



Ca²⁺ Signaling in Pancreatic Acinar Cells

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I. Introduction

Intracellular Ca²⁺ 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 Ca²⁺ 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 lead 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 Ca2+ signals and activation of effectors are elaborately orchestrated in time and space. The importance of the physiological Ca²⁺ signal is reinforced by increasing evidence that disordered Ca²⁺ signaling is causally associated with pancreatic pathology (45). The aim of this chapter is two-fold; first to provide the reader with an overview of the spatiotemporal features of intracellular Ca²⁺ signals in acinar cells as measured by contemporary high-resolution fluorescence measurements. Secondly, a review will be presented of the current understanding of the molecular mechanisms underpinning Ca2+ release, Ca²⁺ influx and clearance; processes that are fundamental for the specific properties of intracellular Ca²⁺ signals in acinar cells. The reader is referred to various "Method" submissions in The Pancreapedia regarding current imaging

techniques used to monitor intracellular Ca²⁺ changes with high spatial and temporal resolution in acinar cells.

A. 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 [Ca²⁺]_i. It is well established that each agonist binds a specific, cognate, 7-transmembrane domain receptor in the plasma membrane (PM) that couples to the heterotrimeric G protein, Gaq. Activation of Gaq results in the increased activity of phosphoinositide (PI)- specific phospholipase C which cleaves phosphatidylinositol, 4.5bisphosphate (PIP₂) in the PM and subsequently generates the second messengers inositol 1,4,5trisphosphate (IP₃) 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 (137) was the first to demonstrate that IP₃ could induce Ca²⁺ release in studies using permeabilized pancreatic acini (137). Notably, release could only be evoked from rough ER vesicles but not mitochondria or vesicles prepared from plasma membrane (135). These

data confirmed earlier studies that secretagogues stimulation resulted in substantial Ca²⁺ loss from ER but not zymogen granules or mitochondria (32). Finally, the causative "loop" was then closed by the demonstration that stimulation with pancreatic secretagogues resulted in the rapid formation of IP₃ (136). Notably, this pathway has subsequently been shown to be ubiquitous and accounts for the generation of Ca²⁺ signaling events important for a multitude of cellular events in all tissue systems.

II. The Characteristics of Secretagogue - Induced Cytosolic Calcium Signals in Acinar Cells

A. Temporal Properties at High Concentrations of Secretagogues

In common with many non-electrically excitable cell types, the spatiotemporal pattern of Ca²⁺ 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 global intracellular Ca²⁺ monitoring the $([Ca^{2+}]_i)$ concentration using fluorometric techniques in single rodent cells reported that maximum concentrations of CCK, ACh or bombesin evoked a similar Ca²⁺ signal. Exposure to either agonist, results in a rapid 5-12 fold increase in [Ca²⁺], from a basal value of [Ca²⁺] ~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 around 100 nM above basal, which is maintained as long as the agonist is present (89, 118, 138, 177). The initial peak was shown to be the result of Ca²⁺ release from intracellular stores, since the early transient response was essentially unaffected by removal of extracellular Ca²⁺ while the later plateau phase was absent (Figure 1B) (107, 138, 152, 177). Removal of extracellular Ca²⁺ during the plateau phase resulted in the rapid attenuation of the signal (Figure 2B), thus indicating an absolute dependence on extracellular Ca²⁺ for this maintained phase and implying the presence of a mechanism for Ca²⁺ influx from the extracellular milieu. A study monitoring AChinduced 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. (83)



Figure 1. [Ca²⁺]_i changes evoked by maximal concentrations of secretagogues in single cells. maximal Stimulation with concentrations of secretagogues results in a characteristic "peak and plateau" type Ca²⁺ signal. (A) In a single fura-2 loaded rat pancreatic acinar cell, stimulation with 10 µM CCh resulted in a sharp increase in [Ca2+] which subsequently declines to a new plateau level which was maintained throughout the period of secretagogue application. (B) In the absence of extracellular Ca²⁺ the initial peak can be initiated, but is only transient indicating that the initial phase of the response is a result of Ca2+ release from intracellular stores. Readmission of extracellular Ca2+ restores the "plateau" phase of the response indicating that Ca2+ influx is required to maintain this portion of the response.

