and reduction of food intake.62,63 CCK exerts its effects both via paracrine actions on vagal sensory neurons and via actions in the dorsal vagal complex.32 In addition, CCK1 receptors are also present on acinar cells and CCK can therefore directly influence acinar cell function, at least in rodents (reviewed in references49,64,65). CCK-1 receptors are expressed on neurons of the dorsal vagal complex and are activated by exogenous administration of CCK. Intraduodenal infusions of casein, a protein known to release endogenous CCK, increased pancreatic exocrine secretion even after vagal afferent fibers were surgically removed, although the response was attenuated. Furthermore, the casein-induced increase in pancreatic exocrine secretion was attenuated after application of CCK-1 receptor blocker in the dorsal vagal complex, suggesting that CCK increases pancreatic exocrine secretion via centrally mediated mechanisms.48 Electrophysiological studies from our laboratory have shown that CCK excites pancreas-projecting neurons via direct effects on DMV neurons and via effects on excitatory synapses impinging onto these neurons. Neurons that were excited by CCK were also inhibited by PP, suggesting that these peptides affect the same population of pancreas-projecting neurons.58

**Glucagon-like peptide-1**

Glucagon-like peptide-1 (GLP-1) is released from intestinal cells into the circulation, where it binds to receptors on the pancreatic β-cells to stimulate insulin release. In addition to its actions on pancreatic β-cells, GLP-1 acts via central mechanisms to decrease food intake and increase insulin secretion.67,68 GLP-1 increases the discharge of fibers from the hepatic branch of the vagus nerve and selective hepatic branch vagotomy attenuated GLP-1-induced increase in insulin secretion.69,70 GLP-1 administration also increases the expression of c-fos, an intermediate early gene and a marker of neuronal activation, in the NTS,71 providing further evidence for central effects of GLP-1. Studies from our laboratory have shown that GLP-1 increases the frequency of excitatory and inhibitory synaptic inputs to pancreas-projecting neurons in the DMV46,57 and that microinjections of exendin-4, a GLP-1 analogue, into the dorsal vagal complex increased plasma insulin levels.46 Taken together, these findings suggest that GLP-1 increases pancreatic endocrine secretions via actions on DMV neurons as well as pancreatic β-cells.

**Serotonin**

Serotonin (5-hydroxytryptamine [5-HT]) modulates pancreatic secretions via both direct and indirect actions. Serotonin-containing neurons innervate the pancreas, stomach, and small intestine and it has been suggested that serotonin inhibits pancreatic exocrine secretion via activation of presynaptic receptors on cholinergic neurons, although this mechanism has not been fully investigated.1 Serotonin also modulates pancreatic exocrine secretion via excitation of vagal afferent fibers.72,73 Vagal deafervation and serotonin-3 receptor antagonists have been shown to block an increase in pancreatic exocrine secretion induced by intraduodenal carbohydrates or mucosal stimulation.72 It has also been demonstrated that serotonin and CCK have synergistic actions in the regulation of pancreatic secretion. This suggestion is supported by the finding that the CCK-1 receptor antagonists attenuate the ability of serotonergic agonist to excite pancreatic vagal afferent fibers.74 This interaction between serotonin and CCK may
provide a means to finely tune the regulation of the neural control of pancreatic functions.

**Thyrotropin-releasing hormone**

Thyrotropin-releasing hormone (TRH) receptors, as well as TRH-immunoreactive axons that originate from the medullary raphe, the parapyramidal nuclei, and the hypothalamus, are expressed in the dorsal vagal complex. Intracerebroventricular and intra-DVC injections of TRH increase pancreatic exocrine secretion and this effect is prevented by vagotomy, ganglionic blockade with hexamethonium, blockade of postganglionic transmission with atropine, or by a VIP antagonist, suggesting that TRH-induced increase in pancreatic exocrine secretion is vagally mediated.

**Acetylcholine**

The hypothalamus plays an important role in modulation of pancreatic secretions. Electrical stimulation of the ventromedial anterior hypothalamus increases, whereas stimulation of the posterior hypothalamus decreases pancreatic secretions. It has been suggested that hypothalamic nuclei that modulate pancreatic secretions receive cholinergic inputs from higher centers in the CNS. Microinjections of muscarinic receptor antagonists into the lateral hypothalamic area increased pancreatic exocrine secretion and central depletion of neuronal acetylcholine stores had similar effects. In contrast, microinjection of muscarinic receptor agonists into the hypothalamus increased pancreatic exocrine secretion. Cholinergic inputs to the hypothalamus originate in the lateral septum and the lateral parabrachial nucleus, which provide a major influence on hypothalamic neurons that project to the dorsal vagal complex.

**Orexin**

Neurons in several regions in the CNS, including the ventromedial hypothalamus, NTS, and the DMV, can directly sense changes in glucose levels. The lateral hypothalamic area contains neurons that are activated by hypoglycemia (glucose-inhibited neurons) and those that are activated by hyperglycemia (glucose-excited neurons) and is known to modulate the efferent outflow to the pancreas. Orexin-containing neurons in the lateral hypothalamic area project to the parasympathetic and sympathetic preganglionic neurons that innervate the pancreas and microinjection of orexin-A antagonist into the lateral hypothalamic area decreases pancreatic vagal nerve activity. These observations suggest that changes in peripheral glucose levels activate glucose-sensitive orexin neurons in the lateral hypothalamic area, which, in turn, activates pancreas-projecting neurons in the DMV.

**Evidence for Distinct Regulation of Endocrine and Exocrine Pancreas**

Several lines of evidence suggest that vagal circuits that modulate pancreatic exocrine secretion are separate from those that regulate pancreatic endocrine secretions. At the level of the pancreas, vagal innervation shows an anatomical gradient, with innervation being more dense at the head compared to the tail of the pancreas. The influence of vagal stimulation on pancreatic exocrine secretion and endocrine secretions depends on either the frequency of vagal stimulation or the frequency of action potentials in DMV neurons. Furthermore, although vagal celiac branches innervate the splenic end of the pancreas, electrical stimulation of the hepatic and gastric branches of the vagus are solely responsible for insulin and glucagon secretion. This finding suggests that celiac branches innervate targets other than pancreatic α- and β-cells. Taken together, these observations suggest that vagal circuits are organized in a highly specific manner and that separate circuits may regulate different pancreatic functions.

Further evidence for distinct circuits regulating exocrine and endocrine pancreatic secretions came from recent studies in our laboratory. Pancreas-projecting neurons in the DMV that regulate pancreatic exocrine secretion can be distinguished from those regulating insulin secretion based on their neurochemical and pharmacological properties. Electrophysiological studies from our laboratory have shown that pancreas-projecting neurons that respond to GLP-1 do not respond to PP or CCK, whereas the majority of neurons that respond to CCK also respond to PP. This observation suggested that pancreas-projecting neurons in the DMV comprise at least two distinct neuronal populations, one of which responds to GLP-1 and the other to CCK and PP. This finding also raised the possibility that the two populations of neurons may regulate separate pancreatic functions. In support of this suggestion, CCK and PP have been shown to modulate PES, whereas GLP-1 modulates insulin release. We have shown that microinjections of GLP-1 into the DVC increase plasma insulin levels, but have no effect on PES, whereas microinjections of CCK and PP in the DVC increase pancreatic exocrine secretion. Furthermore, we have demonstrated that in rats with copper deficiency, which selectively destroys the exocrine pancreas while leaving the islets of Langerhans unaffected, DMV neurons display a diminished responsiveness to CCK and PP, peptides that selectively regulate pancreatic exocrine secretion. This evidence further supports the
idea that separate neuronal populations within the DMV regulate pancreatic exocrine secretion and insulin release.

DMV neurons that regulate pancreatic exocrine secretion can also be distinguished from those that regulate insulin release based on their responses to metabotropic glutamate receptor (mGluR) agonists and antagonists. Using single-cell patch-clamp, we demonstrated that both group II and group III mGluRs are present on excitatory (glutamatergic) and inhibitory (GABAergic) synapses impinging on identified pancreas-projecting neurons in the DMV. Application of a group II mGluR (mGluRII) agonist reduced the frequency of postsynaptic currents in the vast majority of excitatory (89%) and inhibitory (71%) synaptic terminals, whereas application of mGluRIII agonist affected a smaller proportion of excitatory (65%) and inhibitory (58%) synapses. All neurons that responded to the mGluRIII agonist also responded to the mGluRII agonist, whereas another population of neurons responded only to mGluRII agonist. Further analysis revealed that a majority of neurons that responded to the mGluRIII agonist also responded to the GLP-1 analogue exendin-4 but not to CCK or PP. Conversely, neurons that did not respond to the mGluRIII agonist responded to PP and CCK but not to exendin-4. These findings suggested that group III mGluRs modulate the activity of a specific subpopulation of pancreas-projecting neurons in the DMV that has a unique neurochemical phenotype and raised the possibility that this population of neurons modulates a specific pancreatic function, namely insulin secretion.

In order to determine the roles of these neuronal populations in modulating pancreatic functions, we conducted a series of in vivo experiments using DVC microinjections while monitoring pancreatic exocrine secretion and insulin secretion. Microinjections of the mGluRII agonist into the DVC dose-dependently increased pancreatic exocrine secretion and decreased plasma insulin levels, whereas microinjections of the mGluRIII agonist decreased insulin levels but had no effect on PES. Taken together with the patch-clamp data described earlier, these findings suggested that DMV synaptic terminals that express mGluRIII modulate insulin release, whereas terminals that express mGluRII modulate both pancreatic exocrine secretion and insulin release.

Further support for the regulation of endocrine and exocrine function by separate pathways came from studies using models of pancreatic disorders. A study from our laboratory has shown that copper deficiency, which selectively destroys the exocrine pancreas while leaving the endocrine pancreas intact, affects DMV neurons that regulate pancreatic exocrine secretion. Intraduodenal infusions of CCK or casein, potent stimulators of pancreatic exocrine secretion in control conditions, failed to increase pancreatic exocrine secretion in copper-deficient rats. This lack of an effect was accompanied by a reduction in the number of tyrosine hydroxylase-immunoreactive neurons in the DMV, suggesting that there was a reduction in catecholaminergic regulation of pancreatic exocrine secretion. Furthermore, electrophysiological evidence showed that fewer pancreas-projecting DMV neurons responded to CCK and PP in copper-deficient rats compared to controls. Interestingly, while copper deficiency affected postsynaptic responses to these peptides, it did not affect presynaptic responses, suggesting that copper deficiency selectively affects pancreas-projecting neurons in the DMV, while leaving the sensory synaptic inputs onto these neurons intact.

Synaptic inputs to pancreas-projecting DMV neurons are also affected by acute pancreatitis, a severe, and sometimes fatal, disorder of the exocrine pancreas. Acute pancreatitis is characterized by premature activation of zymogens leading to acinar cell injury, release of chemokines and cytokines, and an inflammatory response. Although early events that lead to the development of acute pancreatitis are initiated in the pancreas, it has also been shown that severity of acute pancreatitis is modulated by the CNS. Our laboratory, for example, has demonstrated that acute pancreatitis alters the sensitivity of pancreas-projecting DMV neurons to group II mGluR agonist, which, in turn, changes the balance of glutamatergic and GABAergic synaptic inputs to DMV neurons. Specifically, we demonstrated that acute pancreatitis decreases the response of glutamatergic synaptic terminals in the DMV to group II mGluR agonist. In contrast, group III mGluRs do not appear to be affected by acute pancreatitis. These findings suggest that acute pancreatitis selectively affects DMV neurons involved in the regulation of pancreatic exocrine secretion and further supports the notion that exocrine and endocrine pancreatic secretions are regulated by separate neuronal populations.

Summary

The pancreas plays an important role in the control of nutritional homeostasis. Pancreatic functions are regulated by finely tuned inputs from the sympathetic and parasympathetic branches of the autonomic nervous system, which perform as an integrated neural circuit to adapt exocrine and endocrine secretions to constantly change environmental and physiological conditions.

An increasing amount of experimental evidence indicates that autonomic pathways involved in regulation of pancreatic function are organized in a highly specific manner, with distinct pathways regulating endocrine and exocrine secretions. It is therefore important to understand how specific neural pathways regulate pancreatic secretions and to identify neurotransmitter and receptor...
phenotypes involved in regulation of specific pancreatic functions. Data from our laboratory have shown that DMV neurons that regulate exocrine and endocrine secretions can be differentiated by their responses to CCK, PP, and GLP-1, as well as their responses to group II and group III mGluRs. Thus, in order to completely understand the role of the central nervous system in the regulation of pancreatic functions, future studies should be aimed at further characterizing neuropeptides and receptors involved in regulation of various pancreatic functions. Data from animal models suggests that pathological conditions that affect the pancreas, including diabetes and acute pancreatitis, induce neurochemical changes in DMV neurons. Therefore, understanding of specific pathways that regulate exocrine and endocrine secretions would provide novel targets for the treatment of these disorders. Further studies of neuropeptides, their receptors and receptor pharmacology in pathological conditions are needed to fully understand the contribution of neural regulation in disorders of the pancreas.

References


Chapter 17

Regulation of pancreatic exocrine function by islet hormones

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Introduction

The exocrine pancreas is regulated by nerves, mainly the vagus, and gastrointestinal hormones, especially cholecystokinin and secretin. A third specific source of regulation is by peptide hormones from the islets of Langerhans. Islet hormones are relevant because of structural relations within the pancreas. Except for some fishes that have a single larger collection of endocrine cells, islets are dispersed within the pancreas and have the ability to regulate exocrine cells both by local diffusion that manifests as peri-insular halos of acinar cells and by a specific vascular relationship, the islet-acinar portal system. The latter results in acinar cells being exposed to high concentrations of islet hormones and other regulatory molecules. Of these the best defined relationship is with insulin produced in islet beta cells, which serves as a trophic factor for the exocrine pancreas, as a short-term signal promoting digestive enzyme synthesis at the translational level, and as part of a long-term control system where dietary carbohydrate leads to the synthesis of the carbohydrate-targeted digestive enzyme, pancreatic amylase. Insulin also facilitates digestive enzyme secretion and regulates the membrane transport of glucose and Ca2+. Earlier reviews of this topic have been published.1–3 Evidence for a direct effect of other islet peptides is less clear and some may exert an effect through regulating insulin secretion.

Structure and Vascular Supply of the Pancreas

Structure and distribution of islets of the pancreas

Pancreatic islets are collections of endocrine cells scattered through the exocrine pancreas making up 2–3 percent of the gland. Islets are made up of five types of endocrine cells known as α-, β-, δ-, ε-, and PP/F cells that synthesize and secrete, respectively, glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide, all of which are considered peptide hormones. Beta cells are the most numerous cell type (60–70%) and form the core in rodent islets but are scattered throughout the human islet. Alpha cells make up about 20 percent and along with δ-cells (1–2%) form a mantle on the outside of rodent islets. Some of the cell types are geographically distributed with PP/F cells primarily in the ventral lobe of the pancreas. By contrast, ε-cells are developmentally regulated, making up about 10 percent of the fetal islet but then differentiate into α- and β-cells so that in the adult they are rare. Some of the endocrine cells also express a second biologically active peptide. Amylin and galanin are synthesized in β-cells and are cosecreted with insulin. Pancreastatin is present in α-, β-, and δ-cells where it is derived from chromogranin, and adrenomedullin is produced in PP/F cells. In understanding the effects of these islet peptides on the exocrine pancreas, it is important to realize that some (insulin, glucagon, and PP) are essentially unique to the islets but others like somatostatin are more broadly distributed in the GI tract, which could affect the exocrine pancreas locally or from systemic sources. Because islet peptides can regulate the other cells within the islet, effects of a particular peptide could be mediated directly on exocrine cells or indirectly by affecting the secretion of insulin or another islet peptide. Furthermore, some of these peptides can affect the CNS and vagal regulation of the exocrine pancreas. Receptors on exocrine pancreatic cells along with direct in vitro effects of islet peptides have been identified for insulin and somatostatin but not for glucagon or PP. Further details with references will be given in the sections on individual peptides.

Islet—acinar portal system

Blood flow to the pancreas has been reported to be 0.4–1.1 ml/g/min with most data from rats, rabbits, mice, and dogs.4 Early studies injecting dye or ink into arteries feeding the pancreas showed rapid and intense perfusion of glomerular structures that were identified as islets of Langerhans.5–7 Islet blood flow has been most often quantitated using microspheres. It shows that 5–20 percent of blood flow goes to the islets which have a relative blood flow 5–10 times higher than the exocrine pancreas.8,9 The regulation

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of blood flow to the two portions of the pancreas is distinct with exocrine secretagogues such as CCK and VIP increasing blood flow to the exocrine pancreas\textsuperscript{10,11} and glucose increasing islet blood flow.\textsuperscript{12} In a study in rabbits, Lifson and colleagues concluded that essentially all blood flow to the islets goes subsequently to the acinar capillaries.\textsuperscript{13} Considerable attention has also been paid to the pathway of capillaries within the islets as it pertains to which islet cells are upstream of the others.\textsuperscript{14,15}

Early dye injection vascular perfusion studies also showed that small capillary vessels exited the islets and carried blood to the exocrine pancreas and these have been named the insulo-acinar portal system. This directional flow is facilitated by the fact that islet capillaries are slightly larger in diameter, which should lead to higher capillary pressure.\textsuperscript{16,17} In some species, there are also direct arterioles feeding the acinar tissue and venules draining the islets so not all islet effluent passes to the exocrine tissue. Similar results have been seen for rabbits, rats, mice, guinea pigs, cats, dogs, and baboons.\textsuperscript{18} Evidence for the portal system also comes from scanning electron microscopy of vascular casts and from functional studies. Vascular casts are made by injecting resin or acrylate into the arteries and after polymerization the residual tissue is digested away leaving a three-dimensional vascular cast which is coated with gold and viewed in a scanning electron microscope. Each islet has one or sometimes two afferent vessels which break down into a glomus-like capillary network. Efferent vessels similar in size to capillaries leave the islet and connect to the acinar capillaries.\textsuperscript{19–23} Similar findings have been made in the rat, rabbit, monkey, horse, baboon, and human. Some controversy exists over the extent of this phenomenon especially in rats where Bonner-Weir reported that a large extent of islet blood was drained directly into venules\textsuperscript{24,25} but this was subsequently disputed.\textsuperscript{25} Lifson and Lassa also described an acinar-ductal portal system in the rabbit pancreas.\textsuperscript{26} In any case, all blood from exocrine pancreas exits in venules which combine to form the pancreatic veins.

A different type of evidence for the islet-acinar portal system comes from physiological studies particularly using the perfused rat pancreas. Insulin added to the vascular perfusate enhances the exocrine response to CCK and secretin.\textsuperscript{27–30} Glucose, which by itself does not affect exocrine secretion, when added to the vascular perfusate, enhances the release of insulin and potentiates exocrine secretion.\textsuperscript{30,31} Other sugars that do not stimulate insulin secretion had no effect, and epinephrine, which blocks insulin secretion, blocked the potentiating effect of glucose. Since insulin can only come from the islets and would be carried away in the venous drainage in the single-pass perfusion system, an effect on exocrine secretion can only come from endogenous insulin reaching exocrine cells by the portal system. Similar studies depleting endogenous somatostatin with cysteamine have also provided evidence for endogenous somatostatin acting through the portal system.\textsuperscript{32}

Based on the presence of the insulo-acinar portal system, an important question is what is the concentration of islet peptides in the exocrine interstitial compartment? Two estimates indicate that the concentration of insulin and somatostatin is in the nanomolar range. The first comes from a study evaluating saturable binding of iodinated tracer for insulin and somatostatin in the rat pancreas perfused anterograde or retrograde.\textsuperscript{33} Binding was higher for retrograde perfusion, which was attributed to endogenous hormone released during anterograde perfusion. Displacement curves during retrograde perfusion indicated an interstitial concentration of insulin during anterograde perfusion with glucose of 7.5 nM and for somatostatin of 1.1 nM. A second study used a microdialysis technique which showed a unstimulated concentration of insulin of 0.4 nM during both retrograde and anterograde perfusion of dog pancreas.\textsuperscript{34} The response to stimulation with glucose plus arginine was markedly less during retrograde perfusion. Although the stimulated concentrations did not reach steady state, if insulin release went up 10-fold, the interstitial exocrine concentration during glucose stimulation would be about 4 nM. Unfortunately, reported plasma insulin levels in fasting rats and dogs are quite variable with most ranging from 50 to 400 pM. Much better data are available for humans where most fasting levels are reported to be 25–150 pM.

In summary, although the islet-acinar portal system does not account for all blood flow to exocrine pancreas cells, it does allow a significant fraction of exocrine acinar and duct cells to be exposed to higher concentrations of islet hormones than is the case for other organs. In addition, islet hormones can act directly on acinar cells via specific receptors or indirectly by affecting the release of other islet hormones.

**Action of Insulin on the Exocrine Pancreas**

Insulin appears to be the most important of the classical islet hormones as a regulator of the exocrine pancreas and especially acinar cells. Evidence exists from both human studies and animal models and includes both physiology and disease. Experimental studies have been carried out at different levels of integration from the intact organism to cellular and molecular studies of acinar cells.

**Clinical studies of the effects of diabetes on the exocrine pancreas**

For over 100 years, the pancreas of diabetic patients has been known to be smaller with increased fibrosis consistent with pathology of the entire pancreas and not just the
islets.\textsuperscript{35} Diabetes is now divided into type 1 or insulin-dependent diabetes mellitus (IDDM) where destruction of beta cells leads to lack of insulin, type 2 whose hallmark is insulin resistance and often does not require additional insulin, and most recently type 3 which is due to exocrine pathology such as chronic pancreatitis. It has long been recognized that type 1 and 2 patients may also suffer exocrine abnormalities ranging from subclinical to pancreatic exocrine insufficiency (PEI).

Loss of pancreatic mass in diabetes
Autopsy studies of type 1 diabetes have shown decreased pancreatic weight and volume.\textsuperscript{36,37} Methods have been developed to use CT and MRI imaging to determine pancreatic volume and distinguish fat within the pancreas. Ultrasound has also been used but is not as quantitative and is more operator dependent. Studies of diabetic patients with CT and MRI have consistently shown a decrease in pancreatic volume of 30–55 percent in type 1 and 15–30 percent in type 2 diabetes.\textsuperscript{38–42} Some studies have shown a dependence on the duration of diabetes but others have not. Several studies in which type 3 diabetes or a history of alcoholism have been omitted have shown a smaller loss in pancreatic volume. These changes have been confirmed in a recent meta-analysis of 17 studies.\textsuperscript{43} The authors noted, however, that many of the individual studies were small and classified as low- to moderate-quality data. Only type 2 diabetes has shown increased pancreatic fat which reduces the volume of parenchymal tissue from the total volume.\textsuperscript{40,42} In some studies, with functional as well as imaging data, the decreased pancreatic volume correlated with decreased function as measured by fecal elastase.\textsuperscript{41} To date, there are no longitudinal studies, although this could be done with MRI since there is no ionizing radiation.

Effects of diabetes on pancreatic function
Direct measurement of pancreatic juice bicarbonate ion and digestive enzymes in response to secretin and CCK using multilumen tube collection has been applied to diabetes. Ewald and Hardt list nine such studies from 1943 to 1996.\textsuperscript{44} These studies all show decreased exocrine pancreatic secretion with a bigger effect seen in type 1 diabetes. An effect of the duration of diabetes was seen in some\textsuperscript{45} but not in other studies.\textsuperscript{46} Most studies show a bigger effect on amylase than on other digestive enzymes and the smallest effect is on bicarbonate.

Indirect pancreatic function testing on stool samples has allowed study of greater number of patients. The most commonly used test is fecal elastase measured by an ELISA assay where <200 µg/g is considered evidence for pancreatic exocrine insufficiency (PEI) and <100 µg/g severe deficiency. Elastase is used because it is more resistant to protease digestion, although some studies have measured fecal trypsin or chymotrypsin activity, which yields similar results relative to controls. In the first large study of more than 1,000 diabetics, 40.7 percent showed fecal elastase levels of less than 200 µg/g with 22.9 percent being <100 µg/g.\textsuperscript{47} Other studies have reported similar results,\textsuperscript{48–50} although the frequency of PEI is lower when patients with type 3 diabetes were excluded.\textsuperscript{51,52} Type 1 diabetes with reduced fecal elastase show a higher frequency of steatorrhea up to 60 percent, although the amount of stool fat is modest\textsuperscript{53,54} and clinical PEI requiring enzyme supplementation is rare.\textsuperscript{35}

Overall, diabetes is accompanied by decreased pancreatic mass and reduced secretion of digestive enzymes along with a diffuse pancreatic fibrosis characterized by intracinar fibrosis whose pattern is distinct from that of chronic pancreatitis. These changes may be the result of reduced number of acinar cells, loss of insulin as a trophic factor, or alteration in neural control.\textsuperscript{56} These authors proposed that these changes be recognized as distinct from chronic pancreatitis and be termed “diabetic exocrine pancreatopathy.”

Animal studies of the effects of insulin and diabetes on the exocrine pancreas
Insulin receptors in the exocrine pancreas
Insulin receptors (IRs) on pancreatic acinar cells were first demonstrated by the binding of \textsuperscript{125}I-insulin in a saturable manner to isolated acini from mouse, rat, and guinea pig.\textsuperscript{57–59} Scatchard-plot analysis of binding were biphasic and showed high-affinity sites with a Kd of about 1.5 nM and capacity of about 10,000 receptors per cell; low-affinity sites were much more numerous with a Kd of 88 nM for mouse and 998 nM for rat. Addition of unlabeled insulin accelerated dissociation from rat acini consistent with negative cooperativity rather than two distinct sites.\textsuperscript{58} The receptor bound insulin analogs with varying potency (insulin > desdipeptide insulin > proinsulin > desoctapeptide insulin) that was similar to the ability of the analogs to increase protein synthesis in acini from diabetic rats.\textsuperscript{60} Guinea pig insulin is significantly different from other species and guinea pig acinar receptors bound bovine insulin with a higher affinity than guinea pig insulin or porcine proinsulin.\textsuperscript{59} Mouse acini were also shown to possess distinct receptors for IGF-I and IGF-II.\textsuperscript{61}

The localization of insulin binding to acinar and other cells in the pancreas has been demonstrated by light and EM autoradiography. When \textsuperscript{125}I-Insulin was injected intravenously, saturable uptake was observed by the pancreas.\textsuperscript{62–64} EM autoradiographs showed silver grains localized both over the plasma membrane and intracellularly in acinar cells. Saturable binding was also observed over duct cells.\textsuperscript{64} In an \textit{in vitro} study of \textsuperscript{125}I-Insulin binding to rat
acini, silver grains from $^{125}$I were primarily over the plasma membrane at 3 minutes but by 30 minutes were primarily intracellular. Studies in other cell types have shown that both insulin and its receptor are internalized, although the function of internalized insulin is unclear.

Insulin receptors on acinar cells are also subject to regulation. Insulin downregulates its own receptor in a variety of cells, including acinar cells, when they are exposed to high concentrations of insulin through the islet-acinar portal blood system. Acini prepared from diabetic mice showed an increased number of IRs compared to normal mice and addition of insulin to 24-hour cultured acini decreased insulin binding. Using pancreatic AR42J cells, insulin decreased the biosynthesis of insulin receptors in part by decreasing IR mRNA levels. The binding of insulin was also affected by CCK and other secretagogues. CCK, carbamylcholine, active phorbol ester, and calcium ionophore (A23187) all decreased high-affinity binding without affecting insulin internalization or degradation.

The occupancy of insulin receptors has also been related to the biological effects of insulin on acinar cells. Insulin increased glucose transport (uptake) by acini from normal and diabetic mice with a greater effect on acini from diabetic animals that was seen at lower concentrations of insulin. This greater effect is due both to lower-contaminating insulin levels and IR upregulation. In acini from diabetic rats and mice, insulin increased protein synthesis, indicating insulin levels and IR upregulation. In acini from diabetic animals that was seen at lower concentrations of insulin. This greater effect is due both to lower-contaminating insulin levels and IR upregulation. In acini from diabetic rats and mice, insulin increased protein synthesis, indicating insulin downregulates its own receptor in a variety of cells, including acinar cells, when they are exposed to high concentrations of insulin through the islet-acinar portal blood system. Studies in other cell types have shown that both insulin and its receptor are internalized, although the function of internalized insulin is unclear.

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Insulin regulation of exocrine pancreas biosynthetic and metabolic effects

**GENE EXPRESSION**

Insulin is known to regulate both the amount and activity of a number of anabolic processes and specific metabolic enzymes. In the exocrine pancreas, the best-studied tissue-specific regulation is that of the digestive enzyme amylase which acts on dietary starch and glycogen. The pancreatic amylase content is known to decrease by over 90 percent in experimental diabetes in rats and the fall can be reversed by administration of insulin. This has been studied primarily in rats given alloxan or streptozotocin that induces beta cell death and diabetes. At the same time, other digestive enzymes including trypsinogen, chymotrypsinogen, and lipase increase slightly and ribonuclease decreases slightly. Amylase also decreases in mouse pancreas in experimental diabetes but by only about 50 percent. Pancreatic amylase also decreases in the Zucker fatty rat and the ob/ob mouse, well-characterized models of insulin resistance.

The decrease in pancreatic amylase in experimental diabetes is accompanied by a decrease in amylase synthesis. Moreover, insulin increased amylase biosynthesis in rat pancreatic-derived AR42J cells. The major cause of the decreased amylase synthesis and tissue content is a decrease in amylase mRNA, which was first reported by Korc et al. The decrease in amylase mRNA could be reversed by insulin. Chymotrypsinogen mRNA showed a small change in the opposite direction and there was essentially no change in salivary amylase or its mRNA. Subsequently, Brannon and colleagues showed that dietary glucose and insulin together regulated pancreatic amylase (Amy2) gene expression. More detailed information on the mechanism of amylase gene regulation comes from studies in mice. A 30-bp region in the proximal amylase promoter overlapping with the PTF1-binding site present in most pancreatic digestive enzymes contains the insulin-dependent element and can transfer insulin sensitivity to the elastase promoter. This regulatory region is not present in all mouse amylase alleles and this can explain why some mouse strains are not as sensitive to diabetes and insulin. Unfortunately, there has been little subsequent work defining how insulin receptor signaling regulates the insulin response element.

**MEMBRANE TRANSPORT**

Insulin is known to stimulate membrane transport of many substances usually by effects on the amount of or properties of specific transport proteins. The best studied and probably the most important physiologically is the uptake of glucose into fat and muscle cells that is mediated by the glucose transporter, GLUT4. GLUT4 is present at rest in intracellular vesicles that translocate to the plasma membrane in response to insulin, increasing glucose uptake 5–20-fold. Most other cell types contain GLUT1 that is regulated by another mechanism but to a lesser extent. In mouse pancreatic acini, insulin stimulated the uptake of the nonmetabolizable sugar 2-deoxy-glucose (2DG) about twofold. A more robust effect of lower concentrations of insulin was seen when acini were prepared from diabetic mice and uptake of both 2DG and 3-O-methyl glucose, a nonmetabolizable glucose analog, was increased. Glucose uptake was not affected by inhibitors of protein synthesis but was reduced by the actin inhibitor, cytochalasin B.

In some tissues, insulin also stimulates the uptake of amino acids through effects on specific amino acid
transporters. Uptake of amino acids have been studied in many pancreatic preparations. One of the earliest studies using microdissected pieces of mouse pancreas showed that amino acids were taken up and oxidized in preference to glucose. Neither CCK or insulin affected uptake and transport did not appear to be separated from protein synthesis. Latter studies used perfused pancreas or isolated acini and showed that the basolateral membranes of pancreatic acinar cells possess four to five distinct transporters as measured by physiological studies or gene expression.

Insulin stimulation studies in the perfused rat pancreas show effects of exogenous insulin and diabetes on two Na+-independent transporters, the Asc transporter used by serine and a basic amino acid transporter termed y′ for lysine/arginine while no effect was seen on the Na+-dependent transporters such as the system A transporter for AIB and glutamine. However, increasing glucose in the perfusate that released endogenous insulin had no effect. Studies in isolated rat pancreatic acini also showed no effect of insulin on AIB and cyclolceutine uptake, which are not used for protein synthesis, although it stimulated incorporation of leucine into protein. In a more recent study of the mouse pancreas, Rooman et al. analyzed the expression of 37 genes encoding transporters and found expression of a number with the highest expression of slc7a8 and slc3a2 and confirmed the expression of five by western blot.

PROTEIN SYNTHESIS
Insulin is known to stimulate protein synthesis by translational effects in many tissues. Early studies in the pancreas involved in vivo studies of the incorporation of radioactive amino acids into total protein or specific enzymes of normal and diabetic rodents. While most of the studies showed a positive effect of insulin, the results are complicated by changes in the precursor pool or changes in the levels of mRNAs. When protein synthesis in mice was quantitated by autoradiography, there was more incorporation into peri-insular acinar cells then into tele-insular acinar cells. This difference was lost following treatment with streptozotocin and the authors ascribed it to insulin. One study overcame some of these issues by changing plasma insulin and glucose in vivo but measuring protein synthesis in vitro using pancreatic lobules incubated in a constant medium. It showed that either glucose infusion in vivo or treatment with a sulfonylurea drug both of which stimulate insulin release increased protein synthesis by 25–40 percent. When Zucker fatty rats were studied as a model of insulin resistance, overall pancreatic protein synthesis was reduced by nearly 50 percent. There was considerable difference in synthesis between different digestive enzymes separated by 2-D gel electrophoresis. A morphological study of prolonged diabetes in rats showed gross abnormality in the secretory pathway 28 days after STZ treatment that was partially reversed by insulin administration. However, shorter studies have not shown significant structural alterations 1 week after STZ other than the appearance of cytoplasmic lipid droplets.

More recent studies have been able to overcome some of the methodological issues by measuring the effects of insulin over short times to prevent changes in mRNA and under conditions where the precursor pool is large and constant. In vitro studies have used isolated pancreatic acini under dilute incubation to keep the precursor pool constant. Insulin stimulates the incorporation of multiple different amino acids (leucine, methionine, phenylalanine) in isolated acini from diabetic rats. Similarly, insulin increased methionine incorporation into total protein and immunoprecipitated amylase in rat pancreatic-derived AR42J cells. The effect of insulin on both cell types occurred over concentrations from 30 pM to 100 nM and was mediated by the insulin receptor as insulin analogs stimulated in parallel to their ability to bind to the insulin receptor. Insulin was also shown to have nonparallel effects on different digestive enzymes and structural proteins such as myosin and LDH.

Although the mechanism of insulin signaling is well studied in a number of target tissues especially liver, fat, and muscle, only a few studies have been carried out in the exocrine pancreas. The main signaling pathway regulating protein synthesis is the mTOR pathway and this pathway has been documented in pancreatic acinar cells primarily in mediating the actions of CCK and acetylcholine to stimulate digestive enzyme synthesis. Insulin has been shown to activate S6 kinase and S6 phosphorylation downstream of TORC1 in rat and mouse acini. Akt is upstream of mTOR and insulin activates the phosphorylation of Akt on S473 and T308 in rat acini. This action appeared mediated by PI3K. That these actions are important physiologically is shown by over a 50 percent reduction in mTOR pathway activation after feeding in mice without acinar cell insulin receptors.

SECRETION
The effects of insulin administration and diabetes on pancreatic exocrine secretion have been studied in a number of species and systems ranging from intact awake animals down to isolated acinar cells. The more isolated systems have better-defined parameters but lack neural control and other integrative aspects found in the intact organism.

In vivo studies have largely been carried out in rats with some studies in dogs and other species and often have compared streptozotocin (STZ) treated and intact animals. STZ and alloxan treatment damage islets, thus lowering insulin levels and leading to hyperglycemia. In STZ-diabetic rats, pancreatic juice flow has been reported to be moderately increased. In two studies, this effect was reversed by
transplanting syngeneic islets into the liver.\textsuperscript{115,116} However, in mice STZ treatment or genetic models of diabetes lead to reduced juice flow,\textsuperscript{74} and in sheep, alloxan treatment led to a substantial decrease that could be restored by insulin treatment.\textsuperscript{117} In all of these studies the effects of the diabetic state on plasma glucose and lipids are not clear. The effects of CCK on digestive enzyme secretion in diabetes are complicated by changes in the pancreatic content of specific digestive enzymes. The concentration of secreted amylase is decreased and trypsin increased, although both respond to CCK.\textsuperscript{112,114} The most valid results may be in animals with chronic pancreatic fistulas where juice can be collected in the unanesthetized state but such studies have not been carried out in diabetes.

Other studies have focused on the effects of insulin in normal animals with intact islets. When a high concentration of insulin (4 U per 100 g) was injected into the femoral vein, subsequent pancreatic stimulation by CCK was potentiated.\textsuperscript{27} In another study, insulin (1 U as a bolus) induced hypoglycemia, increased fluid flow, and increased amylase and total protein output; this effect was blocked by atropine and ascribed to hypoglycemia-induced vagal cholinergic activation.\textsuperscript{113} In a third study, an intravenous infusion of insulin also increased pancreatic secretion.\textsuperscript{118} Not all studies however have reported this effect.\textsuperscript{119} In a different approach using unanesthetized rats, IV injection of rabbit anti-insulin serum but not control serum blocked the pancreatic response to a liquid meal and to exogenous CCK or secretin stimulation.\textsuperscript{120} In a study in conscious dogs which secrete a high-bicarbonate pancreatic juice, most of which comes from the ducts, insulin administration diminished secretin-stimulated bicarbonate secretion and this effect was blocked by prior pancreatic denervation.\textsuperscript{121} This study illustrates the complexity of administering insulin in vivo and shows that some of the effects of high-dose insulin in vivo are mediated by neural pathways. However, overall they suggest a positive effect of insulin on pancreatic exocrine secretion.

Studying an in vitro pancreas preparation removes the effects of plasma glucose and the brain-initiated neural control. While some studies have been carried out with isolated pancreas segments from animals with thin pancreas, the perfused pancreas provides better oxygenation and when single-pass perfusion is used the secretion of both endocrine and exocrine secretions can be separately measured. Because the islets are present in their normal position, they can be stimulated independent of the exocrine pancreas and the islet hormones will reach the exocrine cells by local diffusion and even more importantly by the islet-acinar portal system. Most studies of the perfused normal rat pancreas or isolated pancreatic segments incubated with normal glucose have shown that exogenous insulin has little or no effect on basal fluid and protein secretion but potentiates the secretory effect of CCK, ACh, or secretin.\textsuperscript{27–31,122–125} In STZ-induced diabetic animals with high plasma glucose, the in vitro response to secretin and CCK was reduced compared to normal rats.\textsuperscript{126,127} This change could be partially reversed by treatment with insulin. To study the effect of endogenous insulin, investigators have used a low-plasma glucose background and then a pulse of high glucose to stimulate insulin release; this endogenous insulin which must reach the acinar cells by the islet-acinar portal system stimulated secretion of fluid, protein, and amylase in response to CCK or ACh.\textsuperscript{29–31} This effect was induced only by sugars that stimulate insulin release and could be blocked with epinephrine or somatostatin which blocks glucose-induced insulin release. In an alternative approach, Chey and colleagues added anti-insulin antibody to the perfusate in the rat- or dog-perfused pancreas and showed that this resulted in reduced juice, bicarbonate, and protein secretion.\textsuperscript{120,128,129} All of these data are consistent with insulin having an action on acinar and duct cells to facilitate secretion. However, other studies have reported that both high glucose and exogenous insulin inhibit CCK-stimulated amylase secretion in the mouse\textsuperscript{130} or caerulein-stimulated perfused rat pancreas.\textsuperscript{131} In the perfused cat pancreas, insulin had no effect on the basal fluid or enzyme secretion.\textsuperscript{132} While a stimulatory effect of insulin on acinar cells is consistent with the presence of insulin receptors on acinar and duct cells and the ability of insulin to act on isolated pancreatic acini (see below), other studies have suggested effects within the islet can explain much of the actions of insulin. In the studies of immunoneutralization of insulin in perfused rat and dog pancreas, Chey’s group showed that insulin antibody increased the concentration of somatostatin and pancreatic polypeptide in the venous effluent. In their rat study, addition of a antisomatostatin antibody partially reversed the effect of the anti-insulin and the addition of somatostatin partially reversed the effect of combined stimulation by CCK and secretin.\textsuperscript{123,129} Using a perfused canine pancreas, insulin antiserum blocked secretion but when SS and PP antibodies were added together, the effect on fluid and bicarbonate was fully reversed and the inhibition of protein secretion was partially reversed.\textsuperscript{128} These investigators concluded that the inhibitory effect of insulin antiserum was mediated by the local release of and action on the exocrine pancreas of somatostatin and PP.\textsuperscript{128} In a related study, Nakagawa et al. concluded that the net effect of islet peptides on the exocrine pancreas was negative because exocrine secretion was greater with retrograde than anterograde perfusion.\textsuperscript{133} Thus, the action of insulin could be mediated at least in part by suppressing the secretion of inhibitory islet peptides. One study of duct function showed that insulin increased pancreatic juice secretion and that this effect was blocked by ouabain.\textsuperscript{28}

Isolated pancreatic acini and ducts can be used to study cellular function in the absence of neural, hormonal, and islet control. Isolated acini are exquisitely sensitive to CCK, acetylcholine, bombesin, and respond through an increase in intracellular Ca\textsuperscript{2+} to increase digestive enzyme
secretion. Acini also respond through an increase in cyclic AMP to secretin and VIP, which can increase enzyme secretion or potentiate the actions of agonists that mobilize Ca²⁺. Isolated acini from STZ-induced diabetic rats show reduced secretion of amylase and ribonuclease in response to CCK and cholinergic agonists. This decreased secretion is also seen with alloxan-induced diabetes and can be reversed by treatment of the animals with insulin. One cause of the decreased secretion is a decrease in the synthesis and content of digestive enzymes. When secretion is normalized to content and expressed as percent of content released per 30 minutes, maximal secretion is similar but the dose response to CCK is shifted to the right while the response to carbachol is similar. Thus, there is also an effect on CCK receptors, which was confirmed by radioactive binding of CCK showing changes in affinity and capacity of the two affinity states. Interestingly, RT-PCR of the CCK1 receptors showed no change in pancreatic receptor mRNA in rat STZ-induced diabetes. Acinar cell post-receptor signaling is also altered in diabetes with changes in CCK-induced IP₃ formation. This is accompanied by changes in the amount and phosphorylation of IP₃ receptors. The increase in intracellular free Ca²⁺ measured in suspension or single cells is also reduced in diabetes.

An effect of insulin in vitro on both diabetic and normal rat acini has also been demonstrated. An effect of insulin to increase amylase release was seen as early as 30 minutes and increased to a maximum at 2 hours. Over this time, the number and affinity of CCK receptors was also changed. In another study in acini from normal rats, insulin after 90 minutes potentiated the secretion in response to CCK plus secretin but had no effect on either alone; this potentiating effect of insulin was inhibited by the Na⁺-K⁺ ATPase inhibitor, ouabain. Insulin also has been shown to directly activate the plasma membrane Ca²⁺ ATPase (PMCA) in rat.

These studies along with other metabolic effects of insulin on isolated acinar cells indicate that the pancreas can directly affect acinar cell secretion. However, the mechanisms of this effect are not fully established. Moreover, all studies have been carried out in rats and rat tissue and the results need to be extended to other species. The cellular sites where insulin acts on pancreatic acinar cells are shown in Figure 1. Finally, data are needed from isolated ducts to establish whether there is a direct effect of insulin on ductal bicarbonate secretion.

**Effects of insulin on pancreatitis and other pancreatic diseases**

**Clinical and animal studies linking diabetes with pancreatitis**

There is emerging evidence from clinical studies and animal studies that preexisting diabetes may predispose, increase the risk, or make pancreatitis more severe. This implies that endogenous insulin may have a protective role during pancreatitis. Type-2 diabetics have an approximately threefold increased risk of developing acute pancreatitis (AP), which could be explained by a loss of direct protection of insulin on pancreatic acinar cells, due to insulin resistance. Preexisting diabetes increases the severity of acute pancreatitis and diabetes increases the mortality in patients with chronic pancreatitis. Around half of type-1 diabetic patients exhibit lesions within the exocrine pancreas that are reminiscent of chronic pancreatitis.

