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Regulation of Physiologic and Pathologic Exocytosis in Pancreatic Acinar Cells

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Abstract

The pancreatic acinar cell is one of the best-studied cell models of regulated secretion. On activation by neural or hormonal secretagogues, pancreatic acinar cells secrete a variety of inactive digestive enzyme precursors from zymogen granules (ZGs) undergoing exocytosis at the apical pole of this polarized cell. The acinar cell is also an excellent model to study pathologic membrane fusion events which underlie clinical pancreatitis. This includes apical exocytotic blockade along with ectopic fusion events including formation of large cytoplasmic vacuoles and redirected exocytosis to the basolateral plasma membrane; in these compartments zymogens become prematurely activated to initiate pancreatic tissue injury. Over the past two decades, my laboratory has been exploring the central role of SNARE [Soluble N-ethylmaleimide-sensitive factor (NSF) Attachment Protein (SNAP) Receptors] proteins in regulating physiologic and pathologic fusion events in the pancreatic acinar cell. SNARE proteins on cognate vesicles (v-SNARE) and target membrane (t-SNARE) mediate membrane fusion by their highly interactive coiled domains called SNARE motifs that form a trans-complex facilitated by Sec1/Munc18 (SM) and other accessory proteins. This forces the secretory granule (ZG) to come in proximity to the target membrane (plasma membrane) by the zippering action of the trans-SNARE complex that culminates in membrane fusion. This review provides an overview on how these proteins mediate normal regulated exocytosis in the pancreatic acinar cell and pathologic fusion events underlying pancreatitis.

1. Regulated Secretion in Pancreatic Acinar Cells

While all eukaryotic cells are capable of basal constitutive secretion, some specialized secretory cells like pancreatic acinar cells release their secretory products by agonist-evoked regulated exocytosis (35). The pancreatic acinar cell possesses one of the most robust protein synthetic machinery, synthesizing digestive proteases as inactive proenzymes that are sorted out from the *trans*-Golgi into condensing vacuoles (CVs), which then undergo additional maturation steps (3). These mature ZGs, which are among the largest secretory vesicle vesicles (~1 □m) accumulate at the apical pole of the acinar cell, occupying 10-30% of the total cell volume. These

ZGs are in an aggregate "ready-to-release" state waiting for fusogenic Ca^{2+} to induce their exocytosis with the apical plasma membrane and release of their cargo into the ductal lumen. Stimulation by secretagogues such as acetylcholine and cholecystokinin (CCK) activate respective G protein–coupled plasma membrane receptors to trigger a cascade of cellular events leading to the generation of intracellular messengers, primarily diacylglycerol (DAG) which activates protein kinase C (PKC), and inositol trisphosphate (IP_3) which releases Ca^{2+} from IP_3- sensitive stores, although Ca^{2+} is also released from other storage compartments, including those sensitive to nicotinic acidic adenine dinucleotide (22, 51, 53). As well, there is generation of cyclic adenosine monophosphate (cAMP) which activates protein kinase A (PKA) and sequential activation of Rho and Rab family of small G proteins and other protein kinases (29, 40, 52). The concerted actions of these signalling events on downstream substrate proteins, most importantly SNARE and associated proteins that constitute the exocytotic apparatus, culminating in exocytosis of ZGs. However, the apical plasma membrane constitutes less than 10% of the total cell surface area even under maximal physiologic stimulation, and thus primary exocytosis of ZGs with apical plasma membrane would not be sufficiently effective in exporting the very large amount of zymogens needed to be delivered into the duodenal lumen to efficiently digest the food continually being emptied from the stomach during a meal. Remarkably, pancreatic acinar cells are equipped to effect an orderly (within 10 minute) fusion of majority (>30%) of the ZGs within the apical pole, termed sequential exocytosis (32). Sequential and compound exocytosis are not unique to acinar cells and are also observed to be even more efficient in other cell types such as eosinophils and mast cells, as such exocytotic efficiency are required to effect the allergic response. However, it seems unique to the acinar cells that the fusion pores between this extensive network of homotypically-fused ZGs remain open for very long periods (> 10 min) (32, 46), enabling a very efficient emptying of zymogen cargo from the deepest lying ZGs in the apical pole. This provides an exquisitely regulated metered machinery that matches digestive enzyme output to the varying amounts of food being ingested.