B. 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 (118, 129, 138, 153, 177).

These oscillations are characterized by repetitive, regular cycles of elevated and subsequently decreasing Ca²⁺ 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 30s -2 min fairly regular Ca²⁺ oscillations at a frequency of between 1- 6 cycles per minute. The maximal global [Ca²⁺]_i 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 (50, 72, 105, 179). Oscillations stimulated by peptide secretagogues tend to be characterized by slow, relatively long-lived transients originating and returning to basal levels Ca²⁺ spikes while between ACh-induced oscillations are characterized by faster short lasting transients originating from an elevated plateau (Figure 2A and B).



Figure 2. Physiological concentrations of agonists evoke Ca^{2+} oscillations. In single mouse pancreatic acinar cells low concentrations of secretagogue (50-400 nM ACh or 1-50 pM CCK) evoke repetitive Ca^{2+} transients termed Ca^{2+} oscillations. In small clusters of mouse acinar cells muscarinic receptor and CCKstimulation resulted in distinct global temporal patterns of Ca^{2+} signal. (A) CCh-stimulation results in sinusoidal oscillations superimposed on an elevated baseline, while as shown in (B), CCK stimulation results in much less frequent, broader transients which originate and return to basal $[Ca^{2+}]_i$ levels between transients. (From reference (133)).

C. Mechanisms Underlying Oscillating Ca²⁺ Signals

Consistent with a prominent role for receptor stimulated, $G\alpha q$ -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 GTP γ S, sodium fluoride or mastoparan and by introduction of IP₃ into the cell for example via dialysis from a whole-cell patch clamp pipette (105,

161, 179). 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 (74, 153, 177). Although Ca^{2+} release during each cycle only minimally depletes the intracellular Ca^{2+} store (111) and reuptake is efficient (96), 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 (177, 179).

Oscillating levels of IP₃ are not necessary per se for oscillatory behavior, since non-metabolizable IP_3 is capable of initiating Ca^{2+} oscillations in mouse pancreatic acinar cells (161). These data indicate that the mechanism underlying Ca2+ oscillations is most likely the result of an inherent property of the Ca²⁺ release mechanism. Nevertheless, these data do not preclude the possibility that upon agonist stimulation that the [IP₃] itself fluctuates and contributes to the kinetics of Ca²⁺ release. Indeed mathematical predictions based on the experimental behavior of Ca2+ oscillations when Ca²⁺ or IP₃ is artificially raised during agonist exposure predict that the [IP₃] is itself oscillating during CCK stimulation (130). One mechanism whereby oscillating IP₃ could occur is through periodic activation cycles of RGS proteins. RGS proteins stimulate the GTPase activity of Ga subunits thereby terminating the stimulus for activation of effectors such as PLC B. In rat pancreatic acinar cells infusion of RGS proteins via the patch-pipette results in dampening of Ca2+ signals (85, 171, 184, 185). Interestingly, the common catalytic core of RGS proteins, the socalled RGS box is much less effective than infusion of full-length RGS proteins (185). In addition, specific RGS proteins appear to affect the Ca²⁺ signals generated by different secretagogues differentially (171). 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 IP₃ production and contribute to the agonist–specific characteristics of secretagogue-stimulated Ca^{2+} signals in pancreatic acinar cells.

D. Spatial Properties of Ca²⁺ Signals

Measurements of global Ca2+ signals have provided a wealth of data regarding the general temporal properties of Ca2+ signals in pancreatic acinar cells. However, by the nature of the measurements, the experiments report the $[Ca^{2+}]_i$ as a mean value integrated from throughout the cell. Modern imaging techniques however, allow the monitoring of [Ca²⁺]_i 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 Ca2+ and with the ability to be targeted to various cellular compartments (24). Importantly, transgenic animals have been generated expressing a variety of these probes (104, 182). Highlighting the promise of these techniques to enable the study of Ca²⁺ signals in living animals and thus in their native environment, an initial study using intra vital two-photon microscopy in YC3.60 expressing animals reported Ca²⁺ signals following the injection of the muscarinic adonist bethanechol with characteristics similar to that seen in isolated acini (64). The combination of the flexibility of the probes plus the imaginative use of digital imaging techniques, such as confocal, multi-photon and total internal reflection fluorescence (TIRF) microscopy has revealed that the Ca²⁺ 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 Ca²⁺ signal following stimulation with a maximal concentration of ACh (Figure 3A). Stimulation resulted in the initiation of Ca2+ release in the apical region of the cell immediately below the luminal plasma membrane and the subsequent spread of the signal as a wave towards the basal aspects of the cell (65). This Ca^{2+} wave has generally been reported to travel across the cell at a speed of between 5 ~ 45 µm/s, consistent with the velocity observed in other cell types (52, 53, 65, 101, 134, 150).