Moreover, acute pancreatic patients with hyperglycemia are at higher risk of multiple organ failure. Hyperglycemia frequently accompanies severe acute pancreatitis and is used in the Ranson score as a predictor of disease severity. Furthermore, the incidence of AP is higher among type 2 diabetics compared to the normal population and the risk of AP was reduced among diabetic patients treated with insulin or metformin compared to those treated with other drugs such as sulphonylurea-based antidiabetic drugs.

For many years, there had been numerous anecdotal histological observations of the pancreas in animals that the acinar cells surrounding the islets (peri-islet acinar cells) were morphologically distinct from acinar cells distant from the islets. This was further investigated in L-arginine-induced experimental animal models of pancreatitis in which peri-islet acinar cells remained relatively intact compared to distal injured acinar cells. This peri-islet acinar cell protection was abolished in streptozotocin (STZ)-induced diabetic rats, with impaired insulin secretion. These studies suggest that insulin release has a paracrine protective role in the pancreas. Moreover, in addition to the acute injury, the regeneration of exocrine pancreatic tissue was abolished in diabetic rats and restored following the administration of exogenous insulin. This was further investigated in the seminal study by Zechener et al. (2012), in which carulein-induced pancreatitis was aggravated in streptozotocin (STZ)-induced type 1 diabetic mice. Specifically, they showed that numerous markers of the acute phase of pancreatitis injury (within 24 hours) were markedly potentiated—including plasma amylase, lipase, and trypsinogen, pancreatic edema (wt/wt weight ratio), pancreatic histological tissue injury score (H&E), inflammation, and cell death. In addition, regeneration of the pancreas (7 days later) was delayed. Moreover, this diabetic-induced potentiation of pancreatitis phenotype was partially corrected by exogenous administration of insulin (slow-release pellet implant). They also suggested that the mechanism for this more severe pancreatitis phenotype in STZ-induced diabetic mice was due at least in part to depleted pancreatic regenerating islet-derived 3β (REG3β), which has important anti-inflammatory and antimicrobial properties. REG3β
Figure 1. Sites of insulin action on pancreatic acinar cells. Insulin has receptor-dependent action to regulate protein synthesis at the translational level, gene expression at the transcriptional level, membrane transport of glucose and calcium, and potentiation of digestive enzyme secretion. Not all of the steps between receptor occupancy and the end biological effect are known and most of the data are from rodent pancreas (see text for details and references).

is upregulated during pancreatic injury and is suggested to act as an acute emergency program to circumvent the inflammatory response during pancreatitis. However, REG3β expression was found to be severely blunted in the pancreas of STZ-induced diabetic mice, suggesting that this may contribute to the more severe pancreatitis phenotype.

Insulin protection during cellular models of pancreatitis
From a clinical perspective, and to some extent the diabetic animal studies, it is very difficult to separate the confounding effects of hyperglycemia or reduced systemic effects of insulin from a loss of direct insulin protection of acinar cells. This is important because hyperglycemia
is associated with a more severe pancreatitis, by providing a pro-inflammatory environment and by facilitating sepsis. Moreover, insulin itself exhibits anti-inflammatory properties, therefore the systemic loss of insulin secretion or insulin effectiveness would be predicted to promote systemic inflammation. However, studies using acutely isolated pancreatic acinar cells provide compelling evidence that insulin directly protects acinar cells from cellular injury induced by pancreatitis-inducing agents, such as oxidative stress (H$_2$O$_2$) or the alcohol/fatty acid metabolite, palmitoleic acid (POA) (cellular models of acute pancreatitis). Specifically, insulin markedly attenuated ATP depletion, the inhibition of plasma membrane calcium ATPase (PMCA) that transports Ca$^{2+}$ out of cells, cytotoxic Ca$^{2+}$ overload, and necrotic cell death. This protection was partially PI3K/Akt-dependent and due to an acute metabolic switch from mitochondrial to glycolytic metabolism. Consistent with this, insulin enhanced PMCA inhibition by glycolytic inhibitors and abolished PMCA inhibition by mitochondrial inhibitors. Therefore, this switch to glycolysis appears to maintain cytosolic ATP concentration sufficiently to fuel the PMCA and thus prevent cytotoxic Ca$^{2+}$ overload, even in the face of impaired mitochondrial function.

The most likely downstream signaling pathways responsible for insulin’s protective effects during pancreatitis are likely to be mediated by tyrosine kinase or PI3K/Akt. It’s also interesting to note that several related growth factors (GFs), that also couple to tyrosine kinase and PI3K/Akt, similar to insulin, are also reported to be protective in animal models of pancreatitis. These include insulin-like growth factor (IGF-1), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and epidermal growth factor (EGF). Although, altered expression of signaling proteins and metabolic enzymes may contribute to insulin protection in vivo, most of the protective effects of insulin observed in acinar cells occurred over a relatively short term (15–30 minutes), suggesting a more rapid effect of posttranslational effects, such as tyrosine kinase or PI3K/Akt phosphorylation.

Arguably, the most important mechanism for insulin’s protection is the regulation of glycolytic ATP. Glycolytic flux is primarily regulated by the activity of phosphofructokinase-1 (PFK1), which catalyzes the conversion of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F1,6BP) and represents the first rate-limiting irreversible step in glycolysis. Insulin has been shown to lead to the Akt-mediated phosphorylation and direct activation of phosphofructokinase-2 (PFK2)—otherwise known as phosphofructokinase-fructose bisphosphatase (PFKFB). PFKFB consists of four separate bifunctional glycolytic enzymes (PFKFB1–4), with varying catalytic and functional activities. PFKFB catalyzes the conversion of F6P to fructose-2,6-bisphosphate (F2,6BP), via their kinase activity, but also the reverse reaction, by converting F2,6BP back to F6P, via their bisphosphatase activity. F2,6BP is a potent positive allosteric activator of PFK-1, which maintains high glycolytic flux. Therefore, Akt-mediated phosphorylation of PFKFB2 may represent the major molecular mechanism by which insulin increases glycolytic ATP supply in the face of impaired mitochondrial metabolism during acute pancreatitis.

The effect of insulin on the gut microbiome-pancreatitis link

Severe acute pancreatitis is frequently accompanied by infected pancreatic necrosis and systemic bacteremia or sepsis. The source of the bacteria is thought to be from “leaky” gut—particularly the colon which houses a large reservoir of pathogenic and commensal bacteria. This caused by gut dysbiosis, inflammation, altered mucus secretion, and the consequent loss of gut barrier function and bacterial translocation into the blood. The gut microbiome consists of a delicate balance of trillions of bacteria from thousands of different bacterial species that can be broadly categorized into “good” (anti-inflammatory) or “bad” (pro-inflammatory). Moreover, bacteria can aid in the breakdown and absorption of undigested carbohydrates and produce numerous beneficial anti-inflammatory metabolites, such as short-chain fatty acids (SCFAs), such as butyrate, acetate, and propionate that promote epithelial integrity and gut barrier function.

This delicate balance of the gut microbiome and bacterial diversity is maintained by numerous anti-microbial peptides (AMPs) secreted mainly from the Paneth cells within the crypts of Lieberkühn of the small intestine. However, several studies show that AMPs are secreted from pancreatic acinar cells, which may contribute to maintaining a healthy gut microbiome. These include the related C-type lectin family members: lithostathine (REG1α/β), REG3 family members (REG3α/β/γ)—sometimes referred to as pancreatitis-associated peptide (PAP)/hepatocarcinoma-intestine-pancreas (HIP), cathelicidin-related AMP, (CRAMP$^{175}$) and defensins. PAP was originally discovered in pancreas homogenates, secretory granules, and in the pancreatic juice during experimentally induced acute pancreatitis in rats but was absent in control rats. PAP expression was also increased in pancreatic acinar cells in response to both acute and chronic pancreatitis. Moreover, PAP/REG3β expression is regulated by dietary carbohydrates and is blunted in diabetic mice, suggesting that insulin regulates the expression of PAP/REG3β. Therefore, during pancreatitis the loss of PAP/REG3β secretion into the gut may accentuate the gut dysbiosis, inflammation, and loss of barrier function and bacterial translocation, thereby further contributing to the severity of acute pancreatitis.
It is also possible that gut dysbiosis in diabetics may be caused by reduced pancreatic secretion of amylase. Amylase is a highly abundant digestive enzyme that breaks down starch in the gut and its expression and secretion is controlled by insulin and high-carbohydrate diet and is reduced in diabetic animals. Such loss of amylase secretion may also alter the gut microbiome, due to huge amounts of undigested starch entering the colon leading to overgrowth of bacteria that break down undigested starch (Bacteroides spp). However, this has never been fully and specifically investigated and it remains unclear whether reduced amylase per se in the gut would be beneficial or detrimental. Even though pancreatic enzyme insufficiency (PEI) leads to numerous comorbidities that might be explained by an altered gut microbiome, disentangling the specific effects of reduced amylase secretion from the loss of other digestive enzymes would be difficult.

It is also interesting that the gut microbiome is able to signal to and thus contribute to the maintenance of a healthy inflammatory and immune landscape within the pancreas and when disrupted may ultimately leading to autoimmune type 1 diabetes. Specifically, SCFAs, such as butyrate, released from gut bacteria are able to leak into the circulation and reach sufficient concentration to promote the secretion of CRAMP from pancreatic β-cells. CRAMP induced a positive immunoregulatory phenotype in pancreatic resident macrophages by promoting the production of anti-inflammatory cytokine, TGFβ, to maintain immune homeostasis via Treg induction. However, the loss of SCFAs following gut dysbiosis disrupts this process, leading to reduced CRAMP secretion which promotes a pro-inflammatory environment in which activated macrophages secrete TNFα and the ultimate autoimmune destruction of pancreatic β-cells and the consequent type 1 diabetes. This therefore suggests a complex reciprocal regulation between the gut microbiome, the exocrine, and the endocrine pancreas.

**Insulin as a therapy to treat acute pancreatitis**

Insulin therapy has been used to specifically treat hypertriglyceridemia (HTG)-induced pancreatitis with promising outcomes. HTG occurs when plasma lipids exceed 1,000 mg/dL (normal range, 101–150 mg/dL). HTG-induced pancreatitis is relatively rare, accounting for 2.3 percent to 10 percent of all cases of acute pancreatitis but is a well-documented etiological risk factor leading to severe disease. The rationale for using insulin therapy to treat HTG-induced pancreatitis is that it lowers plasma triglycerides, by activating lipoprotein lipase (convert triacylglycerides into free fatty acids) and inhibiting the hormone-sensitive lipase (liberates adipocyte triglyceride), thereby limiting inflammation. However, given the evidence presented above, it’s entirely possible that the beneficial effects of insulin therapy in HTG-induced pancreatitis patients could be due to a direct protection of acinar cells.

Insulin is also used as the standard of care for all critical care patients, including those with severe acute pancreatitis, with the aim of targeting hyperglycemia associated with the acute phase of injury, which facilitates inflammation and sepsis. However, this is likely because in patient groups receiving insulin, fewer patients die from sepsis, but more die from the complications of inadvertent hypoglycemia, an independent risk factor of mortality.

However, given the direct protective effect of insulin on acinar cells described above there is a strong rationale to test high-dose insulin infusion with very tight moment-to-moment glucose control to specifically target the acinar injury. This could be achieved using the hyperinsulinemic euglycemic clamp with automated insulin minipumps combined with continuous closed-loop subcutaneous glucose-monitoring devices. It could be argued that there is a greater requirement for high-dose insulin because pancreatic acinar cells receive a portal blood flow with ~10 times higher insulin concentration than the systemic circulation. Furthermore, stress hormones, such as adrenaline, cortisol, and glucagon, and inflammatory cytokines (TNFα and IL-1β) reduce tissue sensitivity of insulin, so a higher dose of insulin may be necessary to overcome this. Moreover, high-dose insulin infusion (8 mU/kg/min; adjusted for surface area to weight ratio) with tight physiological glucose control has been tested in healthy human volunteers during endurance exercise studies with no reported adverse effects.

**Action of Other Islet Hormones/Peptides on the Exocrine Pancreas**

**Glucagon**

Glucagon was originally identified in pancreatic extracts as a hyperglycemic-glycogenolytic factor that came from the α-cells of the pancreatic islets. Glucagon was originally identified in pancreatic extracts as a hyperglycemic-glycogenolytic factor that came from the α-cells of the pancreatic islets. By contrast, glucagon was originally identified in pancreatic extracts as a hyperglycemic-glycogenolytic factor that came from the α-cells of the pancreatic islets. By contrast, glucagon was originally identified in pancreatic extracts as a hyperglycemic-glycogenolytic factor that came from the α-cells of the pancreatic islets. The subsequent development of a radioimmunoassay for glucagon by Unger made more detailed studies of the physiology and pathophysiology of glucagon possible. Glucagon is a 29-amino acid peptide derived from proglucagon in islet α-cells through the tissue-specific processing by prohormone convertase 2 (PCSK2). By contrast, in intestinal enteroendocrine cells proglucagon is processed to GLP-1, GLP-2, oxyntomodulin, and glicentin. The
major role of glucagon is to antagonize the effects of insulin and maintain plasma glucose homeostasis by promoting hepatic gluconeogenesis and glycogenolysis and inhibiting glycogen synthesis. The major regulators of glucagon secretion by islet alpha cells are glucose which inhibits and amino acids as well as parasympathetic and sympathetic nerves which stimulate glucagon release. The suppression by high glucose, however, is not direct but mediated by insulin/GABA secreted by islet beta cells. Somatostatin also suppresses glucagon secretion.

The effect of exogenous glucagon on exocrine pancreatic secretion has been studied in vivo with a consistent effect of high concentrations of purified crystalline glucagon to inhibit exocrine secretion of fluid, bicarbonate, and protein stimulated by food, CCK, or secretin in dogs, cats, rats, and humans. The mechanism of this inhibition is unclear in these studies. In vitro studies have been carried out in the perfused pancreas, pancreatic segments, and lobules with mixed results. With the development of isolated pancreatic acini and acinar cells, natural purified glucagon was shown to stimulate amylase secretion and increase cyclic AMP in rat, mouse, and guinea pig acini. However, the stimulatory principle did not elute with synthetic glucagon, so its nature is unknown. Most importantly, synthetic glucagon has no effect on amylase secretion by isolated acini. Glucagon receptor mRNA has been identified in the pancreas but not in isolated acini or ducts. Thus, these studies do not support a direct effect of glucagon on acinar cells. Whether the islets or the nervous system is involved is not yet established.

**Somatostatin**

Somatostatin (SS) was originally identified in hypothalamic extracts as a factor able to inhibit growth hormone release and was subsequently purified as a cyclic peptide containing 14 amino acids (SS-14). A second form extended at the amino terminal and containing 28 amino acids (SS-28) was later characterized. Subsequently, somatostatin was found to be widely distributed in the body including other regions of the brain, the small intestine, pancreatic islets, and the stomach. For information on the mRNA sequence and the somatostatin precursor, see reference 226. Outside the nervous system, including islets, somatostatin is produced in D cells and is now considered to be both a hormone and a paracrine regulator and generally acts as an inhibitor of specific physiological processes such as gastrin secretion in the stomach and insulin secretion in the islet. The effect of somatostatin is mediated by specific receptors which most often act to inhibit adenyl cyclase but can also regulate ion channels and activate tyrosine phosphatase.

In all species studied, plasma somatostatin goes up after a meal; in humans, the basal concentration is about 10 pM and this doubles after eating. The majority of released SS comes from the gut and not the islets. However, somatostatin released from islet D cells can have direct effects on other islet cells and pancreatic exocrine cells. Somatostatin release is controlled by cholinergic and adrenergic nerves as well as gastrointestinal hormones including CCK, secretin, and VIP. The half-life of SS-14 in plasma is 1–2 minutes while that of SS-28 is 3–4 minutes.

Pancreatic exocrine secretion of fluid and enzymes is inhibited by in vivo administration of SS-14 or SS-28 in conjunction with stimulation by meal feeding or administration of CCK and/or secretin in dogs, cats, rabbits, and humans. Additional studies using long-acting SS analogs such as Octreotide (SMS201–995) and RC160 have shown similar results. That an effect of endogenous SS release on the pancreas may have physiological importance is suggested by the fact that exogenous SS mimicking postprandial levels is able to inhibit insulin and amylase secretion. Moreover, immunoneutralization of circulating SS leads to an enhancement of CCK-stimulated pancreatic amylase secretion.

The site at which SS inhibits exocrine pancreatic secretion and its physiological importance is unclear. Suggested sites include the central or peripheral nervous system, within the islet through effects on insulin secretion and at the level of pancreatic exocrine cells. It is of course possible that SS acts at more than one site. Because this review focuses on regulation of exocrine pancreas by islet peptides and because somatostatin receptors have been identified in the exocrine pancreas, that possibility will be considered first.

Using iodinated analogs of SS-14 and SS-28, high-affinity binding sites with the characteristics of receptors have been identified in isolated pancreatic acini, pancreatic membranes, and purified plasma membranes. Covalent cross-linking studies showed that the binding protein was a 90-kDa glycoprotein. Saturable binding sites were also demonstrated in the perfused rat pancreas with autoradiography showing uptake by islets and acinar cells with the acini showing the highest density. Molecular cloning has identified six different SS receptors, SSRT1–SSRT5 with SSRT2 having two subtypes. All of these are seven transmembrane proteins that are G protein-coupled whose action is mediated by G_i/G_o. SSTR2 and SSTR5 appear to be the main SS receptor isoforms in acinar and islet cells, although further work is needed.

Studies directed at actions of somatostatin on pancreatic exocrine cells have primarily been carried out using isolated pancreatic acini that have been separated from islets. SS-14, SS-28, and synthetic analogs have consistently been shown to inhibit cAMP formation through a pertussis toxin-sensitive mechanism and in most cases to inhibit amylase secretion stimulated by VIP or secretin that acts through cAMP. However, most studies reported no inhibition by somatostatin of amylase release stimulated
by CCK or cholinergic analogues. Rather SS inhibited the potentiating effect of secretin or VIP on CCK stimulation. This effect whose size is species-dependent may explain a portion of SS inhibition in vivo but it does not appear to be a major effect. Moreover, it is not clear if it involves islet SS or systemic SS.

The major in vitro technique that allows study of islet SS on the exocrine pancreas is the isolated perfused pancreas where secretion of both islet hormones and exocrine secretion can be measured. Insulin in the perfused pancreas potentiates CCK-stimulated exocrine secretion and somatostatin has been established to inhibit both exocrine secretion and insulin secretion. SS on the exocrine pancreas is the isolated perfused pancreas where secretion of both islet hormones and exocrine secretion can be measured. In the perfused rat pancreas, high glucose is known to induce secretion of both insulin and SS, and a SS antagonist enhanced amylase secretion induced by glucose plus CCK. Depleting SS with cysteamine pretreatment also enhanced CCK-stimulated secretion that could be inhibited with exogenous SS. Interestingly, endogenous SS did not modify exocrine secretion stimulated by CCK alone. In the perfused pancreas, SS also appears to inhibit duct function as fluid secretion stimulated by secretin was potentiated by insulin and inhibited by somatostatin in a manner dependent on Na$^+$- K$^+$ ATPase function.

The other proposed locus of somatostatin action to inhibit the exocrine pancreas is at the level of efferent neural control, which is initiated in the dorsal motor nucleus of the vagus (DMV) and travels through the vagus nerve to reach pancreatic parenchymal cells. Studies suggesting inhibition at a central vagal site include the finding in rats that somatostatin inhibits the action of 2-deoxyglucose, a known central vagal stimulator and that microinjection of somatostatin into the DMV inhibits pancreatic secretion evoked by CCK or 2DG. However, in dogs and rats, other investigators have reported that somatostatin still blocked CCK-stimulated secretion after complete pancreatic denervation. These and other authors concluded that the inhibitory action of somatostatin is not dependent on the extrinsic nervous system but that the intrinsic nervous system could be involved.

**Pancreatic polypeptide**

Pancreatic polypeptide (PP) was discovered as a contaminant in the purification of insulin in 1975. It contains 36 amino acids and is part of a family of peptides that includes peptide YY (PYY) and neuropeptide Y (NPY) and has about 50 percent homology to these other peptides. The biologically active part of the molecule is in the carboxyl terminal and this part of the molecule extends out from the main globular portion. PP is immunogenic and immunohistochemistry has localized it in the islets to a specific cell type originally known as F cells, and now referred to as PP cells. These cells are most abundant in the original ventral lobe of the pancreas and some are present outside the islet in the duct epithelium. Small amounts of PP are found in other tissues including the brain.

In humans, fasting pancreatic polypeptide plasma levels are 10–30 pM; they increase rapidly after feeding and remain elevated for 4–5 hours. The vagal nerve is the main stimulator of PP secretion and secretion can be blocked with atropine. Electrical stimulation of the vagus, sham feeding, 2-deoxyglucose, and insulin-induced hypoglycemia all stimulate PP secretion in a vagal-dependent manner. The half-life of PP in plasma is about 6 minutes. Reported actions of PP include effects on the GI tract, metabolism, most notably reversal of hepatic insulin resistance, and as a satiety factor. The latter is based in part on Prader-Willi syndrome where loss of PP secretion is associated with obesity. The actions of PP are mediated by specific receptors that belong to a family of receptors that bind NPY, PYY, and PP. The various receptors are denoted by a capital Y with a numerical subscript. The Y$_4$ receptor has specificity for PP with a high affinity and a hundred-fold lower affinity for PYY.

Purified bovine PP was shown by Lin et al. in 1977 to reduce exocrine pancreatic secretion in dogs with pancreatic fistulas. This was confirmed by Taylor et al. who showed that inhibition of exocrine secretion occurred at doses of porcine PP that raised plasma levels less than seen after a meat meal. Similar actions in dogs have been reported by others. Shiratori et al. showed a similar effect of human synthetic PP to inhibit canine pancreatic secretion and also showed that immunoneutralization of endogenous PP enhanced both interdigestive and postprandial secretion. Similar effects of exogenous PP have been reported in humans and in rats.

Although PP acts to inhibit pancreatic secretion in vivo, this effect appears to be indirect as exogenous PP had no effect on amylase release from isolated rat or mouse pancreatic acini. The perfused cat pancreas, or incubated uncinate pancreas of young rats or pancreatic fragments Binding studies with $^{125}$I-PP also failed to reveal high-affinity binding sites on rat pancreatic acini. Although this lack of in vitro effects is generally accepted, there are several differing reports all using isolated rat pancreatic acini showing a small amount of inhibition. PP stimulation by high concentrations of human PP and inhibition of carbachol but not CCK stimulation by bovine PP. Some of these effects could possibly have been due to contaminants in purified PP.

More recent studies have focused on a neural locus for the action of PP to inhibit pancreatic exocrine secretion. Most studies indicate a site of action in the brain stem. Receptors for PP are present in the area postrema, the nucleus tractus solitarius (NTS), and the dorsal motor nucleus of the vagus, and intravenous PP inhibits pancreatic enzyme secretion in the rat stimulated with...
2-deoxyglucose which acts centrally. More definitively, PP microinjected into the DMV in a site-specific manner inhibited pancreatic secretion. PP directly spritzed on individual DMV neurons revealed a subset where PP reduced postsynaptic currents. This finding suggest that PP in the circulation gains access to the brain through the area postrema and reaches the adjacent DMV where it inhibits vagal excitatory output to the pancreas. As an alternative neural site for PP inhibition, Jung et al. presented data that rat PP inhibited potassium-stimulated amylase release and the presynaptic release of acetylcholine in rat pancreatic slices. They suggested that PP acts on postganglionic cholinergic neurons to prevent acetylcholine release. The function of inhibition of pancreatic secretion by PP is unclear, although several authors have suggested it is to prevent overstimulation of the pancreas.

**Adrenomedullin**

Adrenomedullin (AM) is a 52-amino acid peptide that was isolated from a human pheochromocytoma and has slight primary homology but stronger tertiary resemblance to calcitonin gene-related peptide (CGRP). When injected intravenously, AM causes a potent and long-lasting hypotensive effect in anesthetized rats. AM is present in various organs but most abundantly in the adrenal medulla. It is present diffusely in endocrine cells of the pancreatic islet but most abundantly colocalizes with PP cells where it is present in secretory granules. These authors also showed that it is a strong inhibitor of insulin secretion both in vivo and with isolated islets. The AM receptor is made up of a previously orphan receptor, the calcitonin receptor-like receptor (CTLR), combined with a receptor-activity-modifying protein (RAMP). There are two AM receptors: AM1 which includes CTLR and RAMP2 and AM2 which is made up of CTLR and RAMP3; the CGRP receptor is CTLR plus RAMP1. In most cells that respond to AM, there is an increase in cAMP. In aortic endothelial cells, AM stimulates two signal transduction pathways that increase cAMP and mobilize Ca\(^{2+}\).

Because AM released from PP cells might affect the exocrine pancreas via local blood flow, Tsuchida et al. studied the effect of AM on pancreatic acini. Radioiodinated AM was shown to bind to pancreatic acini in a manner inhibited by picomolar concentrations of AM but not by CGRP. AM at concentrations of 1 pM to 1 nM inhibited CCK-stimulated amylase secretion. AM did not affect CCK binding to acini. In contrast to some other cell types, AM did not affect intracellular cAMP or the rise in Ca\(^{2+}\) induced by CCK. Rather, the effect of AM was blocked by pertussis toxin (PTX) suggesting the participation of a PTX-sensitive G protein in the action of AM. Additionally work on this effect of AM would seem warranted. More recent work on AM in the pancreas has focused on AM produced by pancreatic cancer cells as a mediator of diabetes that often associates with pancreatic cancer, possibly by inhibiting insulin secretion.

**Amylin**

Amylin or islet amyloid polypeptide (IAP) was isolated from amyloid-rich pancreatic extracts of type 2 diabetic pancreas and shown to be a 37-amino acid peptide that can form oligomers, fibrils, and amyloid deposits. The amyloid deposits are injurious to the pancreas and are also seen in the brain where they resemble the plaques of Alzheimer’s disease. Amylin is a term for protein aggregation state in which the proteins form a β-sheet. The amyloid deposits are injurious to the pancreas and are also seen in the brain of type 2 diabetics. Although amylin is not homologous to the Alzheimer Aβ protein, both can interact with the Amylin receptor which is widely distributed in the brain.

Amylin is produced by islet beta cells, has been localized to the secretory granules, and is secreted along with insulin at a ratio of 1:10 to 1:100. Amylin in vivo can inhibit glucagon secretion that occurs at low glucose concentrations and inhibits glucagon secretion from isolated mouse islets. The amylin receptor was identified as a heterodimer of a calcitonin seven transmembrane receptor and a single transmembrane receptor activity-modifying protein or RAMP. These receptors are widely distributed in the brain. Amylin receptor antagonist increased glucagon secretion. The acute effects on insulin secretion are more complex as amylin at low concentrations enhanced insulin release while high concentrations inhibited insulin release from mouse islets.

A few reports have evaluated the effect of amylin on the exocrine pancreas. In one report, amylin had a small effect to increase basal pancreatic secretion in conscious rats. In a study in anesthetized rats, Young et al. found no effect on basal secretion but a dose-dependent inhibition of CCK-stimulated fluid, amylase, and lipase secretion up to 67 percent. This effect was seen at low concentrations of amylin (ED50 was 0.11 µg) comparable to effects reported on gastric emptying. However, multiple studies have shown no effect of amylin on secretion by isolated pancreatic acini or AR42J cells. Thus, there does not appear to be a direct effect of amylin on pancreatic exocrine cells and the in vivo effects may be on the CNS. There is also no information available on whether amylin receptors exist in the exocrine pancreas.

**Pancreastatin**

Pancreastatin (PST) was first isolated from pig pancreas as a result of screening for peptides with a C-terminal amide structure and shown to inhibit stimulated insulin release.
While porcine PST is a 49-amino acid peptide, the biological activity resides in the carboxyl half and requires the C-terminal glycine-amide. PST is believed to be derived from chromogranin A as the result of further processing by prohormone convertase 1. The 52-amino acid sequence predicted by the gene structure of human chromogranin A is homologous to porcine PST and when synthesized the full-length peptide and its 29-amino acid carboxyl terminal had similar activity to porcine PST to inhibit insulin release. Subsequent studies confirmed the inhibition of insulin secretion in vivo, in the perfused pancreas and in isolated pancreatic islets. For reviews, see references 323, 324. By immunocytochemistry, pancreastatin was shown to be present in the pituitary, adrenal glands, pancreas, and throughout the gastrointestinal tract in cells known to contain chromogranin. In the pancreas, PST colocalizes with all four major endocrine cell types and elsewhere is found in neuroendocrine cells. PST can be measured in plasma by RIA and may serve as a predictive marker for neuroendocrine tumors where PST is elevated.

Along with the inhibition of insulin secretion, multiple natural and synthetic forms of PST (human, porcine, bovine, rat) have been shown to inhibit pancreatic digestive enzyme secretion stimulated by CCK in vivo in rats and dogs. PST also inhibited pancreatic exocrine secretion stimulated by 2-deoxy-D-Glucose, a central vagal activator. By contrast to CCK, pancreatic exocrine secretion stimulated by the cholinergic analog bethanechol in vivo was not blocked by PST. Studies of isolated rat pancreatic acini showed no inhibitory action of PST on CCK stimulation, although there is one report of inhibition of CCK stimulation in guinea pig acini. Because PST inhibited exocrine pancreatic secretion in vivo but not on isolated cells, the effect of PST is presumed to be indirect.

One possible site of action is on pancreatic blood flow as shown in anesthetized rats using the hydrogen clearance method. These investigators found that carerulein enhanced pancreatic blood flow and this increase was dose-dependently inhibited by PST at 100–500 pmol/kg/h. However, another study in anesthetized dogs showed no effect of PST at similar concentrations using the laser Doppler flowmeter method, although protein and amylase secretion were inhibited. The other possible site is on the neural stimulatory pathway. Herzig et al. showed that when pancreatic lobules which contained neural elements were incubated in vitro, high K+ concentrations stimulated the release of acetylcholine and amylase secretion—both of which were partially inhibited by PST. Further studies are necessary to establish pancreastatin as a regulatory peptide and to follow up these potential mechanisms of action. It is also not clear whether PST from a pancreatic or systemic source plays a physiological role in the regulation of exocrine pancreatic function. Absent at present is any information about a PST receptor molecule or PST-sensitive ion channel. Moreover, it is not clear what is a physiological concentration of PST. Finally, there is little new information carried out in the last twenty years beyond using PST levels as a tumor marker.

**Galanin**

Galanin is a 29-amino acid peptide isolated from porcine intestine in 1983 and named for its amino terminal glycine and carboxyl terminal alanine. It is considered to be a neuropeptide and is found throughout the body with the highest concentrations in the brain, spinal cord, and enteric nervous system. Galanin is often coexpressed with other peptides or neurotransmitters, including norepinephrine, serotonin, GABA, and acetylcholine. Galanin acts via three major G protein-coupled receptors, GalR1, GalR2, and GalR3. In the pancreas, galanin has been localized mainly to nerves present in both the exocrine and endocrine components but also in some studies to islet cells where colocalization with insulin has been observed.

In dogs, rodents, and humans, galanin suppresses insulin release both in vivo and in vitro. A number of studies have evaluated the effect of galanin on isolated rodent acini and the results are presented in the review by Barreto. Most show no effect or inhibition. In a study of the isolated, perfused rat pancreas, galanin at low concentrations enhanced both insulin secretion and CCK-stimulated amylase secretion. On the other hand, using isolated mouse pancreas lobules, galanin had no effect on carbachol stimulation but inhibited CCK-stimulated release suggesting an effect on cholinergic transmitter release. Galanin also inhibited acetylcholine release stimulated by veratridine in rat pancreatic lobules. Overall, these studies suggest galanin is more likely to affect the neural control of exocrine secretion rather than directly affecting acinar cells. Galanin could also affect amylase secretion by blocking somatostatin release.

**References**


Regulation of pancreatic exocrine function by islet hormones


somatostatin analogue octreotide on amylase secretion from mouse pancreatic acini. Digestion 1993; 54:207–212.


Chapter 18
Regulation of pancreatic fluid and electrolyte secretion
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Introduction

Pancreatic juice is the product of two distinct secretory processes. Enzymes are secreted by exocytosis from acinar cells. Fluid and electrolyte secretion is achieved primarily by the vectorial transport of ions across the ductal epithelium accompanied by water in isotonic proportions (Figure 1A). Each day, the human pancreas delivers 6–20 g of digestive enzymes to the duodenum in approximately 2.5 liters of HCO$_3^-$-rich fluid. The HCO$_3^-$-rich fluid acts as a vehicle for transporting enzymes to the duodenum where the HCO$_3^-$ neutralizes gastric acid. Moreover, pancreatic HCO$_3^-$ secretion is thought to aid disaggregation of secreted enzymes following their exocytosis in the lumen of pancreatic duct.

Species-Dependent Regulation of Pancreatic Fluid and Electrolyte Secretion

The regulation of pancreatic fluid and electrolyte secretion and the volume and composition of the secreted fluid differ considerably from species to species (Table 1). In all species, secretin evokes the secretion of HCO$_3^-$-rich pancreatic juice. However, the amount of fluid is small in the rat and mouse (approximately fivefold less than in the cat, per gram of tissue). Maximal HCO$_3^-$ concentration ([HCO$_3^-$]) reaches 130 mM or more in all species except the rat and mouse, in which 70 and 40 mM are the values. The secreted fluid is nearly isotonic ([HCO$_3^-$] + [Cl$^-$] = 150–160 mM) because of the high transepithelial water permeability and the Na$^+$ conductance of the paracellular pathway.

[HCO$_3^-$] in human pancreatic juice reaches ~140 mM under stimulation. It is generally thought that most of the HCO$_3^-$ secretion originates from epithelial cells lining the proximal pancreatic ducts (centroacinar cells, intralobular ducts, and small interlobular ducts) (Figure 1A). Pancreatic HCO$_3^-$ secretion depends on the activity of cystic fibrosis transmembrane conductance regulator (CFTR) anion channel localized in the apical membrane of pancreatic duct cells (Figure 1B). While acinar cells add a small volume of Cl$^-$ and protein-rich secretion to the lumen, luminal [HCO$_3^-$] quickly increases with time and distance along the duct as a result of HCO$_3^-$ secretion by ductal epithelium. Thus, pancreatic duct cells can transport HCO$_3^-$ against five to sixfold concentration gradients.

Vectorial Transport in Isolated Pancreatic Duct

Studies using isolated interlobular pancreatic ducts from guinea pig pancreas (Figure 1C) demonstrated that pancreatic duct cell is capable to secrete HCO$_3^-$ into already HCO$_3^-$-rich fluid under cAMP stimulation (Figure 2A). The isolated pancreatic ducts respond to physiological concentrations (1–10 pM) of secretin (Figure 2B).

Measurement of intracellular pH in luminally microperfused pancreatic duct demonstrates a polarity that is necessary to achieve vectorial transport of HCO$_3^-$ While basolaterally applied HCO$_3^-$ easily gains access to the cell, intraluminally applied HCO$_3^-$ does not. HCO$_3^-$ accumulation across the basolateral membrane is largely mediated by an electrogenic Na$^+$--coupled HCO$_3^-$ transporter (NBCe1-B, SLC4A4) and the contribution of Na$^+$--H$^+$ exchanger 1 (NHE1, SLC9A1) is small (Figure 3). Although the CFTR-dependent HCO$_3^-$ permeability of the apical membrane is large enough to mediate the observed HCO$_3^-$ secretion, the cell is relatively hyperpolarized and the electrochemical gradient for HCO$_3^-$ is outwardly directed (does not allow entry of luminal HCO$_3^-$) (Figure 3). Cholinergic stimulation also stimulates HCO$_3^-$ secretion in guinea pig pancreatic duct. Ca$^{2+}$-activated Cl$^-$ channels (CaCCs) in the apical membrane instead of CFTR are thought to be involved in HCO$_3^-$ secretion. The molecular identity of CaCCs in pancreatic duct cell is not known at present.

Regulation of Pancreatic Ductal Secretion

This section focuses on the regulatory mechanisms of pancreatic juice secretion, which directly act on pancreatic duct cells. In vitro studies using isolated pancreatic ducts or
Figure 1. Pancreatic duct system. (A) Schematic architecture of pancreatic ductal tree. Centroacinar cells belong to duct cells and represent the terminal cells of the ductal tree. (B) Immunostaining of CFTR in guinea pig pancreas indicates apical membrane of pancreatic ductal epithelium. (C) Interlobular duct segments isolated from guinea pig pancreas (adapted from reference 1).

Figure 2. Fluid and HCO$_3^-$ secretion by interlobular duct segments isolated from guinea pig pancreas. (A) When isolated segments of interlobular pancreatic ducts are cultured overnight, both ends of the ducts seal spontaneously. The lumen of sealed ducts is micropunctured and membrane-impermeable dextran conjugate of the pH sensor BCECF is injected. Time course changes in luminal pH (upper panel) and fluid secretory rates, determined from expansion speed of the lumen (lower panel), in ducts filled with high-HCO$_3^-$ solution (125 mM HCO$_3^-$-24 mM Cl$^-$, pH ~8.2), before and after cAMP stimulation (0.1 μM forskolin), are shown (means ± SE of four experiments) (adapted from reference 3). Before cAMP stimulation, there is a slight decrease of luminal pH accompanied by fluid secretion (likely Cl-rich). Upon stimulation, luminal pH starts to rise, which is accompanied by increase of fluid secretion (HCO$_3^-$-rich). (B) Effects of secretin concentration on the rate of fluid secretion into the lumen of sealed ducts (means ± SE of five experiments) (adapted from reference 4).

Table 1. Species-dependent patterns of pancreatic HCO$_3^-$ and fluid secretion. This table shows the response to stimuli given alone. Potentiation often occurs when stimuli are given together. Most data were obtained from studies on anesthetized animals. Quantitative differences may occur in conscious animals, especially in the rat, in which secretion is increased fivefold in conscious animals (adapted from reference 1).

<table>
<thead>
<tr>
<th>Species</th>
<th>Stimulus</th>
<th>Volume</th>
<th>[HCO$_3^-$] (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog, cat, human</td>
<td>Spontaneous</td>
<td>0 (+)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+ Secretin</td>
<td>+++++</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>+ Secretin</td>
<td>+++</td>
<td>40–70</td>
</tr>
<tr>
<td></td>
<td>+ CCK</td>
<td>+</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>+ Vagus</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Rat, mouse</td>
<td>Spontaneous</td>
<td>+</td>
<td>25–30</td>
</tr>
<tr>
<td></td>
<td>+ Secretin</td>
<td>++</td>
<td>40–70</td>
</tr>
<tr>
<td></td>
<td>+ CCK</td>
<td>+++</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>+ Vagus</td>
<td>++</td>
<td>?</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>Spontaneous</td>
<td>+</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>+ Secretin</td>
<td>+++++</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>+ CCK</td>
<td>+++</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>+ Vagus</td>
<td>+++</td>
<td>120</td>
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Figure 3. A model of $\text{HCO}_3^-$ secretion by guinea pig pancreatic duct cell. (A) Unstimulated duct cell whose apical membrane faces low-$\text{HCO}_3^-$, high-$\text{Cl}^-$ pancreatic juice. Accumulation of $\text{HCO}_3^-$ in the cell is achieved mainly by basolateral NBCe1, while the contribution of NHE1 is small. Some $\text{HCO}_3^-$ is lost via basolateral AE2. $\text{HCO}_3^-$ secretion across the apical membrane is mediated by SLC26A3/6 and CFTR. CFTR mediates both $\text{Cl}^-$ and $\text{HCO}_3^-$ secretion. (B) Cyclic AMP-stimulated duct cell of which the apical membrane faces high-$\text{HCO}_3^-$, low-$\text{Cl}^-$ pancreatic juice. NBCe1, SLC26A3/6, and CFTR are activated, while AE2 is inhibited. At the basolateral membrane, $\text{HCO}_3^-$ accumulation via NBCe1 is increased, while $\text{Cl}^-$ uptake (and $\text{HCO}_3^-$ loss) via AE2 is reduced. As luminal $\text{[HCO}_3^-]$ increases, the contribution of SLC26A3/6 becomes smaller and CFTR mediates most of $\text{HCO}_3^-$ secretion. The $\text{Na}^+:\text{HCO}_3^-$ stoichiometry of NBCe1 is 1:2. SLC26A3 and SLC26A6 are reported to be electrogenic with opposite polarities: SLC26A3 mediates 2$\text{Cl}^-$-$1\text{HCO}_3^-$ exchange, while SLC26A6 mediates 1$\text{Cl}^-$-$2\text{HCO}_3^-$ exchange (adapted from reference 1).
cell lines derived from human pancreatic ductal adenocarcinoma (Capan-1, PANC-1, CFPAC-1 from a patient with cystic fibrosis homozygous for F508del) demonstrated the presence of receptors of various hormones and neurotransmitters and their effects on intracellular signaling, ion transport, and HCO$_3^-$/fluid secretion. Enhancement of HCO$_3^-$/fluid secretion is usually associated with accumulation of intracellular cAMP or elevation of intracellular Ca$^{2+}$([Ca$^{2+}$]$_i$). However, we should note that elevation of [Ca$^{2+}$]$_i$ is not necessarily associated with the increase in HCO$_3^-$/fluid secretion and that pancreatic ductal regulation is also regulated by various intraluminal substances (Table 2).

The major regulators are secretin, vasoactive intestinal peptide (VIP), and acetylcholine, which increase intracellular Ca$^{2+}$/cAMP and stimulate HCO$_3^-$ and fluid secretion. Cholecystokinin (CCK) and bombesin stimulate not only enzyme secretion by acinar cells but also HCO$_3^-$ and fluid secretion by duct cells. In isolated vascularity perfused guinea pig pancreas, arginine vasopressin (AVP) increases the vascular resistance and inhibited fluid secretion. But AVP also directly acts on pancreatic duct cell and inhibits fluid secretion which is associated with [Ca$^{2+}$]$_i$ elevation. Reduction of pancreatic juice secretion by AVP may be physiologically important for body fluid conservation.

The long-term regulation of HCO$_3^-$ and fluid secretion by pancreatic duct cells is thought to be under neurohormonal control and mediated by receptors localized in the basolateral membranes. However, in some occasions, the ion composition and pH of the luminal fluid as well as intraductal pressure needs to be controlled to avoid mechanical injury to ductal epithelium, autoactivation of trypsin, or precipitation of pancreatic stone protein and CaCO$_3$ in the lumen. Table 2 summarizes the candidate list of local (stimulatory and inhibitory) regulators which are locally produced in the pancreas and play autocrine or paracrine roles.

Pancreatic duct epithelium expresses purinergic (P2Y and P2X) and adenosine receptors. Thus, pancreatic ductal secretion is likely influenced by luminal ATP and ATPase (ectonucleotidase) secreted by acinar cells together with the exocytotic release of digestive enzymes as well as basolateral ATP released as neurotransmitter. In isolated pancreatic ducts, ATP causes [Ca$^{2+}$], increase when applied to either the apical or basolateral membrane. However, apical and basolateral purinoceptors have opposite effects on secretion: luminally applied ATP enhances HCO$_3^-$ and fluid secretion while basolaterally applied ATP inhibits secretion. Luminal adenosine activates CFTR in Capan-1 cells.