2. The SNARE Hypothesis for Membrane Fusion

Exocytosis as we know today comes from the convergence of insights from primitive yeast constitutive secretion to the most highly regulated mammalian neurotransmitter release, where molecules mediating membrane fusion called soluble NSF (N-ethyl maleimide sensitive factor) attachment protein receptor (SNARE) proteins were shown to be remarkably and evolutionarily conserved (39). This SNARE Hypothesis has continued to evolve over the past two decades as new molecules have been discovered along with better methods for spatio-temporal resolution of exocytosis. Since SNARE motifs of different SNARE proteins are highly conserved and can in fact undergo promiscuous assembly *in vitro*, the fidelity of the numerous membrane fusion events that exist *in vivo* in a cell requires strict compartmentalized targeting of the different SNARE isoforms (39). Distinct spatial pairing and assembly of v-SNAREs (vesicle-associated membrane protein or VAMPs) and t-SNAREs (Syntaxins (Syn) and synaptosomal associated protein (SNAP) of 25 kDa or SNAP-25) into fusion-competent *trans*-SNARE complexes are selectively activated by a host of regulatory proteins including large families of Sec1/Munc18 or SM proteins, calcium sensors and other proteins, many of which are coupled to second messengers such as Ca^{2+}, cAMP and protein kinases (C and A) triggered by different agonists (45). The pancreatic acinar cell has been an ideal model to examine these tenets of the SNARE Hypothesis.

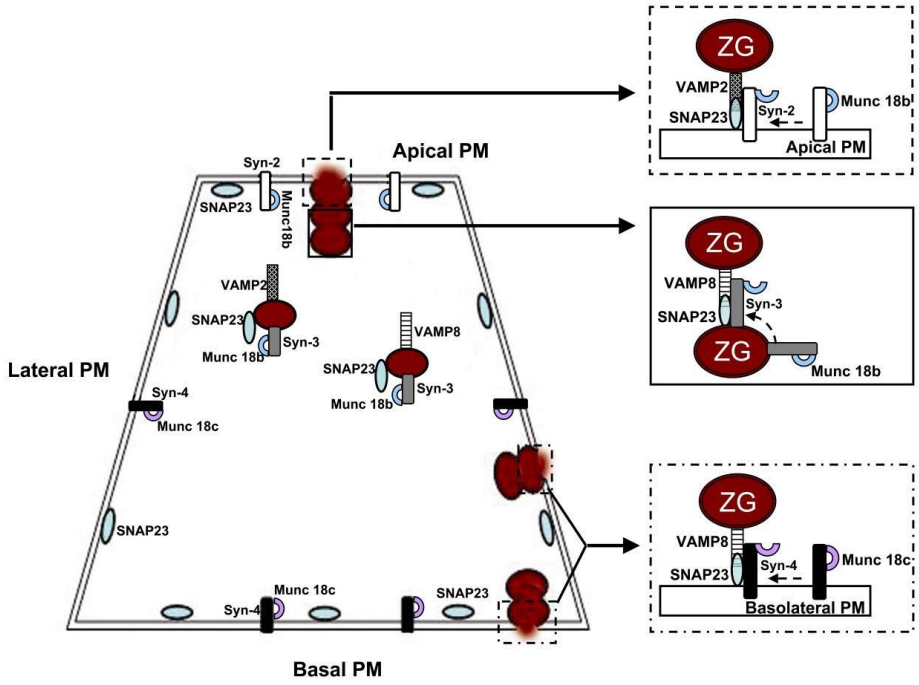

Figure 1. Distinct sets of cognate SM and SNARE proteins mediating apical and basolateral exocytoses, and ZG-ZG fusion. Respective SM proteins bind to syntaxins at the specific compartments at basal state (*left*). Upon stimulation (*right*), the SM proteins activate syntaxins into open conformation capable of binding the cognate VAMPs and SNAP-23 to form the distinct SNARE complexes that mediate the different exocytoses