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 towards the basal aspects of each cell (From reference (65)). The Pseudocolor scale indicates the levels of $[Ca^{2+}]_i$. In (B),

a single pancreatic acinar cell is stimulated with a threshold concentration of ACh. Ca2+ 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 [Ca²⁺]_i elevations are only observed in the apical pole of the acinar cell under these conditions (From reference (66)). (C) Shows the changes in Ca²⁺ following photolytic liberation of 1,4,5 InsP₃ 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-InsP₃ Ca²⁺ 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 Ca2+ 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 (50)).

These data were somewhat counterintuitive since contemporary studies had demonstrated that secretagogues receptors were expressed on the basolateral face of the cell (123) 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 (101).

At physiological concentrations of agonists using similar techniques, Ca²⁺ signals are also shown to initiate in the apical region of acinar cells (66, 148). This initial site of Ca²⁺ release has been termed the "trigger-zone" (66) (Figure 3B). Ca²⁺ 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 photolysis caged-carbachol focal flash of contained in a whole cell patch clamp pipette isolated in the base of the cell (3). 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 (79, 126).

At threshold concentrations of ACh, repetitive short lasting Ca²⁺ transients are initiated which strikingly are contained to the apical third of the cell and do not propagate to the basal region (66, 148). These spikes, although short lived have been shown using low-affinity Ca²⁺ indicators to be of large amplitude in the order of 1-4 μ M Ca²⁺ (62).

At intermediate concentrations of ACh, apically initiated global Ca^{2+} transients dominate, often superimposed on a slight global elevation of $[Ca^{2+}]_i$. 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 Ca²⁺ signals, which are of longer duration (105, 179). In studies of whole cell patch-clamped acinar cells, where the Ca2+ 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 (115). Studies indicate that the precise site of initiation of each transient by specific agonists is very similar, but possibly not identical (126). The site of initiation of each Ca²⁺ transient in the trigger zone is nevertheless tightly coupled functionally to both the exocytosis of zymogen granules and the activation of Clchannels required for the process of fluid secretion from the pancreatic acinar cells (62, 110). Notably, the entire spectrum of signals evoked by secretagogues, from localized apical release to propagation of global Ca2+ waves can be mimicked by global, uniform application of IP₃ either through the patch pipette or via flash photolysis from a caged precursor (Figure 3C) (38, 50, 134, 148). It is envisioned that future work utilizing intra vital microscopy in animals expressing genetically encoded Ca2+ indicators will further our understanding of the spatial and temporal properties of Ca²⁺ signals evoked by physiological stimulation.

E. The Impact of Cell-Cell Communication on Ca²⁺ Signaling

Individual cells in the pancreatic acinus are extensively coupled by the expression of gap junctional proteins (91). 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 Ca²⁺ dependent closure of these junctions (63, 180), at physiological levels of CCK and bombesin where global Ca²⁺ transients predominate the junctions remain open and Ca2+ signals appear to spread as waves between individual cells (131, 180). In contrast, brief, apically confined transients initiated by threshold concentrations of ACh have been reported not to propagate between coupled cells (180). Each cycle of CCK-induced intercellular Ca²⁺ signaling has been reported to be initiated by a "pacemaker cell" (180). This pacemaker presumably represents the individual cell within the acinus most sensitive The propagation of a Ca²⁺ wave to agonist. between adjacent cells obviously requires relatively 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 $[Ca^{2+}]_i$ in neighboring cells (180). These data are consistent with Ca²⁺ acting to facilitate further Ca²⁺ release from intracellular stores as will 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 gapjunctional 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 (131).