### Table 2. Local regulation of pancreatic ductal secretion

<table>
<thead>
<tr>
<th>Substances</th>
<th>Receptor</th>
<th>Membrane localization</th>
<th>Effects</th>
<th>Physiological/pathological role</th>
<th>References</th>
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<tbody>
<tr>
<td>ATP</td>
<td>P2Y$_2$</td>
<td>Basolateral</td>
<td>Inhibitory</td>
<td>ATP released from nerve endings</td>
<td>13, 14, 18</td>
</tr>
<tr>
<td></td>
<td>P2X and P2Y$_2$</td>
<td>Apical</td>
<td>Stimulatory</td>
<td>ATP secreted from acinar cells</td>
<td>13, 14, 18</td>
</tr>
<tr>
<td>Adenosine</td>
<td>A$_2b$</td>
<td>Apical</td>
<td>Stimulatory</td>
<td>Adenosine produced by the hydrolysis of ATP</td>
<td>15</td>
</tr>
<tr>
<td>Serotonin (5-HT)</td>
<td>5-HT$_3$</td>
<td>Basolateral</td>
<td>Inhibitory</td>
<td>Released from EC cells in ductal epithelium upon elevation of intraluminal pressure</td>
<td>19</td>
</tr>
<tr>
<td>Substance P</td>
<td>NK2, NK3</td>
<td>Basolateral</td>
<td>Inhibitory</td>
<td>Released from sensory nerve endings in inflammatory condition</td>
<td>22</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>AT1</td>
<td>Basolateral and apical</td>
<td>Stimulatory</td>
<td>Local renin-angiotensin system is activated in response to hypoxia, pancreatitis, etc.</td>
<td>26</td>
</tr>
<tr>
<td>Trypsin</td>
<td>PAR-2</td>
<td>Basolateral</td>
<td>Stimulatory</td>
<td>Transiently evoke HCO$_3^-$ secretion</td>
<td>27, 28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Induce exocytosis of mucins to the lumen</td>
<td>27, 28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Apical</td>
<td>Inhibitory</td>
<td>Reduce HCO$_3^-$ secretion and enhance trypsinogen activation</td>
<td>29</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>CaSR</td>
<td>Apical</td>
<td>Stimulatory</td>
<td>Stimulate HCO$_3^-$ and fluid secretion and prevent the formation of pancreatic stone</td>
<td>31</td>
</tr>
<tr>
<td>Guanylin</td>
<td>GC-C</td>
<td>Apical</td>
<td>Stimulatory</td>
<td>?</td>
<td>33</td>
</tr>
</tbody>
</table>
Regulation of pancreatic fluid and electrolyte secretion

5-hydroxytryptamine (5-HT, serotonin)-immuno-reactive cells with the morphological characteristics of enterochromaffin cells (EC cells) are found scattered in the pancreatic duct epithelium (Figure 4D). These cells may sense the elevation of intraductal pressure (as EC cells do in the intestine), and the released 5-HT may inhibit fluid secretion by pancreatic duct (Figure 4C). In fact, basolateral application of 5-HT at relatively low concentrations (IC50: ~30 nM) inhibited fluid secretion (~75%) by isolated pancreatic ducts (Figure 4A). A small elevation (+3 cm H2O) of intraluminal pressure reversibly reduced pancreatic fluid secretion in vivo (anesthetized guinea pig), and the effect was attenuated by intravenous granisetron, a 5-HT3 receptor antagonist (Figure 4B). The intraductal pressure of the human pancreas increases after feeding by as much as ~20 cm of H2O. The pressure is elevated in some patients with chronic pancreatitis. Thus, 5-HT may regulate pancreatic fluid secretion under physiological and pathological conditions.

During the course of acute and chronic pancreatitis, substance P and calcitonin gene-related peptide (CGRP) are released from intrapancreatic endings of sensory nerves, which cause vasodilatation and mediate hyperalgesia. Substance P strongly inhibits HCO3- and fluid secretion by isolated pancreatic duct. Pancreatic fluid hypersecretion was observed during the early stage of caerulein-induced

Figure 4. Serotonin (5-HT) inhibits pancreatic fluid secretion in an autocrine manner. (A) Effects of serotonin (5-HT 0.1 μM) on secretin (1 nM)-stimulated fluid secretion by isolated guinea pig pancreatic ducts (means ± SE of four experiments). (B) Effects of small elevations of intraductal pressure and 5-HT3 receptor antagonist (granisetron 40 μg/kg/h) on in vivo secretin (1 μg/kg/h)-stimulated fluid secretion from guinea pig pancreas (means ± SE of four experiments). (C) A hypothetical mechanism. EC cells in the duct epithelium sense the elevation of intraductal pressure and release 5-HT into the interstitium. The released 5-HT binds to 5-HT3 receptor in the basolateral membrane of neighboring duct cells, which facilitates Na+ entry into the cell. That reduces the inward gradient of Na+, which is necessary for intracellular HCO3- accumulation. (D) A 5-HT-immunoreactive cell embedded in pancreatic duct epithelium (adapted from reference 19).
Pancreatic Ductal Secretion and Lifestyle Disease

Effects of ethanol

Ethanol abuse is the leading cause of acute and chronic pancreatitis. The more the daily amount of alcohol drinking, the larger the risk of developing chronic pancreatitis. However, there is no known threshold dose of ethanol that induces chronic pancreatitis and patients drinking even small quantities of ethanol (1–20 g/day) run a higher risk. Studies using isolated pancreatic ducts from guinea pig show that low and high concentrations of ethanol have opposite effects on fluid secretion. While low concentrations of ethanol (0.1–30 mM relevant to normal drinking conditions) enhance fluid secretion, high concentrations of ethanol (100 mM relevant to lethal level) reduce fluid secretion.

The objective effects of ethanol on the central nervous system (CNS) appear at a blood ethanol level of ~5 mM, and they are attributed to the modulation of ion channels. Ethanol reversibly and strongly augments secretin-stimulated fluid secretion in guinea pig pancreatic duct (Figure 5A) where the maximal augmentative effect is achieved with 1 mM ethanol (Figure 5B). The augmentation of fluid secretion involves a transient increase of [Ca$^{2+}$], (Figure 5D) likely via the activation of plasma membrane Ca$^{2+}$ channels. The effects of n-alcohols on pancreatic duct cells are dependent on the length of alkyl chain. While methanol (C1) and ethanol (C2) augment fluid secretion and induce a transient [Ca$^{2+}$], increase, propanol (C3) and butanol (C4) inhibit fluid secretion and reduce [Ca$^{2+}$], (Figure 5C and D). The observation is similar to the so-called “cutoff” effects of n-alcohols on neurotransmitter-gated ion channels in CNS neurons that increase in potency, with an increasing alkyl chain length of n-alcohol up to a point but then disappears with a further increase in chain length. Concerning the pathogenesis of alcoholic chronic pancreatitis, the ductal fluid hypersecretion by ethanol may raise the intraductal pressure when the flow of pancreatic juice is blocked by the presence of highly viscous juice or protein plugs.

Ethanol (100 mM) inhibits cAMP-stimulated CFTR currents, apical Cl-HCO$_3$ exchange, and fluid secretion in guinea-pig pancreatic ductal epithelial cells. Moreover, prolonged treatment with 100 mM ethanol decreases membrane expression of CFTR. Those effects of ethanol involve sustained elevation of [Ca$^{2+}$], and depletion of ATP, which is probably associated with the pathogenesis of alcoholic pancreatitis.

Effects of glucose

Pancreatic exocrine dysfunction is frequently found in patients with type 1 and type 2 diabetes mellitus. Both enzyme secretion from acinar cells and fluid/HCO$_3^-$ secretion by duct cells are impaired. It was reported that exposure to high glucose (for 72 hours) activates polyol metabolism and decreases the activity of Na$^+$.K$^+$-ATPase in Capan-1 cells. The resultant elevation of [Na$^+$] would attenuate the inward Na$^+$ gradient across the basolateral membrane,
which reduces HCO$_3^-$ uptake via Na$^+$-HCO$_3^-$ cotransport and Na$^+$-H$^+$ exchange (Figure 3). This leads to a reduction of fluid secretion.

Even acute exposure to high glucose concentrations (15 minutes) inhibits fluid secretion and reduces basolateral HCO$_3^-$ uptake in secretin-stimulated rat pancreatic ducts. This inhibition is most likely attributed to transepithelial absorption of glucose. Rat interlobular pancreatic ducts express Na$^+$-glucose cotransporter (SGLT1) and glucose transporters (GLUT1 and GLUT2) (Figure 6A) and absorb luminal glucose iso-osmotically (Figure 6B). The absorption of luminal glucose is abolished by phlorizin, an inhibitor of SGLT1 in the lumen. Absorption of luminal Na$^+$ and glucose via SGLT1 would increase [Na$^+$], and depolarize the apical membrane. [Na$^+$] elevation reduces HCO$_3^-$ uptake and apical depolarization reduces the driving force for HCO$_3^-$ secretion via CFTR, both of which leads to a reduction of fluid secretion.

The glucose concentration in human pancreatic juice (0.5–1 mM) is much lower than in plasma. Under physiological conditions, pancreatic duct epithelium probably absorbs luminal glucose via apical SGLT1 to maintain the glucose concentration at a low level in the pancreatic juice.

**Computer Simulation of Pancreatic Ductal Secretion**

On the basis of previous modelling studies, a new computational model has been developed as a set of simultaneous ordinary differential equations in MATLAB (MathWorks, Natick, MA, USA) using the Simulink interface to provide a modular structure and facilitate the simulation of time-course experiments. The activities and
permeabilities of individual ion channels and transporters are estimated by least-squares fitting of the model predictions to the experimental data of isolated guinea pig pancreatic duct (intracellular pH, Cl\(^-\), membrane potential, luminal pH, and volume).

The pancreatic duct epithelium is represented as a four-compartment system comprising the bath, the lateral intercellular space, the cytoplasm, and the lumen. In the perfused duct model, the composition of the luminal fluid is set to predefined values. Alternatively, in the secreting duct model (Figure 7A), the composition of the luminal fluid is allowed to evolve with time and defined by the fluid secreted by the epithelial cells. Stimulation is replicated by increasing the activities of the basolateral Na\(^+\)-HCO\(_3\)\(^-\) cotransporter (NBCe1-B, SLC4A4) and apical Cl\(^-\)-HCO\(_3\)\(^-\) exchanger (SLC26A6), increasing the basolateral K\(^+\) permeability and apical Cl\(^-\) and HCO\(_3\)\(^-\) permeabilities (CFTR) and reducing the activity of the basolateral Cl\(^-\)-HCO\(_3\)\(^-\) exchanger (AE2, SLC4A2). Under these conditions, the model secretes ~140 mM HCO\(_3\)\(^-\) at a rate of ~3.5 nl min\(^{-1}\) mm\(^{-2}\), which is consistent with experimental observations.

Figure 7A shows steady-state fluxes of HCO\(_3\)\(^-\), H\(^+\), and Cl\(^-\) at rest and following cAMP stimulation in the model. Intracellular/luminal/bath Cl\(^-\)/HCO\(_3\)\(^-\) concentrations, osmolarities, and basolateral potential differences

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**Figure 6. Expression of glucose transporters and transepithelial glucose absorption by isolated pancreatic ducts.** (A) mRNA expression of glucose transporters (SGLT1, SGLT2, GLUT1, GLUT2) in the pancreas (P), pancreatic duct (D), pancreatic acini (A), small intestine (I), kidney (K), and lung (L) of the rat. (B) Time course shrinkage of the lumen indicates transepithelial absorption of luminal glucose by isolated rat pancreatic ducts. The lumen is filled with the HCO\(_3\)\(^-\)-free HEPES-buffered solution containing high glucose (44.4 mM). The bath is also perfused with the HCO\(_3\)\(^-\)-free HEPES-buffered solution containing 44.4 mM glucose. The left panel shows images of a duct at the beginning and end of a representative experiment (15 minutes) showing 33 percent shrinkage of the lumen. Time-course changes of the luminal volume (right panel) demonstrate that phlorizin (0.5 mM) in the lumen completely inhibits glucose absorption (means ± SE of four experiments) (adapted from reference 39).
Figure 7. Computational model of HCO$_3^-$ secretion by pancreatic duct cell. (A) A secreting model of guinea pig pancreatic duct epithelium shows steady-state fluxes of HCO$_3^-$ (red font), H$^+$ (blue font), and Cl$^-$ (green font) mediated by (clockwise from top left) NHE1, AE2, NBC1, SLC26A6, and CFTR. Also shown are the steady-state intracellular/luminal/bath Cl$^-$/HCO$_3^-$ concentrations, osmolarities, and basolateral potential difference (black font). The left panel represents the unstimulated duct and the right panel shows the effect of altering the transporter/channel activities to represent cAMP stimulation. The Cl$^-$:HCO$_3^-$ stoichiometry of the SLC26A6 exchanger is assumed to be 1:2. Fluxes are given in nmol min$^{-1}$ cm$^{-2}$ (normalized to the luminal surface area of the epithelium). (B) Effects of varying the HCO$_3^-$/Cl$^-$ permeability ratio of CFTR on HCO$_3^-$ and fluid secretion in the secreting duct model. Steady-state values in the cAMP-stimulated condition of secreted HCO$_3^-$ concentration ([HCO$_3^-$]$_l$), secretory volume flow ($J_v$), and net apical HCO$_3^-$ fluxes via CFTR and SLC26A6 ($J_{HCO3}$) as a function of the HCO$_3^-$/Cl$^-$ permeability ratio ($P_{HCO3}/P_{Cl}$) of CFTR. The sum of the permeabilities ($P_{HCO3} + P_{Cl}$) is maintained at a constant value. (C) Effects of CFTR activity on HCO$_3^-$ and fluid secretion in the secreting duct model. Steady-state values in the cAMP-stimulated condition of [HCO$_3^-$]$_l$, $J_v$, and $J_{HCO3}$ via CFTR and SLC26A6 as a function of the CFTR activity (adapted from reference 42).
are shown as well. In the steady state, ion compositions of the effluent (pancreatic juice), the luminal fluid, and the fluid secreted by the epithelial cells are identical. While the apical HCO$_3^-$ flux at rest is divided between CFTR and SLC26A6 (assumed to be 1Cl$^−$:2HCO$_3^-$ stoichiometry in the standard model), the apical HCO$_3^-$ flux under cAMP stimulation is predominantly (~90%) via CFTR rather than SLC26A6. The luminal fluid is ~3 percent hypertonic compared to the bath under stimulation.

The HCO$_3^-/Cl^-$ permeability ratio ($P_{HCO3}/P_{Cl}$) of CFTR has been estimated to be 0.2–0.5 and reported to be regulated by Cl$^-$-sensitive kinases.$^{43}$ $P_{HCO3}/P_{Cl}$ is set at 0.4 in the standard model (Figure 7A). Increasing the ratio to 1.0 has a little effect on the volume ($J_v$) and HCO$_3^-$ concentration ([HCO$_3^-$]) of the secreted fluid (Figure 7B). When the ratio is decreased to zero, SLC26A6 compensates for the defective HCO$_3^-$ transport of by CFTR and the model still secretes ~120 mM HCO$_3^-$ at a rate of ~2 nl min$^{-1}$ mm$^{-2}$. CFTR Cl$^-$ permeability is set at 60 × 10$^{-6}$ cm s$^{-1}$ in the standard model. Figure 7C shows the effects of varying CFTR activity on [HCO$_3^-$]$_v$, $J_v$, and apical HCO$_3^-$ fluxes ($J_{HCO3}$) via CFTR and SLC26A6. When CFTR activity is completely lost, SLC26A6 by itself cannot support HCO$_3^-$ and Cl$^-$ secretion and there is no fluid secretion. Residual CFTR activity as low as 10 percent in combination with SLC26A6 accomplishes fluid secretion containing ~120 mM HCO$_3^-$ at a rate of ~1.5 nl min$^{-1}$ mm$^{-2}$. Effective treatment of cystic fibrosis requires restoration of CFTR function and different organs have different requirements for CFTR function. Ion transport in airway epithelium is normalized when 6–10 percent of cells have corrected CFTR function.$^{44}$ Recent studies in young children with cystic fibrosis suggest improvement of pancreatic exocrine function by CFTR modulators.$^{45}$

Although the computational model is simple and lacks molecular interactions between CFTR and other transporters and regulation by intracellular Cl$^-$, it well reproduces the experimental data including the redundancy of acid/base transporters: CFTR and SLC26A6 in the apical membrane and NBC and NHE in the basolateral membrane.$^{42}$

Acknowledgments

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References

Regulation of pancreatic fluid and electrolyte secretion


Chapter 19

Molecular mechanisms of pancreatic bicarbonate secretion

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Introduction

The human exocrine pancreas secretes 1–2 liters of pancreatic juice each day. When stimulated, the pancreas secretes alkaline pancreatic juice containing copious amounts of bicarbonate \( \text{HCO}_3^- \).1,2 \( \text{HCO}_3^- \) plays essential roles in the digestive system. \( \text{HCO}_3^- \) determines the pH of bodily fluids as the major buffer system that guards against toxic pH fluctuations.3 \( \text{HCO}_3^- \) in pancreatic juice neutralizes gastric acid and provides an optimal pH environment for digestive enzymes to function in the duodenum.4 In addition, \( \text{HCO}_3^- \) acts as a moderate chaotropic ion that facilitates the solubilization of macromolecules, such as digestive enzymes and mucins.5 The importance of pancreatic \( \text{HCO}_3^- \) secretion is highlighted in the abnormal \( \text{HCO}_3^- \) secretion in several forms of pancreatitis6,7 and in cystic fibrosis (CF), which causes poor mucin hydration and solubilization leading to obstruction of ductal structures of the pancreas, intestine, vas deferens, and the lung.7,8

The exocrine pancreas is composed of three major cell types, acinar, duct, and stellate cells. Acinar cells secrete a small volume of isotonic, plasma-like, NaCl-rich fluid and digestive enzymes. Duct cells modify the ionic composition of the fluid and secrete the bulk of the fluid and \( \text{HCO}_3^- \) of the pancreatic juice. Stellate cells may aid the pancreas recovery from injury.9 As the main \( \text{HCO}_3^- \) secretor, the duct has key roles in the development of acute and chronic pancreatitis. At pH 7.4 and 5 percent CO\(_2\), the \( \text{HCO}_3^- \) concentration in plasma is approximately 25 mM. In human, dog, cat, and guinea pig, \( \text{HCO}_3^- \) concentration in postprandial pancreatic juice is higher than 140 mM.1,2 This remarkable transport performance has attracted much attention from pancreatologists and physiologists. Current understanding of the molecular mechanism of pancreatic \( \text{HCO}_3^- \) secretion was improved by the recent identification of ion transporters and channels, including the cystic fibrosis transmembrane conductance regulator (CFTR),10 the electrogenic Na\(^+\)-\( \text{HCO}_3^- \) cotransporter NBCe1-B (also known as pNBC1),11 and the solute-linked carrier 26 (SLC26) transporters,12,13 together with regulatory proteins, such as with-no-lysine kinase 1 (WNK1),14 STE20/SPS1-related proline/alanine-rich kinase (SPAK),15 and the inositol-1,4,5-triphosphate (IP\(_3\)) receptor-binding protein released with IP\(_3\) (IRBIT)16 and their role in pancreatic \( \text{HCO}_3^- \) secretion.

Control of Pancreatic \( \text{HCO}_3^- \) Secretion

Pancreatic \( \text{HCO}_3^- \) secretion increases in response to ingestion of a meal and is regulated by multiple neurohumoral inputs. Fluid and enzyme secretion by acinar cells are controlled predominantly by an increase in cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)).17–19 Fluid and \( \text{HCO}_3^- \) secretion by duct cells are regulated by the second messengers cAMP20–22 that synergizes with Ca\(^{2+}\) to generate the physiological response.21–23 Pancreatic ductal cells express receptors for a battery of hormones and neurotransmitters. The two major hormones controlling pancreatic fluid and \( \text{HCO}_3^- \) secretion are the G\(_s\)-coupled, cAMP-generating hormone secretin and the G\(_q\)-coupled, Ca\(^{2+}\)-mobilizing hormone cholecystokinin (CCK), which are released from gastrointestinal endocrine cells in the upper duodenum. Cholinergic vagal output via an enteropancreatic vagovagal reflex also has an important role in controlling ducal fluid and \( \text{HCO}_3^- \) secretion. In addition to these classic stimuli, several other humoral agents are released by the pancreas for fine-tuning its secretion, including insulin, somatostatin, purines, and prostaglandins.24 Additional information on hormonal control of pancreatic secretion can be found in a previous review2 and the “Regulation of Pancreatic Secretion” section in Pancreapedia.25

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**Humoral control**

Secretin

The low pH (below 4.5) of gastric chyme stimulates the release of secretin from duodenal S cells into the blood. Secretin stimulates ductal fluid and HCO$_3^-$ secretion and synergizes with Ca$^{2+}$-mobilizing agonists to potentiate enzyme secretion by acinar cells. Plasma secretin levels rise after a meal and correlate with HCO$_3^-$ output. Secretin-stimulated fluid and HCO$_3^-$ secretion is modulated directly or indirectly by both peptide hormones, such as CCK and somatostatin, and by vagal stimulation.

CCK

CCK is a major stimulator of acinar cell enzyme and fluid secretion, which is mediated by the Ca$^{2+}$-dependent exocytosis of zymogen granules and activation of apical (luminal) Cl$^-$ channels, respectively. The synaptotagmins are the Ca$^{2+}$ sensors that convey the Ca$^{2+}$ signal for pancreatic exocytosis and Ca$^{2+}$ activates the Ca$^{2+}$-activated anoctamin 1 (TMEM16A) to initiate acinar cell fluid secretion. CCK also acts on pancreatic duct secretion; however, the effects of CCK on pancreatic duct differ among species. In humans, the effect of CCK alone on ductal fluid secretion is weak; however, CCK greatly potentiates the effects of secretin.

Purines

Pancreatic duct cells express multiple purinergic type 2 receptor (P2R) types, including ionotropic P2X and metabotropic P2Y receptors at the apical and basolateral membranes. P2Rs are stimulated by purinergic ligands released from nerve terminals at the basolateral space, zymogen granules of acinar cells into the luminal space, or efflux by ductal ATP transporters to both the basolateral and luminal compartments. Stimulation of P2Rs increases [Ca$^{2+}$]$_i$ in duct cells. Several studies have examined effects of P2Rs on ion transporters in ductal cell lines, but there are almost no studies on ductal fluid and HCO$_3^-$ secretion. Ishiguro et al. demonstrated that luminal ATP stimulates while basolateral ATP inhibited fluid and HCO$_3^-$ secretion in guinea pig pancreatic duct. More recent studies examined the effect of various stimuli and ion channels of ATP release from ductal cell lines that will be important to verify in native ducts.

Neuronal control

Pancreatic secretion is regulated by the enteric nervous system, which is composed of a gut-brain axis and an intrapancreatic system. The major neurotransmitter acting on pancreatic duct cells is acetylcholine released by vagal parasympathetic fibers. Duct cells express both M1 and M3 muscarinic receptors, which act through changes in [Ca$^{2+}$]$_i$. The M3 receptors maybe more prominent based on their higher expression level relative to the M1 receptors. In humans, cholinergic stimulation enhances ductal secretion stimulated by secretin, likely by synergistic mechanism that is mediated by IRBIT. Vasoactive intestinal peptide (VIP) and ATP are also localized in parasympathetic nerve terminals. Vagal stimulation causes VIP release that is associated with fluid and HCO$_3^-$ secretion.

Key Transporters Involved in Pancreatic HCO$_3^-$ Secretion

Pancreatic HCO$_3^-$ secretion is mediated by a coordinated function of transporters expressed in the apical and basolateral membranes of duct cells. Pancreatic HCO$_3^-$ secretion can be divided into two steps. The first step is uptake of HCO$_3^-$ into duct cells from the blood through the basolateral membrane. The second step is efflux of HCO$_3^-$ across the apical membrane of duct cells. Regulatory mechanisms in the cytosol that include ions like Cl$^-$ and several kinases and phosphatases act on the transporters to coordinate and integrate the secretory process. Recent advances in molecular, cellular, and physiological techniques have enhanced our understanding of the molecular identity, localization, function, and regulatory mechanisms of ductal ion transporters. The major ion transporters expressed in the apical and basolateral membranes of the pancreatic duct cells are summarized in Table 1 and Figure 1.

Na$^+$/K$^+$ ATPase and K$^+$ channels

The main driving force for fluid secretion is achieved by the Na$^+$/K$^+$ ATPase pump and K$^+$ channels which generate the transmembrane Na$^+$ and K$^+$ gradients and the negative intracellular membrane potential. The Na$^+$/K$^+$ ATPase pump is expressed in the basolateral membrane of the ducts and generates the Na$^+$ and K$^+$ gradients by extruding three Na$^+$ ions in exchange for uptake of two extracellular K$^+$ ions using the energy of ATP hydrolysis. K$^+$ channels in both the basolateral and apical membranes use the K$^+$ gradient generated by the pump to generate a negative membrane potential. The Na$^+$ gradient is used to drive several Na$^+$-coupled solutes, including HCO$_3^-$ absorption by the basolateral Na$^+$/HCO$_3^-$ cotransporter NBCe1-B and basolateral and luminal Na$^+$/H$^+$ exchangers (NHEs). The negative membrane potential aids in controlling HCO$_3^-$ uptake by NBCe1-B and in HCO$_3^-$ efflux through luminal electrogenic transporters. MaxiK channels (KCNMA1) have been identified on the basolateral membrane of rat pancreatic duct cells and are likely candidates for maintaining the membrane potential during agonist-stimulated
HCO₃⁻ secretion. A Ba²⁺-sensitive channel of 82 pS conductance (KCNN4) appears to be a basolateral K⁺ channel, which is responsible for the resting K⁺ permeability. Apical membrane K⁺ channels were identified in acinar cells and in pancreatic duct cells, with the later having a role in ductal HCO₃⁻ secretion.

**Na⁺-HCO₃⁻ cotransporters (NBCs)**

The main ductal basolateral membrane HCO₃⁻ accumulation transporter is NBCe1-B. NBCe1-B was cloned from pancreas and was named pNBC1. It was later renamed NBCe1-B as part of classification of the NBC family. NBCe1-B is an electrogenic transporter with a 1 Na⁺:2 HCO₃⁻ stoichiometry in pancreatic duct cells. NBCe1-B can be regulated by cAMP-dependent protein kinase A (PKA) phosphorylation at Ser1026 and Thr49. In principle, Na⁺/H⁺ exchangers in the basolateral membrane (e.g., NHE1) can also mediate HCO₃⁻ influx during secretin-induced ductal fluid and HCO₃⁻ secretion in guinea pig. Indeed, NBCe1-B contributes up to ~75 percent of the HCO₃⁻ influx during secretin-induced ductal fluid and HCO₃⁻ secretion in guinea pig.

**Table 1. Transporters of pancreatic duct**

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Molecular mechanisms of pancreatic bicarbonate secretion

Figure 1. A schematic diagram depicting the transporters and channels in the apical (luminal) and basolateral membranes of pancreatic duct cells. The main driving force for HCO$_3^-$ secretion is achieved by the Na$^+$ gradient generated by the Na$^+/K^+$ ATPase pump and K$^+$ channels at the basolateral membrane, which generate the intracellular negative membrane potential. HCO$_3^-$ is loaded mainly through the electrogenic (1Na$^+/-2$HCO$_3^-$) NBCe1-B and partly by NHE1 located in the basolateral membrane. Basolateral AE2 may act to supply Cl$^-$ in to maintain the secretion. Apical HCO$_3^-$ secretion is performed by the interacting and functionally interrelated CFTR and Slc26a6. Transcellular HCO$_3^-$ movement generates a lumen-negative electrical potential that results in paracellular Na$^+$ secretion through the paracellular pathway. Water follows Na$^+$ and HCO$_3^-$ osmotically via paracellular and transcellular (aquaporins) pathways. In the resting state, luminal NHE3 and NBCn1-A function to salvage luminal HCO$_3^-$ (modified from reference 46).

by the WNK and CaMKII (Ca$^{2+}$- and calmodulin-activated kinase II) kinases and the SPAK along with the calcineurin phosphatase that dephosphorylate the serine residues phosphorylated by the respective kinases. The kinases/phosphatases pairs determine regulation of NBCe1-B by Cl$^-$, which emerges as a new general form of signaling ion. In addition to NBCe1-B, the duct expresses electroneutral NBCn1-A (NBC3) on the apical (luminal) membrane. This transporter may mediate HCO$_3^-$ salvage in the resting state to maintain acidified pancreatic juice.

**CFTR**

The discovery of acidic pancreatic juice in patients with cystic fibrosis (CF) was a milestone in understanding the mechanism of pancreatic HCO$_3^-$ secretion. The CF transmembrane conductance regulator (CFTR) was discovered as the protein mutated in patients with CF. Although CFTR is a member of the ATP-binding cassette transporter superfamily that usually acts as membrane pumps that transport their substrates against the electrochemical gradient, CFTR functions as an anion (Cl$^-$ and HCO$_3^-$) channel, through which ions diffuse down the electrochemical gradient. CFTR is located at the apical membrane of pancreatic ducts and all secretory epithelia and is activated by the cAMP/PKA pathway. At [Cl$^-$]i higher than 10 mM, CFTR functions as a Cl$^-$ channel that has limited permeability to HCO$_3^-$, but when [Cl$^-$]i drops to below 10 mM, CFTR anionic selectivity changes to increase HCO$_3^-$ permeability and mediate luminal HCO$_3^-$ exit. Indeed, as has been shown in patients with CF, CFTR is critically involved in epithelial HCO$_3^-$ secretion. This leads to revision of the original model of ductal HCO$_3^-$ secretion, in which Cl$^-$/HCO$_3^-$ exchangers mediate apical HCO$_3^-$ efflux and CFTR facilitates the apical Cl$^-$/HCO$_3^-$ exchangers by recycling the Cl$^-$. This continues to be the case at high Cl$^-$i. However, at low [Cl$^-$]i, HCO$_3^-$ efflux via CFTR driven by the membrane potential has essential role in HCO$_3^-$ efflux and HCO$_3^-$-driven fluid secretion in the pancreatic duct.
other members of the WNK kinases are regulated by Cl$^{-}$ in$, with high Cl$^{-}$ in$ in the low (WNK4) and high (WNK1) range inhibiting the WNKs. Reduction in Cl$^{-}$ in$ activates the WNKs that act directly, or through SPAK, on CFTR and other HCO$_3$-, Na$^+$, Cl$^-$, and K$^+$ transporters$^{53}$ to regulate their activity and selectivity. If is of interest that the WNKs show differential sensitivity to Cl$^{-}$ in$, and binds to PDZ domains of adapter proteins, such as severity of the CF disease.

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CFTR has a more global role in ductal fluid and HCO$_3$- secretion. In addition to functioning as a Cl$^-$ and HCO$_3$- channel, CFTR functions as a scaffold forming macromolecular complexes with other transporters and regulatory proteins at the apical membrane.$^{46}$ CFTR has a PSD95/Discs-large/ZO-1 (PDZ) ligand at the C-terminus and binds to PDZ domains of adapter proteins, such as Na$^+$/H$^+$ exchanger regulatory factors (NHERFs). It also has SH3 and multiple ankyrin repeat domains 2 (Shank2),$^{89,90}$ through which CFTR interacts and regulates the activity of slc26a6, slc26a3,$^{93}$ NHE3,$^{92}$ and NBCn1-A.$^{64}$ Other interactions of CFTR are with soluble NSF attachment protein receptor (SNARE) proteins, A-kinase anchor proteins (AKAPs), kinases, and phosphatases$^{89}$ that may serve to regulate CFTR activity and the activity of the transporters interacting with CFTR. The interaction with the SLC26 transporters is of particular significance since the two transporters are mutually activated when interacting.$^{91,94}$ The mutual regulation is mediated by interaction of the CFTR R domain with the SLC26 transporters STAS domain.$^{94}$

**Cl$^-$/HCO$_3$- exchangers**

Cl$^-$/HCO$_3$- exchangers mediate the bulk of HCO$_3$- exit across the apical membranes of the pancreatic duct cells until the last portion of HCO$_3$- exit that is mediated by CFTR once it gains HCO$_3$- permeability. In humans, members of the solute-linked carrier 4 (SLC4) and the SLC26 families function as Cl$^-$/HCO$_3$- exchangers. Among the SLC4 transporters, duct cells express AE2 (SLC4A2) at the basolateral membrane that regulates pH$_i$ and protects against alkaline load.$^6$ However, our studies revealed an essential role for AE2 in ductal fluid and HCO$_3$- secretion.$^{95}$ Intuitively, basolateral HCO$_3$- efflux mechanism should inhibit rather than stimulate ductal HCO$_3$- secretion. It is not clear why AE2 is essential for ductal fluid secretion. Maintaining stable pH$_{in}$ that neutralize acid load by the Na$^+$/H$^+$ exchangers and high pH next to the plasma membrane is one potential critical function of AE2. Another possibility is that AE2 may provide the duct with Cl$^-$ that is needed to keep the luminal slc26a6 functioning in a face of limited Cl$^-$ provided by acinar secretion.$^{95}$

Among the SLC26 family transporters, SLC26A3 and SLC26A6 are located on the apical membrane of the pancreatic duct cells and mediate Cl$^-$/HCO$_3$- exchange. Interestingly, SLC26A3 has a 2Cl$^-$/1HCO$_3$- stoichiometry,$^{94,96}$ while SLC26A6 functions as a 2HCO$_3$-/1Cl$^-$ exchanger.$^{96,97}$ A persistent osmotic gradient is needed to support the copious fluid secretion by the pancreatic duct. This is satisfied by the coupled action of NBCe1-B and SLC26A6 that results in a continuous net HCO$_3$- (osmolyte) transcellular transport and thus transcellular H$_2$O flow.$^{98-100}$ In addition, as indicated above, SLC26 transporters interact with CFTR through the sulfate transporter and anti-sigma factor antagonist (STAS) domain and regulate pancreatic secretion by activating CFTR.$^{96}$ This form of regulation is critical for pancreatic and other exocrine glands HCO$_3$- secretion, including the pancreas, salivary glands, the kidney, and the lung.$^{101}$

**Other transporters, channels, and carbonic anhydrases**

**Na$^+$/H$^+$ exchangers (NHEs)**

The SLC9A NHE family contains electroneutral 1Na$^+$/1H$^+$ exchangers. The ubiquitous NHE1 (SLC9A1) is essential for intracellular pH homeostasis and supplies Na$^+$ to the Na$^+$/K$^+$ ATPase pump on the basolateral membrane of the pancreatic duct.$^{102}$ Diffusion of CO$_2$ from the blood into the duct and CO$_2$ generated by metabolism is hydrated by the action of carbonic anhydrases to generate HCO$_3$- and H$. Consequently, H$^+$ efflux by NHE1 may contribute to basolateral HCO$_3$- uptake. However, NHE1 does not have a major role in basolateral HCO$_3$- influx as revealed by minimal inhibition of fluid and HCO$_3$- secretion by inhibition of NHE1 in pancreatic duct of most species.$^{103,104}$ The NHE3 isoform is expressed in the apical membrane of pancreatic duct and is thought to mediate HCO$_3$- salvage at the resting state.$^{105}$ At the resting state, the pancreatic juice is acidic, indicating an active H$^+$ secretion$^{66,67}$ that may be mediated by the combined action of NHE3 and NBCn1-A. Similar to NBCn1-A,$^{64}$ NHE3 interacts with CFTR via PDZ domain containing proteins$^{92}$ and is
regulated by IRBIT.\textsuperscript{106,107} However, the physiological significance of these transporters awaits evaluation in mouse models with targeted pancreatic deletion of ductal NHEs and NBCn1-A.

\textbf{Ca$^{2+}$-activated Cl$^-$ channels (CaCCs)}

Several members of the anoctamin (TMEM16/ANO) family function as CaCCs.\textsuperscript{108–110} TMEM16A/ANO1, TMEM16B/ANO2, TMEM16F/ANO6, TMEM16H/ANO8, and TMEM16K/ANO10 are expressed in pancreas.\textsuperscript{111} However, ANO1 is expressed in acinar but not duct cells,\textsuperscript{109} ANO6 functions as a flipase and as a Cl$^-$ channel,\textsuperscript{50} and ANO8 is a tether at the ER/PM junctions that controls assembly of Ca$^{2+}$-signaling complexes.\textsuperscript{111} The function of ANO2 and ANO10 in the pancreas is not clear at this time. Nevertheless, ample evidence shows that the pancreatic duct (and ducts of other secretory glands) has CaCC activity in the apical membrane.\textsuperscript{112–115} The molecular identity of this channel is not known at present, nor its function in HCO$_3^-$ secretion. ANO6 appears to function as a Ca$^{2+}$-activated Cl$^-$ channel in the intestine that participates in fluid and electrolyte secretion\textsuperscript{116} and may have a similar function in the pancreatic duct. Several other CaCCs are known and are candidates for the ductal CaCC. In pancreatic acinar cells and other serous cells, ANO1 may have a role in HCO$_3^-$ transport. At physiological [Ca$^{2+}$] concentrations, ANO1 functions as a Cl$^-$ channel. However, at high [Ca$^{2+}$], and perhaps at high [Ca$^{2+}$] microdomains, ANO1 HCO$_3^-$ permeability is increased by Ca$^{2+}$/calmodulin,\textsuperscript{117,118} raising the possibility that ANO1 can provide an alternative Cl$^-$ and HCO$_3^-$ conduction in acinar cells.\textsuperscript{119}

\textbf{Aquaporins}

Although the paracellular pathway is permeable to H$_2$O, H$_2$O flows mostly transcellularly via the water channels aquaporins (AQP) family. This is best illustrated in salivary glands, where knockout of AQP5 markedly reduces salivation.\textsuperscript{120} Among the 13 AQPs, AQP1 and AQP5 are the major aquaporins in pancreatic duct.\textsuperscript{121–123} AQP1 is expressed in the luminal membrane of human acinar and duct cells and is significantly reduced in chronic pancreatitis.\textsuperscript{124} Moreover, deletion of AQP1 in mice prominently inhibits ductal and pancreatic fluid and HCO$_3^-$ secretion, due to both reduction in fluid transport and in CFTR expression and activity and thus HCO$_3^-$ secretion.\textsuperscript{124} The role of AQP5 in the duct and pancreatic secretion has not been established yet.

\textbf{Carbonic anhydrases}

A poorly studied topic that deserve more attention is the role of the ductal carbonic anhydrases (CAs) in fluid and electrolyte secretion, in particular with the emerging secretory epithelial diseases due to mutations in CAs. Mutations that affect the action of CA4 cause retinitis pigmentosa\textsuperscript{125} and a mutation in CA12 causes salt wasting.\textsuperscript{126,127} All transporters involved in fluid and HCO$_3^-$ secretion are affected by HCO$_3^-$ concentration at the cellular compartments and microdomains that determine HCO$_3^-$ availability at plasma membrane inner and outer surfaces. Hydration of CO$_2$ by CAs determines local HCO$_3^-$ concentration both at the outer and inner plasma membrane surfaces.\textsuperscript{128} Several CAs are localized in the cytoplasm (such as CA2 and CA7) and several are anchored at the plasma membrane (such as CA4, CA12, and CA14) with the catalytic site at the extracellular surface and regulate HCO$_3^-$ concentration at the basolateral (CA4 and CA12) or the luminal (CA4) membrane surfaces.\textsuperscript{129}

CAs localized in the plasma membrane and cytoplasm interact with H$^+$ and HCO$_3^-$ transporters that mediate ductal fluid and HCO$_3^-$ secretion and regulate their activity. CA4 interacts with the C terminus of NBCe1-A to increase its activity.\textsuperscript{130} The C terminus of NBCe1-A and NBCe1-B are conserved and thus it is likely that CA4 regulates NBCe1-B. NBCn1-A recruits the cytoplasmic CA2 to the plasma membrane, where CA2 increases the activity of NBCn1-A.\textsuperscript{131} CA2 is closely associated with NHE3 and increases NHE3 activity.\textsuperscript{132} CA2 interacts with a novel site at the C terminus of NHE1 to regulate NHE1 activity.\textsuperscript{133} CA2 has been reported to interact with the C terminus of slc26a6 to increase its activity. However, the role of other CAs, in particular the plasma membrane-anchored CAs, on the activity of the slc26a6 and other SLC26 transporters has not been investigated yet. Finally, CA2 also interacts with AQP1 to increase water flux by AQP1 by an unknown mechanism.\textsuperscript{134} A particularly interesting CA is the basolateral membrane-anchored CA12 with its catalytic site at the extracellular membrane surface. A human mutation in CA12(E143K) is the cause of an autosomal recessive form of salt wasting, which leads to hyponatremia with hyperkalemia, high sweat Cl$^-$, dehydration, and failure to thrive.\textsuperscript{126,127,135} A recent work to understand the cause of the disease established a prominent role for CA12 in ductal fluid and HCO$_3^-$ secretion. Thus, CA12 increased, while CA12 (E143K) markedly reduced ductal fluid secretion in isolated ducts and \textit{in vivo}. This could be attributed to a potent stimulation of ductal and topically expressed AE2 by CA12.\textsuperscript{95} The E143K mutation is a folding mutation that resulted in retention of CA12(E143K) in the ER.\textsuperscript{95} How exactly CA12 with an external catalytic site activates AE2 is not obvious. CA12 may clear the extruded HCO$_3^-$ from the membrane surface to prevent its buildup at the mouth of the AE2. If this can be established, it will be a new mode of regulating HCO$_3^-$ transporters by CAs.
Regulation and Mechanism of Pancreatic HCO$_3^-$ Secretion

**Intracellular signaling pathways: cAMP and Ca$^{2+}$**

The cAMP/PKA pathway is central in inducing ductal HCO$_3^-$ secretion. Secretin is the major hormone that activates the cAMP pathway. VIP also signals to increase cAMP via VIP receptors (VPAC1). At maximal receptor stimulation, the cAMP/PKA pathway can fully activate fluid and HCO$_3^-$ secretion by activation of the apical CFTR and the basolateral Na$^+$/HCO$_3^-$ cotransporter, NBCe1-B. However, at physiological conditions the cAMP/PKA pathway synergizes with the Ca$^{2+}$ signaling pathway to activate the secretory process (see below).

Several agonists that act on the pancreatic duct engage the Ca$^{2+}$ signaling pathway. These include CCK, cholinergic stimuli, P2Rs, and protease-activated receptor 2 (PAR2) receptors. When activated, the CCK and muscarinic receptors activate PLC$\beta$ to generate IP$_3$ that releases Ca$^{2+}$ from intracellular stores, mainly the endoplasmic reticulum (ER), and activates the membrane Ca$^{2+}$ influx channels, Orai and TRPC. P2Rs and PAR2 receptors also act through activation of the Ca$^{2+}$ signaling pathway at physiological stimulus intensity, the cAMP and Ca$^{2+}$ signaling pathways synergize to activate ductal secretion. Early studies in vivo already noted the synergistic action of ductal stimuli. Application of secretin at a level observed in the postprandial state only produces modest HCO$_3^-$ and fluid output. Application of CCK and stimulation of M1 and M3 receptors markedly augmented secretin-stimulated pancreatic fluid secretion, although alone CCK and muscarinic stimulation have minimal effect on ductal secretion.

The molecular mechanism of synergism was resolved with the discovery of regulation of ductal secretion by IRBIT, which is discussed below. The cAMP and Ca$^{2+}$ signaling pathways crosstalk on several additional known domains of IRBIT are PP1 and calcineurin-binding motif, a PEST domain, a coiled-coil domain, and a PDZ sequence while it lacks the hydrolase activity.