3. SNARE Protein Regulation of Physiologic Exocytosis in the Acinar cell

We first mapped out the cellular locations of the v- SNARE (VAMPs) and t-SNAREs (syntaxins (Syn) and SNAP-25) in the pancreatic acinar cell (Figure 1). We found three exocytotic syntaxin isoforms in different exocytotic compartments: syntaxin 2 (Syn-2) on the apical plasma membrane, syntaxin 3 (Syn-3) on ZG membrane, and syntaxin 4 (Syn-4) on the basolateral membrane (14, 19). A smaller SNAP-25 isoform, SNAP-23, was the dominant SNAP-25 isoform present in ZGs and the entire acinar plasma membrane (16, 21). Although VAMP-2 and VAMP-3 were both present in acini, VAMP-2 was the dominant form for regulated exocytosis, while VAMP-3 may play a role in constitutive secretion (19). Different strategies were used to assess the exocytotic functions of these SNARE proteins. The first strategy was by employing botulinum neurotoxins or BoNTs, including tetanus neurotoxin or TeNT, which have been long known to block neurosecretion; and the discovery that exocytotic blockade was by proteolytic cleavage of SNARE proteins was instrumental in catapulting the field forward (18). However, unlike neurons, pancreatic acinar cells do not have plasma membrane receptors to internalize BoNTs. Thus, cell permealization strategies (ie. streptolysin O) were employed to internalized the BoNTs (18), which allowed us and others to show that TeNT and BoNT/C1 selectively cleaved pancreatic acinar VAMP-2 and syntaxins (Syn-2 and Syn-3 only), respectively, blocking Ca^{2+}-evoked enzyme release (18, 23). Of note, our initial observation in 1994 showed that in spite of the complete proteolysis of VAMP-2 by TeNT, there was only a 30% inhibition of enzyme secretion (18). It was 15 years later when we discovered that another VAMP, *VAMP-8*, when genetically-deleted, abrogated the majority of regulated apical exocytosis (7). This was primarily by VAMP-8's role in mediating ZG-ZG fusion,

shown by epifluorescence and confocal imaging of the fluorescent dye FM1-43, and with higher spatial resolution employing multi-photon microscopy (4, 7). Thus, primary exocytosis of ZGs with apical plasma membrane is mediated by VAMP-2 *per se* (4, 7). Interestingly, the *VAMP-8*-deleted mice showed no symptoms of malabsorption suggesting that the remaining zymogens secreted by the restricted primary exocytosis into the gut were sufficient for digestion. The exocytosis role of SNAP-23, which was resistant to BoNTs, was shown by adenovirus expression of dominant- negative carboxyl-terminal deleted SNAP-23 (27). The current thinking of the SNARE Hypothesis indicates a critical role for SM proteins (6) to bind and activate syntaxins to bind cognate SNAREs proteins to form fusion-competent SM/*trans*- SNARE complexes (45). SM (nSec1/Munc18) proteins are a group of hydrophilic 60–70-kDa polypeptides, first identified as *UNC-18* in *C. elegans* (5) and *Sec1* in yeast (34). The mammalian homologue neuronal Munc18-1 (also known as Munc18a, nSec1 or RbSec1) was first identified and found to bind syntaxin-1A (26), then later shown to activate syntaxin-1A to bind cognate SNAREs proteins to form fusion- competent SM/*trans*-SNARE complexes (45). Neuronal Munc18a is not present in acinar cells, whereas Munc18b and Munc18c are (7,16). Using immunoprecipitation assays on physiologic CCK- and carbachol-stimulated pancreatic acini, distinct quaternary SM/SNARE complexes were captured (4, 7), including Munc18b binding to the *trans*- SNARE complex – Syn-2/VAMP-2/SNAP-23 we postulate to mediate primary exocytosis of ZGs with apical plasma membrane, and Munc18b binding to the *trans*-SNARE complex – Syn- 3/VAMP-8/SNAP-23 we postulate to mediate sequential ZG-ZG fusion (4, 7). While the basic tenets of the different above- mentioned exocytotic processes mediated by distinct SM/SNARE complexes in the pancreatic acinar cell seem to mimic the well-studied synaptic vesicle exocytosis in the neuron (39, 45), the pancreatic acinar cell does exhibit some distinct features. Unlike neurons and other secretory cells, it seems that most of the acinar ZGs do not completely collapse into the plasma membrane during exocytosis; thus the complete disassembly and recycling of SNARE complexes postulated to occur in neuro-exocytosis (39, 45) may not be completely mimicked in the acinar cell. Future work will also be needed to elucidate how this architecture of intact ZGs is maintained during exocytosis. Furthermore, in the acinar cell, the fusion pores between the sequentially-fused ZGs remain open for great lengths of time and then close after partial emptying of the zymogen cargo (32, 46). The partially emptied ZGs then reload with zymogens to get ready for the next round of exocytosis. Much work will also be needed to elucidate the molecular machinery that maintains the fusion pores to remain open for such extended periods.