IRBIT was isolated as a protein that interacts with the IP$_3$ binding pocket of the receptors (IP$_3$Rs) and it can be disassociated from the IP$_3$Rs by IP$_3$$_{3.50}$ IRBIT competes with IP$_3$ for binding to the IP$_3$Rs$_{51}$ to inhibit Ca$^{2+}$ release. In fact, the IP$_3$Rs appear to function as IRBIT buffers to prevent IRBIT access to many transporters and targets regulated by IRBIT.$^{46}$ The C-terminal region of IRBIT family proteins shows ~50 percent homology with the ubiquitous housekeeping enzyme S-adenosyl-l-homocysteine hydrolase (AHCY), with IRBIT having additional N terminal sequence while it lacks the hydrolase activity.$^{152}$ The main known domains of IRBIT are PPI and calcineurin-binding motif, a PEST domain, a coiled-coil domain, and a PDZ ligand at the end of C terminus.$^{46,63}$

IRBIT plays an important role in pancreatic ductal secretion by regulating multiple transporters and mediating the synergistic action of the cAMP/PKA and Ca$^{2+}$ signaling pathways (Figure 2). Knockdown of IRBIT in ducts and knockout in mice modestly inhibit fully stimulated pancreatic duct fluid and HCO$_3^-$ secretion$^{138}$ and eliminates the physiological synergistic action of the cAMP/PKA and Ca$^{2+}$ signaling pathways.$^{21}$ IRBIT accumulates at the apical pole where IP$_3$Rs are highly expressed, but it can be found all over the cell where IP$_3$Rs are present.$^{153}$ A search for IRBIT-binding proteins identified NBCe1-B as a binding partner, where IRBIT binds to the N terminus autoinhibitory domain of NBCe1-B to activate it by removing the autoinhibition.$^{60}$ Subsequent detailed studies, in particular with the pancreatic duct, revealed that IRBIT at the apical pole potently activates the apical CFTR,$^{16,138}$ SLC26A6,$^{21}$ and NHE3.$^{107}$ At the basal side, IRBIT regulates NBCe1-B.$^{16,66,138}$ IRBIT activates the transporters by multiple mechanisms. First, IRBIT recruits protein phosphatase 1 (PP1) to the transporters to dephosphorylate serine residue 75 in NBCe1-B and yet to be identified residue in CFTR that are phosphorylated by the kinase SPAK. For these phosphorylations, SPAK must be activated by phosphorylated by the two kinases WNK1 and/or WNK4.$^{52}$ IRBIT also recruits the phosphatase calcineurin to dephosphorylate serine residue 12 that is phosphorylated by CaMKII.$^{63}$ This enhances the plasma membrane relocation of NBCe1-B, CFTR,$^{16}$ and slc26a6$^{21}$ from intracellular vesicular pools. At the plasma membrane, IRBIT directly interacts with the transporters to further increase their activity. Moreover, phosphorylation by SPAK and CaMKII and dephosphorylation by the respective phosphatases PP1 and calcineurin determine regulation of NBCe1-B, and likely other IRBIT-regulated transporters, by Cl$^{-}$.$^{65}$ The mechanism by which IRBIT activates the other transporters is not known at this time beyond the need for the PDZ-binding motif of IRBIT for assembling the IRBIT-NBCe1-B and IRBIT-CFTR complex.$^{138}$

IRBIT and synergism

An important action of IRBIT is mediating the synergistic action of the cAMP/PKA and Ca$^{2+}$ signaling pathways$^{21}$ (see Figure 2). Physiological stimulus intensity must be quite weak to prevent cell toxicity that occurs under strong stimulation of all signaling pathways. Indeed, at
physiological stimulus intensity the secretory process is activated only by about 5–10 percent or less of maximal stimulation. Synergism between weakly stimulated signaling is used to generate the maximal response while avoiding cell toxicity and increasing fidelity. IRBIT mediates the synergism between the cAMP/PKA and Ca\(^{2+}\) signaling pathways by translocation between cellular compartments and transporters. At the resting state, IRBIT is sequestered by the high level of IP\(_3\)Rs at the ductal ER apical pole and is not available for interaction with the transporters. The affinity of the IP\(_3\)Rs for IRBIT and IP\(_3\) is regulated by PKA-mediated phosphorylation of specific IP\(_3\)Rs serine residues. Phosphorylation of the serine residues increases the affinity for IP\(_3\) and at the same time decreases the affinity for IRBIT. Now, a small increase in IP\(_3\) evoked by weak stimulation of the Ca\(^{2+}\) signaling pathway is sufficient to dissociate IRBIT from the IP\(_3\)Rs.\(^{21}\) The released IRBIT can bind to CFTR and Slc26a6 first in intracellular vesicles to dephosphorylate them by the IRBIT-recruited PP1 and calcineurin and promote their translocation to the luminal membrane. IRBIT also binds to the autoinhibitory domain of NBCe1-B to activate it. Increased surface expression of the transporters also aids pancreatic ductal HCO\(_3^-\) secretion (modified from reference \(^{146}\). See text for details).

**Figure 2. A model for IRBIT-associated pathway of pancreatic ductal fluid and HCO\(_3^-\) secretion.** Key domains of IRBIT related to HCO\(_3^-\) secretion are illustrated at the top of the figure. In the resting state, IRBIT is bound to IP\(_3\)Rs, and SPAK phosphorylates NBCe1-B, Slc26a6, and CFTR located at intracellular organelle. When the duct cells are stimulated, IP\(_3\) is released and bound to IP\(_3\)Rs, while IRBIT is disengaged from IP\(_3\)Rs. PP1 recruited to IRBIT dephosphorylates transporters located at the plasma membrane. IRBIT also binds to the autoinhibitory domain of NBCe1-B to activate it. Increased surface expression of the transporters also aids pancreatic ductal HCO\(_3^-\) secretion (modified from reference \(^{146}\). See text for details).
Regulation by $[\text{Cl}]_i$

WNK1 and dynamic regulation of CFTR HCO$_3^-$ permeability

The WNK proteins consist of four members (WNK1–WNK4) with a conserved kinase domain that is noted for the unique position of the catalytic lysine residue.\textsuperscript{154} The discovery that mutations in WNK1 and WNK4 cause hypertension in humans has attracted much attention to these kinases function and regulation.\textsuperscript{155} The main function of the WNKs is the regulation of Na$^+$, K$^+$, Cl$^-$, HCO$_3^-$, and Ca$^{2+}$ transporters in epithelia and brain.\textsuperscript{146,156–158} The WNKs act either by regulating surface expression of membrane transporters through modulation of their endocytosis or by phosphorylating the transporters and other target proteins directly or indirectly through affecting the effect of other kinases.\textsuperscript{157} Several functions of WNKs are mediated by phosphorylating and activating the downstream oxidative stress-responsive kinase 1 (OSR1) and SPAK.\textsuperscript{159} WNK1, WNK3, WNK4, SPAK, and OSR1 are expressed in the pancreatic ducts\textsuperscript{14,16} and participate in the regulation of HCO$_3^-$ transporters and channels.\textsuperscript{46} Accordingly, knockdown of WNK4 alone or a combined knockdown of WNK1, WNK3, and WNK4 increase pancreatic duct fluid secretion by removing a tonic negative effect on ductal HCO$_3^-$ transporters.\textsuperscript{16} However, the role of the WNKs, in particular WNK1, changes at the terminal portion of the duct when $[\text{Cl}]_i$ is reduced to below 10 mM. WNK1 and the other WNKs bind $[\text{Cl}]_i$ and their activity is regulated by $[\text{Cl}]_i$.\textsuperscript{85,86,160}

The role of WNK1 in pancreatic HCO$_3^-$ secretion is illustrated in the left portion of Figure 3. Osmotic stress or

![Figure 3](image-url)
low \([\text{Cl}^-]\) activates WNK1.\(^{85}\) Notably, activation of WNK1 by low \([\text{Cl}^-]\) greatly increases the \(\text{HCO}_3^-\) permeability of CFTR.\(^{14,117}\) During active pancreatic \(\text{HCO}_3^-\) secretion, lower \(\text{Cl}^-\) concentration in the pancreatic juice progressively reduces \(\text{Cl}^-\) absorption. Because of the low basolateral and high luminal \(\text{Cl}^-\) permeability,\(^{14,161}\) \([\text{Cl}^-]\) rapidly decreases in response to the reduction in luminal duct \(\text{Cl}^-\) concentration. At a membrane potential of -60 mV, \([\text{Cl}^-]\) is less than 1/10 of luminal \(\text{Cl}^-\). Indeed, ductal \([\text{Cl}^-]\) was estimated to be about 5 mM during cAMP-induced active secretion.\(^{14,161}\)

WNK1 modulates the anion selectivity of CFTR by changing its pore size.\(^{117}\) Stimulation by WNK1 increases CFTR pore size from 4.8 Å to 5.3 Å, which facilitates the passage of \(\text{HCO}_3^-\) (4.3 Å, diameter) more than the smaller anion, \(\text{Cl}^-\) (3.7 Å, diameter). Changes in pore size affect the energy barrier of ion dehydration by altering the electric permittivity of the water-filled cavity in the pore. Dielectric constant (relative permittivity, \(\varepsilon\)) is a unit of electric permittivity, and the dielectric constant of water \((\varepsilon_w)\) is approximately 80 at room temperature. Water molecules in confined geometry like ion channels exhibit a space-dependent reduction in the pore water \(\varepsilon_w\) down to 20, due to the restriction of the translational and rotational mobility of water molecules.\(^{162}\) Pore dilation relieves this restriction of water molecule movement and increases \(\varepsilon_w\), which eventually leads to an increase in the overall \(\varepsilon\) of the anion channel pore. Indeed, the pore dilation induced by WNK1 activation increased the \(\varepsilon\) of the CFTR pore from 16 to 43.\(^{117}\) In general, ions pass through the channel after dehydration (at least partial dehydration). Asymmetrically charged ions, such as \(\text{HCO}_3^-\), show lower permeability than the symmetrically charged ions, such as \(\text{Cl}^-\), due to the high hydration/dehydration energy barrier. The increase in anion channel pore \(\varepsilon\) greatly alleviates the dehydration penalty of the asymmetrically charged \(\text{HCO}_3^-\) and increases \(P_{\text{HCO}_3^-}\times P_{\text{Cl}^-}\) (Figure 4). In an initial study, WNK-OSR1 or WNK1-SPAK complex was suggested to increase CFTR \(P_{\text{HCO}_3^-}\times P_{\text{Cl}^-}\).\(^{14}\) However, subsequent study showed that WNK1 alone was sufficient to increase CFTR \(P_{\text{HCO}_3^-}\times P_{\text{Cl}^-}\).\(^{84}\)

Molecular dissection of the WNK1 domains revealed that the WNK1 kinase domain is responsible for CFTR \(P_{\text{HCO}_3^-}\times P_{\text{Cl}^-}\) selectivity by direct association with CFTR, while the surrounding N-terminal regions mediate the \([\text{Cl}^-]\) sensitivity of WNK1.\(^{84}\)

Although the precise WNK1-binding sites on the CFTR are not fully defined, examining pancreatitis-causing CFTR mutations revealed that R74 and R75 located in the first

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**Figure 4.** WNK1 modulates the anion selectivity of CFTR by changing the pore size. Stimulation by WNK1 increases the pore size of CFTR from 4.8 Å to 5.3 Å and the pore dilation increases the dielectric constant (\(\varepsilon\)) of the CFTR pore from 16 to 43. The increase in pore size facilitates the passage of the larger anion, \(\text{HCO}_3^-\) (4.3 Å, diameter), more than the smaller anion, \(\text{Cl}^-\) (3.7 Å, diameter) by reducing the energy barriers of size exclusion. More importantly, the dielectric constant increase enhances the \(\text{HCO}_3^-\) permeability of CFTR by reducing energy barriers required for ion dehydration of \(\text{HCO}_3^-\).\(^{117}\) (see text for details).
elbow helix region of the CFTR are involved in the WNK1-CFTR association [39]. A computational protein-protein docking analysis using the Protein Data Bank-deposited structures of human CFTR and the WNK1 kinase domain showed that WNK1 S231–T234 and I384–E388 can potentially bind to an intracellular CFTR region near R74–R75 in the elbow helix 1 (Figure 5). The handle-like elbow helix 1 is located immediately ahead of transmembrane domain 1 and contacts a proximally located lasso motif that has been suggested to play a role in CFTR gating and regulation of the R domain. Therefore, it appears that the binding of WNK1 kinase domain to the elbow helix 1 region of CFTR affects the CFTR open structure to facilitate \( \text{HCO}_3^- \) permeation.

Interestingly, activated WNK1 while increasing CFTR \( P_{\text{HCO}_3^-/\text{Cl}} \) and \( G_{\text{HCO}_3^-} \) does not lose the inhibitory effect on SLC26A6 and SLC26A3. When the luminal \( \text{HCO}_3^- \) concentration is greater than 140 mM, continuous activation of apical \( \text{Cl}^-/\text{HCO}_3^- \) exchange would reverse to absorb \( \text{HCO}_3^- \) from the lumen. This is more of a problem for the 2\( \text{Cl}^-/1\text{HCO}_3^- \) exchange by SLE26a3 and less, if at all, for the 1\( \text{Cl}^-/2\text{HCO}_3^- \) SLE26a6, especially at membrane potential of -60 mV across the luminal membrane. However, inhibition of the apical \( \text{Cl}^-/\text{HCO}_3^- \) exchangers is required to prevent the reverse mode of \( \text{Cl}^-/\text{HCO}_3^- \) exchange activity if SLE26a3 dominates the exchange when ductal \([\text{Cl}]_i\) is below 10 mM and ultimately achieves \( \text{HCO}_3^- \) concentration above 140 mM in pancreatic juice.75,164

Figure 5. Structural model for the molecular complex between hCFTR and the WNK1 kinase domain in the presence of a lipid bilayer. The R74 (yellow balls) and R75 (orange balls) residues from hCFTR participate in the binding interface. The figure shows the hCFTR-WNK1 complex predicted by ClusPro, after equilibration in the MD simulation system where it is embedded into the membrane lipids (lines with their phosphorus atoms shown as purple spheres) and solvated by 0.1 M NaCl solution. The snapshot was taken after 100 ns MD simulations. The inset figure shows a close-up view of interfacial interactions. WNK1 residues at the interface include S231, K233, T234, T386, and E388 (modified from reference 84).
that mediate high- and low-affinity inhibition by Cl\(^-\) emerged as a signaling ion by regulating several ion transporters and channels. A comprehensive review of Cl\(^-\) as a bona fide signaling ion can be found in reference \(^23\). Here we discuss the signaling function of Cl\(^-\) in pancreatic ductal function. By virtue of regulating the function of the WNK kinases [Cl\(^-\)], may affect other transporters regulated by these kinases. A significant discovery is that [Cl\(^-\)] profoundly regulates the activity of several Na\(^+\)-HCO\(_3\) cotransporters (NBCs) at the [Cl\(^-\)]-physiological range. \(^62,63\) [Cl\(^-\)] regulates the activity of all NBCs tested NBCe1-B, NBCe1-C, and NBCe1-A. The IRBIT-independent activity of NBCe1-B is inhibited by [Cl\(^-\)], between 60 and 140 mM that is outside the physiological range and may function to inhibit NBCe1-B activity under pathological conditions. Most notably, when activated by IRBIT, NBCe1-B activity is reduced by [Cl\(^-\)], in the range of 5–20 mM, where at 20 mM [Cl\(^-\)], NBCe1-B activity is reduced to the basal, IRBIT-independent level. Molecular analysis identified two Cl\(^-\) interacting motifs at the N terminus of NBCe1-B that mediate high- and low-affinity inhibition by [Cl\(^-\)\]. Regulation of NBCe1-B is mediated by sites that contain the GXXXP motif. The first site mediates the high [Cl\(^-\)] affinity (5–20 mM) regulation of NBCe1-B and the second site mediates the low [Cl\(^-\)] affinity (60–140 mM) regulation of NBCe1-B. \(^62\) NBCe2-C activity is not regulated by IRBIT and in this case regulation of NBCe1-C is mediated by a single site containing the GXXXP motif and takes place at [Cl\(^-\)], between 10 and 30 mM. Regulation of NBCe1-A by [Cl\(^-\)] is mediated by a cryptic Cl\(^-\) interacting site containing the GXXXP motif. The cryptic NBCe1-A [Cl\(^-\)]-interacting sites were unmasked by deletion of residues 29–41. Further analysis showed that interaction of Cl\(^-\) with the GXXXP sites is regulated by phosphorylation/dephosphorylation events with SPAK and PP1 acting on serine 65 to affect Cl\(^-\) sensing by the \(^32\)GXXXP\(^36\) site, while CaMKII and calcineurin acting on serine 12 to affect Cl\(^-\) sensing by the \(^194\)GXXXP\(^198\) site. \(^63\) Other phosphorylation sites affecting NBCe1-B activity are Ser232, Ser233, and Ser235 with the phosphorylation status of Ser232, Ser233, and Ser235 is regulated by IRBIT to determine whether NBCe1 transporters are in active or inactive conformations. \(^63\)

Hence, cells have a [Cl\(^-\)] sensing mechanism that plays an important role in the regulation of Na\(^+\) and HCO\(_3\)\(^-\) transporters that mediate the critical step of HCO\(_3\)\(^-\) influx in the process of ductal fluid and HCO\(_3\)\(^-\) secretion. At [Cl\(^-\)], of up to 20 mM, CFTR functions mostly as a Cl\(^-\) channel and slc26a6 mediates most ductal HCO\(_3\)\(^-\) secretion. As [Cl\(^-\)] is reduced below 20 mM and additional HCO\(_3\)\(^-\) secretion takes place in the face of unfavorable Cl\(^-\) and HCO\(_3\)\(^-\) gradients across the apical membrane, there is an increased demand for HCO\(_3\)\(^-\) entry across the basolateral membrane. Pancreatic duct cells achieve this by [Cl\(^-\)]-mediated regulation of NBCe1-B and CFTR, at which NBCe1-B activity and CFTR HCO\(_3\)\(^-\) permeability gradually increase as [Cl\(^-\)] is reduced toward 5 mM.

### A model for pancreatic HCO\(_3\)\(^-\) secretion

Electrogenic HCO\(_3\)\(^-\) transporters can generate higher gradients of HCO\(_3\)\(^-\) than electroneutral transporters when the electrorepulsive force generated by the negative membrane potential is coupled to the basolateral uptake of HCO\(_3\)\(^-\) by NBCe1-B and the luminal efflux of HCO\(_3\)\(^-\) through SLC26A6 and CFTR. The electrogenic SLC26A6 exchanger with the stoichiometry of 1Cl\(^-\)\::2HCO\(_3\)\(^-\) can achieve luminal HCO\(_3\)\(^-\) concentration of up to about 120 mM at apical membrane potential of ~60 mV. \(^75\) To drive luminal HCO\(_3\)\(^-\) concentration to 140 mM, the physiologic HCO\(_3\)\(^-\) concentrations in pancreatic juice, another mechanism is needed. \(^75\) Such a mechanism should be Cl\(^-\) independent, since significant fraction of pancreatic HCO\(_3\)\(^-\) secretion is retained in the absence of luminal Cl\(^-\). \(^82,165\) The WNK1-activated CFTRs satisfy these requirements. At HCO\(_3\)\(^-\) in stimulated duct cells above 25 mM and membrane potential of ~60 mV, CFTR mediates HCO\(_3\)\(^-\) efflux even at luminal HCO\(_3\)\(^-\) concentrations of above 140 mM. Transport by NBCe1-B and Slc26a6 results in osmotic transport of HCO\(_3\)\(^-\) that is essential for the transcellular water transport by the duct. The transcellular basal to luminal electrogenic HCO\(_3\)\(^-\) transport by both Slc26a6 and CFTR generates a lumen-negative electrical potential that results in paracellular Na\(^+\) secretion. Water flows down the osmotic gradient generated by the Na\(^+\) and HCO\(_3\)\(^-\) fluxes via paracellular and transcellular (aquaporins) pathways (Figure 3). Overall, these processes generate an efficient mechanism for HCO\(_3\)\(^-\)-driven ductal fluid secretion to generate the volume and HCO\(_3\)\(^-\) content of the pancreatic juice.

### Conclusions

The mechanism by which the human pancreatic duct secretes nearly isotonic HCO\(_3\)\(^-\) solution has long been an enigmatic question for both physiologists and clinicians. \(^25\) When Bayliss and Starling first noticed that the exocrine pancreas secretes alkaline fluid, they assumed that carbonate is responsible for the strong alkalinity of the pancreatic juice. \(^166\) Later, with better understanding of the carbonate/HCO\(_3\)\(^-\)/CO\(_2\) buffer system, \(^167\) it became clear that the exocrine pancreas secretes fluid in which the dominant anion is HCO\(_3\)\(^-\), and HCO\(_3\)\(^-\) secretion is coupled to fluid secretion. \(^144,168,169\) Current understanding indicates that activation of three key transporters, the basolateral NBCe1-B (and likely AE2), and the luminal SLC26A6 and CFTR, and their synergistic regulation by the cAMP and Ca\(^{2+}\) signaling pathways through IRBIT
and WNK1 perform vectorial pancreatic HCO$_3^-$ secretion that drives fluid secretion. NBCe1-B, with a 1 Na$^+/2$ HCO$_3^-$ stoichiometry, is the main HCO$_3^-$-concentrating transporter in the basolateral membrane and can achieve the necessary HCO$_3^-$ influx. Basolateral AE2 activity is also required to support ductal HCO$_3^-$ fluid and HCO$_3^-$ secretion probably by controlling cytoplasmic and near membrane pH$_{in}$, although the exact role of AE2 is not known at present. The electrogenic SLC26A6, with a 1 Cl$^-$/HCO$_3^-$ exchanger, which mediates most HCO$_3^-$ efflux in the early step of pancreatic HCO$_3^-$ secretion. Activated WNK1 increases HCO$_3^-$ permeability and conductance of CFTR, allowing further apical HCO$_3^-$ efflux and setting the pancreatic juice HCO$_3^-$ concentrations above 140 mM. Ductal fluid and HCO$_3^-$ secretion is essential for the function of the pancreas and is severely altered in all forms of pancreatitis. Our understanding of the mechanism of pancreatic fluid and HCO$_3^-$ secretion will continue to improve as our knowledge of existing pathways increases and new mechanisms are identified and delineated, to provide a better scientific basis for therapeutic approaches to treat diseases like cystic fibrosis and acute and chronic pancreatitis.

**Acknowledgments**

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**References**


93. Stewart AK, Yamamoto A, Nakakuki M, Kondo T, Alper SL, Ishiguro H. Functional coupling of apical Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchange with CFTR in stimulated HCO\textsubscript{3}\textsuperscript{−} secretion by guinea pig interlobular pancreatic duct. Am J Physiol Gastrointest Liver Physiol 2009; 296:G1307–G1317.
95. veel T, Villanger O, Holthe MR, Cracoe EJ, Jr., Raeder MG. Na\textsuperscript{+}/H\textsuperscript{+) exchange is not important for pancreatic HCO\textsubscript{3}\textsuperscript{−} secretion in the pig. Acta Physiol Scand 1992; 144:239–246.


Chapter 20

Ion channels in pancreatic duct physiology

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Physiology of Pancreatic Ductal Cells

The main function of pancreatic ductal cells is to secrete a HCO$_3^-$-rich, isotonic fluid that washes out the inactive form of digestive enzymes from the ductal system, as well as provides pH conditions that are essential for normal pancreatic function. The rate of HCO$_3^-$ secretion is influenced by several factors (such as secretory rate, species, or the location of the cell in the ductal tree), and with stimulation, HCO$_3^-$ can reach up to 140 mM. This means a significant concentration difference exists between the outside and the inside of the ductal cell, which poses a physiologic challenge to duct cell homeostasis. The high level of HCO$_3^-$ secretion is achieved through the coordinated action of ion transporters and channels, in which the Cl$^-$/HCO$_3^-$ exchanger and the cystic fibrosis transmembrane conductance regulator (CFTR) Cl$^-$ channel play a central role.

Ion transporters and channels on ductal cells are differentially expressed on the luminal and basolateral membranes, resulting in functional polarization of the ductal cells (Figure 1). The major ion transporters on the duct cell basolateral membrane include the Na$^+/H^+$ exchanger (NHE), the Na$^+/HCO_3^-$ cotransporter (NBC), the Na$^+/K^+$ ATPase, and various types of K$^+$ channels. The electroneutral, NHE1 isoform can be found on the basolateral membrane of ductal cells and acts as a proton extruder with a 1 Na$^+$:1 H$^+$ stoichiometry. In addition to playing an important role in the regulation of intracellular pH, NHE1 promotes the formation of HCO$_3^-$ from carbonic acid by removing excess H$^+$ from the cell. The NHE3 isoform is located on the luminal membrane of ductal cells and, unlike NHE1, is involved in HCO$_3^-$ salvage. HCO$_3^-$ also enters the cell directly through NBC. The electrogenic NBC isoform NBCe1B has been identified on the basolateral membrane of ductal cells, with a 1 Na$^+$/2 HCO$_3^-$ stoichiometry. Through this transporter, more HCO$_3^-$ enters the cell than is formed during the dissociation of carbonic acid. The electroneutral form of NBC, the NBCn1 or NBC3, has been shown to be active on the luminal membrane of ductal cells, where it plays a role in HCO$_3^-$ salvage. In addition, Na$^+/K^+$ ATPase, together with basolateral K$^+$ channels, maintains a negative membrane potential using the energy from the hydrolysis of ATP, which provides a driving force for anion secretion across the luminal membrane.

There are two major transporters on the luminal side of the ductal cells: the CFTR chloride channel and the Cl$^-$/HCO$_3^-$ exchanger. Several mechanisms have been proposed to explain how ductal cells can secrete up to five times as much HCO$_3^-$ as is present in the cytosol. The most generally accepted view is that HCO$_3^-$ secretion occurs through both the Cl$^-$/HCO$_3^-$ exchanger and the CFTR channel. Among the Cl$^-$/HCO$_3^-$ exchangers, the Slc26a6 (PAT1) and the Slc26a3 (DRA) isoforms are present on pancreatic ductal cells. PAT1 and DRA have different Cl$^-$/HCO$_3^-$ stoichiometry (2:1 for DRA and 1:2 for PAT1) and are expressed in different parts of the ductal tree. In the initial stage of HCO$_3^-$ secretion, HCO$_3^-$ is secreted into the lumen through the Cl$^-$/HCO$_3^-$ exchanger in exchange for Cl$^-$ that is returned to the lumen through the CFTR Cl$^-$ channel. Luminal concentration of HCO$_3^-$ and Cl$^-$ follows a reciprocal pattern that is stimulation dependent. With increased secretory volume the concentration of HCO$_3^-$ increases in the lumen and Cl$^-$ decreases. At the same time, the WNK1-OSR1/SPAK signaling pathway is activated, which increases the permeability of the CFTR channel to HCO$_3^-$, which allows HCO$_3^-$ concentration to reach 140 mM HCO$_3^-$ in the lumen.

Pathophysiology of Pancreatic Duct Cells

Normal electrolyte secretion is required to maintain pancreatic exocrine cell homeostasis and solubilization of secretory protein; impairment of pancreatic fluid and electrolyte secretion contributes to tissue destruction in diseases such as cystic fibrosis (CF). An important consequence of impaired
HCO₃⁻ secretion is an acidic pancreatic juice (less than 6.5). This increases mucus viscosity and decreases the solubility of secreted digestive enzymes, which predisposes to the formation of mucin/protein plugs and eventually cysts within the ductal tree. A more acidic pH may also induce premature activation of digestive enzymes within the ductal tree, leading to the development of pancreatitis. Therefore, intensive research is underway to develop drug molecules capable of restoring the function of transporters, especially in CF.

Cystic fibrosis

CF is the most common, life-limiting, inherited disease in Caucasian populations (1 in 3,500 newborns in Europe).¹⁶,²⁰ Over 2,000 CF-causing mutations have been identified in the cftr gene (http://www.genet.sickkids.on.ca/), although to date only ~360 of these variants have been fully annotated (https://cftr2.org/). However, 70–90 percent of CF individuals harbor the F508del mutation on at least one allele,²¹ which results in misfolding and incorrect processing of CFTR to the apical membrane. For those mutations that have been studied in detail, the genetic alteration leads to a variety of functional defects in the CFTR protein. These functional defects have been grouped into six classes.²² Classes 1–3 cause severe CFTR dysfunction, while Classes 4–6 produce less severe effects on CFTR, and, in general, the mutated protein retains some level of channel activity. In relation to pancreatic pathology, ~85 percent of people with CF are born pancreatic insufficient (PI), which equates to a reduction in pancreatic function of more than 95 percent. In these people, there is a very good correlation between pancreatic disease severity and the class of mutation with “severe” CF mutations, such as the most common Class 2 CF mutation, F508del, and the Class 3 gating mutant, G511D, strongly correlate with PI. For those with “milder” mutations (some residual channel activity such as Class 4, R117H), pancreatic function is preserved (pancreatic sufficient (PS)), albeit to differing levels. However, in general, these PS individuals require less pancreatic enzyme replacement supplements but can become PI with increasing age. Mutations that cause moderate deficiencies in CFTR activity (10–50% of normal function) can increase the risk of developing pancreatitis, alone or when combined with other risk factors such as alcohol.

As described in the introduction, CFTR plays a fundamental role in pancreatic ductal NaHCO₃ and fluid secretion, where it regulates HCO₃⁻ secretion in two fundamentally different ways: first, as a direct exit pathway for HCO₃⁻ secretion and, second, as a regulator of SLC26A-mediated Cl⁻/HCO₃⁻ exchange.¹ For the latter process, this involves physical interaction between CFTR and the SLC26A6 anion exchanger,¹⁴,²⁶–²⁸ and loss of functional CFTR leads to loss of anion exchange activity. The exact mechanism underlying this complete loss of anion exchange...
activity is not fully understood, but studies from polarized cultures of CFPAC cells, a human CF pancreatic ductal cell line homozygous for F508del, showed that apical SLC26A3 Cl⁻/HCO₃⁻ exchange activity was absent, despite evidence for mRNA expression. Importantly, viral-mediated CFTR transduction of the CFPAC cells restored anion exchange activity, suggesting that CFTR may be required for the trafficking of SLC26A6 to the apical membrane. Furthermore, it is interesting that the anion exchanger is activated by a number of CFTR mutants that lack Cl⁻ channel activity and that this correlates with a good retention of pancreatic function in patients carrying those mutations. Taken together, these results strongly suggest that a functional CFTR at the apical plasma membrane is a prerequisite for SLC26A6-mediated anion exchange and that mild CFTR mutations are likely to preserve Cl⁻/HCO₃⁻ exchange activity, although this needs formal demonstration.

Strategies for improving HCO₃⁻ secretion in the CF pancreas are limited because of the marked tissue destruction at birth in the majority of people with CF. However, the last decade has seen a major improvement in the treatment of the basic defect in CF, through the development of small molecule CFTR modulators. Clinically approved drugs include the CFTR potentiator, Ivacaftor, which enhances CFTR open probability for a number of gating mutants, as well as CFTR correctors, which help restore processing of CFTR trafficking mutants (including F508del-CFTR) to the apical membrane. These include the first-generation corrector drug Lumacaftor as well as more efficacious second-generation correctors, such as tezacaftor and elexacaftor. These drugs are given either alone or in combination (double and triple combinations), depending on the functional defects(s), and have produced substantial improvements in lung function, number of hospitalizations and exacerbations, as well as BMI. Since lung dysfunction is the major cause of morbidity and mortality in people with CF, median survival rates are predicted to significantly improve. However, there are only a few studies that have directly assessed if these CFTR modulators also improve pancreatic function. For example, pancreatic function measurements in young children with CF taking Ivacaftor over 24 weeks showed a significant restoration of enzyme-secreting capacity (increased fecal elastase-1 levels) and, by inference, pancreatic tissue regeneration, which is an extremely exciting finding that warrants further research. A more recent but limited study also provided evidence that Ivacaftor restored some pancreatic function in an adult with CF. 

**Acute pancreatitis**

Acute pancreatitis (AP) is a sudden inflammation of the pancreas, which in most cases is mild, but in approximately 20 percent of patients, a life-threatening, severe form can develop in which the mortality rate can reach up to 20–40 percent. Large individual differences can be observed in the development and course of the disease, in which the disturbed balance of protective and damaging factors presumably plays a significant role. Pancreatic ductal cells are considered as protective mechanism in the pancreas by the secretion of a HCO₃⁻ rich, isotonic fluid. The two main etiological factors in the development of AP are gallstone obstruction and excessive alcohol consumption. Both etiologies associated with marked changes in HCO₃⁻ and fluid secretion based on in vitro intracellular pH and fluid transport studies from isolated microdissected ducts. At low concentrations, both agents increased HCO₃⁻ secretion, a response that required CFTR and Cl⁻/HCO₃⁻ exchange activity. However, higher levels of these agents led to a severe inhibition of CFTR-dependent HCO₃⁻ secretion, which was due to profound mitochondrial damage and a consequent reduction in intracellular ATP levels. These studies were the first to suggest that ductal HCO₃⁻ secretion could play a protective role against these noxious agents.

**Bile acids**

Under normal conditions, bile cannot enter the pancreatic ductal system; however, in the case of gallstone obstruction it may regurgitate into the pancreas through the common bile duct. Chenoxychololate (CDCA) is the most abundant hydrophobic, unconjugated bile acid in human bile. Using isolated guinea pig pancreatic ducts, it has been shown that luminal administration of CDCA at low concentrations (0.1 mM) significantly increases ductal HCO₃⁻ secretion, in which CDCA-induced intracellular Ca²⁺ oscillation plays an important role. The major source of CDCA-induced Ca²⁺ is the endoplasmic reticulum (ER), from which Ca²⁺ is released via an IP₃-mediated pathway. The released Ca²⁺ activates large-conductance Ca²⁺-activated K⁺ channels (BKCa) on the luminal membrane of ductal cells, and opening of these channels hyperpolarizes the cell membrane, thereby enhancing the electrochemical driving force for anion secretion through the luminal membrane. This stimulatory effect of CDCA has also been demonstrated in the CFPAC-1 cell line and has been shown to be highly dependent on CFTR expression. In contrast, high concentration of this bile acid (1 mM) causes a toxic increase in Ca²⁺ and strongly inhibits HCO₃⁻ secretion. The inhibitory effect of high concentrations of bile acids presumably results from the fact that at this concentration CDCA damages mitochondria, resulting in ATP depletion and ultimately apoptosis. This dual effect of bile acid is thought to be important in the pathogenesis of AP. In the early stages of the disease, when bile acids reach ductal cells at low concentrations, the increased fluid secretion tries to wash out the toxic bile acids from the ductal...
Table 1. The effects of bile, ethanol, and trypsin on pancreatic ductal HCO$_3^-$ secretion.

<table>
<thead>
<tr>
<th>Agent</th>
<th>concentration</th>
<th>Ductal HCO$_3^-$ secretion</th>
<th>Mechanism of action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile acids</td>
<td>0.1 mM</td>
<td>Increase</td>
<td>Induction of physiological Ca$^{2+}$ signaling and activation of BK$_{Ca}$ channels at the luminal membrane of the ductal cells</td>
<td>41, 42, 43</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>Decrease</td>
<td>Induction of a huge, sustained (Ca$^{2+}$)$^j$ signal, damage of mitochondria and consequently ATP depletion</td>
<td>40, 42, 46</td>
</tr>
<tr>
<td>Ethanol</td>
<td>10 mM</td>
<td>Increase</td>
<td>Induction of physiological Ca$^{2+}$ signaling</td>
<td>38, 39, 48</td>
</tr>
<tr>
<td></td>
<td>100 mM</td>
<td>Decrease</td>
<td>Induction of a huge, sustained (Ca$^{2+}$)$^j$ signal, damage of mitochondria and consequently ATP depletion. Inhibition of CFTR activity and expression</td>
<td>38, 39, 48</td>
</tr>
<tr>
<td>Trypsin</td>
<td>10 µM</td>
<td>Decrease</td>
<td>Elevation of (Ca$^{2+}$)$^j$ and inhibition of the Cl-/HCO$_3^-$ exchanger and CFTR, which leads to the acidification of the luminal pH and autoactivation of trypsinogen</td>
<td>49</td>
</tr>
</tbody>
</table>

tree to avoid pancreatic damage. If the concentration of bile acids further increases, it energetically destabilizes the cell, inhibits the function of ion transporters and induces apoptosis. Bile acids reach the acini where they induce inflammatory processes. Interestingly, the toxic effect of bile acids highly depends on their hydrophobicity. The hydrophilic bile acid, Ursodeoxycholic acid, the 7-alpha enantiomer of CDCA, is able to counteract the cell-damaging effects of a high dose of CDCA through stabilization of the mitochondrial membrane that raises the possibility of therapeutic use of this bile acid. 46, 47

Ethanol

Ethanol (EtOH) also dose-dependently affects ductal HCO$_3^-$ secretion (Table 1). Low concentration of EtOH (0.3–30 mM) enhances both basal and secretin-stimulated ductal fluid secretion from intra-interlobular ducts isolated from guinea pigs, in which cAMP activation and Ca$^{2+}$ release play a key role. 48 The stimulatory effect of low concentrations of EtOH has also been demonstrated in the Capan-1 cell line and has been shown to be dependent on Ca$^{2+}$ release from the ER via an IP$_3$-mediated pathway. 39 In contrast, high concentrations of EtOH (100 mM) strongly inhibited the rate of HCO$_3^-$ secretion and the activity of CFTR. 39, 48 The inhibitory effect of EtOH is presumably mediated by fatty acids (FAs) and fatty acid ethyl esters (FAEEs) formed during the non-oxidative metabolism of EtOH. Similar to the effect of bile acids, EtOH and its non-oxidative metabolites induce toxic Ca$^{2+}$ signaling in ductal cells by completely depleting ER stores and promoting extracellular Ca$^{2+}$ uptake into cells. Persistently elevated Ca$^{2+}$ causes mitochondrial Ca$^{2+}$ overload, resulting in decreased mitochondrial membrane potential and ATP production. Chelation of Ca$^{2+}$ abolished the inhibitory effect of EtOH and FA on HCO$_3^-$ secretion, suggesting that the inhibitory effect of high dose of these agents is mediated by toxic intracellular Ca$^{2+}$. 39 Moreover, EtOH and its metabolites profoundly inhibit CFTR function on the ductal cells which can be prevented by the supplementation of intracellular ATP (ATP$_i$), indicating that the inhibitory effect of EtOH on CFTR is mediated by ATP$_i$ depletion. Since CFTR works in close coordination with the Cl$^-$/HCO$_3^-$ exchanger, improper CFTR function may contribute to decreased fluid and HCO$_3^-$ secretion and thus to the pathogenesis of AP. Consequently, maintenance of ATP$_i$ may represent a therapeutic option in the treatment of the disease. 38, 39 Decreased expression of CFTR has also been observed on the luminal membrane of human pancreatic ductal cells in alcohol-induced acute and chronic pancreatitis, and in response to FAs and FAEEs in which the accelerated turnover and decreased biosynthesis of the channel play a role. The importance of CFTR in the alcohol-induced pancreatic damage has been further confirmed in CFTR knockout mice, where the absence of CFTR caused much more severe pancreatitis. 39

Trypsin

One of the most important roles of ductal HCO$_3^-$ secretion is to prevent intraductal activation of trypsinogen. Although there is no significant trypsin in the lumen under physiological conditions, it may leak from the acinar cells in the early stages of pancreatitis. By binding to PAR-2 receptors on the luminal membrane of ductal cells, trypsin or trypsin-activating peptide (PAR-2-AP) increases [Ca$^{2+}$], levels and inhibits Cl$^-$/HCO$_3^-$ exchange and CFTR function, resulting in lower luminal pH. 49 Low luminal pH favors premature activation of trypsinogen, which will activate additional trypsinogen molecules. This process leads to a vicious cycle in which the more trypsin is formed, the more trypsinogen will be activated, resulting in even more inhibition of the activity of the luminal transporters. 49 The importance of PAR-2 activation in the pathobiology of pancreatitis has been also demonstrated using PAR-2 knockout mice, in
which luminal administration of either trypsin or PAR-2-AP had significantly lower effect on both \([\text{Ca}^{2+}]\) and \(\text{pH}_i\).

**Therapeutic Perspectives and Clinical Significance**

The CFTR chloride channel is clearly the most investigated and the most utilized ductal channel that has been targeted for therapy.\(^{50,51}\) In the first decades after the discovery of the CFTR gene, only symptomatic therapy was available. Difficulties over the years have been caused by the heterogeneity of CFTR gene mutations. Therefore, it is almost needless to say, that CF is typically a disease where personalized therapy needs to be considered. However, one approach that would be potentially suitable for all people with CF is gene therapy. The first randomized clinical trial, of a non-viral-based gene therapy for CF, was performed by E. Alton et al. They found that a 12-month treatment by pGM169/G67A gene therapy formulation improved lung function among the CF patients.\(^{52}\) Although the results were very promising, no further trials have taken place since the original observation, although preclinical development of a viral-mediated gene therapy treatment for CF is underway (https://www.cff.org/Trials/Pipeline/details/10160/Spirovant-Sciences). However, in the last decade the orally bioavailable correctors, potentiators, and suppressors of CFTR gene mutations have become available for treatment.\(^{53}\)

CFTR-directed therapies may also be useful for the treatment of pancreatitis, since recent animal studies have suggested that strategies that help maintain levels of \(\text{HCO}_3^-\) secretion would limit the extent of pathology induced by bile and alcohol.\(^{39,40,54}\) Furthermore, the effects of ethanol and ethanol metabolites on CFTR are consistent with reduced biogenesis, accelerated plasma membrane turnover, as well as channel inhibition.\(^{39}\) Thus, restoring cell surface expression and activity of CFTR could partially alleviate the ethanol-induced damage. This potentially could be through the use of CFTR correctors (Lumacaftor, Tezacaftor) and potentiators (Ivacaftor) to improve channel activity. We have recently found that Ivacaftor (VX-770) and Lumacaftor (VX-809) restore CFTR expression defect associated with alcohol in pancreatic ductal cells, suggesting that their combination sold as Orkambi® may serve as a therapeutic option in acute, recurrent, or chronic pancreatitis.\(^{55}\) Akshintala et al. recently showed that CFTR modulators, alone or in combination, reduced the risk of recurrent acute pancreatitis within a three-year follow-up therapy in adult CF patients.\(^{56}\) A 24-year-old CF patient with R117H/7 T/F508del mutations with recurrent acute pancreatitis were reported pancreatitis free during Ivacaftor therapy.\(^{57}\) Carrion et al. also found a reduced frequency and recurrence rate of pancreatitis in patients with CF during Ivacaftor therapy.\(^{58}\) Both the basic research results and the pancreatitis-free periods achieved in CF patients suggest that the drugs used for treating CF patients should be tested in randomized clinical trials in non-CF patients with recurrent pancreatitis as well.

Another possible way to compensate for defective CFTR would be to target alternative ion channels, such as the calcium-activated Cl channel, TMEM16A,\(^{59,60}\) or transporters such as the SLC26 family members (A3, A6, or A9), or short-circuiting their regulation by CFTR and rebalancing exocrine homeostasis. It has been shown that variants (SNPs) in the SLC26A9 anion transporter influence disease severity in the CF lungs and intestinal tract and therefore act as gene modifiers. Importantly, a recent study has suggested that SNPs in SLC26A9 also influence the degree of pancreatic insufficiency.\(^{61}\) Furthermore, variants of SLC26A9 also influence the extent of CF-related diabetes, which may be due to beneficial effects of restoring ductal bicarbonate secretion on endocrine (islet) function in CF.\(^{62}\) This opens up the possibility of targeting this anion transporter as a potential therapeutic target to slow the progression of exocrine dysfunction in CF. One important advantage of this “alternate non-CFTR approach” is that it would benefit all CF patients regardless of genotype.

**Summary**

One of the most important functions of ductal cells is their ability to neutralize acidic \(\text{pH}\) within the pancreas and duodenum. This ability of ductal cells is due to the secretion of a \(\text{HCO}_3^-\)-rich fluid, which results from the coordinated action of ductal ion channels and transporters. Impairment of transport processes can result in a decrease in both the volume and the \(\text{pH}\) of pancreatic fluid, which can predispose to inflammatory processes and consequently the development of various diseases. In the case of CF, it is well known that the inadequate function of the CFTR channel underlies the disease; however, it is only recently that research has shed light on the pathological role of ductal cells in pancreatitis. Various etiological factors such as bile acids and EtOH are now known to impair ductal \(\text{HCO}_3^-\) and fluid secretion, which are likely to play an important role in initiating pancreatitis by creating an unfavorable \(\text{pH}\) environment. Consequently, drugs that enhance the function of ion transporters may be of great importance not only in CF therapy but also in the treatment of pancreatitis.

**References**


43. Maleth J, Hegyi P, Venglovecz V, Dockins J, West NE, Jennings MT, Dezube R, Whitcomb DC, Lechtzin N, Merlo CA, Singh VK. Cystic fibrosis transmembrane conductance regulator modulators reduce the risk of recurrent...


Chapter 21

Structure-function relationships of CFTR in health and disease: The pancreas story

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Introduction

In 1938, Dr. Dorothy Anderson published a paper describing the characteristics of cystic fibrosis (CF) in the pancreas; the term “cystic fibrosis” refers to the autopsy findings of fibrosis with cysts in the pancreas of children who died early in life with this disease. In 1949, Anderson also discovered that CF is a genetic condition. More recently, this was understood to be caused by mutations in the CF transmembrane conductance regulator (CFTR).1 Other than the pancreas, CF also affects the lungs, liver, kidneys, male reproductive system, and gastrointestinal tract.2,3 The disease leads to shortened life expectancy, most often due to respiratory failure resulting from airway obstruction, bacterial infections, and inflammation.4,5

CFTR, a member of the ATP-binding cassette (ABC) transporter protein family, is the cAMP-dependent Cl channel at the apical membranes of most epithelial cells.6 Mutations of CFTR gene cause CF, which is the most common fatal autosomal recessive disorder with a disease frequency of 1 in 2,500 live births and a carrier rate of approximately 5 percent in Caucasian population.7,8 The disease is characterized by a malfunction of exocrine tissues due to dysregulation of an epithelial chloride (Cl) channel. The major clinical features include chronic pulmonary disease, pancreatic exocrine insufficiency, intestinal disease (especially constipation), and an increase in the concentration of sweat chloride.9 In the lungs, airways become colonized with bacteria, and repeated pulmonary infections ensue. The recurrent infections and inflammation result in submucosal gland (SMG) hypertrophy and excessive mucus secretion. The impaired mucociliary clearance and plugging of small airways cause progressive bronchiectasis and ultimately lead to respiratory failure.10 Following the lung, the pancreas is the most affected organ in CF. It has been documented that most of the CF patients have pancreatic exocrine insufficiency, which leads to malabsorption and potentially malnutrition.11 In this context, malabsorption of fat and fat-soluble vitamins are the most common nutritional deficient seen in this disease.

In this chapter, we will focus on how decreased CFTR function leads to protein plugging of the ducts and pancreatic atrophy. We will also shed light on the latest animal models to better understand the CF pancreatic disease and its relationship to chronic pulmonary disease and intestinal disease.

CF Animal Models

Mice, rats, pigs, and ferrets for CF research

Since the discovery of the CF gene, many animal models have been generated to mimic the CF symptoms in human patients. The earliest models were mice with ∆F508 mutation—cftr mutation.12–15 CF mouse models have made significant contributions toward our understanding of the disease and the development of therapies.14 Different CF mouse models have been developed, such as the exon 10 knockout (KO) model, the ∆F508 model,15,16 and the G551D model.17 However, significant limitations have been acknowledged in translating the information gained from CF mice to the humans. For example, unlike human CF patients, CF mice show neither pulmonary pathophysiology nor obvious pancreatic pathology or liver problems.18 Recently established CFTR KO rats recapitulate several features of human CF disease; however, they do not develop spontaneous lung infections.14 CFTR KO ferrets and CFTR KO and ∆F508 pigs generated by nuclear transfer have shown a similar pathology to that observed in human CF patients,19 including lung, pancreatic, and liver phenotypes that were not often found in CF mice. However, neither pigs nor ferrets are convenient laboratory species. Both CF ferrets and CF pigs suffer from meconium ileus (MI), which causes these animals to die within a few days after birth; therefore, they are associated with high maintenance cost and require special animal-handling skills.20 These factors have limited the applicability of CF pigs and ferrets almost exclusively to the labs originally producing these animals and a few groups closely associated with them (Table 1). An ideal animal CF model would

Chapter 21

Structure-function relationships of CFTR in health and disease: The pancreas story

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With emphasis on the rabbit model the table summarizes the differences of phenotypes of CF in all known animal models. In addition, we showed the type of laxatives that will help overcome the gut impaction. The pancreas defect was also shown in the different models. * represents the cost of the animal model on a 1–5 scale.

### Table 1. Characteristics of CF and key clinical consequences noted in animal models

<table>
<thead>
<tr>
<th></th>
<th>Knock-out</th>
<th>DF508 Kl</th>
<th>Pancreas</th>
<th>Lung, liver Phenotypes</th>
<th>Meconium ileus</th>
<th>Laxatives</th>
<th>Cost</th>
<th>Special Care</th>
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<tbody>
<tr>
<td>CF Mice</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes/mild</td>
<td>?</td>
<td>Yes</td>
<td>Golytely</td>
<td>*</td>
<td>Yes</td>
</tr>
<tr>
<td>CF Pigs</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes/severe</td>
<td>Yes, 100% death &lt;48hrs</td>
<td>Surgery, or gut-corrected</td>
<td>*****</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>CF Ferrets</td>
<td>Yes</td>
<td>NO</td>
<td>Yes/severe</td>
<td>Yes, 75% death &lt;72hrs</td>
<td>gut-corrected</td>
<td>****</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>CF Rats</td>
<td>Yes</td>
<td>NO?</td>
<td>No</td>
<td>?</td>
<td>Yes</td>
<td>Golytely</td>
<td>*</td>
<td>No</td>
</tr>
<tr>
<td>CF Zebrafish</td>
<td>Yes</td>
<td>?</td>
<td>Yes/Severe</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>*</td>
<td>No</td>
</tr>
<tr>
<td>CF Rabbits</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes/Severe</td>
<td>Yes</td>
<td>Yes</td>
<td>Golytely</td>
<td>**</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Although CFTR−/− KO rabbits eventually develop distal intestinal obstruction, MI is rarely observed in the animal within the first month after birth because rabbit has a large functional cecum. To some extent, our work challenges the traditional view on the importance of SMGs in CF pathology, suggesting that SMGs may not be a critical player for the development of CF lung disease. In support of this view, overexpression of βENaC in mice can produce mucus obstruction in the small, non-glandular airways, which are thought to be the site of disease initiation in CF neonates.

In CF pigs, the mucus appears to arise from goblet cells in the surface of the epithelium of the airways. Mucus accumulation in CF ferret airways is associated with variable levels of goblet and mucus cell hyperplasia in the surface airway epithelium and SMGs. More recent data indicated that defective goblet cell exocytosis in CFTR KO mice contributes to CF-associated disease in the intestine. In fact, though SMGs in airways might be a primary site for CF pathogenesis, a critical role for mucus-producing goblet cells in CF airway pathology has not been excluded. Indeed, our preliminary data revealed that the goblet cells, which exist in rabbit airways, maybe the primary contributor to mucus accumulation in the airways of CFTR KO rabbits (Tables 1 and 2).

### AF508 mutation

Mutations of the gene encoding CFTR lead to CF, and more than 2,023 CF mutations (disease related or not) have been identified (http://www.geneticsickkids.on.ca/cftr/) in the CFTR gene. The most common mutation in CF is the deletion of the phenylalanine residue at position 508 (ΔF508), which is in the first nucleotide-binding domain of CFTR. The ΔF508 mutation is present in more than 90 percent of CF patients. A critical issue in CF disease is the inability of ΔF508CFTR to achieve the native, folded state required
for its export from the endoplasmic reticulum (ER) and traffic to the cell surface. Instead, ΔF508 protein is exclusively retained in the ER and degraded by the ubiquitin-proteasome system.\textsuperscript{27–29} A therapeutic strategy aimed at facilitating ΔF508 folding and trafficking is highly desirable for treatment of the disease because ΔF508 mutant has a substantial CFTR Cl current if it reaches the cell surface.\textsuperscript{30} A new drug, Orkambi, that combines a CF corrector (Lumacaftor, which acts as a chaperone for a correct protein folding which increases the number of CFTR proteins to the cell surface) and potentiator (Ivacaftor, increases the activity of the CFTR (conductance) at the cell surface) recently has received breakthrough therapy designation to treat CF patients with ΔF508CFTR. However, this drug only improves lung function assayed by forced expiratory volume in 1 second (FEV1) by 2.6–4 percent. Therefore, further research on ΔF508 mutation is needed to develop a better drug to treat CF patients.

### CFTR Deficiency in CF

The Cfr gene encodes the CFTR protein, a member of the ABC transporter superfamily, which is the cAMP-dependent Cl channel at the apical membranes of most epithelial cells, making it unique among members of this protein family.\textsuperscript{6} The CFTR protein migrates to the surface of cells that line the pancreatic duct, airways, gastrointestinal tract, biliary tract, part of the male reproductive tract, and cells that are part of sweat glands.\textsuperscript{5,31,32} CFTR forms a pore or channel that allows ions, including chloride and bicarbonate, to move from one side of the cell membrane to the other (Figure 1). Channel activation is mediated by cycles of regulatory (R) domain phosphorylation by PKA/PKC, ATP binding to the nucleotide-binding domains, and ATP hydrolysis (Figure 1). Demonstration that CFTR functions as a chloride channel regulated by cyclic AMP\textsuperscript{34}-dependent phosphorylation is consistent with the ion transport disturbances documented in CF tissues (for review, see\textsuperscript{15}). These disturbances in ions change the concentration of molecules in the fluid within the ducts or organs.\textsuperscript{11}

### CFTR Protein Structure

The CFTR protein is comprised of 1,480 amino acids organized into five functional domains.\textsuperscript{7,9} As other ABC transporters, CFTR has two membrane-spanning domains (TMD1 and TMD2), two nucleotide-binding domains (NBD1 and NBD2), and one regulatory domain (R) (Figure 1). For more insights regarding the structure of CFTR, see the review by Patrick and Thomas.\textsuperscript{36} The two TMDs, each composed of six transmembrane segments, form the CFTR channel pore, and the two NBDs interact with nucleotides to regulate channel activity opening and closing of the TMDs (Figure 1).\textsuperscript{7,36} The R domain, through interactions with the N-terminal cytosolic region of TMD1, also controls the channel activity.\textsuperscript{7,37,38} NBDs are responsible for the binding and hydrolysis of the ATP, which causes a conformational change in the TMDs leading to the transport of substrates across cell membranes.\textsuperscript{39} CFTR mutations can occur in any of the five protein domains. However, many mutations occur in NBD1, including the ΔF508 mutation. The location of the CFTR mutations can affect the formation or function of the CFTR protein (Figure 2, Table 3).\textsuperscript{7} In Table 3 we summarized the role of not only the major domains described for CFTR but also the connecting sequences, which include N-terminal, intracellular loops (ICLs), extracellular loops (ECLs), transmembrane (TM) helices 1 through 12, C-terminal domain. The N-terminal domain was shown to be involved in the folding and the trafficking of the CFTR protein through protein–protein interactions (e.g. syntaxin 1A), and mutations in this domain reduced the function of the channel.\textsuperscript{40,41} Some TM helices have been studied more than the others. For instance, the results from many studies suggested that transmembrane segments TM1, TM2, TM6, TM11, and TM12 form the pore lining and regulate the pore function.

<table>
<thead>
<tr>
<th>Table 2. CFTR-related characteristics among species.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of aminoacid</strong></td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>Human</td>
</tr>
<tr>
<td>Mouse</td>
</tr>
<tr>
<td>Rat</td>
</tr>
<tr>
<td>Pig</td>
</tr>
<tr>
<td>Ferret</td>
</tr>
<tr>
<td>Zebrafish</td>
</tr>
<tr>
<td>Rabbit</td>
</tr>
</tbody>
</table>
Figure 1. Activation of the CFTR channel. The CF transmembrane conductance regulator (CFTR) protein channel is a member of the ABC transporter superfamily. It acts in apical part of the epithelial cells as a plasma membrane, cyclic AMP-activated chloride, and bicarbonate anion channel. CFTR has two membrane-spanning domains (TMD1 and TMD2), two nucleotide-binding domains (NBD1 and NBD2), and one regulatory domain (R). CFTR is a key regulator for cell surface water-salt homeostasis of the apical membranes of epithelial cells in multiple organs, including the pancreas that produces alkaline fluid in pancreatic ducts. The open status of the CFTR is initiated by ATP binding at the NBD domains. The activation of the channel is dependent on phosphorylation by cyclic AMP-dependent protein kinase (PKA) at multiple sites in the R domain. The magnitude of response to PKA is amplified by phosphorylation of CFTR by protein kinase C (PKC).

by selecting the anions,\textsuperscript{42–44} whereas TM5 plays a role in the anion binding.\textsuperscript{43} Also, V232D mutation in TM4 leads to a loss of function because it forms a bond with Q207 in TM3, which does not occur in the WT form of CFTR.\textsuperscript{45,46} The mutation V232D is not the only mutation that involves a change from a neutral/hydrophobic residue to a polar or charged residue, causing CF. Therien et al. studied more than 31 mutations in TMD1 including all six TM helices and concluded that CFTR mutations in the TMs lead to a loss of function through the formation of membrane-buried interhelical hydrogen bonds.\textsuperscript{45} ECLs 1 through 6 represent about 4 percent of the CFTR protein, whereas 77 percent is in the cytoplasm and the rest, 19 percent, includes TM1 to 12. CF-associated mutations in the ECLs have been shown to affect channel gating\textsuperscript{47} and interactions with extracellular anions.\textsuperscript{48,49} For example, mutations D110H/E and R117C/H/L/P in ECL1 are associated with CF disease. These mutations affect the stability of the CFTR ion pore, resulting in reduced conductance of CFTR.\textsuperscript{47,50} Q890X and K892C mutations in ECL4 have been reported to affect channel gating and extracellular anion interaction.\textsuperscript{50–52} ECL4 is the only ECL that contains N-linked glycosylation sites (N894 and N900).\textsuperscript{51} Many studies have been performed on the function of CFTR’s intracytoplasmic loops (ICLs), including their roles in regulating inter-molecular interactions as well as CFTR interactions with other proteins. E193K and I148T mutations in ICL1 have been reported to affect the pore opening.\textsuperscript{53,54} Recent studies showed that ICL2/3-NBD2 interface and ICL1/4-NBD1 interface have a role in protein folding and processing in the ER.\textsuperscript{55,56} In addition, S945L, H949Y, and G970R mutations in ICL3; L1065P, R1070Q, and Q1071P mutations in ICL4; and D1152H, D1154G, and W1204X mutations in ICL5 have been shown to affect the conductance of CFTR.\textsuperscript{52,57,58} As for the rest of the domains, ATP-binding events occurring in both NBDs allow the hydrolysis of intracellular ATP to ADP.\textsuperscript{59} This event allows the conformational changes, and that change in structure allows the CFTR channel to transition from an opened to closed state, thus controlling the gating kinetics of the channel.\textsuperscript{59} NBD1 was studied more than NBD2 due to the presence of frequent ΔF508 deletion of NBD1 in the CF patients. The ΔF508 is present in more than 70 percent of the CF patients, resulting in destabilization of the CFTR protein.\textsuperscript{59–61} Also, mutations ΔF508 and G551D modify
the interactions between NBDs and NBDs–ICLs. Other well-studied NBD1 mutations such as G542X and G551D result in channel-gating problems. Similarly, NBD2 mutations like N1303K, p.Ile1234_Arg1239del, G1244E, S1251N, S1255P, and G1349D are CF disease related and are considered to be gating mutations. The R domain, along with NBDs, controls the channel activity of CFTR. The activation of the channel is dependent on the phosphorylation by cyclic AMP-dependent protein kinase (PKA). To date, more than 15 phosphorylation sites in the R domain have been attributed to PKA phosphorylation, which contributes in varying proportions to the response to activation of the CFTR channel. It has been reported that CFTR also can be phosphorylated by several other protein kinases, including protein kinase C (PKC), casein kinase II, cyclic GMP-activated protein kinase, and Src kinase. The R domain has multiple phosphorylation sites for PKC, which modulate PKA-induced domain–domain interactions. Many CF-related mutations like D648V, E664X, E656X, and 2108delA in the R domain disrupt the normal function of the R domain, for example, the transport of HCO₃⁻ in secretory epithelia and in CF. The same authors showed that mutants reported to be associated with CF with pancreatic insufficiency do not support HCO₃⁻ transport and

Figure 2. Classes of CFTR mutations. Class I mutations lead to no protein synthesis, which includes mutations that involve premature stop codons and nonsense mutations. Class II mutations include the most frequent mutation of CF disease, ΔF508, which lead to trafficking, improper folding, and processing defects of the CFTR protein. This class is the primary target in the CF research and the main target by the pharmaceuticals companies. Class III mutations affect the ATP binding at one of the two binding sites in the NBDs. The CFTR protein reaches the cell surface, but the mutations render the CFTR channel nonfunctional, which impairs the opening of the channel. Class IV mutations also involve CFTR protein reaching the cell surface but with reduced ion passage through the channel because of the structural defect caused by the mutations in the CFTR channel. Class V mutations affect the amount of CFTR protein that reaches the cell surface because of splicing problems or inefficient trafficking. Class VI mutations lead to a rapid turnover of the CFTR channel at the cell surface. Examples of CFTR mutations regarding their pancreatic defects, PI or PS. The mutations mentioned in the table are representative of each class, but we have to keep in mind that according to the new classification some mutations are classified in more than one class, which will result in having more than one defect.
Table 3. Domains structure of CFTR. A detailed description of the CFTR domains consists of an N-terminal, 6 ICLs, 6 ECLs, 12 transmembrane (TM) arranged into TMD1 and TMD2 consisting of 6 TM for each domain, NBD1 and NBD2, and the c-terminal domain. The specified aa for each domain, their known function, positive charge residues, some mutations causing CF, and their effects on the pancreas are as well described.

<table>
<thead>
<tr>
<th>Domains</th>
<th>Amino acids</th>
<th>Functions</th>
<th>Positive charge residues</th>
<th>Mutations causing CF</th>
<th>References</th>
<th>Pancreas</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal</td>
<td>1 to 81</td>
<td>Folding and/or trafficking; Protein interaction</td>
<td></td>
<td></td>
<td></td>
<td>PI</td>
</tr>
<tr>
<td>TM1</td>
<td>82 to 103</td>
<td>Regulation of pore function; Pore lining</td>
<td>K95</td>
<td>R117C/H/L/P</td>
<td>42–44, 67</td>
<td>PS/PI</td>
</tr>
<tr>
<td>ECL1</td>
<td>104 to 117</td>
<td>Stability of the CFTR ion pore</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TM2</td>
<td>118 to 138</td>
<td>CFTR pore lining</td>
<td>R134</td>
<td></td>
<td>42–44, 69</td>
<td>PS/PI</td>
</tr>
<tr>
<td>ICL1</td>
<td>139 to 194</td>
<td>Pore opening</td>
<td>E193K, I148T</td>
<td></td>
<td>53–55</td>
<td>PS/PI</td>
</tr>
<tr>
<td>TM3</td>
<td>195 to 215</td>
<td>ND</td>
<td>Q207 form a bond with the mutation V232D</td>
<td></td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>ECL2</td>
<td>216 to 220</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>47</td>
</tr>
<tr>
<td>TM4</td>
<td>221 to 241</td>
<td>Loss of function of pore</td>
<td>V232D</td>
<td></td>
<td>45, 46, 70</td>
<td>PS/PI</td>
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<tr>
<td>ICL2</td>
<td>242 to 307</td>
<td>Protein folding, Processing in the ER</td>
<td></td>
<td></td>
<td>55,56</td>
<td></td>
</tr>
<tr>
<td>TM5</td>
<td>308 to 328</td>
<td>Anion binding</td>
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<td>329</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TM6</td>
<td>330 to 350</td>
<td>Pore lining; anion selectivity</td>
<td>R334, K335, R347</td>
<td></td>
<td>43, 44</td>
<td>PS/PI</td>
</tr>
<tr>
<td>ICL2.5</td>
<td>351 to 432</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NBD1</td>
<td>433 to 586</td>
<td>Hydrolyzation of ATP, Channel opening, regulation of the sodium ion channel</td>
<td>Delta(F508); G551D; G542X</td>
<td></td>
<td>60, 61</td>
<td>PI</td>
</tr>
<tr>
<td>R</td>
<td>587 to 859</td>
<td>Phosphorylation sites for PKA/PKC</td>
<td>D648V, E664X, E656X and 2108delA</td>
<td></td>
<td>66</td>
<td>PI</td>
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<tr>
<td>TM7</td>
<td>860 to 870</td>
<td>ND</td>
<td></td>
<td></td>
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<tr>
<td>ECL4</td>
<td>881 to 911</td>
<td>Glycosylation at N894 and N900</td>
<td>Q890X, K892C</td>
<td></td>
<td>50–52</td>
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<td>TM8</td>
<td>912 to 932</td>
<td>ND</td>
<td>S912L</td>
<td></td>
<td>52</td>
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<tr>
<td>ICL3</td>
<td>933 to 990</td>
<td>Conductance</td>
<td>S945L, H949Y, G970R</td>
<td></td>
<td>52, 57</td>
<td>PS/PI</td>
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<tr>
<td>TM9</td>
<td>991 to 1011</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECL5</td>
<td>1012</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TM10</td>
<td>1013 to 1034</td>
<td>Processing</td>
<td>R1030</td>
<td></td>
<td></td>
<td>PI</td>
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<tr>
<td>ICL4</td>
<td>1035 to 1102</td>
<td></td>
<td>L1065P, R1070Q, Q1071P</td>
<td></td>
<td>52</td>
<td></td>
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<tr>
<td>TM11</td>
<td>1103 to 1123</td>
<td>Pore lining; anion selectivity</td>
<td></td>
<td></td>
<td>42–44, 67</td>
<td>PS/PI</td>
</tr>
<tr>
<td>ECL6</td>
<td>1124 to 1128</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TM12</td>
<td>1129 to 1150</td>
<td>Pore lining; anion selectivity</td>
<td>M1137V, M1137R, I11139V and deltaM1140</td>
<td></td>
<td>43, 44, 58</td>
<td>PS/PI</td>
</tr>
<tr>
<td>ICL5</td>
<td>1151 to 1218</td>
<td>Conductance</td>
<td>D1152H, D1154G, W1204X</td>
<td></td>
<td>52, 58</td>
<td>PS/PI</td>
</tr>
<tr>
<td>NBD2</td>
<td>1219 to 1386</td>
<td>Maturation, gating</td>
<td>N1303K, G1349D, G1244E, S1251N, S1255P, and G1349D</td>
<td></td>
<td>58</td>
<td>PI</td>
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<td>C-terminal</td>
<td>1387 to 1480</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

those associated with pancreatic sufficiency show reduced HCO₃⁻ transport.⁶⁵

Classification of CF Patients

Traditional classification

As mentioned earlier, more than 2023 CF mutations have been identified (http://www.genletsickkids.on.ca/cftr/) in the CFTR gene. These mutations are categorized based on the dysfunctions of CFTR at different levels of the maturation and function of the CFTR channels (Figure 2). Traditionally, these dysfunctions were divided into 6 groups based on function (Classes III, IV, and VI) and processing (Classes I, II, and V) of the CFTR (Figure 2).³³,⁷¹,⁷² The class I mutations include a nonsense, frame-shift, or splicing mutations which prevent CFTR biosynthesis by introducing a premature termination codon (PTC). The most common mutation in this class is the ΔF508 (Figure 2).

The class II mutations include a missense mutation which causes misfolding of the CFTR to lead to its degradation in the ER by quality-control machinery, resulting in the absence of functional protein at the cell surface. The most important mutation in this class is the ΔF508, present in more than 90 percent of CF patients (Figure 2).

The class III mutations include a missense mutation which leads to a non-functioning CFTR at the cell surface, resulting in unstable and reduced channel gating characterized by a lower open probability. The most common mutation representing this class is the G542X (Figure 2).

The class IV mutations include missense mutation which leads to a reduced CFTR channel conductance. The decrease in conductance is caused by an abnormal conformation of the pore resulting in disruption of the ion flow. The most common mutation in this class is the R117H (Figure 2).

The class V mutations introduce splicing or promoter defects in the CFTR gene, resulting in a reduced amount of CFTR protein at the cell membrane caused by reduced protein synthesis. Those mutations affect the gene expression but do not change the conformation of the channel. The most representative mutations in this class are the 3849 + 10kbC-T and A455E (Figure 2).

The class VI mutations include missense mutation which leads to a decrease in the CFTR stability. These mutations result in an accelerated turnover of CFTR protein at the cell membrane and reduced apical cell surface expression.⁷² The most representative mutation in this class is Q1412X (Figure 2).

Though more than 2,023 mutations/variants have reported for CFTR, whether each of these can cause channel dysfunction and disease is largely unknown. However, studies to predict the functional consequences and clinical outcome of individual patients carrying these mutations are being conducted.⁷³,⁷⁴ These interpretations of such studies have been challenged by the general lack of correlation between the genotype and the clinical severity⁷³ (Table 1).

New classification

The lack of correlation between the genotype and the phenotype of the CF patients led to a new classification based on the severity and the clinical symptoms of the CF patients. Recently, Dupuis et al. studied meconium ileus (MI) in CF patients and reported that only a subset of patients with CF develop MI.⁷³ Furthermore, MI demonstrates notable heritability. Although studies have shown that non-CFTR genes contribute to susceptibility, the CFTR genotype itself affects the occurrence of this complication; only patients with the more severe CFTR variants are at risk for MI.⁷³ It was hypothesized that the susceptibility to MI is influenced by specific CFTR genotypes, and that the prevalence of MI can be used to discriminate among severe CFTR mutations.⁷³ The pleiotropic molecular defects of a single mutation in the CFTR have limited the drug therapy effects for some mutants which have been categorized as class I, II, or II/IV.⁷² The authors proposed a modification of the traditional class I–VI CF mutations classification. This expanded classification of the major mechanistic categories⁷³,⁷⁵ accommodates the unusually complex, combinatorial molecular/cellular phenotypes of CF alleles. The new classification consists of 31 possible classes of mutations, including the original classes I, II, III/IV, V, and VI, as well as their 26 combinations.⁷² For example, according to the expanded classification, G551D will be designated as a class III mutation as before,⁷ while ΔF508 will be classified as class II–III–VI, W1282X as class I–II–III–VI, P67L as class II–III, Q1412X as class III–VI, and R117H as class II–III–IV, reflecting the composite defects in mutant CFTR biology.⁷² More evidence supporting the new classification came from a study by Vertex Pharmaceuticals where they tested 54 missense mutations and found that 24 of them have both processing and gating defects.⁷⁶

CFTR Function and Its Role in Pancreas

CF and exocrine pancreas

As mentioned earlier, CFTR is predominantly expressed on the apical membrane of epithelial cells in the small pancreatic ducts. CFTR acts as a selective ion channel involved in chloride and bicarbonate (HCO₃⁻) transport across the apical membranes of epithelial cells in multiple organs, including the pancreas, which produces alkaline fluid in the pancreatic ducts.¹¹,⁷⁷,⁷⁸ HCO₃⁻ is an important ion in the pancreatic juice. It also facilitates solubilization of the digestive enzymes and mucins.⁸⁹ Indeed, aberrant HCO₃⁻ transport has a crucial role in human diseases.³¹,⁷⁹ In his
review, Quinton proposed that in CF patients, the HCO3− is required to form normal mucus. His explanation is that once a granule is released, HCO3− sequesters Ca2+ and H+ ions away from the mucin anions to form a complex with them. Therefore, the lack of secreted HCO3− in CF patients impairs Ca2+ removal, prevents normal mucin expansion, and promotes stasis of mucus in the ducts or on the luminal surfaces of affected organs. In addition, reduced secretion of HCO3− and chloride (Cl−) leads to a more acidic and viscous luminal content. MUC6 is one of the pancreatic mucins expressed by 13 weeks of gestation and shows a very similar distribution to that of CFTR. In addition, MUC6 mucin is the main constituent of the complexes that form in small ducts and cause obstruction. Therefore, CF patients carrying mutations in the CFTR gene showed a lower pH, low flow of secretions, and high protein concentration in the pancreas duct secretions, which lead to precipitates, obstruction, and injury in the duct lumina. Meyerholz and his colleagues showed in the CF pig model that the changes (obstruction) could be detected in gestations as early as week 17. They showed that the site of obstruction ranged from the distal jejunum to the proximal spiral colon, similar to that reported in humans with MI. The obstruction in the acini and ducts lead to dilatation which causes epithelial injury and destruction, inflammation, fibrosis, and fatty infiltration. Tucker and colleagues reported that acinar plugs developed before mucous metaplasia and found that early acinar plugs are composed of zymogen granules and were distinct from mucus in pancreatic tissue of CF patients. These findings then indicate that zymogen material from the acinar cell, not mucus, may become inspissated in the acinus in early CF and that subsequent mucous metaplasia occurs as the obstruction and exocrine atrophy progress.

### CF and Hyperinflammation

As mentioned earlier, airways of the lungs become colonized with bacteria, and repeated pulmonary infections ensue. The recurrent infections and inflammation result in SMG hypertrophy and excessive mucus secretion. The impaired mucociliary clearance and plugging of small airways cause progressive bronchiectasis and ultimately lead to respiratory failure. Many studies have been done to explain the cause of the hyperinflammation in the CF patients. Many have suggested that the balance between Th1, Th2, and Th 17 could play a role in the CF disease. It has been established that Th17 is known to be a key player in autoimmune diseases. In addition, the authors showed that Th17 is regulated by miR-183C via inhibition of Foxo1. For a long time, it has been thought that CFTR mutations in epithelial cells have an indirect effect on the immune system which causes the inflammation in the lungs, because of the colonization of bacteria like Pseudomonas aeruginosa, Burkholderia cenocepacia, and Mycobacterium abscessus which causes the infections in the CF patients. Recently, emerging evidence points the problem to be directly affecting the immune cells. Since it has been shown that CFTR is expressed in lymphocyte T cells (like Th2, Th17, and Tregs) and macrophages, CFTR mutations causing CF may have a direct impact on these cells. Grumlli et al. summarized in their review that CFTR mutations have a direct effect on the T cells function which results in an enhanced Th2 response, a reduced Treg population, and elevated Th17 response which translate by an increase of neutrophils and recruitments by IL-17 to the lung which leads to the destruction of alveoli in the lungs of CF patients. In addition to the lymphocytes and neutrophils, macrophages also have been shown to participate in the lung decay found in CF patients through activation of MMP12. Understanding better the mechanisms behind the infections and the immune response could lead to a better drug therapy targeted to each patient depending on the severity of the CF disease.

### CF and Endocrine Pancreas

CF is also recognized to affect the endocrine pancreas. There is a correlation between glucose abnormalities, morbidity, and mortality in CF patients. Glucose abnormalities include cystic fibrosis–related diabetes (CFRD) and impaired glucose tolerance (IGT). CFRD is one complication in the CF patients occurring in more than 40 percent of adults and 25 percent of adolescents, which is preceded by episodes of IGT. Both reduced insulin secretion and insulin resistance are observed in CFRD. CFRD has characteristics of both type I and II diabetes and does not belong to either one of the diabetes classes. It is characterized by the loss of functional β-cell mass and varying degrees of insulin resistance. It is believed that cross-talk between pancreatic exocrine and endocrine components can also contribute to the CFDR. Destruction of pancreatic exocrine tissue caused by a decrease in islet cell mass and dysfunction in the β-cell are the cause of CFRD. It is very well documented that glucose deprivation leads to ER stress. Some CFTR mutations (like AF508) can cause the accumulation of unfolded proteins in the ER, triggering an evolutionarily conserved response, termed the “unfolded protein response” (UPR). In addition, aberrant Ca2+ regulation in the ER lumen causes protein unfolding, and rapid degradation of mutated CFTR proteins may contribute to the complex multi-organ CF pathology. It is known that ER stress triggers β-cell death typically by apoptosis.
when protein misfolding is persistent or excessive. As Harding et al. stated, the special sensitivity of insulin-producing cells to a mutation (like CFTR mutations) that affects a signaling protein responsive to ER stress may also be relevant to the development of more common forms of human diabetes mellitus. The major abnormality in most patients with CFRD is resistance to the action of insulin. However, glucose intolerance develops only after β-cell decompensation renders the endocrine pancreas unable to keep up with the demand imposed by IR. Because of their high rates of protein synthesis, β-cells are particularly susceptible to ER stress, which may trigger CFRD. The expression of CFTR was reported to be required in β-cells for glucose-induced secretion. Therefore, CFTR plays a significant role in the normal function of pancreatic β-cells. Ntimbane and his colleagues have summarized the factors leading to an abnormal glucose homeostasis in CF patients: (a) impairment of β-cell function with progressive fibrosis of islets of Langerhans with resultant distortion, ischemia, cell death, and a decrease in islet numbers; (b) impairment of other islet cell functions; (c) impairment of the insulinotropic gut hormone secretin; (d) changes in insulin sensitivity; and (e) altered insulin clearance rate. The exact cause and mechanism of CFRD are still largely unknown. The most probable cause of CFRD is a combination of many events unlikely to be attributable to one defect. The clinical effects and disease states associated with CF patients with CFRD include chronic pancreatic inflammation, dysfunction of the immune system, oxidative stress, impaired insulin production and secretion, variable state of IR, and altered entero–insular axis hormones.
**Insulin secretion and CFTR**

All the proposed causes mentioned earlier are supported by evidence to explain the pathogenesis of impaired insulin secretion in the β-cells. The expression of CFTR has been reported in cultured β-cells derived from mice (MIN6) or rat (RINm5F). But the earliest report came in 2007 from studies by Boom and his colleagues showing the expression of CFTR protein in rat islet cells and the significantly higher level in non-β than in β-cell populations. They also showed by immunohistochemistry studies that CFTR expression also occurs in glucagon-secreting alpha cells. Guo and his colleagues demonstrated that glucose-induced whole-cell currents, membrane depolarization, electrical bursts or action potentials, Ca\(^{2+}\) oscillations, and insulin secretion in β-cells are dependent on CFTR, indicating an essential role of CFTR in the regulation of insulin secretion. Their studies showed that specific inhibitors of CFTR ( GlyH-101 and CFTRinh-172) blocked a CFTR Cl\(^{-}\) gating needed for insulin secretion in primary β-cells and ΔF508-CFTR mutant mouse islets.

In addition, Edlund and her colleagues detected small CFTR conductance in both human and mouse β-cells. The augmentation of insulin secretion by activation of CFTR by cAMP (forskolin or GLP-1) in the presence of glucose was significantly inhibited by the specific CFTR inhibitors. They also demonstrated reduced cAMP-dependent exocytosis upon CFTR inhibition, concomitant with fewer docked insulin granules. These reports and others from the patients with CFTR mutations showed insufficiency of secreted insulin. However, these studies did not describe the molecular mechanism that causes a decrease in insulin secretion. In addition to the role of CFTR in regulating insulin secretion and exocytosis after glucose-induced membrane depolarization leading to insulin secretion, the study also demonstrated that CFTR molecules act upstream of the chloride channel Anoctamin 1 (ANO1; TMEM16A) in the regulation of cAMP- and glucose-stimulated insulin secretion. Thus, the impaired insulin secretion seen in patients with CF would be caused by the lack of glucose-induced Cl\(^{-}\) efflux through both CFTR Cl\(^{-}\) channels and ANO1 due to a decreased membrane depolarization.

In summary, these studies showed that CFTR is an important regulator of pancreatic β-cell insulin secretion, exocytosis, and membrane depolarization and is induced by glucose via elevation of cytosolic Ca\(^{2+}\) concentration.

Marunaka in his recent review summarized new studies connecting the role of CFTR and ANO1 in insulin secretion. Briefly, it has been established that intracellular Cl\(^{-}\) concentration ([Cl\(^{-}\)]) is a very useful marker of channel activity. In these recent studies, the authors showed that [Cl\(^{-}\)] measured using N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) is about 100 mM under the basal condition in RINm5F β-cell line, and application of CFTRinh-172 (an inhibitor of the CFTR channel) increased [Cl\(^{-}\)] about 26 mM. This means that CFTR indeed mediates Cl\(^{-}\} efflux under basal condition, which may maintain a relatively depolarized membrane potential in the β-cells at rest, and the electrochemical potential of Cl\(^{-}\} in the intracellular space is larger than that in the extracellular space.

This was confirmed by showing that membrane potentials of pancreatic β-cells expressing wild-type CFTR Cl\(^{-}\} channels are −61~−67 mV [70] but hyperpolarized to −75 mV when using CFTRinh-172 or ΔF508. Thus, CFTR has an important role in determining the resting membrane potential of the β-cells by acting as a Cl\(^{-}\} channel to maintain the membrane depolarization. The same study also showed that ΔF508 CFTR Cl\(^{-}\} channel decreases membrane depolarization induced by glucose and increases [Ca\(^{2+}\}] due to the activation of voltage-sensitive Ca\(^{2+}\} channels.

In his review, Marunaka concludes that the loss of CFTR function leads to insulin insufficiency which is caused by the higher intracellular Cl\(^{-}\} electrochemical potential in pancreatic β-cells. In general, Cl\(^{-}\} uptake into the intracellular space is mediated via active Cl\(^{-}\} transporting systems, such as Na\(^{+}\}Cl\(^{-}\} cotransporter (NCC) and/or NKCC, driven by the Na\(^{+}\}, K\(^{+}\}-ATPase-generated Na\(^{+}\} chemical potential difference between the intracellular and extracellular spaces: the intracellular Na\(^{+}\} chemical potential < the extracellular Na\(^{+}\} chemical potential. Therefore, if we could increase the [Cl\(^{-}\}] by elevating the NCC- and/or NKCC-mediated Cl\(^{-}\} uptake, the insufficiency of insulin secretion would be improved via membrane depolarization due to an elevation of Cl\(^{-}\} efflux from pancreatic β-cells of ΔF508 CFTR-expressing CF patients.

Recent studies contradict the findings by Guo et al. by presenting some evidence using the ferret’s pancreas that β-cells do not express CFTR. The authors showed that CFTR RNA is expressed in exocrine and not in endocrine cell types of islets and pancreas. They used a different approach, smFISH, to show the expression of CFTR. WT and CFTR-KO neonatal ferret pancreas were used to perform CFTR and INS dual smFISH. As expected, the INS was present in both genotypes, but CFTR was present in the WT pancreas. More importantly, CFTR RNA was not co-expressed in INS (β-cell), GCG (α-cell), PPY (PP cell), or SST (δ-cell) expressing cells but was expressed in KRT7-expressing ductal cells in the WT pancreas. The same findings were shown in dissociated cells from isolated adult ferret and human islets. These findings contradict the previous findings by Guo et al. and demonstrate that exocrine-derived duct cells associated with isolated islets express the highest levels of CFTR and support a mechanism by which CFTR-dependent duct/islet crosstalk might influence β-cell insulin secretion.
Although there is evidence that supported each of these contradicting studies, a clear resolution to the question of whether CFTR directly or indirectly functions within the β-cells or other islet cell types to support insulin secretion needs further clarifications.

Role of glucose transporters (GLUTs) in CF and their impact in the pancreas

As mentioned earlier, glucose abnormalities in CF include CFRD and IGT. The relationship between CFTR and the causes of CFRD is still not very well established. Studies are also emerging regarding the implication of GLUT transporters in developing the diabetic state in CF patients. In studies of obesity and diabetes in mice, it was reported that CFTR was significantly decreased, while GLUT5 and Villin were increased in the jejunum. It is known that CFTR Cl− channel provides the major route for Cl− exit across the apical membrane in normal murine intestine, and disturbance in the anion exchange and recycling of K+ is thought to be one of the causes of diabetes.

Recently, it was reported that GLUT-4 is expressed in normal human airway epithelial cells. The authors also showed that cells expressing F508del-CFTR have impaired glucose uptake, elevating action on the transepithelial resistance and diminishing action on paracellular flux of small molecules after insulin stimulation. In a different study, GLUT-4 subfractionation demonstrated that, despite insulin stimulation, the GLUT-4 content of intracellular-associated subfraction was significantly higher in CF subjects compared with controls, corresponding to significantly lower GLUT-4 content in cell surface–associated subfraction. These findings are consistent with the abnormal subcellular localization of GLUT-4. Impairment of GLUT-4 translocation in CF correlated with higher TNF-α levels in all CF subjects than in controls. CF patients that have CFRD with a decreased insulin secretion could be explained by the elevation of TNF-α and impaired translocation of GLUT-4. In addition, the results indicate that the function of CFTR Cl− channels is required for insulin to stimulate glucose uptake, elevate the transepithelial resistance, and diminish the paracellular flux of small molecules in airway epithelial cells.

Pancreatic Defect and CF

In general, the exocrine pancreatic disease and its progression correlates well with the genetic factors of the CF patients. CF patients have been divided into two classes, pancreatic insufficient (PI) and pancreatic sufficient (PS). Approximately 85 percent of patients with CF have PI which is categorized as the “severe” CF phenotype, the rest 15 percent are PS patients and thus “mild” CF phenotype. The exocrine pancreas in PI patients no longer secretes the required digestive enzymes. Therefore, CF patients often require an oral pancreatic enzyme supplement each meal. Pancreatic damage from CF can be detected in utero in subjects with PI and show obstruction of the small ducts and acini which will lead to the destruction of the pancreas with only a few islets or ducts left in a sea of adipose tissue. In contrast, PS-CF patients do have pancreatic damage, as measured by the high levels of serum immunoreactive trypsinogen (IRT), but retain normal digestion due to a sufficient endogenous function of exocrine pancreatic ducts. As we have mentioned earlier, CF patients are classified into six traditional classes from I to VI based on their CFTR mutations. The exocrine pancreatic phenotypes PI and PS are directly linked to genotype.

Wilschanski and his colleagues describe that CF patients homozygous or compound heterozygous for severe alleles belonging to classes I, II, III, or VI confer PI. Whereas a mild class IV or V allele sustains pancreatic function in a dominant fashion even if the second mutation is severe and falls into PS. They go further and explained that this observation appears plausible because all known mild alleles belong to class IV or V, all of which are (or predicted to be) associated with some residual chloride channel activity at the epithelial apical membranes. On the other hand, in a recent review by Gibson-Corley and his colleagues, they described the PI CF patients to be in the classes I, II, III, IV, and VI because they have mutations that render CFTR to be absent or nonfunctional. The remainder of the patients belonging to class V or mild class IV considered PS, due to less severe CFTR mutations.

In both reviews, it was mentioned that this classification of the PI and PS does not entirely fit into the six classes of CFTR mutations. Those who are considered PS still show pancreatic destruction as the serum level of IRT is elevated but will not require enzyme replacement for normal digestion. Some class I mutations with the stop codon at the end of the gene are CF patients with PS. In addition, a small portion (~3%) of CF patients with a severe mutation on both alleles are considered PS at diagnosis but eventually transition from PS to PI. To further exemplify G85E a missense mutation and few other mutations do show variable pancreatic phenotypes. PS CF patients are more susceptible to developing pancreatitis than the PI patients. It is known that pancreatic ducts have an essential part in CF and chronic pancreatitis, and only PS patients develop pancreatitis, suggesting that partially impaired the function of pancreatic ducts is retained in PS patients. Druno and colleagues showed that there is a strong correlation between genotype and phenotype in patients with CF and pancreatitis. They showed from a CF cohort study of about 1,000 patients followed over a period of 30 years that PS CF patients carry at least one
mild mutant allele and are at a significant risk of developing pancreatitis. Symptoms of pancreatitis may precede the diagnosis of CF. Pancreatitis is associated with an otherwise mild CF phenotype. A larger CF cohort study of about 10,071 patients had reported that out of 331 patients with PS, 34 cases had pancreatitis, whereas the occurrence of pancreatitis among patients with PI was 15 cases out of 2,971 patients. More evidence goes toward the correlation of PS CF patients and the development of pancreatitis, with a novel pancreatic insufficiency prevalence score where they divided the patients into three groups: severe, moderate-severe, and mild, with the mild mutations more susceptible to the risk of developing pancreatitis. The new classification of CFTR mutations into 31 new classes reflects the composite defects in mutant CFTR biology.

We will need to take into consideration the complexity of the disease and the severity of the CFTR genotype and their relationship with risk of pancreatitis. In a very recent study, for example, three sibling patients with a novel missense mutation, the R248G in exon 6 of the CFTR gene, present a recurrent acute pancreatitis. A similar missense mutation, R248T, has been previously reported as a mild CFTR-RD mutation that is not associated with pancreatitis. The R248G mutation may alter the normal function of CFTR more than the R248T mutation based on the clinical phenotypes of the three patients. As the authors conclude, future structure-functional studies on the CFTR protein can provide further insight into the impact of the R248G mutation at the molecular level.

**CFTR Mutations and Pancreatitis**

It is very well established by now that CFTR is a key protein in the pancreatic duct, which regulates the exchange of anions between the luminal surface and the cytoplasm of the duct cells. As mentioned, in CF patients the pancreas is one of the first organs to fail because mutations in CFTR play a critical role in pancreatic pathophysiology. The large number of mutations known to date in CFTR led scientists to tackle this complex disease with so many symptoms from a different angle, that is, to make a specific correlation between CFTR mutations with certain symptoms such as pancreatitis in its both forms, chronic and acute, to determine the severity of the disease. In an Austrian cohort study of 133 pancreatitis patients, the frequency of CFTR mutations was 11.2 percent. In patients classified as “idiopathic definitive chronic pancreatitis,” the frequency of mutations was 12.7 percent, whereas patients with “acute pancreatitis” or “possible chronic pancreatitis” had a frequency of CFTR mutations of 10 percent and 9.1 percent, respectively. The authors concluded that the frequency of CFTR mutations is highest in patients with definitive chronic pancreatitis and may, therefore, be regarded as a risk factor for the development of chronic pancreatitis. Another large Canadian CF cohort study of 2,481 subjects with PS-CF (with and without pancreatitis) showed some correlation between the severity of CFTR genotype and the risk of pancreatitis. They showed that patients carrying mild mutations are more likely to develop pancreatitis than those who had moderate-severe mutations. Therefore, patients with mild mutations had 71 percent increase in the risk of developing pancreatitis at any given time than those with moderate-severe mutations. Thus, approximately 20 percent of PS-CF patients develop pancreatitis. Coffey et al. in their review summarized the complexity of the correlation between the CFTR mutations and the development of pancreatitis by categorizing the mutations into four groups based on the clinical status of the patients: (i) CF-causing mutations, (ii) mutations associated with CFTR-related disease, (iii) mutations with no known clinical consequence, and (iv) mutations with unknown clinical relevance. Also, Ooi and colleagues found that certain diseases that resemble CF at an organ-specific level (e.g., pancreatitis) are also strongly associated with mutations in the CFTR gene. In conclusion, pancreatitis in the CF patients and the relationship with multiple mutations of CFTR are very complex due to the multiple levels of the disease symptoms, which are different from mutation to another due to the extended classification of those different mutations into the 27 different classes according to the new classification.