4. Other Proteins in Regulated Apical Exocytosis

Complexins (complexins 1 and 2), Synaptotagmins (Syts) and cysteine string protein (CSP)-α have been recently described to be accessory SNARE-interacting effectors of neural exocytosis (38, 45). Complexins preferentially bind to assembled SNARE complexes and act both as clamp and an activator of SNAREs by pulling the complex closer to the membrane, leading to a “super-primed” state ready for immediate fusion (31). It is believed that the Ca^{2+} sensing Syts finally triggers fusion (38,45). Ca^{2+} binding to Syts promote their oligomerization and binding to the SNARE complex, allowing the formation of a quaternary SNARE-synaptotagmin-Ca^{2+}-phospholipid complex and thus promote membrane fusion (38). CSPα is a synaptic vesicle protein which forms a complex with heat shock protein cognate 70 (Hsc70) and complexin in neurons (44). These proteins are all present in the

pancreatic acinar cell (10). In pancreatic acinar cells, complexin 2 is in the apical pole (10) and CSP-α is on the ZG, the latter further shown to interacted with VAMP-8 and Hsc70 (50). Recently, several isoforms of Syt (Syt 1, 3, 6 and 7) were also found in pancreatic acini (11), with Syt 1 found in the ZGs and apical membrane, and Syt 3 found in both acinar membrane and microsomes (11). It is possible that some of these proteins, particularly Syts and complexins, may act as either fusion clamps and/or calcium sensors capable of responding to the different Ca^{2+} release events, including apical oscillatory Ca^{2+} spikes versus global Ca^{2+} rise (36), mediating physiologic (above) and pathologic (below) exocytoses, respectively.

5. SNARE Proteins Mediate Pathologic Basolateral Exocytosis Underlying Pancreatitis

The basolateral plasma membrane accounts for 90% of the acinar cell surface area where little or no exocytosis occurs normally. However, the basolateral plasma membrane of the pancreatic acinar cell contains a complete set of t-SNARE proteins (Syn-4, SNAP-23) and cognate SM protein Munc18c indicating that this plasma membrane domain has the membrane fusion machinery for exocytosis to potentially occur (17). An early morphological study showed that supraphysiological CCK or cholinergic stimulation caused apical exocytotic blockade and induced ectopic exocytosis at the lateral plasma membrane (42), releasing enzymes into the interstitial space, which when activated led to cellular destruction ie. interstitial pancreatitis (24). This report (42) was largely ignored until more recently when we elucidated the exocytotic machinery mediating this pathologic fusion event (15), thus re-establishing the thinking that this is a major event contributing to pancreatitis (15). Employing FM1-43 fluorescence imaging, basolateral exocytosis was observed in dispersed rat pancreatic acini after supramaximal CCK or carbachol stimulation; and more remarkably, also after treatment with clinically-relevant concentrations of alcohol and putative alcohol metabolites followed by physiologic CCK or carbachol stimulation (8, 9, 28), thus simulating alcoholic pancreatitis. We recently reported an improved exocytosis imaging technique (**Figure 2**) whereby adenoviral expressed pH-sensitive fluorophore, syncollin-pHlourin, targeted to ZGs, fluoresced upon exocytosis observed by spinning disc microscopy, enabling high spatial resolution and real time visualization of both apical and basolateral exocytoses (9, 12). In these reports, electron microscopic analysis of pancreatic tissues of rodents subjected to similar supramaximal stimulation showed aberrant exocytosis occurring at the lateral plasma membrane and consequential interstitial pancreatitis. We found SM protein Munc18c on the basolateral plasma membrane, which on supramaximal stimulation resulted in specific PKC-α phosphorylation of Munc18c, inducing its assembly and activation of *trans*-SNARE complex Syn-4/VAMP-8/SNAP-23 (8, 28), which we postulated to be the putative SM/SNARE complex mediating pathologic basolateral exocytosis (7). PKC phosphorylation of Munc18c activates Syn-4 into open conformation conducive to forming SNARE complexes with SNAP-23 and VAMP8, thus rendering the basolateral plasma membrane receptive to exocytosis with approaching VAMP8 containing ZGs. Activation of Munc18c reduces its affinity to open conformation Syn-4. Interestingly, in a human case of quiescent chronic alcoholic pancreatitis, this resulted in Munc18c becoming displaced into the cytosol of residual intact acinar cells, suggesting that this might be a possible contributing mechanism predisposing to recurrent pancreatitis often observed in these patients (20). Whereas ZGs are sparse in the vicinity of the basolateral surface, we found that upon supramaximal carbachol or alcohol (or alcohol metabolite) plus submaximal agonist stimulation of pancreatic acini, VAMP-8-labeled ZGs were redirected to approach the basolateral membrane (7, 9).