**Conclusion**

In CF patients the pancreas is one of the first organs to fail because mutations in CFTR have a critical role in pancreatic pathophysiology. CFTR is the key regulator of the pancreatic duct that regulates the anion exchange between the luminal surface and the cytoplasm of the duct cells. The large number of CFTR mutations is leading scientists to approach an understanding of their functional impact from a different angle with the goal of making a specific correlation between CFTR mutations and certain symptoms such as PI and PS. The lack of correlation between the genotype and the phenotype of the CF patients has led to a new classification (31 possible classes of mutations) based on the severity and the clinical symptoms of the CF patients. The pleotropic molecular defects of a single mutation in CFTR have limited the effects of drug therapy for some mutants which have been categorized as class I, II, or II/IV. The expanded classification of the major mechanistic categories will accommodate the unusually complex, combinatorial molecular/cellular phenotypes of CF alleles. In addition to the new proposed classification, one more level of complexity is to categorize the mutations into four groups based on the clinical status of the patients: (i) CF-causing mutations, (ii) mutations associated with CFTR-related disease,
(iii) mutations with no known clinical consequence, and (iv) mutations with unknown clinical relevance. All the recent discoveries and the new hypothesis will help shed light on the complex CF disease from a new perspective, which will help develop a new combined therapy to rectify the mutation or mutations at different levels of CFTR defects.

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References

28. Jensen TJ, Loo MA, Pind S, Williams DB, Goldberg AL, Riordan JR. Multiple proteolytic systems, including the


Structure-function relationships of CFTR in health and disease: The pancreas story


120. Ooi CY, Dorfman R, Cipolli M, Gunska T, Castellani C, Keenan K, Freedman SD, Zielenski J, Berthiaume Y, Corey...


Chapter 22

Regulation of normal and adaptive pancreatic growth

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Introduction

It is now established that almost all vertebrate organs show dynamic replacement or turnover at the level of individual proteins, organelles, and cells. This allows replacement of damaged components and also regulation of functional mass. Regulatory loops where the expression of a mitogen is modulated by a feedback mechanism are common to many tissues. This is consistent with the observation that many tissues show both autonomous and regulated growth. Furthermore, in vertebrates, cell division and cell size are independent processes.

Regulation of pancreatic size is similar to other organs and occurs through genetic programming and environmental influences (especially food intake), with both directed at assuring that the organ can carry out its function in an energy-efficient manner. In most vertebrates, the islets of Langerhans are scattered through the gland but make up only 2–3 percent of the volume. Acinar cells make up about 85 percent of volume and ducts 5 percent, so overall pancreatic size reflects the exocrine component. However, acinar cells make up a smaller fraction of total cells and nuclei (approximately 50%) as individual acinar cells are larger than myofibroblasts and duct cells. In the adult, islet mass and exocrine mass appear to be regulated independently. Assessment of islet volume generally requires histological analysis and will not be further considered here.

Measurement of Pancreatic Size

Overall pancreatic size is expressed as cm$^3$ if volume is measured or g if weighed. Because the pancreas increases in size with age, the weight or volume is usually normalized to body weight. Until recently, the most common technique was dissecting and weighing the pancreas. This yields values for young adults, usually males, fed a control diet, of 8–12 mg pancreatic weight/g body weight for mice,$^5$–$^8$ 22.5 mg PW/gBW for Syrian hamster,$^{10}$ 2.5–4 mg PW/g BW for rats,$^{11}$–$^{16}$ and 0.5–1.1 g PW/kg BW for adult humans.$^{17,18}$ Note that the relative pancreas size gets smaller as the animal gets larger. Smaller animals have a larger body surface area and higher metabolic rate and food consumption relative to body size. It seems reasonable that the higher food consumption requires more digestive enzymes and hence a larger pancreas.

Recently, imaging studies using CT or MRI have been used and give similar values for pancreatic size in humans as earlier autopsy studies.$^{19}$–$^{22}$ A study in pigs validated the method by comparing the pancreatic volume obtained by MRI to water displacement after pancreas removal.$^{23}$ A recent study adopted MRI to determine the volume of mouse pancreas, and the results were similar to water displacement.$^{24}$ The main advantage of the imaging techniques is that they are noninvasive and can be carried out repetitively. Also, the volume for pancreatic parenchyma and fat can be determined separately. Such studies have shown that both total pancreatic mass and pancreatic fat increase with increasing BMI in humans$^{21}$ and decrease in both type 1 and type 2 diabetes.$^{25}$–$^{28}$ The technique has been adapted to mice by use of a micro-MRI with a 7 Tesla magnet and worked best with the Rapid Acquisition with Relaxation (RARE) protocol.$^{24}$ In this study, mice were perfusion-fixed initially, so further work is necessary to establish the technique in living mice. In another study the pancreas architecture including the islet component was carried out by micro-magnetic resonance microscopy.$^{29}$

Prenatal Pancreatic Growth

We can divide the lifespan of the pancreas into the prenatal, postnatal to adolescence and adult phases. The prenatal phase is dominated by organogenesis in which outgrowth from the foregut produces undifferentiated branching tubules expressing the transcription factor Pdx1 (pancreatic and duodenal homeobox 1). These cells, particularly the buds at the end of the tubules, are considered to be transient progenitor cells. They will develop into acini, islets,
and mature ducts under the influence of mesenchyme and a number of other transcriptional regulators. However, these progenitor cells are not totipotent and probably should not be considered stem cells. During development, cell division and differentiation result in an increase in organ size, and at birth, or within a few days, the pancreatic cells are able to assume their physiological function in response to environmental stimuli which activate neurohormonal control mechanisms. Some aspects of the pancreas change in mammals with weaning when a shift from a high fat to a high carbohydrate or high-protein diet occurs, and these changes can be considered as either completion of development or an environmental (dietary) adaptation.

The size of the pancreas at birth depends on the original number of progenitor cells as ablation of part of these cells during embryogenesis by targeting diphtheria toxin to the Pdx1 expressing cells reduces the size of the pancreas at birth and through postnatal life. This implies that each progenitor cell can give rise to a fixed amount of pancreas. At the time of birth the rat pancreas weighs about 5–10 mg and the relative size is 3–3.8 mg/g body weight.

Prenatal pancreatic growth has recently been shown to be regulated by the Hippo pathway. Originally discovered in Drosophila, the mammalian pathway consists of mammalian sterile 20-like kinase 1/2 (MST1/2), which phosphorylates large tumor suppressor 1/2 (LATS1/2) which then phosphorylates Yes associated protein (YAP) and its homolog TAZ leading to inactivation and cytosolic retention. When the pathway is activated by cell–cell interaction or certain chemical factors, hypophosphorylated YAP translocates to the nucleus and induces gene expression. YAP1 increases growth and inhibits differentiation in most cell types. In pancreatic cells the pathway is primarily active before birth, especially in the secondary transition to differentiated cells. Activation in the adult liver leads to a fourfold increase in mass, but a similar increase in pancreas has little or no effect. Functional deletion of this pathway by gene deletion of the upstream kinase MST1/2 leads to acinar cell dedifferentiation. YAP expression is lost in adult endocrine pancreas but remains in the exocrine compartment, especially ducts, and may play a role in pathology and recovery from acute pancreatitis.

Postnatal pancreatic growth

In the period from birth to adulthood the pancreas grows roughly in proportion to body growth. This has been quantitated both by dissecting and weighing the pancreas and by imaging using CT or MRI followed by volume calculation. In a detailed study in Wistar rats, Iovanna et al. evaluated pancreatic weight, protein, and RNA from birth to 90 days. Pancreas weight increased faster than body mass until reaching a peak at 35 days and then declined (Figure 1A). A similar pattern of pancreas weight increase can be derived from the data of Snook carried out using CD strain rats which showed a peak PW/BW at 28 days. Using micro-MRI, Paredes et al. showed a linear relation in mice between pancreas volume and body weight between 20 days and 9 months of age (Figure 1B). By contrast, Stanger et al. demonstrated a hyperbolic relationship with pancreatic growth faster than body growth in the first three weeks of life with gradual slowing thereafter. These differences are not major and may reflect differences in species, strain, or diet. In mice postnatal growth is not dependent on two of the major pancreas regulators, cholecystokinin (CCK) and secretin, as the pancreas in young adult mice is similar when these peptides or their receptors undergo gene deletion. Similarly, in rats, pancreatic growth with age is not altered by a CCK antagonist. By contrast, the growth of the neonatal pancreas can be increased by glucocorticoids and thyroxine which probably reflects a role of these hormones on development. The human pancreas also shows a linear relationship between size and age from birth to 25 years, after which there is little change in size in adults until it begins declining around age 60. In humans, due to the larger size, imaging can distinguish fat from parenchymal tissue, and from midlife on the amount of fat in the pancreas has been reviewed.

**Figure 1.** Pancreatic and body weight as a function of age. (A) Data for rats from 1 day postnatal to 87 days (replotted from Iovanna et al.). (B) Data for mice showing pancreatic volume and body weight from 20 to 280 days (from Paredes et al.)
increases with a concomitant decrease in the mass of exocrine tissue. During the growth phase, the increase in pancreatic mass primarily reflects cell division, and the size of individual cells changes little after the first few days of life.44

During the postnatal growth phase, pancreatic DNA increases in parallel to pancreatic weight. Acinar cell size shows a transient increase around birth due to the cells being packed with zymogen granules before secretion matures, but it is otherwise relatively constant for a particular species, with cell volume inversely related to the size and longevity of the animal—for example, cell volume varying from 4,288 \( \text{um}^3 \) for the tree shrew to only 457 \( \text{um}^3 \) for a rhinoceros.45 In mice, cell size increases at the time of weaning.45 Humans, however, maintain a constant acinar cell size over the lifespan. In support of the concept that pancreatic growth is primarily driven by cell proliferation, a number of studies have evaluated \(^3\)H-thymidine incorporation by autoradiography or the incorporation of the thymidine analog BrdU visualized by immunohistochemistry. Other studies evaluated specific proteins expressed by cells undergoing mitogenesis including proliferating cell nuclear antigen (PCNA) and Ki67. Finally, some early studies counted cells undergoing mitosis to obtain the mitotic index. These approaches generally parallel each other, although showing different absolute numbers where the percentage of cells expressing PCNA is higher than those synthesizing DNA. Almost all of the earlier studies up to the year 2000 or later were carried out in rats. All reported measures showed a high rate of acinar cell division in the last week of gestation, with the rate falling around birth and then increasing postnata lly to a peak index of 5–20 percent from 5 to 20 days, and then a decline starting around weaning to adult rates at 30–60 days.44,46–49 In adult rats and mice on a nutritionally sound diet, the indices of proliferation are under 1 percent.44,46,48,50 Lineage tracing has shown that acinar cell growth is due to self-duplication.51 Similar to acinar cells, new adult islet beta cells also arise by self-duplication from existing beta cells.52 In a study of thymidine incorporation by autoradiography in ICR mice, the authors estimated a half-life for adult acinar cells of 70 days, with 40 days for duct cells.53

One additional complication is that starting around day 20 in rats the number of bi-nucleate cells increases to near 50 percent at 50 days, and these cells synthesize little more DNA.54 In a study in mice, polyploidy first began at 20 days and increased to 9 percent at 90 days.55 Recently, most studies of pancreatic growth and regeneration are being carried out in mice, and immunostaining with antibody to Ki67 has become the most common way to show which cell types are proliferating. However, there is much less quantitative data over the life span in mice, compared to rats, and only a little in other species.

Adaptive Pancreatic Growth

Need for regulation of pancreatic size

As discussed earlier, the size of the pancreas and the spectrum of digestive enzymes that it produces are generally related to the animal’s size and diet. The pancreas must provide sufficient enzymes for digestion, and for trypsin, a specific feedback system mediated by CCK is present to maintain the activity of trypsin in the intestinal lumen.56 Thus, the conditions that induce adaptive pancreatic growth are generally related to changes in diet. An increase in food intake such as occurs in pregnancy, lactation, and cold exposure leads to an increase in pancreatic size in rodents.57–60 Whether heavy exercise, which can double caloric intake, would affect pancreatic size would also be worthy of study. Also, there is little information as to whether pancreatic size adjusts to increased caloric consumption in humans.

The converse of increased food intake is starvation. Starvation is accompanied by loss of body and organ weights in all species examined, from fish to mammals.61 While the pancreas has not always been evaluated, all organs in the GI tract decrease their weight during starvation, especially the liver and intestine. In rats starved for 4 or 7 days, pancreatic weight decreased slightly more than body weight.62–64 Tissue weight and protein decreased more than DNA65 consistent with cellular atrophy. Smaller acini with fewer ZG have also been observed by electron microscopy with starvation.66–68 This was most prominent in phase 3 of starvation, when fat reserves have been utilized. Similar results have been reported in catfish69 and sparrows.70 A study in obese humans who were fasted for 20 days showed a decrease in pancreatic juice volume and enzyme content upon pancreatic function testing consistent with pancreatic atrophy, although pancreatic size was not measured.71 An extreme case is seen in snakes such as pythons that feed on a large meal and then carry out an extended fast.72 In this case, the involution of the GI tract occurs, while the animal has adequate nutrients but food is not entering the gut. That the latter is important is shown by the results of parenteral feeding in rats where the intestine and pancreas atrophy even though normal nutrition is maintained.73,74 Total pancreatic weight and protein were decreased in total parental nutrition (TPN), while results for DNA were not clear. This effect of TPN has been ascribed to the loss of GI hormones that normally would be released in response to feeding as well as the absence of nutrients in the lumen. Both, exogenous CCK and bombesin have been shown to reduce the pancreatic atrophy induced by TPN in rats,75,76 and the atrophy was shown to be due to the loss of luminal protein, as the combination of luminal fat, carbohydrate, and amino acids could not reverse the pancreatic atrophy induced by TPN.77

Another case of altered pancreatic function as a result of altered gut function is celiac disease. In celiac disease,
ingestion of gluten, present in wheat and some other grains, triggers an immune attack on the duodenal mucosa which loses its villi, resulting in a decreased surface area leading to malabsorption. Twenty to thirty percent of patients showed reduced exocrine pancreatic function, and a portion of these show pancreatic insufficiency. While the mechanism is not clear, the most favored explanation is a reduction of GI hormone secretion, especially CCK. Other postulated mechanisms include reduced response to CCK, amino acid deficiency, and protein malnutrition. There is no evidence at present, however, that the pancreas is smaller in celiac disease.

Models for studying adaptive growth

Exogenous secretagogue stimulation

A large number of studies have shown that exogenous CCK can stimulate pancreatic growth. Because of the short half-life of CCK in vivo, CCK or its analogue caerulein have been given as two or three subcutaneous injections per day or by continuous infusion. Studies in rats, mice, and hamsters all show an increase in pancreatic size of 30–50 percent. Secretin administration potentiates the increase in response to CCK in some but not all studies. While somatostatin inhibits growth stimulated by CCK, this secretagogue driven increase in pancreatic mass was accompanied by increased protein content, thymidine incorporation into acinar cells peaking at 2–3 days accompanied by an increase in pancreatic DNA content. However, higher doses of CCK can induce apoptosis and a reduction in pancreatic weight. The trophic action of CCK is mediated through CCK1 (CCK-A) receptors.

The effect of exogenous administration of other secretagogues has also been studied. Bombesin, an analog of gastrin-releasing peptide, when administered to rat, mouse, and rabbit induced an increase in pancreatic wet weight and protein with or without an increase in DNA. Because bombesin can stimulate the release of CCK, several studies showed that the growth effect of bombesin was not blocked by a CCK antagonist which blocked the effect of exogenous CCK. The effects of gastrin on pancreatic growth has been studied, but no consistent effect has emerged. Secretin by itself has no effect but may potentiate the action of CCK. The effect of administration of cholinergic analogs such as bethanecol and carbachol has been studied and shown to induce modest pancreatic growth which is less than the response to CCK. However, it is not clear whether this effect is direct on acinar cells or mediated by release of some other regulator.

Although having considerable differences from in vivo pancreatic growth, primary cultures of acinar and duct cells have shown that CCK can directly stimulate acinar cell growth. Caerulein, CCK, and gastrin stimulated H-thymidine incorporation into monolayer cultures of mouse acinar cells on a collagen matrix (Figure 2), while carbachol, bombesin, and substance P had no effect. The effect involved both high- and low-affinity CCK receptors in rat acini and could be inhibited by TGF-β. CCK has also been shown to stimulate the growth of pancreatic cancer cell lines. Growth stimulatory effects of IGF-1 on mouse cells cultured on a laminin-coated membrane have also been reported. However, the acinar cells in these cultures appear to have dedifferentiated, so they may be more of a model for recovery from damage than adaptive growth. Similar cultures of isolated duct cells have been studied, but only growth factors and not secretagogues stimulated their growth.

Trypsin inhibitor feeding

It has been known for many years that chicks and rats fed raw soybean flour developed large pancreases with increased capacity to secrete digestive enzymes. This was found due to the presence of active trypsin inhibitor (TI), and the effect could be reproduced by a variety of purified TIs and in a variety of animal species, but with most subsequent work in rats. Green and Lyman showed that soybean trypsin inhibitor (SBTI) stimulated pancreatic secretion and presented evidence for a feedback mechanism in which active trypsin in the gut lumen inhibited pancreatic secretion. Thus, the feedback loop works to maintain an adequate level of trypsin and other proteases in the intestinal lumen. With the development of sensitive and specific CCK assays, it was shown that release of CCK is part of the feedback loop. Diversion of bile pancreatic juice from the duodenum to the lower intestine also stimulates CCK release and pancreatic growth by removing active trypsin from the duodenum. Further information and references on this feedback loop can be found in Liddle, RA (1995) and Logsdon, C (1999).

The feeding of either natural or orally active chemical trypsin inhibitors such as camostat (also known as FOY-305) can be used as a model to stimulate CCK-driven pancreatic adaptive growth. The effects of TI are bigger than the response to exogenous CCK and will stimulate pancreatic growth when given two or three times a day probably because of desensitization to large exogenous boluses of CCK. Rodent and hamster pancreases will double in size in about a week in response to feeding TI (Figure 3), and the growth consists of hypertrophy and hyperplasia with the relative amount dependent on species. Current evidence suggests islet morphology and insulin content of the pancreas are unaffected. Co-administration of CCK antagonists blocks the effect of TI in rats. In mice the action of CCK was shown to be directly on acinar cells. In mice the effect of TI is not seen following the genetic deletion of either
CCK or the CCK receptor. In both rats and mice the adaptive growth is reversible, and when the TI is removed the pancreas returns to its original size. No dependence on secretin was seen in mice as TI had similar effects in mice where secretin signaling was genetically deleted. The evidence as to whether such a feedback loop involving CCK occurs in humans is mixed and may involve proteases other than trypsin.

High-protein diet
Consumption of a high-protein diet is well established to cause pancreatic growth, although most of the information is from rodents. Switching from a 5–10 percent casein diet to one containing 70–75 percent casein caused an increase in pancreatic weight, protein, and DNA content reaching a plateau in 7–14 days. This was accompanied by a transient increase in plasma CCK, and the growth response could be partially blocked with a CCK antagonist. Protein is a primary stimulus for CCK release in the rat, with casein being the most potent protein tested after trypsin inhibitor. These experiments led to the concept that protein stimulated pancreatic growth through CCK and that this growth involved both hypertrophy and hyperplasia of acinar cells and was accompanied by an increase in pancreatic protease content.

However, it has been demonstrated that feeding a diet high in amino acids to rats stimulated pancreatic growth without stimulating CCK release. This growth was also not blocked by a CCK antagonist. More definitively, a CCK-deficient mouse fed a high-protein diet showed an increase in pancreatic weight and protein content and a smaller increase in DNA (Figure 4). That the growth in CCK-deficient mice was primarily due to cellular hypertrophy was shown by the increase in protein-to-DNA ratio, an increased acinar cell size, and no increase in DNA synthesis as assessed by BrdU incorporation.

The converse of feeding a high-protein diet is feeding a protein-free or low-protein diet in the presence of adequate calories. Normal rodent chow contains 18–22 percent protein, most often casein, but what should be the normal amount is not always clear. Rat studies of high protein feeding have most often used 5 or 10 percent protein as the control or low protein to maximize the effect of the
high protein. In rats on 5 percent protein the body weight failed to increase over the 14-day experimental period and the pancreas weight decreased slightly. In rats fed trypsin inhibitor or injected with caerulein to stimulate pancreatic growth, there was no pancreatic growth in animals on the 5 percent protein diet. It has also been shown that gavage feeding of 9 percent casein increased plasma CCK but that 2 percent did not. Thus, rodents on diets with less than 5 percent protein may be compromised due to shortage of amino acids and lack of secretion of the pancreatic trophic hormone CCK. Rats fed a protein-free diet lost pancreatic weight in excess of body weight, and there was an increased apoptosis of acinar cells. A more detailed study has been carried out in mice where feeding protein-free diet was restricted to four days at which time there was a 11 percent decrease in body weight, no change in heart weight, but pancreas weight decreased by 24 percent. Most strikingly, the protein content of the pancreas decreased almost 60 percent, while there was little fall in DNA (Figure 5).

These changes were reversible when protein was returned to the diet. Electron microscopy has shown decreased acinar size and a large decrease in the number of zymogen granules in mice, rats, rabbits, and monkeys subjected to protein deficiency. The changes were reversible and interpreted as cellular atrophy. Small-sized acinar cells and failure of digestion have also been reported for children with Kwashiorkor. This maldigestion was interpreted as a failure of the pancreas to secrete digestive enzymes.

Signaling Pathways Mediating Adaptive Growth

Pathways regulating cell size

The primary pathway regulating adaptive cell size growth is centered on the mechanistic target of rapamycin complex 1 (mTORC1). This pathway was discovered in yeast where TOR1 and TOR2 were identified as the targets for the macrolide rapamycin which was isolated from a soil bacterium discovered on Rapa Nui (Easter Island) and found to inhibit growth. Mammals have one form of TOR named mTOR (m originally stood for mammalian but now for mechanistic), which forms two distinct complexes: mTORC1 being primarily related to growth and is sensitive to rapamycin, while mTORC2 regulates the actin cytoskeleton and is relatively insensitive to rapamycin. Each complex has a number of additional regulatory proteins. TORC1 contains six known proteins with the scaffolding protein Raptor, and the inhibitory protein proline-rich Akt substrate of 40 kDa (Pras40) being unique to mTORC1. Other subunits including DEP-domain-containing mTOR interacting protein (Deptor, a negative regulator of mTOR) and mammalian lethal with sec13 protein 8 (MLST8) and are shared with mTORC2 which also contains its own unique protein, Rictor. mTOR the catalytic subunit is a 280 kDa protein that belongs to the phosphoinositide 3-kinase-related kinase family and contains multiple domains, including one binding FKBP-12, the receptor for both FK506 and rapamycin. The mTORC1 pathway integrates input from growth factors, energy status, oxygen, and amino acids. When these are present, mTORC1 stimulates
protein synthesis, lipid synthesis, nucleotide synthesis, and cell cycle progression and inhibits autophagy.\textsuperscript{147,150}

A simplified diagram of the pathway as known for pancreatic acinar cells is shown in Figure 6.

The CCK1 receptor, M3 muscarinic receptors and insulin, and other growth factor receptors all activate phosphatidylinositol 3-kinase (PI3-K). PI3-K phosphorylates and activates Akt, also known as PKB, which phosphorylates and inactivates the tuberous sclerosis complex (TSC). In other cells hypoxia and low cellular energy status are known to activate TSC through REDD and AMPK, respectively. The TSC complex inhibits activation of the G protein Rheb, which when liganded by GTP activates TORC1.\textsuperscript{151} This activation process in cells that have been studied involves migration to the surface of lysosomes, which is the site where amino acids, especially leucine and arginine, activate TORC1 by a process involving a number of other proteins.\textsuperscript{152,153} TORC1 in pancreatic acinar cells is known to be activated by branched chain amino acids.\textsuperscript{154} Moreover, in acini incubated in amino acid–free media, basal TORC1 activity declines to near zero and cannot be activated by CCK (Crozier, S, and Williams, JA; unpublished data).

The best characterized substrates of TORC1 are ribosomal protein S6 kinase (S6K) 1 and initiation factor 4E binding protein 1 (4E-BP1). S6K1 phosphorylates a number of substrates including ribosomal small subunit 6 (S6), while phosphorylation of 4E-BP1 releases IF4E which binds the 5’ cap on mRNA to initiate cap-dependent translation which accounts for about 90 percent of mRNA species. Both of these events are readily demonstrable in intact pancreas and pancreatic acini in response to CCK, growth

\textbf{Figure 4.} Effect of a high-protein diet on pancreatic size and composition in normal and CCK-deficient mice. Changes are shown for pancreas weight (A), protein (B), DNA (C), and protein-to-DNA ratio (D) after seven days on normal or high-protein diet (from Crozier et al.).
factors, and amino acids. A third action in acini contributing to protein synthesis is the activation of elongation factor 2 (eEF2).  

The importance of TORC1 for adaptive pancreatic growth is shown by the fact that pancreatic growth stimulated by feeding TI is blocked by rapamycin or by genetic deletion of raptor in acinar cells (Crozier, S, and Williams, JA; unpublished data), both of which selectively block the TORC1 pathway without affecting other pathways such as the activation of ERK and JNK leading to

Figure 5. Feeding a protein-free diet induces pancreatic atrophy as shown by a decrease in pancreatic weight (A) and protein content (B) (from Crozier et al.).
Interestingly, rapamycin blocks adaptive growth but has little effect on the baseline size of the pancreas. By contrast, feeding a protein-free diet blocks the TORC1 pathway and causes a reduction in pancreatic size due to cellular atrophy. This difference may be due to the fact that some actions of TORC1 are known to be insensitive to rapamycin. When raptor is deleted in acinar cells leading to a loss of TORC1 components, there is both an increase in apoptosis and a dedifferentiation of acinar cells. Thus, there is clearly more to learn of the importance of the TORC1 pathway in acinar cells. Although not presented in detail here, islet beta and alpha cells also are dependent on TORC1 to maintain their mass and demonstrate adaptive growth.

**Pathways regulating pancreatic mitogenesis**

Because of the prominent role of CCK in inducing adaptive pancreatic growth, attention has focused on how CCK receptors activate acinar cell mitogenesis. Mature acinar cells appear to exist in a Go state, and mitogenic stimuli cause them to enter the cell cycle. Early response genes are a class of genes known to be rapidly upregulated following cellular stimulation and function as mediators coupling short-term signals to longer-term cellular responses such as growth by regulating other genes. Initial work showed that CCK induced various early response genes in rat pancreas or isolated acini including c-Fos, c-Jun, Egr-1, and c-myc. This led to enhanced activity of AP-1 transcription factor, which in its most common form is a Fos-Jun heterodimer.
A more complete study of the response of the mouse pancreas to feeding TI in chow evaluated mRNA for 18 early response genes, with 17 showing a rapid increase with a maximum effect seen at 1 to 4 hours followed by a decline (Figure 7). Protein expression for c-Jun, c-Fos, ATF-3, Egr-1, and JunB peaked at two hours. Immunohistochemistry showed nuclear localization, and AP-1 activity was increased in nuclei. The effect of feeding TI was largely blocked by a CCK antagonist, and there was no effect of refeeding regular chow. That this is a direct effect of CCK was supported by similar findings in monolayer cultures of acinar cells. In the in vitro study CCK also induced c-Jun, AP-1 activity, and cyclin D1 expression. Dominant negative JNK or shRNA blocked the c-Jun increase and prevented enhanced mitogenesis. In this culture system acinar cells dedifferentiate before mitogenesis, so it is not a perfect model but shows that CCK can directly stimulate mitogenesis as shown earlier by Logsdon. For further information on signaling pathways activated by CCK in pancreatic acinar cells, see the review by Williams.

Calcineurin-NFAT pathway
CCK is known to activate a number of signaling pathways, but a central role has been assigned to the activation of intracellular Ca²⁺ signaling for the stimulation of digestive enzyme secretion. A sustained increase in Ca²⁺ is known to activate the Ca²⁺-regulated phosphatase calcineurin (CN) in a variety of cells. Utilizing the pharmacological inhibitors cyclosporine A (CsA) and FK-506, Tashiro et al. showed that active CN was necessary for TI-stimulated pancreatic growth that occurs by activation of mitogenesis. A major target of CN are NFATs (nuclear factor of activated T cells). In the basal state NFATs are phosphorylated and remain in the cytoplasm. Upon stimulation, which increases intracellular Ca²⁺, activated CN dephosphorylates NFAT which move into the nucleus and either alone or in partnership with AP-1 activates specific gene expression (Figure 8). In isolated pancreatic acini, CCK stimulates NFAT translocation into nuclei with the effect dependent on CN. An NFAT reporter was also used to show increased NFAT activity. Similar activation of NFATs with nuclear translocation was seen in vivo in response to feeding TI.

Further evidence for the importance of CN came from the discovery that Rcan1 (Regulator of calcineurin 1), also known as DSCR1, was highly induced by feeding TI, with the induction almost totally inhibitable by FK-506. Rcan1 blocked CN action in cell models, and when induced in pancreatic acinar cells in vivo using a LSL expression
of and Rcan1 construct activated by Ela-Cre, it completely blocked adaptive growth in response to feeding TI (Figure 9).\textsuperscript{167} Thus, both pharmacological and genetic inhibition of CN blocked adaptive growth. Rcan1 functions as an endogenous feedback inhibitor of CN action. Though it is reasonable to presume that adaptive growth is mediated by NFATs, other CN substrates could also participate.

RNA microarray analysis of pancreatic genes revealed 38 genes induced by TI two hours after feeding that were at least 70 percent inhibited by FK-506.\textsuperscript{167} These changes were not observed in CCK-deficient mice. The 38 genes included feedback inhibitors (Rcan1, Socs3, Rgs2, Socs2, Lif), growth factors (FGF21, HBEGF), and transcriptional regulators. About 60 percent had predicted NFAT

**Figure 8.** CCK stimulates nuclear translocation of NFATc1-GFP in mouse pancreatic acini (from Gurda et al.\textsuperscript{166}).
regulation, and CHIP analysis confirmed this for five genes in pancreatic 266–6 acinar cells stimulated with calcium ionophore.

**TORC1 Pathway**

The TORC1 pathway, discussed earlier as playing a role in the increased cell size seen in response to high-protein feeding, is also necessary for adaptive growth involving cell proliferation. This has been shown through the use of rapamycin, which blocks both mitogenesis and the increase in pancreatic weight, protein, and DNA in response to TI feeding. Genetically, deletion of raptor, an essential component of TORC1, also blocks adaptive growth (Crozier, S, and Williams, JA; unpublished data). Similarly, mTORC1 is required for islet beta cell growth. In yeast, rapamycin blocks cell division because mitogenesis cannot occur without the increase in cell protein required to convert one cell into two. This does not appear to be the case in mammalian cells, and attention has focused instead on actions of TORC1 on cell cycle regulators such as Cyclin D1 and CDK4 which are sensitive to rapamycin.

**MAPK Pathways**

CCK and other pancreatic secretagogues activate multiple MAPK pathways including ERK, JNK, and p38 MAPK. Of these, both the ERK and JNK pathways are also activated by feeding TI. Interestingly, the activation of JNK was blocked by FK-506 pretreatment, while activation of ERK, which occurred rapidly with peak activation at 30 min, was unaffected. A later study also showed a more prolonged activation of ERK. JNK activation is known to be important for mitogenesis in cultured acinar cells but has not been studied in normal animals. A requirement for ERK for adaptive growth in vivo in mice has been shown using new ERK inhibitors developed for in vivo administration as potential cancer therapeutic agents (Figure 10).

PD-0325901 and GSK-1120212 (Trametenib) can be mixed in chow or gavage fed and inhibited by binding to the catalytic site of MEK, thereby blocking ERK with low toxicity. The MEK inhibitors blocked pancreatic ERK signaling, adaptive growth, and the increased DNA synthesis stimulated by feeding TI to mice. Interestingly, the MEK inhibitor blocked induction of c-Fos but had no effect on

**Figure 9.** Overexpression of Rcan1 in acinar cells blocks TI-stimulated pancreatic growth (from Gurda et al.).
c-Jun induction. It also blocked the expression of cyclins D1, D3, E, and A along with PCNA. The ERK pathway has previously been shown to play a proliferative role in multiple other cells and tissues, although its mechanistic target is not clearly established.

Stat3
Bioinformatics analysis of 318 genes which were upregulated in pancreas from 1 to 8 hours after feeding TI suggested involvement of Jak-STAT signaling. An increase in phospho STAT3 showed a strong nuclear occupancy peaking at 75 percent of acinar cell nuclei at 2 h. Western blotting showed a large increase in p-STAT3 without a change in total STAT3. Socs2, which acts as a negative feedback regulator of STAT3, is a transcriptional target of STAT3, and its mRNA was found to also be enhanced. Interestingly, in isolated acini CCK fails to activate Jak-STAT signaling while growth factors can. Since growth factors such as HB-EGF are induced by TI feeding, it is possible that CCK does not work directly but rather through a growth factor whose receptor can activate Jak-Stat signaling. At present, there is no direct evidence that STAT3 is involved in adaptive pancreatic growth. However, it seems likely that it will be involved as STAT3 is involved in the malignant transformation of acinar cells. Also, STAT5 and Socs3 have been shown to regulate growth of pancreatic islet beta cells.

Overall model of mitogenesis
It seems clear that acinar cell mitogenesis is under different and more complex control than is secretion of digestive enzymes. Blockage of three different pathways—the calcineurin-NFAT, the TORC1, and the ERK pathways—blocks adaptive growth, indicating that all must be activated for mitogenesis to occur (Figure 11). It is not clear whether all three converge on a single rate-limiting step or that multiple key steps exist and each is activated by a different pathway. It appears that the induction of multiple cyclins is blocked by inhibiting each pathway, so the regulated step is most likely connected with moving cells out of Go and initiating the cell cycle. In addition to completing the understanding of how acinar cell mitogenesis is initiated, work is needed on whether and how there is a parallel increase in ducts and blood vessels as the pancreas grows. Similar studies have also yielded an incomplete map for regulation of islet beta cell growth.
Figure 11. Three CCK-activated signaling pathways (mTORC1, calcineurin-NFAT, and the ERK pathways) required for pancreatic acinar cell growth by proliferation of acinar cells.

References

42. Zucker KA, Adrian TE, Bilchik AJ, Modlin IM. Effects of the CCK receptor antagonist L364,718 on pancreatic growth in adult and developing animals. Am J Physiol Gastrointest Liver Physiol 1989; 257:G511–G516.


McCue MD. Starvation physiology: reviewing the different strategies animals use to survive a common challenge. Comp Biochem Physiol A Mol Integr Physiol 2010; 156:1–18.


Baumler MD, Koopmann MC, Thomas DD, Ney DM, Grobelwski GE. Intravenous or luminal amino acids are insufficient to maintain pancreatic growth and digestive enzyme expression in the absence of intact dietary protein. Am J Physiol Gastrointest Liver Physiol 2010; 299:G338–G347.


Chapter 23
Pancreatic regeneration: Models, mechanisms, and inconsistencies
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Introduction
While skin, liver, and gut have the ability to regenerate and heal, other organs such as heart and brain do not display similar regenerative capacities. The adult pancreas displays a limited capacity to regenerate, although this regenerative capacity declines with age.¹⁻⁵ Thus, with respect to the pancreas, the disagreement is not so much about the overall ability of the adult pancreas to regenerate but rather about which cells may act as cell(s) of origin in this process. For example, it is widely accepted that under physiologic conditions β-cell regeneration in the adult mouse pancreas originates from β-cell self-duplication.⁶,⁷ However, depending on the type of injury model, it appears that new β-cells can arise from cells either residing within the ducts,⁸⁻¹² in proximity to the ductal network,¹³ or from other pancreatic endocrine cells.¹⁴⁻¹⁷ This uncertainty regarding the types of cells that may potentially give rise to new β-cells comes in part from the fact that in each experimental model of regeneration, the exact target cells and the severity of the injury are different. Here, we will first review some of the injury models that have been used to study the mechanisms leading to replacement of acinar- or β-cells, followed by a discussion of the discrepancies in these reports.

Injury Models to Study Pancreatic Regeneration
Over the years multiple models of pancreatic injury have been used by different investigators to explore the regenerative capacity of exocrine or endocrine compartments of the adult pancreas.¹⁸ Among these models, some of the commonly used are pancreatic duct ligation, partial pancreatectomy, caerulein-induced pancreatitis, alloxan- or streptozotocin-induced diabetes, and diphtheria toxin-mediated cell ablation. These models are of varying specificity, and they entail surgical, chemical, or genetic methods.

Pancreatectomy
Pancreatectomy (Px) is the oldest model with which to examine the regenerative capacity of the pancreas.¹⁹ The first documented removal of the pancreas was performed on dogs by Johann C. Brunner in 1683.²⁰ However, it was first in 1890s that Px was reported to result in diabetes, and hence a link between the pancreas and glucose homeostasis was established.¹⁹,²⁰ Px can be used to study acinar and β-cell recovery in both rats and mice; however, because of the increased islet mass, this injury model has been extensively used to study β-cell regeneration.³,⁵,²¹⁻²⁷ Partial pancreatectomy (PPx) involves resection of less than 90 percent (often 50–75%) of the adult mouse pancreas.⁵,⁶,²³,²⁸⁻³³ Here, the remnant of the pancreas displays normal gross morphology. A more severe form of Px that has been performed on rats is subtotal pancreatectomy (SPx) and entails removal of 90–95 percent of the gland through tissue abrasion.²⁵,²⁸,³⁴⁻³⁶ Thus, in contrast to PPx, here the acinar cells in the remnant of the pancreas undergo rapid atrophy, which results in a desmoplastic reaction.²⁵,³⁷ Regardless of the extent of resection, Px is associated with an IGF/PI3K-dependent upregulation of Pdx1 expression in duct cells.⁵,²⁶,³⁶ Interestingly, the limited organ recovery that follows Px appears to be proportional to the size of the excision.²⁸,³⁴,³⁵,³⁸⁻⁴⁰ SPx in rats leads to ductal cell proliferation and induction of an extensive regenerative process that promotes mature duct cells to regress and reexpress embryonic genes such as Pdx1, Ptf1a, and Ngn3 before differentiating to the different pancreatic cell types.²⁵,³⁶ While these data imply that the duct cells might contribute to the observed increased islet mass following Px, other studies would argue against the involvement of duct cells in this process.⁶,³⁰,³²,³³ Using lineage-tracing studies, independent investigators have not been able to find any evidence for β-cell neogenesis following 50–75% PPx.⁶,⁴⁵ Accordingly, 50 percent PPx in Ngn3-GFP transgenic mice failed to

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induce Ngn3-expression in cells within islets or ducts. All together, although the potential contribution of other cell types cannot be ruled out, the current literature supports the notion that the main source for acinar or β-cell regeneration that follows Px is preexisting acinar or β-cells, respectively.6,32,33,40

**Pancreatic duct ligation**

As in the case of Px, ductal obstruction and ligation have historically been used in investigating pancreatic regeneration.19 Pancreatic duct ligation (PDL) involves ligation of one of the main ducts, which leads to acinar cell death and inflammation in the area distal to the ligation. An advantage with PDL is that the unligated portion of the pancreas remains unaffected and thus can be used as an internal control. However, the regenerative process in this model, particularly the acinar regeneration, appears to be species-dependent. PDL in rats is associated with near complete acinar recovery through a process that involves appearance of ductular structures and their differentiation into acinar cells.34,41 In mice, although PDL results in the formation of similar metaplastic ducts, the acinar compartment does not regenerate.32,41-43 Lineage-tracing studies in mice show that both surviving acinar cells32 and Hnf1β-expressing duct cells42 in the ligated part of the pancreas can contribute to the formation of these ductular structures. In other words, the tubular structures observed in the ligated part consist of acinar-derived metaplastic ducts and preexisting ducts that have changed morphology. Similarly, in rats it is believed that preexisting acinar cells transdifferentiate into these ductular structures,34,41 whereas the contribution of duct cells in this process has yet to be determined.

PDL has been primarily used to provide insights on islet β-cell generation, as it is reported to stimulate β-cell regeneration in both mouse and rat.12,13,33,34,41,44-46 Nevertheless, there is a controversy with respect to the mechanism allowing the observed β-cell generation. While some studies favor β-cell proliferation as the main mechanism for β-cell formation after PDL,33,41-43,47 others support the potential contribution of non-β-cells (in particular, cells within or in proximity of ductal network) to β-cell neogenesis in a PDL setting.9,12,13,48

**Caerulein-induced pancreatitis**

Caerulein is a cholecystokinin orthologue, which when administered repeatedly at high concentrations leads to the death and dropout of over half of the acinar compartment which can cause acute or chronic pancreatitis.18,49,50 In mice, the pancreas regains its normal histology within a week after caerulein treatment. The rapid regenerative process associated with this injury model has been used by many investigators to follow the course of acinar recovery.51-55 Lineage-tracing studies have shown that following caerulein-induced pancreatitis the surviving acinar cells contribute to the recovery of the acinar compartment.32,51,53,55 Acinar regeneration in this model is through acinar-to-ductal metaplasia (ADM), a process that requires a transient reactivation of various developmental genes and signaling pathways, including notch, hedgehog, and wnt.51-53,56-58 ADM involves de-differentiation of acinar cells into duct-like cells, proliferation of metaplastic ducts, and finally re-differentiation of duct-like cells into acinar cells.53 A first step toward transdifferentiation of one cell type to another cell type is that cells have to lose their original identity in order to acquire a new one. Accordingly, the de-differentiation of acinar cells is associated with expression of ductal markers such as Hnf6, Sox9, and cytokeratin-19 and concomitant repression of acinar markers Ptf1a, Mist1, amylase, and Cpa.58 Wnt/β-catenin signaling is one of the embryonic pathways, which is reactivated in ADM following caerulein-induced pancreatitis.55,56 However, for the ADM to re-differentiate into acinar cells wnt signaling has to be eventually downregulated, as persistent wnt/β-catenin activity leads to impaired acinar recovery.53,59 The precise mechanism for the dynamic activity of wnt/β-catenin in ADM is not clear, but a recent report implicates HDACs as an important epigenetic switch required for controlling nuclear β-catenin transcriptional activity.59 The transcriptional factor PDX1 has primarily been associated with the embryonic pancreas and mature β-cells in the adult pancreas. However, a more recent study highlights the importance of PDX1 in maintaining acinar cell identity.60 PDX1 displays similar dynamic expression as wnt/β-catenin during ADM, and accordingly its downregulation is necessary for re-differentiation of ADM into acinar cells.60

**Other models of pancreatitis**

In addition to the aforementioned caerulein-induced pancreatitis, other rodent models commonly used to study acute pancreatitis entail bile salt infusion, duct obstruction, the choline-deficient ethionine supplemented diet (CDE), or administration of basic amino acids such as L-arginine. These injury models have been extensively described and reviewed by Lerch and Gorelick elsewhere.18

**Alloxan- or streptozotocin-induced diabetes**

Alloxan and streptozotocin (STZ) are used to induce diabetes by chemical ablation of pancreatic β-cells. Alloxan was first described in the early 1800s, but its diabetogenic property was reported in 1943, and since then alloxan treatment has been used as an experimental model for diabetes.61 STZ was initially used as a chemotherapeutic agent in pancreatic
islet cell tumors and other malignancies, but since its discovery as a diabetogenic agent in 1963, it has been widely used in diabetes research. Alloxan and STZ are both toxic glucose analogues that preferentially accumulate in insulin-producing β-cells via the Glut2 glucose transporter. Diabetes as the result of alloxan or STZ-treatment is not associated with β-cell regeneration. Because of the absence of spontaneous β-cell recovery, these models have been useful tools to study a given treatment on β-cell regeneration. In addition, alloxan- or STZ-treatment can be combined with PDL to study the effect of hyperglycemia on the regenerative process in the ligated portion of the pancreas. While the combination of alloxan- or STZ-treatment and PDL in mice led to transformation of glucagon-producing α-cells or acinar cells into β-cells, no such α-to β-cell conversion could be found when rats were subjected to a combined PDL and STZ-treatment. A relatively new method which enables cell-specific ablation is transgenic activation of the diphtheria toxin cell death pathway using a cell-specific promoter. Mature diphtheria toxin (DT) is composed of subunits A and B (DTA and DTB). DT binds a toxin receptor on the cell surface of toxin-sensitive cells and is endocytosed. Upon entry into the cytoplasm, the DTA subunit is released and it catalyzes the inactivation of elongation factor 2, resulting in termination of all protein synthesis, with rapid apoptotic death of the target cell. The toxicity of DTA is sufficiently high that only one molecule of DTA in the cytosol may be enough to kill the cell. The DT receptor (DTR) is a membrane-anchored form of the heparin binding EGF-like growth factor (HB-EGF precursor). The human and simian HB-EGF precursors bind DT and function as toxin receptors, whereas HB-EGF from mice and rats do not bind the toxin and therefore remain insensitive to DT. Thus, transgenic expression of the simian or human DTR in mice can render naturally DT-resistant mouse cells DT-sensitive. Recently, a mouse strain was generated (R26
dTR), in which a loxP-flanked STOP cassette and the open reading frame of simian DTR had been introduced into the ROSA26 locus. In the R26
dTR strain, the gene encoding DTR is under the control of the potent Rosa promoter, but DTR expression is dependent first on Cre-recombinase removal of the STOP cassette. Following Cre-recombinase activity, the DTR-expressing cells, that is, cells expressing Cre, and all of their progeny, are viable and function normally. However, these cells are rapidly killed upon DT administration. Noteworthy, the HB-EGF is no longer active as an EGFR ligand, as transgenic lines expressing DTR in different pancreatic lineages do not display any abnormal phenotype. In the adult pancreas, DTR/DTA-mediated β-cell ablation has been used to study regeneration following α- or β-cell specific losses. The Inconsistencies
A brief look at Table 1 highlights the inconsistencies that currently exist in the literature regarding the regenerative capacity of the adult mouse pancreas. For example, there is a complete recovery of the acinar compartment within a week after caerulein-induced pancreatitis, whereas there is principally no acinar regeneration following PDL. Additionally, α-cells can differentiate into β-cells; however, this plasticity has been observed only upon total β-cell ablation but not following partial loss of β-cell mass. Variations between different studies are likely due to the disparities in the nature, extent, and, perhaps more importantly, the severity of injury used. In this review, we will argue that the combined effects of these parameters not only may determine whether or not regeneration would occur but also dictate which cell type(s) should contribute to this process.

The nature of injury
Here, the question is not so much about whether the injury is chemically, mechanically, or genetically induced but rather about what kind of cell death it triggers. Apoptosis is programmed cell death generally associated with retention of plasma membrane integrity, condensation, and cleavage of nuclear and cytoplasmic proteins and cell shrinkage or the formation of apoptotic bodies. It is a highly coordinated process which requires significant amount of energy and therefore relies on mitochondrial respiration and ATP production. Necrosis, on the other hand, is invoked in response to external stimuli and ATP deficiency. Pancreatic injury and ensuing regeneration invariably depend on proper clearance of the dead cells. Because of its nature (loss of cytoplasmic membrane integrity, cellular fragmentation, and release of lysosomal and granular contents into surrounding extracellular space), necrosis does not allow for proper removal of cell organelles, and as a result it is followed by reactive inflammation. In contrast, apoptosis involves debriding the tissue without generating massive inflammation that is usually induced by the degeneration of dead cells. The effect of apoptosis or necrosis on regeneration is perhaps best manifested when one compares β-cell regeneration following STZ (or alloxan) treatment with DT-mediated β-cell ablation. In these two models, the target cells (β-cells) and the degree of β-cell loss (75–80% sub-optimal condition for STZ, or 75% β-cell ablation using PdxCreERT) are similar. Interestingly, STZ-induced necrosis is accompanied with a massive inflammatory response and the absence of β-cell regeneration, whereas DT-induced apoptosis leads to almost complete β-cell mass recovery.
Overall, compared to the necrosis, apoptosis provides an environment that would favor β-cell regeneration. Notably, acinar regeneration appears to be less sensitive to the nature of injury, as robust acinar tissue recovery has been reported in both necrotic (caerulein) and apoptotic (DT-mediated) environments.\textsuperscript{1,10,51–56}

**The extent of injury**

Another factor that may influence the regenerative process is the extent of injury. In other words, how many different cell types are affected by the insult? In addition to the ductal, acinar, and five different hormone-producing endocrine cell types, the adult pancreas is home to numerous endothelial, stellate, and neuronal cells. The type of injured cells is important as recent reports have shed light on the role of macrophages in inducing ADM, as well as promoting β-cell, or acinar cell regeneration.\textsuperscript{1,45,87–89} Macrophages appear to have differential functions in diverse phases of regeneration: first, to debride the tissue following injury and, second, to convert injury signals into lineage-specific regenerative signals.\textsuperscript{1,90,91} One exception to this rule is PDL, which as mentioned earlier is associated with acinar atrophy. Thus, one would expect that this model would lead to acinar regeneration, but instead it stimulates β-cell regeneration. Clearly, the effect of PDL on non-acinar cells residing in the ligated part cannot be ruled out. Therefore, it is possible that the combined regenerative signals released by macrophages (as the result of engulfing damaged acinar- and non-acinar cells) would serendipitously create an environment that would promote β-cell regeneration instead of acinar. In fact, combined PDL and β-cell ablation by STZ has been reported to enhance acinar to β-cell transdifferentiation.\textsuperscript{48}

Based on our current understanding of the involvement of macrophages in regeneration, simultaneous ablation of many cell types would make direct interpretation of cellular mechanism of regeneration difficult. Thus, cell-type specific ablation may be a better method for analyzing the in vivo function of cells during regeneration, which can be achieved by using streptozotocin, alloxan (for β-cells), or caerulein (for acinar cells). Alternatively, transgenic activation of the diphtheria toxin (DTR/DTA) cell death pathway, which depending on the promoter, can target one specific (e.g., Elastase promoter for acinar cell ablation) or more than one cell type (Pdx1 promoter to target all pancreatic epithelial cells).\textsuperscript{1,10}

**The severity of injury**

Mounting evidence suggests that the severity of injury is perhaps one of the most important elements that dictate whether the mechanism for repair should include replication of preexisting cells or neogenesis from other cell types. DT-mediated cell ablation has been used by a number of investigators to vary the extent and the severity of injury, while keeping other variables (such as the nature of the injury) relatively constant. Collectively, it appears that regardless of cell type, as long as ablation of a specific cell type does not reach near 100 percent, the mechanism for regeneration mainly involves the preexisting cells.\textsuperscript{1,6,14,51,53,55} Therefore, following 75 percent ablation of β-cells, surviving β-cells proliferate to generate new β-cells, whereas complete loss of insulin-producing cells promotes conversion of other endocrine cell types into β-cells.\textsuperscript{1,6,14,17,92,93} Consistently, acinar cell recovery following caerulein-induced pancreatitis is through preexisting acinar cells. However, near complete loss of both acinar and endocrine cells stimulates cells within the ductal compartment to form new acinar and endocrine cells.\textsuperscript{1,10} One could also argue that the extent of the surgical intervention may be important also in the Px setting and could explain discrepancies in some reports describing absence or vigorous pancreatic regeneration after partial or SPx, respectively.\textsuperscript{6,25,35} However, as mentioned earlier unlike PPx, SPx is associated with acinar atrophy and a desmoplastic reaction. Of note, this inflammatory reaction has been reported to be important for the robust regeneration that follows SPx, as
inhibition of the inflammation prevented regeneration. Therefore, it is likely that the inconsistencies between PPx and SPx are due to the presence or the absence of inflammation rather than the extent of injury.

Conclusions
Pancreatic regeneration relies on a complex interaction between cells that provide necessary regenerative signals and cells that are receptive to those signals. As discussed here, the nature, extent, and the severity of injury are three important parameters that determine whether tissue recovery is achieved. β-cell regeneration seems to be more sensitive to the nature of injury than acinar regeneration. Finally, the extent of injury determines which cell types would respond to these regenerative signals.

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References


40. Strobel O, Dor Y, Alsina J, Stirman A, Lauwers G, Trainor A, Castillo CF, Warshaw AL, Thayer SP. In vivo lineage tracing...


Pancreatic Islet and Stellate Cell Structure and Function
Chapter 24
Structure of islets and vascular relationship to the exocrine pancreas
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\section*{Introduction}

The pancreas is a retroperitoneal organ with its function being dictated by the possession of two morphologically distinct tissues, the exocrine and endocrine pancreas. The endocrine pancreas is organized into islets of Langerhans, consisting of five cell subtypes: alpha, beta, delta, epsilon, and PP (pancreatic polypeptide) cells that secrete glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide, respectively. Islet cells make up only 2 percent of the adult pancreatic mass. The exocrine pancreas, which is composed of acinar and ductal epithelial cells, accounts for nearly 98 percent of the adult pancreatic mass.\textsuperscript{1} The structure of the exocrine and endocrine pancreas has been extensively studied due to the clinical importance of pancreas-specific diseases such as diabetes, pancreatic cancer, and pancreatitis. However, many of these findings have been extrapolated from mice, which remains the most studied animal model in pancreas research. This focus on mouse is due to difficulty in procuring human pancreas. The mouse pancreas contains approximately 1000–5000 islets, whereas the human pancreas contains approximately 1–15 million islets; their size is similar with an upper diameter of 500–700 μm in both mice and humans.\textsuperscript{2} Pancreatic islets are highly vascularized micro-organs with a capillary network that is five to ten times denser than that of the exocrine pancreas (\textbf{Figure 1}).\textsuperscript{3} Other structures within the pancreas that appear highly vascularized are the pancreatic ducts which are enveloped by a dense network of vessels that are much denser than in the surrounding acinar tissue (\textbf{Figure 1}).\textsuperscript{3} Blood vessel formation plays an essential role in adult tissue function as well as during embryonic development.\textsuperscript{4}

\section*{History}

Over time, the pancreas has evolved from relative obscurity to possessing the most studied cell in the world, the beta cell. The term pancreas, “all-flesh,” was first coined by Aristotle (384–322 B.C.) and later described by the Greek anatomist and surgeon Herophilus (335 B.C.–280 B.C.).\textsuperscript{5} However, this organ was then overlooked for many years, partly because of its location as opposed to other more prominent organs such as the liver or kidneys, but mostly because its function was unknown for many centuries. Galen’s (Claudius Galenus, 129–216 A.D.) false dogma that the organ functioned as a mechanical protective cushion for the mesenteric vessels was unchallenged until the 17th century.\textsuperscript{6} In 1642, the main pancreatic duct was discovered by the German anatomist Johann Georg Wirsung (1589–1643) during an autopsy of a human subject, engraving his finding on a copper plate as a method of reproducing the anatomical illustration.\textsuperscript{6} Although its function was never known to him, it was eventually named after him in honor of his discovery. Though Wirsung himself and others did make the observation of occasionally seeing the duct to be double, the discovery of the accessory pancreatic duct as a normal variant is credited to Giovanni Domenico Santorini (1681–1737).\textsuperscript{6} The pancreatic duct discovery was a milestone in pancreatic history because it made Galen’s theory obsolete and led to studies of the pancreas as an exocrine gland. Reignier de DeGraaf (1641–1673) showed that the pancreas was undoubtedly an exocrine gland by cannulating the duct, which abolished the previous theory that the pancreas acted as a cushion of the stomach. Paul Langerhans (1847–1888), a German pathologist, was working on his thesis entitled “Contributions to the microscopic anatomy of the pancreas” when he discovered discrete areas of “clear cells” within the pancreas. Langerhans documented the histology of the pancreas by injecting the ductal system in the rabbit pancreas with Berlin blue dye, identifying and describing the endocrine cells as “small irregular-polygonal structures with a cytoplasm perfectly shiny.” However, the first to discover that these “islands of clear cells” were well vascularized and communicated with the capillary bed of the exocrine tissue were Wilhelm F. Kuhne and A. S. Lea in 1882 who compared these distinct
vascular regions to the renal glomeruli. In 1893, the French histologist Edouard Laguesse suggested that these vascular regions be named “islets of Langerhans” in memory of their original discoverer.

Since then, a variety of injection techniques have been used by a variety of authors to demonstrate the islet microvascular anatomy including Berlin blue dye, methylene blue, dyed collagenase, and fluorescent dyes such as Lucifer Yellow or tomato lectin. Other methods utilized to visualize the microvascular anatomy included corrosion casting coupled with scanning electron microscopy. The latter method allowed a beautiful three-dimensional view of the pancreatic and islet vasculature but was labor intensive. Then came the development of diffusible tracer techniques to estimate blood flow distributions within organs, including the islets. Microspheres measuring 10–15 μm introduced through an intra-arterial injection became the standard marker for islet blood flow measurements. The number of microspheres within each organ is proportional to their blood perfusion. Microspheres are quantified here by either directly counting the spheres or by quantifying a signal (radioactivity, fluorescence, or different colors), that is incorporated into the spheres. However, there was a risk of overestimating blood flow due to shunting, and if the number and size of the spheres exceeded a certain threshold, there would be a risk for emboli and tissue ischemia. At the turn of the 21st century, novel imaging techniques were developed to visualize the intricate islet microvascular anatomy in three dimensions, such as whole-mount immunohistochemistry (Figure 1 A-D) and real-time in vivo imaging. A major challenge now is to quantify the endocrine cell mass in vivo to better monitor beta cell loss in diabetics or after islet transplant. New pancreas-specific biomarkers are being sought for this purpose.

Embryonic Pancreatic Vasculature Development

Despite the well-established role that blood vessels play in the development of different organ systems, their role in pancreatic morphogenesis and differentiation remains poorly understood. However, it is clear that blood vessels play different roles during different stages of pancreatic development, with the potent vasculogenic factor, vascular endothelial growth factor (VEGF), having an especially important function.

In the developing pancreas, there are abundant VEGFR2-positive endothelial cells throughout the early embryonic mesenchyme. Furthermore, VEGF receptor (VEGFR2) inhibition resulted in abnormal epithelial growth and differentiation, whereas Pdx1-driven
overexpression of VEGF resulted in pancreatic growth arrest and islet disruption.\textsuperscript{20,21,23} The avascular dorsal pancreatic bud first evaginates at embryonic day 9.5 (E9.5) toward the aorta and subsequently undergoes extensive morphogenesis, giving rise to a highly branched and tubular epithelium with small capillaries being observed within the surrounding mesenchyme of the pancreatic bud epithelium. At E10.5, some blood flow is seen in the capillaries surrounding the pancreatic bud. Then at E11.5–E12.0, the pancreatic epithelium further remodels, forming bulging “tips” with blood vessels becoming intercalated in between them. These observations suggest that the pancreas gets vascularized in the early stages through a combination of epithelial growth into a preexisting mesenchymal vascular plexus of patent capillaries, as well as peripheral vasculo-genesis (i.e. de novo development of vessels from angioblasts). The latter process is directed by the early expression of VEGF throughout the pancreatic epithelium at E11.5. The developing endocrine islets and ductal trunk exhibit high VEGF expression, whereas the acini at the epithelial tips lack VEGF expression. This explains why both islets and pancreatic ducts appear highly vascular in the postnatal pancreas compared to the surrounding acini (Figure 1). A primitive primary honeycomb-like primary plexus of vessels appears in the surrounding pancreatic mesenchyme at E10.5, which later forms the veins, followed by the formation of a large central vessel, which is formed along the center of the pancreatic bud by E12.5, becoming an artery. By E13.5, the central vessel becomes progressively integrated into the expanding pancreas and by E14.5, the main central artery becomes more established, with the fusion of more vessels progressing from proximal to distal pancreas. Thus, the veins localize to the periphery, and the arteries more centrally within the pancreas.\textsuperscript{20}

Shah et al.\textsuperscript{22} nicely demonstrated in their whole embryo “angiogram,” utilizing a novel in utero intracardiac injection, a relative paucity of blood flow in early pancreatic development (E11.5), with the presence of blood flow correlating with the differentiation of pancreatic epithelial cells in the region of the blood flow. Specifically, during E12.5–E14.0 glucagon-positive cells tended to localize specifically near perfused blood vessels, and by early E14 insulin-positive cells tended to localize near the blood flow. Thus, endothelial cells and blood flow appear to help direct pancreatic differentiation.\textsuperscript{21,22}

**Gross Anatomy of the Pancreas**

The pancreas is an endodermally derived retroperitoneal organ that is formed by the fusion of the ventral and dorsal buds of the foregut. In the human it is divided into the head, neck, body, and the tail, with no clear-cut borders between them. However, it is generally accepted that the border between the head/neck and body is marked by the superior mesenteric artery, whereas the border between the body and tail is considered to be the midpoint between the neck and the tip of the tail. The head has an extension known as the uncinate process that hooks beneath the superior mesenteric artery, with the head nestled in the curvature of the duodenum. The body of pancreas sits beneath the stomach, extending horizontally and crossing over the inferior vena cava, abdominal aorta, superior mesenteric artery, and vein. The neck is the thin section of the gland between head and body overlying the superior mesenteric artery. The pancreatic tail abuts the splenic hilum. The total length of the gland is about 15 cm and it weighs 50–100 gm\textsuperscript{2}.

The mouse pancreas is divided into three major parts: the duodenal, splenic, and gastric lobes, with the splenic lobe making up over half of the total pancreatic volume. The duodenal lobe is homologous with the head of the human pancreas, whereas the splenic lobe is homologous with the body and tail of the human pancreas, which extends horizontally between the duodenum and spleen. The gastric lobe is the third and smallest part of the mouse pancreas.\textsuperscript{2}

The distribution of islets in different lobes of the mouse pancreas is heterogeneous and strain dependent. The number of islets per unit volume is highest in the gastric lobe, followed by the duodenal and splenic lobe. In the human pancreas, the density of islets per unit volume is higher in the tail compared to the head and body.\textsuperscript{2}

**Macroscopic Vascular Anatomy of the Pancreas**

The blood supply of the pancreas is primarily derived from the celiac artery, representing approximately 1 percent of the total cardiac output.\textsuperscript{24} The head of the pancreas is supplied by an arterial arcade system formed by the anterior and posterior superior pancreaticoduodenal arteries arising from the gastroduodenal artery of the common hepatic artery and anastomosing with the anterior and posterior inferior pancreaticoduodenal arteries, which is the first branch of the superior mesenteric artery. The body and pancreatic tail, on the other hand, primarily receive their blood supply from the pancreatic branches of the splenic artery, which is the left most branch of the celiac artery, and by the dorsal pancreatic artery that branches off near the origin of either celiac, hepatic, or splenic artery. Its right branch anastomoses with the anterior superior pancreaticoduodenal artery, whereas its left branch forms the transverse pancreatic artery that runs along the inferior border of the pancreas and usually anastomoses with the pancreatica magna artery, the largest pancreatic branch of the splenic artery in the middle of the gland.\textsuperscript{25,26}

The venous drainage of the pancreas mainly follows the arterial system and drains into the portal vein via either the splenic vein, from the body and tail of the pancreas, or
via the superior mesenteric vein from the pancreatic head venous arcade system.\textsuperscript{2}

Microscopic Vascular Anatomy of the Pancreas

The islets of Langerhans, which compromise nearly 2 percent of the total pancreatic volume, are highly vascularized structures within the pancreas, receiving approximately tenfold the blood supply of the exocrine tissue by volume, with the intra-islet microcapillary network constituting 7–8 percent of the total islet volume\textsuperscript{27,28} (Figure 1). The islets are irregular in shape, but mostly circular to oval, scattered individual endocrine cells, not contained within an islet, are found throughout the pancreatic acinar and ductal tissue (Figure 2). The ratio of beta to alpha cells is higher in mice than in humans, with beta cells making up the majority of endocrine cells within an islet (50–70\% and 60–80\% in humans and mice, respectively). Alpha cells compose 20–40 percent and 10–20 percent of endocrine cells in human and mouse islets, respectively\textsuperscript{2,3} (Figure 3). Depending on the size of the islet, 1–5 arterioles enter each

Figure 2. (A) Whole-mount image of wild-type mouse pancreatic tissue demonstrating the pancreatic duct (DBA) and different shapes and sizes of the islets (red). (B) A pancreatic section from a wild-type mouse showing scattered insulin positive endocrine cells (red) in a pancreatic duct (DBA). DBA: dolichos biflorus agglutinin, a ductal marker.

Figure 3. (A) Whole-mount image of a two-month-old human pancreatic islet. (B–D) Pancreatic section of a 16-year-old human pancreatic islet revealing that up to 40 percent of the islet is composed of alpha cells (C). (E) Section of a two-month-old small (<60\um) human pancreatic islet demonstrating the mantle-core distribution of alpha and beta cells. (F) Section of a wild-type mouse pancreas revealing the typical mantle distribution of the alpha cells with 80 percent of the islet composed of insulin-secreting beta cells. Pancreatic duct cells are labeled by DBA.
islet and divide into a dense fenestrated capillary network\cite{15} (Figure 4). In most rodents, the distribution of endocrine cells within an islet follows a mantle distribution of non-beta cells, including alpha, delta, and pancreatic polypeptide, with beta cells filling up the core (Figure 3F). This unique distribution appears to have functional implications. When beta cells are intermingled with non-beta cells instead of the mantle-core segregation, the beta-cell function was reduced. Bosco et al.\cite{30} demonstrated in their morphological analysis of the human pancreas that the alpha cell distribution was not random. In small human islets (40–60 μm in diameter) the typical mantle-core distribution was seen with alpha cells in the periphery and beta cells occupying the core. In larger human islets (>60 μm in diameter) it was noted that alpha cells were found in a mantle, but they also always lined the inner vascular channels, which are the empty spaces found in the middle of the islet, thus always juxtaposed to endothelial cells. Their three-dimensional analysis revealed that islet cells are organized into a trilaminar plate composed of one layer of beta cells sandwiched between two layers of alpha cells. The islets are folded onto each other with different degrees of complexity and vessels invaginating the “empty spaces” within the islets.\cite{30}

There have been numerous islet vascular perfusion studies conducted and it appears, at least in mice, that there are three microcirculatory flow patterns. Firstly, from center to periphery (i.e. alpha cells are exposed to blood first). Secondly, from center to periphery (i.e. beta cells are exposed to blood first) and lastly, from one pole of the islet to the other. Roughly, 66 percent of islets have the center-to-periphery pattern\cite{16,31} (Figure 5).

References

Figure 5. Three blood flow patterns of the islet microcirculation. (A) From periphery to center; (B) from center to periphery; (C) from one pole to the other.
The Dual Nature of the Pancreas

The pancreas is a complex gland active in digestion and metabolism through secretion of digestive enzymes from the exocrine portion and hormones from the endocrine portion. The exocrine pancreas, which accounts for more than 95–98 percent of the pancreas mass,1 is structurally comprised of lobules, with acinar cells surrounding a duct system. The endocrine pancreas makes up only 2 percent of the pancreatic mass and is organized into the islets of Langerhans—small semi-spherical clusters of about 1,500 cells2 dispersed throughout the pancreatic parenchyme—which produce and secrete hormones critical for glucose homeostasis. The existence of islets was described by Paul Langerhans in 1869, and the functional role of islets in glucose homeostasis was first demonstrated in 1890 when Joseph von Mering and colleagues showed that dogs developed diabetes mellitus following pancreatectomy.3 Though islet mass may vary between individuals—an example is the increase in the setting of adult obesity4—the average adult human pancreas is estimated to contain one to two million islets.5,6 In humans, the concentration of islets is up to two times higher in the tail compared to the head and neck. However, the cellular composition and architectural organization of cell types within the islets are preserved throughout the pancreas.7 Pancreatic islets are composed of α, β, δ, ε, and PP (pancreatic polypeptide) cells; these are primarily endocrine (hormone-secreting) cells, containing numerous secretory granules with stored hormone molecules, ready for release upon receipt of the appropriate stimulus. Insulin-producing β-cells are the most common cell type, making up 50–70 percent of islet mass, with small islets containing a greater percentage of β-cells in contrast to moderate or large islets.8,9 β-cells were first discovered in 1907 by silver staining10 and were the second islet cell type discovered, thus designated “β” cells. In addition to insulin, β-cells also produce islet amyloid polypeptide (IAPP), or amylin, which is packaged and released within insulin-containing granules.11 Amylin reduces post-prandial hyperglycemia by slowing gastric emptying and promoting satiety.

Glucagon-producing α cells were discovered before β-cells, by alcohol fixation, thereby garnering their name “α”–cells.10 As the second most abundant islet cell type, they make up about 35 percent of islet mass in humans12 but less in rodents. Glucagon’s primary function is to prevent hypoglycemia by stimulating glycogenolysis and hepatic gluconeogenesis.13 Somatostatin-producing δ-cells comprise less than 10 percent of islet mass and are evenly distributed throughout the pancreas.14 Somatostatin is an inhibitory peptide hormone, inhibiting both endocrine and gastrointestinal hormones. Pancreatic polypeptide15 producing cells, also known as PP or F cells,1,16 comprise less than 5 percent of islet mass and, like α cells, are most prominent in the head of the pancreas. Pancreatic polypeptide (PP) has roles in exocrine and endocrine secretion functions of the pancreas.17 Ghrelin-producing ε cells are the last discovered islet endocrine cell type. Although present in islets, ghrelin is predominately produced in the stomach; ghrelin suppresses insulin release and plays a role in regulating energy homeostasis.18 The close proximity of the acini and the islets of Langerhans mirrors their functional interplay. The anatomic structure of the pancreatic parenchyme allows for a paracrine effect of the islet hormones on adjacent acinar cells, termed the “islet-acinar” axis.19,20 Notably, the islets are highly vascularized—receiving 15 percent of pancreatic arterial blood flow despite composing only 2 percent of the pancreatic mass.21 Via the islet-acinar portal system, blood bathing the pancreatic islets flows into a capillary bed within the pancreatic acini, thus exposing the acinar pancreas to the islet hormones.22 Insulin binds to an insulin receptor on acinar tissue and potentiates amylase secretion.23 In contrast, somatostatin inhibits pancreatic exocrine secretion;24 endogenous PP is also largely noted to inhibit pancreatic exocrine secretion.17,25 Studies have been inconsistent with regard to the effect of glucagon, some suggesting a
stimulatory effect while many suggesting an inhibitor effect of glucagon on secretion of zymogen granules.\textsuperscript{19}

**Insulin Structure**

The hormone insulin was first isolated in the 1920s by Dr. Frederick Banting and a medical student Charles Best, garnering Banting (jointly with John James Rickard Macleod) the Nobel Prize in Medicine in 1923. This was a critical step forward in diabetes care, as porcine insulin therapy was then made available for human use to treat type 1 diabetes, an otherwise fatal disease. In the 1950s Frederick Sanger determined its primary amino acid structure, consisting of an A and a B chain connected by disulfide bonds.\textsuperscript{26,27} Ten years following this discovery, these chains were found to be from the same polypeptide precursor, preproinsulin. In the 1960s Dorothy Hodgkin defined its tertiary structure. During translation of preproinsulin from its mRNA, the N-terminal signal peptide is cleaved to yield proinsulin. The proinsulin molecule is a single-chain polypeptide containing both the A-chain (21 amino acids long) and the B-chain (30 amino acids long). In proinsulin, two chains are connected by C-peptide, which is cleaved to release C-peptide, and the remaining insulin molecule, which contains the A- and B-chains connected via two disulfide bonds.\textsuperscript{26} Although insulin and C-peptide are co-released from β-cell secretory vesicles into circulation,\textsuperscript{28} only insulin is biologically active in regulating blood glucose. C-peptide, however, can serve as a useful clinical and research measure of endogenous insulin production in patients receiving exogenous insulin injections.

**Insulin Gene Transcription**

The insulin gene on chromosome 11 is primarily expressed in pancreatic β-cells but is expressed in low levels in the brain, thymus, and in the yolk sac during fetal development.\textsuperscript{29–31} It has three exons and two introns, and its transcription results in the 446 base pair preproinsulin mRNA (Figure 1).

![Figure 1. Various levels of glucose regulation of insulin gene expression.](image) Glucose stimulates nuclear translocation of Pdx-1; promotes Pdx-1 and MafA phosphorylation and binding to the insulin promoter; and stimulates transcription of the insulin gene, pre-mRNA splicing, translation, and mRNA stability (used with permission from Poitout et al.\textsuperscript{32}).
Transcription of the insulin gene to proinsulin mRNA is sophisticated and reflects the tight regulation by transcription factors and recruited coactivators. Pdx-1, NeuroD1, and MafA are important transcription cofactors in beta-cell function, and their activity is responsive to elevated glucose levels. Individual β-cells respond to ambient glucose with differential insulin secretion, and these changes are apparent at the level of gene transcription. At the level of the islet, rapid increase in blood glucose results in rapid elevation in proinsulin mRNA in the endocrine pancreas. A rapid decrease in blood glucose results in a slow decline in proinsulin mRNA. This is due to the unusual stability of proinsulin mRNA, further stabilized by increased glucose concentrations. The specific regulation of this molecule’s translation is the primary mechanism of insulin production control.

Mature insulin-containing granules are retained from a few hours up to several days within the β-cell, ready for transport to plasma membrane and exocytosis when stimulated. The storage of insulin in mature β granules is far greater than that secreted. During a one-hour glucose stimulation only ~1–2 percent of insulin within a primary islet β-cell is released. The insulin content within a given β-cell remains relatively constant in the short term, but in the long term it will adapt in response to physiologic demands.

Insulin Function

In an evolutionary milieu of sporadic access to nutrients, insulin became critical in facilitating survival. As an anabolic hormone, insulin controls metabolism of carbohydrates, lipids, and protein. It mediates the availability of energy sources in both fasting and fed states. Insulin promotes energy storage in the fasting state and energy utilization and uptake in the fed state (Table 1). In so doing, it maintains serum glucose levels within a narrow physiologic range despite variation in energy intake and expenditure. Insulin acts at extracellular insulin receptors in multiple organ tissues including the liver, muscle, and adipose tissue, and its effect depends on interstitial insulin concentration, which is influenced by insulin secretion rate from β-cells and clearance from circulation.

The liver serves as the primary storage site for glucose, accounting for 80 percent of glucose production in fasting states, with the kidney only contributing 20 percent. To preserve glucose stores, the low insulin concentrations in the portal venous blood—as seen in the fasting state—allows minimal glucose production, only enough to match the needs of essential glucose-dependent tissues including the red blood cells and the central and peripheral nervous systems. The liver also clears insulin more rapidly in the fasting state, thus maintaining low circulating insulin levels. Low insulin concentrations also contribute to lipolysis in adipocytes, releasing free fatty acids to encourage utilization of lipid over glucose to meet resting energy needs. Hepatic glucose release during fasting states through glycogenolysis and gluconeogenesis is stimulated by counter-regulatory, or “anti-insulin” hormones. Glucagon plays a major role, with synergistic effects from catecholamines, cortisol, and growth hormone.

Table 1. Endocrine effects of insulin.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Effect of insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Catabolic Pathways</td>
</tr>
<tr>
<td></td>
<td>Inhibits glycogenolysis</td>
</tr>
<tr>
<td></td>
<td>Inhibits conversion of fatty acids and amino acids to keto acids</td>
</tr>
<tr>
<td></td>
<td>Inhibits conversion of amino acids to glucose</td>
</tr>
<tr>
<td>Anabolic Pathways</td>
<td>Promotes glucose storage as glycogen (induces glucokinase and glycogen synthase, inhibits phosphorylase)</td>
</tr>
<tr>
<td></td>
<td>Increases triglyceride synthesis and VLDL formation</td>
</tr>
<tr>
<td>Muscle</td>
<td>Protein Synthesis</td>
</tr>
<tr>
<td></td>
<td>Increases amino acid transport</td>
</tr>
<tr>
<td></td>
<td>Increases ribosomal protein synthesis</td>
</tr>
<tr>
<td>Glycogen Synthesis</td>
<td>Increases glucose transport</td>
</tr>
<tr>
<td></td>
<td>Induces glycogen synthetase</td>
</tr>
<tr>
<td></td>
<td>Inhibits phosphorylase</td>
</tr>
<tr>
<td>Adipose Tissue</td>
<td>Triglyceride Storage</td>
</tr>
<tr>
<td></td>
<td>Lipoprotein lipase is induced by insulin to hydrolyze triglycerides in circulating lipoproteins for delivery of fatty acids to the adipocytes</td>
</tr>
<tr>
<td></td>
<td>Glucose transport into cell provides glycerol phosphate to permit esterification of fatty acids supplied by lipoprotein transport</td>
</tr>
<tr>
<td></td>
<td>Intracellular lipase is inhibited by insulin</td>
</tr>
<tr>
<td>Brain</td>
<td>Decreased appetite</td>
</tr>
<tr>
<td></td>
<td>Increased energy expenditure</td>
</tr>
</tbody>
</table>

Adapted from Masharani and German.
By contrast, in the fed state—in response to digestion and absorption of nutrients—circulating insulin concentration increases in the portal vein secondary to insulin secretion from pancreatic β-cells. The increased insulin and glucose concentrations normally limit hepatic glucose production and stimulate liver glucose uptake through glycogen deposition. Insulin causes upregulation of hexokinase, phosphofructokinase, and glycogen synthase within hepatocytes, thus inhibiting glycogenolysis and gluconeogenesis and stimulating glycogen synthesis.

The effect of insulin on gluconeogenesis can be direct (via its effect on the liver) or indirect via its effect on islet α cells (by decreasing glucagon secretion), adipose tissue (by suppressing lipolysis), skeletal muscle (by reducing proteolysis), and the brain (pleiotropic effect).

In situations when there is poor insulin response such as type 2 diabetes mellitus or insulin resistance, the process of gluconeogenesis continues even in the fed state, thus further compounding hyperglycemia. Liver clearance of insulin is decreased in the fed state, thus further increasing the circulating insulin concentration. In adipocytes, insulin upregulates lipoprotein lipase and downregulates hormone sensitive lipase, which inhibits lipolysis and subsequent free fatty acid release. In hepatocytes, insulin instead stimulates hepatic free fatty acid synthesis from glucose, thereby increasing lipid stores. Proteolysis of skeletal muscle is also inhibited by insulin, which along with lipolysis inhibition, limits delivery of glucose precursors (glycerol and amino acids) to the liver. Systemic circulation of insulin stimulates glucose uptake and utilization in skeletal muscle and adipocytes.

In summary, the release of insulin in the fed state (1) promotes accumulation of energy stores through glyco- and lipogenesis, (2) reduces new hepatic glucose output by preventing glycogenolysis and gluconeogenesis (in the non-insulin resistant, non-diabetic individual), and (3) promotes uptake of glucose by skeletal muscle and fat, the net effect of which is to maintain a normal circulating serum glucose levels while storing extra energy for use during later periods of fasting (Figure 2).

Glucose movement into cells is made possible by specific protein transporters within the plasma membrane of glucose-responsive cells that reversibly bind glucose and transport it bidirectionally across the cell membrane. There are 14 known glucose transporters (GLUTs). They are present in different concentrations and in different tissues, with varying sensitivity to insulin (Table 2).

Tissues such as muscle and adipocytes carry the insulin-dependent glucose transporter GLUT-4 and uptake of glucose into these tissues occurs only under conditions of adequate circulating insulin. In contrast, vital organs such as red blood cells, brain, placenta, and kidney carry insulin-independent glucose transporters. Thus, these latter essential organs can continue to function even in states of insulin deficiency. β-cells also depend upon on a

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**Table 2.** Most common glucose transporters (GLUT) in human tissues.

<table>
<thead>
<tr>
<th>Glucose transporter</th>
<th>Insulin sensitivity</th>
<th>Tissue(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT-1</td>
<td>Insulin -independent</td>
<td>RBCs; blood brain barrier; more recently identified in human β-cell</td>
</tr>
<tr>
<td>GLUT-2</td>
<td>Insulin -independent</td>
<td>β-cell; kidney, liver, intestinal cells</td>
</tr>
<tr>
<td>GLUT-3</td>
<td>Insulin -independent</td>
<td>Blood-brain barrier, placenta</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>Insulin -dependent</td>
<td>Skeletal muscle, smooth muscle, cardiac muscle, adipocytes</td>
</tr>
</tbody>
</table>
glucose-independent transporter, GLUT2, to allow ambient blood glucose to freely transverse the β-cell membrane in order to stimulate insulin production.

**Insulin Secretory Pathway**

The pancreatic β-cells act as a self-contained system to secrete insulin in response to changes in ambient blood glucose concentration, in order to maintain glucose homeostasis. Glucose is freely taken up into the β-cell via GLUT transporters, metabolized to produce ATP, which triggers a cascade of signals within the β-cell necessary for glucose-induced insulin secretion. While GLUT2 has been traditionally assumed as the major mediator of glucose uptake, GLUT1 and GLUT3 play a more important role, and are the main glucose transporters in human islet β-cells. This redundancy explains why individuals with variants in the gene encoding GLUT2 (SLC2A2 mutations, or Fanconi–Bickel syndrome) do not have significant abnormalities in insulin secretion. As blood glucose increases (e.g., after a meal), there is a resultant flux of glucose across the GLUT transporters in the β-cell. Subsequently, within the β-cell, glucose is phosphorylated to glucose-6-phosphate by glucokinase. This is the rate-limiting step of insulin secretion, and as such, glucokinase is considered the “glucose sensor” for the β-cell. Because of this critical role of glucokinase, individuals with heterozygous mutations in the glucokinase gene have a mild to moderate non-progressive hyperglycemia (maturity onset of diabetes in the young, type 2). Once in the mitochondria, glucose-6-phosphate is metabolized by the Krebs cycle to produce ATP. The resultant ATP binds and closes the ATP-dependent potassium channel, a pore across the cell membrane, which consists of four Kir6.2 subunits and has four regulatory SUR (sulfonylurea receptor) subunits. Channel closure blocks potassium exit from the β-cell, thus depolarizing the cell membrane. Once the cell is depolarized, the L-type voltage-gated calcium channels are triggered, increasing influx of calcium and resultant cellular calcium concentrations. Increased cytoplasmic calcium concentrations triggers release of insulin and C-peptide from a pool of insulin-containing docked secretory vesicles and stimulates the migration of additional vesicles to the cell membrane. Though simple glucose-stimulated insulin secretion (GSIS), as described earlier, is considered the primary pathway for insulin secretion, the full picture is more nuanced. GSIS is augmented by amplifying pathways including (1) metabolic amplification by amino acids, free fatty acids, and glucose itself; and (2) neurohormonal amplifiers such as GLP-1 and parasympathetic innervation. More recent data from mice suggest a role for skeletal muscle in regulating β-cell insulin secretion via production of an anorexigen factor typically derived from the hypothalamus in the brain called BDNF (brain-derived neurotrophic factor). This effect is mediated via the BDNF receptor (TrkB.T1), which is expressed on β-cells, and is thought to play a potential role in exercise-induced glucose metabolism. These physiologic, and pharmacologic, triggers for insulin secretion are further described in the following sections. About half of insulin secretion occurs as basal insulin release, while the other half occurs as “bolus” insulin responses to a meal. This basal-bolus dynamic of insulin secretion is important in considering clinical management of the patient with diabetes (Figure 4). In those with complete insulin deficiency—for example, type 1 diabetes, late-stage type 2 diabetes, or late-stage chronic pancreatitis diabetes—insulin analogs are administered by multiple daily injections or a continuous subcutaneous insulin infusion (insulin pump) to mimic this basal-bolus pattern of endogenous insulin secretion.

**Regulation of Insulin Release**

Insulin release from pancreatic β-cells is tightly regulated and allows the sensitive response of insulin levels to calorigenic nutrients in the body. Glucose, free fatty acids, and amino acids serve as fuel stimuli for insulin release, promoting insulin granule exocytosis. Additional hormonal factors influence the regulation pathway. Pharmacological agents can also be used to augment insulin release.

**Glucose-stimulated insulin secretion**

Glucose-stimulated β-cell insulin release is the primary mechanism of insulin regulation (Figure 3). In humans, this is illustrated by use of the hyperglycemic clamp technique (Figure 5), in which individuals are made rapidly hyperglycemic by injection of intravenous dextrose, and hyperglycemia is maintained by variable rate dextrose infusion at a predefined target glucose. Hyperglycemic clamp studies demonstrate a dose-response of insulin secretion in response to glucose concentration, with greater degrees of hyperglycemia eliciting a more robust insulin secretory response in the non-diabetic individual. Using this research technique, two distinct phases of insulin secretion are observed. During the first-phase insulin response (otherwise referred to as the acute insulin response to glucose, AIRglu), there is an immediate and transient rise in insulin secretion, peaking by five minutes and lasting no more than ten minutes. This first phase of insulin secretion is hypothesized to largely result from the immediate release of insulin from insulin secretory vesicles that are already docked and
Figure 3. Glucose stimulated insulin-secretion coupling in the β-cell. The drawing shows the main pathway of glucose-stimulated insulin secretion in the beta cell. Glucose enters the beta cell through GLUT transporters. Glucose metabolism results in an enhanced cytoplasmic ATP/ADP ratio which prompts closure of ATP-sensitive K⁺ (K\textsubscript{ATP}) channels in the plasma membrane evoking membrane depolarization and subsequent opening of voltage-gated Ca\textsuperscript{2+} channels. This culminates in an increase in cellular Ca\textsuperscript{2+} influx—a primary driver of insulin exocytosis. Ca\textsuperscript{2+} and vesicle docking and fusion events can also be modulated by agents acting through the phospholipase C (PLC)/protein kinase C (PKC) or adenylate cyclase (AC)/protein kinase A (PKA) pathways, via neuro-hormonal and metabolic amplification (not illustrated) (used with permission from Marchetti et al.\textsuperscript{[58]}).

Figure 4. Diagrammatic illustration of insulin secretion. A low background secretion exists upon which is superimposed insulin secretory bursts stimulated by food intake (used with permission from Thompson et al.\textsuperscript{[65]}).

primed at the β-cell membrane. This first-phase response is lost under conditions of diabetes mellitus, when β-cell reserves are exhausted.\textsuperscript{[71]} The second sustained phase begins at this ten-minute time-point and lasts as long as the glucose elevation is elevated. The second phase results from recruitment of insulin secretory vesicles to the β-cell membrane, and is also controlled by intracellular calcium levels.\textsuperscript{[38]}

It is unclear how significant first- and second-phase insulin responses are in the ‘real world’ setting. In contrast to this scenario of rapid infusion of intravenous glucose, ingestion of a physiologic meal results in a much more gradual rise of serum glucose.\textsuperscript{[72]} However, characterization of first-phase insulin response is critically important in diabetes research. In progression to type 1 and type 2 diabetes mellitus, the earliest abnormality is a loss in the first-phase insulin secretion (measured as the AIR\textsubscript{glu}). Although chronic pancreatitis
Pancreatic β-cells adjust insulin secretion based on other nutrients, including amino acids, fatty acids, and ketone bodies. Oral protein intake and subsequent rise in serum amino acids stimulate insulin release by direct β-cell stimulation. The insulinotropic effect varies among amino acids, and there appears to be a synergistic effect of mixed amino acids versus individual administration.

Some amino acids stimulate insulin secretion by acting as substrates in the Krebs cycle, metabolizing glucose-6-phosphate to create ATP. Enzymes active in β-cell mitochondrial amino acid metabolism have been implicated in hyperinsulinemic hypoglycemic syndromes associated with high-protein containing meals. The ATP binds to and closes the potassium channel, leading to cell depolarization and insulin secretion. There seems to be a direct effect of proteins and amino acids on β-cell glucose sensitivity, because ingestion of amino acids with glucose results in the same plasma insulin concentrations as elicited by a lower level of glucose alone.

### Lipids and free fatty acids

It is generally accepted that lipids play a role in insulin secretion signaling, but the precise pathways and molecules involved in the process remain less well understood. Lipid breakdown and metabolism to signaling molecules have been linked to glucose metabolism through enhanced membrane phospholipid metabolism turnover and other pathways yet to be firmly established. It is thought that free fatty acids (FFA) modulate β-cell insulin secretion either directly via GPR40 (G-protein coupled receptor on the β-cell) leading to insulin secretion, or indirectly via oxidation of FFA to acyl coA, which enters the Krebs cycle and generates ATP.