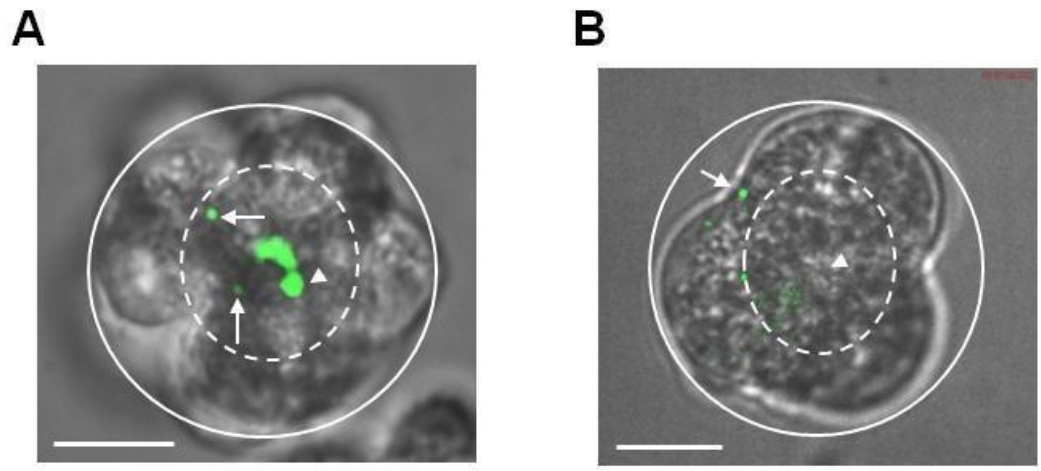

Figure 2. Imaging of apical and basolateral exocytosis by recording syncollin-pHlourin fluorescence hotspots using spinning disk confocal microscopy. Shown are DIC images with superimposed syncollin-pHluorin green images (scale bar=10 □m) of rat pancreatic acini. Two concentric circles are drawn. *Inner dashed circle* encompasses the ZG poles surrounding the apical lumen; exocytosis in this region we consider as apical exocytosis. *Outer solid circle* encompasses from the acinar basal plasma membrane up to the borders of the ZG poles; exocytosis in this region we consider as basal exocytosis. Note in **A**, in a rat pancreatic acinius stimulated with 200 pM CCK-8, syncollin-pHluorin green fluorescent hotspots were confined to the apical region (*inner dashed circle*) with *arrowhead* indicating the apical lumen. The fluorescent hotspots in the deeper regions of the apical pole (*arrows*) indicate sequential exocytosis with these deep-lying ZGs. In **B,** an acinus pretreated with 3 mM acetaldehyde (an alcohol metabolite) then stimulated with 200 pM CCK-8, caused a redirection of much of the apical exocytosis (*arrowhead* pointing to apical lumen) to the basolateral region. The *arrow* indicates a green hotspot at the junction of basal and lateral plasma membrane region. Many of the hotspots are outside the inner dashed circle and distant from the apical lumen and apical poles. These data are similar to those recently reported by us in ref. 9.

Furthermore, employing *VAMP-8* null mouse dispersed pancreatic acini, basolateral exocytosis was indeed completely prevented (7). Remarkably, induction of acute pancreatitis by *in vivo* supramaximal carbachol or alcohol plus submaximal carbachol stimulation did not result in pancreatitis in *VAMP-8* null mice (7). To translate these findings to clinical pancreatitis, we fed mice with an alcohol diet and then stimulated with postprandial carbachol stimulation, which caused pancreatitis in wild type mice, but not in the *VAMP-8* null mice (7). Likewise, the alcohol diet redirected CCK-mediated exocytosis to the basolateral membrane causing alcoholic pancreatitis (7).

6. Formation of large cytoplasmic vacuoles

The current dogma over the past two decades postulated that the dominant key early cellular event leading to pancreatitis is the formation of large cytoplasmic vacuoles where hydrolytic lysosomal enzymes and zymogens become co-localized to cause premature activation of zymogens (33, 41, 49) causing intracellular digestion leading to cell injury. The molecular and cellular mechanisms underlying the formation of these large vacuoles are only now becoming clearer. Studies on *in vitro* model of acute pancreatitis in dispersed acini (43) or *in vivo* studies on cerulein/L-arginine model of acute pancreatitis (30) reveal perturbation in endocytosis (43) and autophagy (30) underlying the process of vacuole formation and intracellular trypsinogen activation.

Table 1

SNARE, SM and accessory proteins in pancreatic acinar cells

SNARE protein	Localization	Binding partners	Function	References
v-SNAREs				
VAM-2	ZG	Syn-2, SNAP-23	Apical exocytosis	18, 19, 23
VAM-8	ZG	Syn-4, SNAP-23 Syn-3, SNAP-23	Basolateral exocytosis ZG-ZG fusion	4, 7 4, 7
t-SNARE				
Syn-2	Apical membrane	SNAP-23, Munc 18b, VAMP-2	Apical exocytosis	4, 7, 9, 14, 23
Syn-3	ZG	SNAP-23, Munc 18b, VAMP-8	ZG-ZG fusion	4, 7, 9, 14, 23
Syn-4	Basolateral membrane	SNAP-23, Munc 18c, VAMP-8	Basolateral exocytosis	7-9, 14, 17,
SNAP2 3	Basolateral and ZG membrane	28 Syn,, Syn-3, Syn-4, VAMP2, VAMP-8	All three exocytoses	7, 17, 21
SNARE Regulators				
SM Proteins				
Munc 18b	Apical membrane & ZG	Syn-2, Syn-3	Promotes ZG-apical PM 16 and ZG-ZG SNARE complex	7, 9,
Munc 18c	Basolateral membrane	Syn-4	Promotes basolateral 28 SNARE complex	7-9, 17,
Complexins				
Complexin 2	Apical membrane	VAMP-2, Syn-3, Syn-4	Regulates apical secretin	10
Synaptotagmins				
Synaptotagmin 1	Apical ZG	VAMP-2	Regulate Ca2+ induced secretion	11
Synaptotagmin 3	Acinar membrane	ND		
Synaptotagmin 6	ND	ND	ND	11
Synaptotagmin 7	ND	ND	ND	11
Cysteine string protein α	ZG complex	VAMP-8, complexin	Augments exocytosis by stabilizing exocytotic	50

ND, Not determined: PM, plasma membrane

The ability of cathepsin B inhibitor to inhibit trypsinogen activity in the endocytic vacuole (43), taken along with the evidence that autophagosomes/autolysosomes accumulate in acute pancreatitis (25, 30) suggest that vacuolar trypsinogen could be delivered to hydrolase-containing vesicles or lysosomes by endocytosis or autophagy. Of note, almost nothing is known about the precise molecules mediating these pathologic vesicular transport and fusion processes. Studies from different groups investigating other cell types have proposed the involvement of SNARE proteins in the fusion of endocytic and autophagy c vacuoles with lysosomes (13, 37). Syntaxin 7 is thought to be the SNARE protein required for both homotypic late endosome fusion and heterotypic fusion with lysosomes (2). The other SNARE proteins in this process were identified using antibody inhibition of cell-free assays, which are Vti1b, syntaxin 8, VAMP-7 and VAMP-8 (1, 47, 48). These reports taken together lead us to postulate that these pathologic processes underlying pancreatitis may involve these candidate membrane fusion

molecules rather than those we have described above for apical and basolateral exocytoses. Much further work from our laboratory and others will be required to pursue these possibilities.

7. Future Directions

From this brief review, much is known about the different fusion molecules mediating physiologic and pathologic exocytosis in the pancreatic acinar cell, which we summarized in **Table 1**. Many of these exocytotic processes have been found to mimic neuronal exocytotic machinery. However, the pancreatic acinar cell exhibits a number of distinct features in physiologic exocytosis such as long fusion pore openings, and in pathologic exocytosis such as the formation of large cytoplasmic vacuoles, whose molecular bases are unknown. From the future work directed at defining the molecular mechanisms underlying these unique features in exocytosis, one may be able to identify strategies to increase the efficiency of secretion of residual pancreatic acinar cells to treat diseases of exocrine insufficiency, or block the pathologic exocytosis that could prevent progression to severe pancreatitis.

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