Glucose and FFA metabolism have been shown to be tightly linked and likely includes malonyl-CoA/carnitine palmitoyltransferase I/fatty acyl-CoA metabolic signaling network and the glycerolipid/free fatty acid (GL/FFA) cycle. The GL/FFA cycle along with the Krebs cycle and pyruvate cycling are the three likely interlinked metabolic cycles that play essential roles in insulin secretion promoted by glucose, FFA, and amino acids. In so doing, FFA work synergistically with glucose-stimulated insulin secretion to enhance insulin secretion in nutrient-abundant states.

Chronic elevation of fatty acids may increase basal insulin secretion levels but inhibits glucose-stimulated insulin secretion. Chain length and degree of saturation affect the role free fatty acids play in regard to insulin plasma levels. Adipose tissue responds to insulin resistance with a persistently elevated rate of lipolysis, thus increasing the plasma free fatty acid levels. This is believed to be important in type 2 diabetes development.

### Proteins and amino acids

Incretin hormones

Though glucose concentrations can account for the majority of changes in insulin concentrations, complex studies
evaluating in vivo insulin concentrations following meals have identified other factors. Indeed, insulin secretion following an oral glucose tolerance test is directly related to blood glucose levels, but is considerably higher than predicted following intravenous glucose infusion. These findings suggest a role for potentiating effects on insulin release by hormones that specifically respond to oral glucose, the “incretin effect”. This terminology is derived from intestinal hormones called incretins, which are credited with facilitating this response.

The most active incretins are glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), but gastrin, secretin, and cholecystokinin may also have minor roles. In response to glucose and other nutrients, intestinal L cells secrete GLP-1 and K cells secrete GIP. These hormones then bind their specific receptors on the pancreatic β-cell membrane. GLP-1 binds a G protein-coupled receptor. This results in direct activation of insulin gene’s cyclic-AMP (cAMP) response element (CRE) of the S-proximal control sequence. It can also augment Pdx-1 binding in the setting of a glucose-stimulus and stimulate transcription of the PDX-1 gene. Finally, it potentiates glucose-induced insulin gene transcription by activating NFAT (nuclear factor of activated T-cell). The incretin effect is also mediated by glucose concentration, stimulating more insulin secretion in more extreme hyperglycemic states. GIP and GLP-1 receptors also exist on neuronal cells (e.g. nodose ganglion of the Vagus nerve), suggesting an additional indirect role in β-cell regulation.

GLP-1 and GIP are cleared by dipeptidyl peptidase-4 (DPP-4) which is present on vascular endothelium. As a result, their half-life in the circulation is 2–3 minutes and 4–5 minutes, respectively.

**Insulin’s Counterregulatory Hormones**

The tight control of energy utilization and stores by insulin is balanced by the counterregulatory hormones glucagon, pancreatic polypeptide, somatostatin, cortisol, catecholamines, and growth hormone. There is asymmetry in the glucose regulation hormones, as insulin is the only hormone to prevent hyperglycemia, while at least three other hormones (cortisol, glucagon, and adrenaline) prevent hypoglycemia. Collectively, these counter-regulatory hormones act to promote glucose release from the liver by glycogenolysis and gluconeogenesis, and inhibit glucose storage during times of starvation.

Glucagon is formed within pancreatic α islet cells and has a hyperglycemic effect on the body. Its name is derived from glucose agonist. Glucagon carries out its effects via activating its G-protein-coupled receptor that is found in various organs/tissues such as the liver, adipose tissue, kidneys, gastrointestinal (GI) tract, brain, and islet α- and β-cells. It stimulates glucose production from amino acids and glycerol through gluconeogenesis and from the liver through glycogenolysis. Glucagon also acts at the adipocyte to upregulate hormone-sensitive lipase, thereby enhancing lipolysis and free fatty acid delivery to the liver. In the brain it increases satiety, and in the GI tract it reduces GI motility. Glucagon, via its autocrine role, stimulates further glucagon secretion through its effect on α-cells. Interestingly, glucagon stimulates insulin secretion via glucagon’s effect on β-cells. It is not clear if this effect is mediated mainly via glucagon’s effect on glucagon receptors or on GLP-1 receptors. This effect on insulin secretion occurs in the fed state.

Mechanisms explaining glucagon secretion are not as well understood as those of insulin secretion, although the direct effect of reduced glucose on cAMP and the sodium-glucose cotransporter (SGLT) are thought to play a role in α-cell glucose transport. Mice and human data suggest that α-cell inhibition can occur, at least in part, due to the paracrine action of somatostatin from δ-cells as a result of gap junction-dependent activation by adjacent β-cells.

Cortisol antagonizes insulin’s function by promoting protein catabolism to provide amino acid substrate for gluconeogenesis and also impairs peripheral insulin-mediated glucose uptake.

Catecholamines directly affect β-cell secretion of insulin, as activation of α-2 receptors inhibits insulin secretion and β stimulation increases it. Catecholamines promote adipocyte lipolysis, hepatic glycogenolysis and peripheral insulin resistance. Epinephrine inhibits insulin secretion through inhibiting the rate of insulin gene transcription. Somatostatin also destabilizes the preproinsulin mRNA, resulting in premature degradation.

Somatostatin is released from pancreatic islet δ-cells and exerts inhibitory effect on pancreatic β-cells. Once bound to specific somatostatin receptors, β-cell membrane repolarization is induced, resulting in reduction of calcium influx and thereby inhibiting insulin release.

Pancreatic polypeptide is secreted by PP, or F, cells in pancreatic islets. In addition to its effects reducing gastric acid secretion, decreasing gastric emptying, and slowing upper intestinal motility, PP acts within the pancreas to self-regulate pancreatic insulin secretion.

**Pharmacologic Modulators of Insulin Response**

There is a plethora of pharmacologic agents designed to target various aspects of glucose metabolism. In this chapter, we provide examples of pharmacologic agents that directly or indirectly modulate insulin response.

**Incretin mimetics**

Diabetes therapeutics have recently utilized the role of incretin hormones for pharmacologic benefit. Due to the
Desirable effect of GLP-1 on hemoglobin A1c (HbA1c) reduction and weight loss, GLP-1 receptor agonists and inhibitors of its degradation via dipeptidyl peptidase-4 (DPP-4) inhibitors have been used to treat type 2 diabetes since 2005.

Short-acting GLP-1 receptor agonists (such as exenatide and Liraglutide) and long-acting GLP-1 receptor agonists (such as weekly exenatide and Semaglutide) potentiate insulin secretion and reduce gastric motility. Given that GLP-1 receptor agonists potentiate glucose-induced insulin gene transcription, they, alone, do not induce hypoglycemia when used as monotherapy. Additionally, sitagliptin has been found to potentiate GSIS independently of GLP-1 via islet peptide tyrosine tyrosine (PYY).

**Sulfonylureas**

Through a direct action on pancreatic islet cells, sulfonylureas are pharmacological agents that stimulate insulin secretion, thereby lowering blood glucose levels. This class of medication was discovered by happenstance in 1942 when Marcel Janbon, a clinician at the Clinic of the Montpellier Medical School in France, found his patients treated for typhoid fever with a new sulfonamide (2254 RP) developed hypoglycemia. Shortly after this, his colleague Professor August Loubatieres established the hypoglycemic property of 2254 RP, and its analogues were by direct action on pancreatic islets. This marked the birth of sulfonylureas for treatment of certain forms of diabetes. It was not until 50 years later that the mechanism of action was discovered. Sulfonylurea was found to bind to and block the potassium ATP channel on the β-cell surface, thus depolarizing the membrane and provoking calcium influx, raising intracellular calcium concentration, and triggering insulin secretion. Sulfonylurea binding to the sulfonylurea receptor associated with the K-ATP channel stimulates events similar to those in response to glucose stimulation. Sulfonylureas are also used in the chronic treatment of type 2 diabetes mellitus for their effects on both insulin release and blood glucose reduction. In contrast to acute use of sulfonylureas, chronic use results in improved blood glucose control, but with less rather than more insulin secretion. Assessments of its chronic effects are difficult to interpret, given that the magnitude of sulfonylurea stimulation of insulin secretion are multifactorial.

**Insulin sensitizers**

Biguanides (such as metformin) and Thiazolidenediones (such as pioglitazone) improve hepatic and peripheral (muscle and fat tissue) insulin sensitivity, respectively. Metformin is by far the most widely used pharmacologic agent as first-line therapy in patients with type 2 diabetes mellitus. Similar to thiazolidenediones, metformin has an effect on improving peripheral insulin sensitivity in addition to reducing hepatic glucose output. Contrary to thiazolidenediones and sulfonylureas, metformin does not cause weight gain, and, in fact, it has a modest weight loss effect. When used as monotherapy, metformin does not induce hypoglycemia.

**Diazoxide**

Diazoxide is a sulfonamide pharmacological agent used in the treatment of hyperinsulinism, insulinoma, and hypoglycemia due to overtreatment with sulfonylureas. It works by opening β-cell membrane potassium ATP channels, hyperpolarizing the β-cells, thus decreasing intracellular calcium concentration and inhibiting insulin secretion.

**Conclusion**

In conclusion, although the pancreatic islets comprise only a small portion of the pancreas, pancreatic islets play a vital role in our well-being and survival through control of glucose homeostasis. Most critically, loss of insulin production from the pancreatic β-cells, whether due to autoimmune destruction in type 1 diabetes mellitus, exhaustion and genetic predisposition to failure in type 2 diabetes mellitus, or bystander fibrotic destruction in pancreatic exocrine disease, results in diabetes. Insulin secretion is tightly regulated in healthy non-diabetic individuals, with both insulin gene transcription and exocytosis from insulin-containing granules responsive to rises in ambient circulating blood glucose. Other nutrients (protein and lipid) play a smaller role. In contrast, the other pancreatic islet cells, particularly the glucagon-producing α-cells, play a key role in glucose counter-regulation to avoid dangerous hypoglycemia.

**References**


77. Nuttall FQ, Gannon MC, Turley SD, McGarry JD. The insulinotropic potency of fatty acids is influenced profoundly by their chain length and islet beta-cell function in health, obesity and diabetes. Mol Cell Endocrinol 2012; 353:88–100.


Introduction
Pancreatic fibrosis is a well-recognized histopathological feature of two major diseases of the pancreas—chronic pancreatitis and pancreatic cancer. In health, the process of fibrogenesis is a well-regulated dynamic process which is necessary for regular turnover of extracellular matrix (ECM) that allows remodeling and maintenance of normal pancreatic architecture. However, during injury, the equilibrium between production and degradation of fibrous tissue is disrupted, leading to excessive deposition of ECM proteins resulting in fibrosis.

Pancreatic stellate cells (PSCs) are now considered to be the key contributors of pancreatic fibrosis. These cells were first observed by Watari et al. in 1982 and later confirmed by Ikejiri in 1990. The development of isolation and culture methods for PSCs in 1998 helped to unravel the mechanisms involved in the process of pancreatic fibrogenesis and also helped researchers to investigate the functions of these cells in both health and disease.

Pancreatic Stellate Cells
PSCs in health
In a healthy pancreas, PSCs make up 4–7 percent of the total parenchyma and are located around the basolateral aspects of the acinar cells (Figure 1A). PSCs are also found around small pancreatic ducts, pancreatic islets, and blood vessels. PSCs in their native state express abundant vitamin A (retinoids) containing droplets in their cytoplasm which is a characteristic feature of the “stellate cell system” in the body. The buoyancy imparted by the vitamin A-containing vesicles was utilized by Apte et al. to develop a density gradient centrifugation method for the isolation of quiescent PSCs from the pancreas. In early culture, PSCs are polygonal in shape with abundant lipid droplets in the cytoplasm (Figure 1B) and express stellate cell selective markers such as desmin, glial fibrillary acidic protein (GFAP), nestin, neural cell adhesion molecule, nerve growth factor, and synemin. Due to the expression of neural markers, PSCs were initially thought to be of neuroectodermal origin. However, lineage-tracing studies with hepatic stellate cells (counterparts of PSCs in the liver) have confirmed a mesenchymal origin for these cells. The fact that a proportion of PSCs are replenished from bone marrow further supports a mesenchymal origin for PSCs.

Islet stellate cells (ISCs) are similar to but differ in certain aspects from PSCs. ISCs have fewer vitamin A-containing droplets and undergo a more rapid activation than PSCs. Upon activation, ISCs express more α-SMA but have reduced rates of proliferation and migration compared with exocrine PSCs.

PSCs are the primary producers of ECM proteins, such as collagen I–IV, fibronectin, and laminin, in the pancreas. At the same time, they also produce ECM-degrading enzymes like matrix metalloproteinases (MMPs) and their inhibitors—tissue inhibitors of matrix metalloproteinases (TIMPs). In the normal pancreas, these enzymes maintain an equilibrium between the deposition and degradation of ECM proteins.

It is now recognized that PSCs have functions beyond ECM remodeling in the normal pancreas. PSCs express receptors for the secretagogue cholecystokinin (CCK1 and CCK2) and have the capacity to synthesize the neurotransmitter acetylcholine (ACh). Upon exposure to physiological levels of CCK, PSCs have been shown to secrete ACh which, in turn, acts on the muscarinic receptors on acinar cells and induces amylase secretion. PSCs also play an important role in innate immunity through the expression of Toll-like receptors (TLRs) 2, 3, 4, and 5 which can recognize pathogen-associated molecular patterns (PAMPs), alarmins, and endogenous molecules released by tissue damage (DAMPs). These cells also possess the ability to phagocytose polymorphonuclear neutrophils and cell debris, which is mediated by CD36 via peroxisome proliferator-activated receptor γ. PSCs may
also possess progenitor-like capabilities since they express several stem cell markers, including CD133, SOX9, nestin, and GDF3.\textsuperscript{17,18} They can differentiate into other cell types including insulin-secreting cells under the influence of relevant growth factors.\textsuperscript{13,18–21}

**PSCs in disease**

In the event of pancreatic injury, PSCs are activated, leading to loss of the vitamin A-containing lipid droplets, and transform to a myofibroblast-like phenotype that expresses alpha smooth muscle actin (αSMA), fibroblast activation protein-α (FAP-α), fibroblast specific protein-1 (FSP-1), and fibrinogen.\textsuperscript{9} These cells also exhibit increased proliferation, migration, and ECM synthesis.\textsuperscript{22,23} Activated PSCs produce collagenous stroma during pancreatic injury\textsuperscript{24–26} which can overwhelm the ECM-degrading capacity of MMPs, leading to fibrosis. A comparison of resting and activated PSCs is given in Table 1.

PSCs can be activated by several factors such as proinflammatory cytokines;\textsuperscript{27} oxidant stress;\textsuperscript{28,29} ethanol and its metabolites, acetaldehyde and fatty acid ethyl esters;\textsuperscript{30,31} fatty acids (oleate);\textsuperscript{32} and endotoxins\textsuperscript{33} (Table 2). In addition, in the setting of pancreatic cancer, factors such as acidosis,\textsuperscript{34} hypoxia,\textsuperscript{35} increased interstitial pressure\textsuperscript{36} and hyperglycemia\textsuperscript{37} are also involved in the activation of PSCs. Recently, epigenetic modifications (increased acetylation of histones) have also been shown to play an important role in the activation of PSCs and collagen synthesis.\textsuperscript{38}

In addition to being activated by exogenous factors, PSCs are capable of sustaining their own activation (even in the absence of the initial triggers) through the production of cytokines (such as transforming growth factor beta (TGFβ), connective tissue growth factor (CTGF), interleukins 6, 7, 8 and 15, CXCR1, monocyte chemotactic protein-1 (MCP1), and regulated-on-activation-normal T cell expressed-and-secreted (RANTES)).\textsuperscript{39–41} These cytokines act via corresponding receptors on the PSCs themselves (autocrine effects) leading to a state of perpetual activation, which further facilitates pathological fibrosis.

Several pathways that mediate PSC activation have now been identified (summarized in Table 3). A number of these pathways interact with each other leading to significant redundancy in terms of the modulation of PSC activation.\textsuperscript{22,42} Furthermore, studies have also shown that numerous signaling pathways can converge, causing sustained increase in intracellular calcium within the PSCs.\textsuperscript{43–45} Recently, microRNAs (small noncoding RNAs), which are implicated in cell functions such as proliferation, differentiation, apoptosis, and protein synthesis, have been shown to be differentially expressed between quiescent and activated PSCs.\textsuperscript{46} Other studies have implicated miR15b and 16 (known to regulate PSC apoptosis)\textsuperscript{47} and miR21 as possible cofactors in connective tissue growth factor (CCN2)-mediated PSC activation.\textsuperscript{48}
The past two decades have seen a considerable improvement in our understanding of the role of PSCs in pancreatic inflammation (both acute and chronic pancreatitis) as well as pancreatic cancer. Acute pancreatitis is usually a self-limiting disease where PSCs aid remodeling and repair of tissue architecture, while both chronic pancreatitis and pancreatic cancer are characterized by pathological accumulation of fibrous tissue.

**Acute Pancreatitis**

Acute pancreatitis is a condition which results from acute inflammation of the pancreatic parenchyma, ranging from mild inflammation to extensive pancreatic necrosis. Most of the cases are mild and self-limiting with a mortality rate <1 percent. The most common causes of acute pancreatitis are gallstones and alcohol excess.\(^{49}\)

In response to inflammatory cytokines and chemokines secreted by damaged acinar cells and inflammatory cells, PSCs are activated and proliferate early in acute pancreatitis.\(^{50}\) The majority of the increased numbers of activated PSCs, in the inflamed areas, are a result of local proliferation of resident PSCs. However, a small proportion (7–18%) is derived from circulating bone marrow cells.\(^{11}\) Activated PSCs secrete increased ECM proteins that act as a scaffold for regenerating ductal and acinar cells during the repair process. These ECM proteins are also important for differentiation and cell growth via integrin-mediated interactions between cell membranes and the surrounding matrix.\(^{51}\) Indeed, using β1-integrin knockout animals, it has been shown that the lack of integrin-mediated interactions results in decreased ECM production by PSCs and increased apoptosis and decreased proliferation of acinar cells, thereby impeding pancreatic repair.\(^{52}\)

### Table 1. Characteristics of quiescent and activated PSCs.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Quiescent PSCs</th>
<th>Activated PSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A lipid containing droplets</td>
<td>Abundant</td>
<td>Absent</td>
</tr>
<tr>
<td>Alpha smooth muscle actin</td>
<td>Not expressed</td>
<td>Expressed</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Basic</td>
<td>Enhanced</td>
</tr>
<tr>
<td>Ability to migrate</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Collagen production</td>
<td>Limited</td>
<td>Increased</td>
</tr>
<tr>
<td>Activity of MMPs and TIMPs</td>
<td>In equilibrium</td>
<td>TIMP &gt; MMP</td>
</tr>
<tr>
<td>Cytokine production</td>
<td>Limited</td>
<td>Increased inflammatory cytokines (PDGF, TGFβ, CTGF, IL-1, IL-6, IL-15)</td>
</tr>
<tr>
<td>Ability for phagocytosis</td>
<td>No</td>
<td>Present (CD6 mediated)</td>
</tr>
<tr>
<td>Protein expression</td>
<td>Basal expression</td>
<td>Differential expression</td>
</tr>
</tbody>
</table>

### Table 2. Factors causing activation of pancreatic stellate cells.

- Angiotensin
- Cyclooxygenase 2 (COX-2)
- Endothelin-1
- Endotoxin
- Ethanol and its metabolites (acetaldehyde, fatty acid ethyl esters)
- Fibrinogen
- Galectin-1
- Hyperglycemia
- Hypoxia
- Inflammatory mediators (cytokines, growth factors, complement)
- Oxidant stress
- Parathyroid hormone-related protein (PTHrP)
- Pigment epithelium-derived factor
- Proteases
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Table 3. Signaling pathways involved in the interactions between PSCs and cancer cells. α-SMA, alpha smooth muscle actin; c-MET, tyrosine-protein kinase of Met; CCL, chemokine ligand; CXCL, chemotactic cytokine ligand; ECM, extracellular matrix; JAK/STAT, Janus kinase/signal transducers and activators of transcription; HGF, hepatocyte growth factor; PSC, pancreatic stellate cells; PCC, pancreatic cancer cells; SDF, stromal derived factor; PDGF, platelet-derived growth factor; SMAD, small worm mothers against decapentaplegic.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Signaling pathway</th>
<th>Functional role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSC-derived CXCL12 (SDF-1)</td>
<td>CXCL12 (SDF-1) signaling</td>
<td>Immunosuppression</td>
<td>154</td>
</tr>
<tr>
<td>PSC-derived SDF-1</td>
<td>Galectin-1</td>
<td>Proliferation of PSC and chemokine production facilitating PCC metastasis</td>
<td>155</td>
</tr>
<tr>
<td>Smo, Gli</td>
<td>Hedgehog pathway</td>
<td>PSC activation, ECM synthesis, migration, desmoplasia, angiogenesis; PCC proliferation, migration and chemoresistance</td>
<td>140</td>
</tr>
<tr>
<td>HGF</td>
<td>HGF/c-MET pathway</td>
<td>PSC promote proliferation and metastasis of tumor cells</td>
<td>123</td>
</tr>
<tr>
<td>CCL2</td>
<td>Hypoxia inducible factor 1 (HIF-1)</td>
<td>PSC activation, Macrophage recruitment</td>
<td>156</td>
</tr>
<tr>
<td>IL-6</td>
<td>IL6/JAK/STAT</td>
<td>PSC activation and proliferation</td>
<td>157</td>
</tr>
<tr>
<td>Kindlin-2</td>
<td>Integron</td>
<td>Cytokines production in PSCs facilitating progression and migration of PCC</td>
<td>158</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase (MAPK) signaling pathway</td>
<td>PSC proliferation, TIMP-1 production</td>
<td>159</td>
</tr>
<tr>
<td>Periostin</td>
<td>Periostin pathway</td>
<td>Periostin secreted by PSCs promoting PCC proliferation, EMT and resistance to nutrient deprivation and hypoxia</td>
<td>160</td>
</tr>
<tr>
<td>PPAR-y ligands</td>
<td>Peroxisome proliferator-activated gamma (PPARy)</td>
<td>Inhibition of PSC activation, proliferation, and collagen synthesis</td>
<td>16</td>
</tr>
<tr>
<td>PDGF</td>
<td>PI3-Kinase pathway</td>
<td>PSC migration and proliferation</td>
<td>42</td>
</tr>
<tr>
<td>Hyperglycemia</td>
<td>Protein kinase C</td>
<td>PSC proliferation, a-SMA, collagen-I production, angiogenesis</td>
<td>161</td>
</tr>
<tr>
<td>Suppression of miRNA-21</td>
<td>Reactive oxygen species (ROS)</td>
<td>PSC activation and induction of glycolysis</td>
<td>91</td>
</tr>
<tr>
<td>Rho- associated protein kinase</td>
<td>Rho-ROCK pathway</td>
<td>Activation of PSC, collagen I synthesis and fibrosis</td>
<td>162</td>
</tr>
<tr>
<td>SMAD-2,3</td>
<td>SMADS</td>
<td>PSC activation, proliferation, ECM deposition, transdifferentiation, TGF-β expression</td>
<td>163</td>
</tr>
<tr>
<td>TLR9</td>
<td>Toll-like receptor (TLR) signaling</td>
<td>Immunosuppression; PSC-derived cytokine production</td>
<td>164</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>Vitamin D receptor</td>
<td>PSC quiescence; Decreased chemoresistance</td>
<td>96</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Wnt/β-catenin signaling</td>
<td>Invasion of PCCs</td>
<td>165</td>
</tr>
</tbody>
</table>

PSCs may also play an important role in remodeling and regeneration after acute necrotizing pancreatitis. Zimmerman et al.53 observed that pilot ductules that originate from hypercellular regenerative spheres (islands of vascular granulation tissue, ductular cells, stellate cells, and residual lobular elements) grow outwards in close association with activated PSCs. The authors have suggested that these PSCs may be able to support differentiation of cells to mature duct and acinar cells, thereby playing a role in the reconstitution of the cell populations after acute injury. The process of regeneration also requires removal of excess ECM, a task that is aided by the fibrinolytic activity of PSCs via the production of matrix degrading enzymes (MMPs).

Chronic Pancreatitis

Chronic pancreatitis (CP) is a progressive disease characterized by fibrosis, acinar cell loss, distorted ducts, and inflammatory cell infiltration, eventually leading to significant exocrine and endocrine dysfunction. The presence of activated PSCs was confirmed in the areas of fibrosis using dual staining techniques (Sirius Red for collagen and immunostaining for αSMA)(Figure 2). While dual staining for αSMA and procollagen mRNA has shown that activated PSCs are the predominant source of collagen I in the fibrotic areas(Figure 3). Interestingly, the fibrosis of chronic pancreatitis was found not to be limited to the exocrine parenchyma. Activated PSCs have also been demonstrated within and surrounding pancreatic
islets and are co-localized in fibrotic areas around the islets in diabetic Goto–Kakizaki rats and db/db mice (obesity, diabetes, and dyslipidemia model due to leptin receptor deficiency). Additionally, activated PSCs can inhibit insulin secretion and cause beta cell apoptosis, effects which are further aggravated by hyperglycemia. These findings suggest that PSCs can modulate islet cell function, suggesting a direct role for these cells in the diabetes of chronic pancreatitis.

Many factors are known to activate PSCs during chronic pancreatitis, including the profibrogenic growth factor TGFβ, platelet-derived growth factor (PDGF), nerve growth factor (NGF), and oxidative stress. It is now known that PSC-activating factors such as TGFβ, PDGF, oxidant stress, and other cytokines are upregulated early during pancreatic injury leading to PSC proliferation and ECM synthesis. As noted earlier, PSCs can be maintained in a perpetually activated state through autocrine effects of endogenous cytokines, leading to pathological fibrosis.

Animal models provide an invaluable tool for understanding the pathogenesis of chronic pancreatitis. Several methods of inducing experimental chronic pancreatitis have been reported in the literature: repeated intravenous injections of caerulein or superoxide dismutase inhibitor, instillation of toxins into the pancreatic duct, and chronic ethanol administration followed by secondary challenge with caerulein, cyclosporin or endotoxin.

Regarding alcoholic chronic pancreatitis, endotoxin is a relevant trigger factor, given that gut permeability is known to be increased by alcohol and elevated serum endotoxin levels have been reported in heavy drinkers. Further, endotoxin (lipopolysaccharide, LPS) challenge was shown to enhance fibrosis in Sprague-Dawley rats fed Lieber de Carli alcohol liquid diets.

Transgenic models of CP include animals overexpressing cytokines or profibrogenic factors such as IL-1β, TGFβ, and heparin-binding EGF-like growth factor (HB-EGF) and WBN–Kob rats and Goto–Kakizaki rats with type 2 diabetes. More recently, the effects of smoking on pancreatic fibrosis have been studied, given the close association of drinking and smoking as lifestyle factors. Lugea and colleagues have shown that smoking significantly worsened tissue injury as well as fibrosis in a rat model of alcoholic pancreatitis. The increased fibrosis could be due to direct activation of PSCs by (i) nicotine and NNK via nicotinic...
The study of mechanisms underlying PSC activation and fibrosis can help in developing novel anti-fibrotic treatments. Several antifibrotic strategies have been successfully evaluated to counter CP in experimental animal models including: (i) inhibition of profibrogenic growth factors TGFβ and tumor necrosis factor alpha (TNFα); (ii) antioxidants such as vitamin E, ellagic acid, a plant polyphenol, and salvianolic acid, a herbal medicine; (iii) protease inhibitors; (iv) modulation of signaling molecules (e.g., troglitazone binding to the peroxisome proliferator receptor gamma, PPARγ); retinoic acid-induced PSC quiescence via suppression of the Wnt–catenin pathway; collagen siRNA, an anthraquinone derivative Rhein and a flavonoid, apigenin; (vii) a prostacyclin mimetic, a PI3K inhibitor decreased fibrosis by decreasing autophagy in PSCs.

Several plant compounds have shown promising anti-fibrotic activity in several studies. Curcumin (a polyphenol found in turmeric) was reported to inhibit activation of PSCs through the inhibition of IL1β and TNFα-induced activation of activator protein-1 (AP-1) and mitogen-activated protein (MAP) kinases (ERK, c-Jun N-terminal kinase (JNK), and p38 MAP kinase). Conophylline, a plant alkaloid, decreased fibrosis through the inhibition of ERK1/2 in PSCs. In an in vitro study, resveratrol, a natural polyphenol, decreased oxidative stress-induced activation and glycolysis in PSCs mediated by ROS/miR-21. Genistein, a natural isoflavone, decreased fibrosis in human PSCs transfected with let-7d to express thrombomodulin 1, a marker of fibrosis. Saikosponin A, the active component of the Chinese medicine 'chaitu', was shown to decrease PSC activation, viability, proliferation, and migration and to promote apoptosis by inhibiting autophagy and the formation of NLRP3 inflammasome via the AMPK/mTOR pathway. Similarly, date palm fruit extract decreased fibronectin-1 and αSMA, markers of fibrosis in PSCs activated by TNF-α.

Vitamins D and its isoforms D2 and D3 are reported to decrease in vitro PSC activation by decreasing IL-6. Furthermore, in vivo studies with the vitamin D ligand calcitriol have shown significant attenuation of the fibrosis of chronic pancreatitis in mice. Since storage of vitamin A is associated with PSC quiescence, administration of vitamin A (retinol), or its metabolites such as retinoic acid has been assessed in models of chronic pancreatitis. In this regard, vitamin A-containing liposomes combined with TLR4-silencing shRNA have been reported to inhibit pancreatic fibrosis in mouse models of chronic pancreatitis.

Other compounds known to induce quiescence of activated PSCs include melatonin, the anthraquinone derivative rhein, bone morphogenic protein, and troglitazone (a ligand for the peroxisome proliferator activated receptor PPARγ). Recently, kinase inhibitors such as sunitinib, trametinib, and dactolisib have been shown to inhibit PSC proliferation and ECM synthesis. Interestingly, trametinib also decreases the expression of two autocrine mediators of PSC activation, IL6 and TGFβ. Coenzyme Q10 suppresses PSC activation by inhibiting autophagy through PI3K/ATK/mTOR signaling. Inhibition of cyclo-oxygenase by indomethacin, an anti-inflammatory drug, also results in decreased activation of PSCs.

Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is characterized by extensive stroma/desmoplasia (Figure 4) which...
constitutes 80–90 percent of the tumor volume. This stroma consists of ECM proteins including collagen type I, fibronectin and laminin, non-collagenous factors such as glycoaminoglycans (e.g., hyaluronan), glycoproteins, and proteoglycans, and several cell types, including stellate cells, endothelial cells, neural elements and immune cells. Just as with the fibrosis of chronic pancreatitis, activated PSCs are now also recognized as the primary source of the collagenous stroma in PDAC. The detection of activated PSCs expressing peristin (a cell adhesion protein), galectin-1 (a glycan binding protein) and αSMA (an activation marker) in precursor pancreatic intraepithelial neoplasms (PanINs) suggests that activation of PSCs is an early event in PDAC. Studies have also shown a positive correlation between extent of PSC activation in the stroma and poor clinical outcome.

It is now known that functionally different subsets of PSCs exist within the stroma of pancreatic cancer. Yuzawa et al. observed the presence of fibroblasts with varying expression of αSMA and PDGFRβ, while Ohlund et al. observed that cancer-associated fibroblasts (CAFs)/pancreatic stellate cells in proximity to cancer cells exhibit higher αSMA expression compared to those located at a distance from cancer cells. The authors termed the PSCs close to cancer cells as (myofibroblastic) myPSCs and those at some distance from cancer cells as (inflammatory) iPSCS, based on their cytokine secretion profile.

Studies involving in vitro and in vivo models have established that PSCs interact bidirectionally with cancer cells to facilitate local tumor growth and distant metastasis. PSCs also interact with other cells in the stroma, including immune cells resulting in a complex cascade of events (Figure 7). PSCs promote cancer cell progression, migration, and cancer cell survival while, in turn, cancer cells promote PSC proliferation, migration, and ECM synthesis. Several in vitro studies have shown that cancer cells increase PSC proliferation and ECM synthesis mediated by PDGF, FGF2, and TGFβ1 and also promote the secretion of MMPs by PSCs through ECM metalloproteinase inducer (EMMPRIN) secretion and TGFβ1 signaling. Cancer cells can also induce autophagy in PSCs leading to release of alanine which acts as an alternative carbon source for the TCA cycle and lipid synthesis in cancer cells, thus improving cancer cell survival in the

Figure 5. Dual staining for alpha smooth muscle actin (αSMA) and collagen mRNA in a human pancreatic cancer section. Immunostaining for αSMA (brown) combined with in situ hybridization for collagen mRNA (blue) reveals colocalization of the two stains in stromal areas of the section with no staining in tumor cells. This pattern of staining indicates that pancreatic stellate cells are the main source of collagen in pancreatic cancer stroma (from reference; reproduced with permission of Wolters Kluwer).

Figure 6. Activated PSCs in early pancreatic intraepithelial neoplasia (PanIN). A section from an eight-week-old transgenic KrasG12D P53R172H Cre (KPC) mouse showing staining for the PSC-activation marker alpha smooth muscle actin (αSMA, brown) around PanINs in a section of the pancreas. Magnification 20×.
nutrient-poor and hypoxic environment of PDAC. Epithelial–mesenchymal transition (EMT) and stemness, two processes that play an important role in cancer metastasis and recurrence, are known to be induced by PSCs. Studies have shown that stromal signaling is indispensable for cancer progression. Sherman and colleagues have reported that KRas mutation alone is insufficient for PDAC and that stromal cues—collagen and cytokines derived from cancer associated PSCs—are essential to this process. Furthermore, in both subcutaneous and orthotopic xenograft models, co-injection of cancer cells and stromal cells (PSCs) resulted in larger tumors with significant desmoplasia compared to injection of cancer cells alone.

An intriguing report published some years ago suggested that PSCs may facilitate seeding of cancer cells at metastatic sites. Using a gender mismatch approach and an orthotopic model of pancreatic cancer, the authors of this study found PSCs from the primary tumor within metastatic nodules at distant sites, indicating that, in addition to cancer cells, PSCs can also disseminate via the circulation. Similar observations were made by Suetsugu et al. who injected a mixture of PSCs and pancreatic cancer cells into the spleen and observed that both cell types co-migrated from the spleen to metastatic nodules. In a recent groundbreaking study, Pang et al. have identified the presence of circulating PSCs alongside circulating tumor cells (CTCs) in the blood of orthotopic tumor-bearing mice.

In recent years, there has been considerable focus on the role of exosomes (nano size vesicles secreted by most cell types) in a variety of diseases. These exosomes carry a cargo of proteins, lipids, DNA, mRNA, and microRNA, which can influence the function of cells remote from the source of the exosomes. Regarding pancreatic cancer, Takikawa et al. have demonstrated that exosomes derived from PSCs contained a variety of microRNAs and an abundance of miR-21–5p and miR-451a which mediated PSC-induced proliferation and migration of PSCs. Similarly, exosomes derived from PSCs were found to stimulate activation, proliferation, and migration of PSCs through upregulation of transforming growth factor β1 (TGFβ1) and tumor necrosis factor (TNF).

Several studies have shown that pancreatic desmoplasia can contribute to chemoresistance by affecting the kinetics of chemotherapeutic agents. Hessmann et al. found that cancer-associated activated fibroblasts are resistant to gemcitabine and tend to accumulate and rapidly convert gemcitabine into an inactive metabolite 2′,2′-difluorodeoxyuridine, thus contributing to decreased efficacy of gemcitabine on tumor cells. Moreover, there is additional evidence that PSCs may also directly alter the response of cancer cells to chemotherapeutic agents. Autocrine secretion of IL-6 by PSCs in response to stromal-derived factor 1α (SDF-1α) demonstrated a protective effect on cancer cells from the apoptotic effect of gemcitabine. Owing to their chemoresistance, post therapy, PSCs can facilitate proliferation of residual cancer stem-cells leading to recurrence.

Strategies to Counter Stromal–Tumor Interactions in Pancreatic Cancer

As PSC-cancer cell interactions play an important role in the progression of PDAC, several strategies have been developed to counter these interactions. Several chemicals including phytochemicals and hormones have been shown to decrease stromal–tumor interactions in experimental studies. Metformin, an activator of AMP-activated protein kinase, reduced the production of fibrogenic cytokines from cancer cells, inhibited PSC activation in co-culture studies, decreased tumor growth, and enhanced chemosensitivity to gemcitabine in an orthotopic xenograft model. ProAgioQ20, which targets an integrin α(v)β (3) overexpressed in PSCs, caused apoptosis of cancer-associated PSCs, reabsorption of collagen, inhibition of tumor growth,
and inhibition of drug penetration in a subcutaneous, orthotopic xenograft and transgenic KPC model.\textsuperscript{131} Similarly, inhibition of cyclic AMP-response element binding protein-binding protein (CBP)/β-catenin signaling using ICG-001 resulted in decreased activation of PSCs and PSC-induced migration of PANC-1 cancer cells in a transwell migration assay.\textsuperscript{132} Curcumin, the active compound of turmeric, was shown to inhibit IL-6 in PSCs under hypoxic conditions, leading to suppression of EMT and PSC-mediated cancer cell migration \textit{in vitro}.\textsuperscript{133} Relaxin-2, an endogenous hormone combined with supermagnetic iron oxide nanoparticles decreased fibrosis and tumor growth besides potentiating the effects of co-administered gemcitabine in an orthotopic xenograft model.\textsuperscript{134}

Several pathways are shown to be key regulators of cancer cell–PSC interactions, and inhibition of these pathways in animal studies has been reported to decrease fibrosis, tumor growth, and metastasis. In this regard, the Hedgehog pathway is significant as the ligand Sonic hedgehog (Shh) is expressed in tumor cells while the receptor, Smootherned (Smo), is located mainly on cancer-associated fibroblasts.\textsuperscript{135} In animal models, inhibitors of this pathway such as ormeloxifene\textsuperscript{136} and IPI-926\textsuperscript{137} decreased desmoplasia and tumor growth and improved chemosensitivity to co-administered gemcitabine. Interestingly, in an orthotopic tumor model where cancer and PSCs are co-injected, a triple combination of CXCR4 antagonist (AMD3100), Hedgehog inhibitor (GDC0449), and gemcitabine resulted in complete remission of tumor growth.\textsuperscript{138}

Similar to the Hedgehog pathway, the HGF/c-MET pathway is involved in cancer proliferation, invasion, and metastasis. The ligand HGF is produced by PSCs while its receptor, c-MET, is located on cancer cells and endothelial cells.\textsuperscript{139} In transgenic KPC mice, dual inhibition of both Sonic Hedgehog and HGF/c-MET pathways were found to sensitize PDAC to gemcitabine.\textsuperscript{140} In a subcutaneous xenograft mouse model, combination of the c-Met inhibitor XL184 with gemcitabine significantly decreased tumor growth compared to individual treatments.\textsuperscript{141} Xu et al.\textsuperscript{142} used a triple therapy approach consisting of HGF and c-Met inhibitors plus gemcitabine in an advanced model of orthotopic pancreatic cancer. Triple therapy not only reduced tumor size but, importantly, eliminated metastasis. Further, the authors reported that the combination therapy was successful in overcoming gemcitabine-stimulated stemness and aggressiveness of cancer cells. These findings corroborated the findings of previous studies that reported that gemcitabine alone increases stemness of cancer cells in PDAC.\textsuperscript{143,144}

Extracellular signal-regulated kinases (ERKs) regulate cellular processes such as proliferation, differentiation, transcription, and development, and this pathway has been shown to be strongly expressed in cancer-associated PSCs. Dual inhibition of PSC ERK1/2 and autophagy with S7101 and chloroquine, respectively, resulted in PSC senescence and suppressed liver metastasis in a splenic pancreatic cancer organoid mouse model.\textsuperscript{145}

Vitamins inducing PSC quiescence have been successfully demonstrated to improve the outcome of pancreatic cancer in both preclinical and clinical studies. PEGylated gold nanoparticles containing all trans retinoic acid (ATRA) combined with siRNA against heat shock protein (HSP-47, known to activate PSCs) was used to induce PSC quiescence and inhibit ECM hyperplasia, thereby promoting drug delivery to pancreatic tumors in an orthotopic model developed by co-inoculation of cancer cells and PSCs.\textsuperscript{146} Similarly, the vitamin D analogue calcitriol, combined with gemcitabine, induced quiescence of activated PSCs and decreased fibrosis in KPC mice.\textsuperscript{96} Based on this study, a vitamin D analogue paricalcitol, in combination with gemcitabine and nab-paclitaxel, is currently being evaluated in a clinical trial (NCT03520790).\textsuperscript{147}

As epigenetic modifications also play an important role in PDAC, some studies have targeted epigenetic pathways to reprogram the tumor microenvironment in a bid to improve outcomes.\textsuperscript{148,149} Common epigenetic targets relevant to PDAC include DNA methylation, histone modifications, and bromodomain and extra-terminal domain (BET) family proteins. A DNA methyl transferase inhibitor, 5-azacitidine, in combination with abraxane and gemcitabine is currently being tested in a Phase II clinical trial in PDAC patients (NCT01845805) undergoing resection. Inhibiting epigenetic bromodomain and the extraterminal domain (BET) family of proteins using iBET151, and SB 203580, a MAPK inhibitor, decreased YAP-1 levels resulting in decreased PSC activation.\textsuperscript{150}

Myeloid-derived suppressor cells (MDSC) help to maintain an immunosuppressive environment in PDAC by eliminating effector T cells.\textsuperscript{150} Therefore, therapies that boost effector T cell infiltration into tumor are gaining attention. The desmoplastic reaction in PDAC is known to sequester cytotoxic CD8+ T cells and hinder drug penetration. In transgenic spontaneous pancreatic cancer-bearing KC mice (Kras\textsuperscript{G12D}, Cre) challenged with caerulein, an inhibitor of rho/MRTF pathway, CCG-222740 not only decreased PSC activation but also decreased infiltration of macrophages, CD4+ T cells, and B cells, suggesting inhibition of PSC activation improves local immunity.\textsuperscript{151} Similarly, inhibiting stroma through an acidic-pH-responsive-nanoparticle cluster containing TGF-β inhibitor (LY2157299) and PD-L1 (siRNA) improved local tumor immunity by increasing CD8+ T cell infiltration in a subcutaneous xenograft orthotopic model.\textsuperscript{152} Tumor-associated macrophages (TAM), which are derived from MDSCs, are shown to rapidly inactivate gemcitabine, and depletion of TAM using clodronate increased the concentration of active gemcitabine in desmoplastic tumors of KPC mice compared to either PanINs or normal pancreas.\textsuperscript{153}
Conclusion

PSCs play manifold roles in both health and disease. In the healthy pancreas they are responsible for maintaining ECM turnover and may also have additional roles in innate immunity and CCK-mediated exocrine secretion. In diseased conditions, PSCs are activated by a variety of factors known to be upregulated in the injured pancreas. The last two decades have seen an explosion of findings regarding the interaction of PSCs with other cells in the pancreas, in health, and in disease. These findings have vastly improved our understanding of the physiology and pathology of pancreatic disease, raising hopes for innovative new treatments. Once activated, PSCs become the primary contributors to the pathological fibrosis of both pancreatic inflammation and PDAC and impact on the function of beta cells of islets. Research efforts are now directed toward understanding the mechanisms underlying PSC-mediated fibrogenesis to develop novel therapies to treat fibrotic diseases of the pancreas.

References


43. Gryshchenko O, Gerasimenko JV, Gerasimenko OV, Petersen OH. Ca²⁺ signals mediated by bradykinin type 2 receptors in normal pancreatic stellate cells can be inhibited by specific Ca²⁺ channel blockers. J Physiol 2016; 594:281–293.


48. Charrier A, Chen R, Chen L, Kemper S, Battiori T, Takigawa M, Bragstock DR. Connective tissue growth factor (CGN2) and microRNA-21 are components of a positive feedback loop in pancreatic stellate cells (PSC) during chronic pancreatitis and are exported in PSC-derived exosomes. J Cell Commun Signal 2014; 8:147–156.


55. Xu W, Li W, Wang Y, Zha M, Yao H, Jones PM, Sun Z. Regenerating islet-derived protein 1 inhibits the activation


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cancer stimulates pancreatic stellate cell proliferation and TIMP-1 production through the MAP kinase pathway. Biochem Biophys Res Commun 2004; 323:1241–1245.