III.MolecularMechanismsUnderlyingCa2+SignalinginPancreatic Acinar Cells

A. Intracellular Ca²⁺ Release

*IP*₃-*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 IP_3 receptors (IP_3R) in the extreme apical region of pancreatic acini (75, 100, 176). IP₃R were first isolated and cloned from cerebellum and have subsequently been shown to represent a family of three proteins named the type-1 IP₃R (IP₃R1), type-2 IP₃R (IP₃R2) and type-3 IP₃R (IP₃R3) which are all related to the ryanodine receptor Ca²⁺ release channel (41, 88, 94). Initially, it was reported that IP₃R3 was expressed in the apical pole (100). Later studies however, showed that all three subtypes, had essentially identical expression; all IP₃R were excluded from areas containing zymogen granules and were apparent immediately below the apical and lateral plasma membrane (75, 176) (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 (99). By this technique no other significant localization of IP₃R was noted except for moderate

expression on perinuclear structures (75, 176). The later distribution is consistent with a recent report of IP₃-induced Ca²⁺ release from isolated nuclei prepared from mouse pancreatic acinar cells (47). Studies where IP_3 was released from a caged precursor in various localized regions of mouse acinar cells has also functionally confirmed that the apical region of the cell is more sensitive to IP_3 than the basal area of the cell (38). These data were later confirmed by a study imaging permeabilized pancreatic acini (70). Quantitative Western analysis and PCR have indicated that there is approximately equal expression of IP₃R3 and IP_3R2 in pancreatic acinar cells making up ~ 90% of the total complement of IP₃R (28, 166). The pivotal importance of IP₃R2/3 for Ca²⁺ signaling and subsequent exocrine secretion has been

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confirmed by studies of transgenic animals in which both subtypes have been knocked out (42). Individual knock down of IP₃R2 or IP₃R3 resulted in a modest reduction in the magnitude of Ca²⁺ release (42, 103) 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 (42). These data also reveal that the residual IP₃R-1 in the IP₃R-2/3 null animal cannot compensate for the loss of the other subtypes in pancreatic acinar cells.



Figure 4. Localization of IP₃R in pancreatic slices. The localization of IP₃R1 (**A**) IP₃R2 (**B**) and IP₃R3 (**C**) was determined with specific antibodies to individual InsP₃R types and visualization by confocal microscopy. IP₃R 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**). IP₃R1 and IP₃R3 also localized to perinuclear structures (arrowheads in **A** and **C**). (From reference (176)). (**E**) IP₃R3 (stained in red) are colocalized with the terminal web of the actin cytoskeleton (stained in green).

IP₃R Structure and Regulation

The functional IP₃R is formed co-translationally by the tetrameric association of four individual receptor subunits (112, 140). In pancreatic acinar cells there is evidence that the channel can form a heterotetramer since multiple types of IP₃R can be detected in immunoprecipitates of specific individual receptor types (1, 23, 167). Indeed, a study has shown by sequential recent immunodepletion of individual subtypes from pancreatic lysates, that homotetrameric IP₃R likely constitute a minority of the functional channels (1). Each subunit has a binding site for InsP₃ towards the n-terminus which is formed by a cluster of positively charged amino acids thought to coordinate the negatively charged phosphate groups of IP₃ (11, 125). The Kd for binding of IP₃ to pancreatic membranes has been reported to be between 1-7 nM, a figure similar to that reported for other peripheral tissues such as liver (39, 50). 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 InsP₃ gated cation channel with many of the characteristics of the release channel (34). The fact that the outer nuclear membrane is continuous with the ER has been exploited in patch-clamp experiments to study IP₃R channel activity in isolated nuclei from Xenopus laevis oocytes, COS and DT40-3KO cells (10, 87, 159). 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 IP₃R in native pancreatic membranes.

While the IP₃ binding pocket and channel pore are highly conserved between IP₃R 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 ~1600 amino acids is thought to be important in modulating the Ca²⁺ release properties of the IP₃R. Indeed, Ca²⁺ release through the IP₃R is markedly influenced by many factors, most importantly by Ca²⁺ itself (9, 36). The majority of studies have indicated that all forms of the IP₃R are biphasically regulated by Ca²⁺. [Ca²⁺] in the range 0f 0.5- 1 μ M increases the steady-state open-probability of the channel while at higher concentrations the activity decreases (9, 36). This property of the IP₃R is thought to be fundamentally important in the generation of the different spatial and temporal pattern of Ca²⁺ signals observed in cells (141). In pancreatic acinar cells IP₃-induced Ca²⁺ release has been shown to be inhibited when Ca2+ is elevated and enhanced when Ca2+ is buffered with chelators (33, 187). The [Ca²⁺]_i, together with the range of action of Ca²⁺ can be manipulated by dialyzing cells with buffers exhibiting differing onrates for Ca²⁺ binding. In pancreatic acinar cells, restriction of the range of action of Ca²⁺ using the slow on-rate buffer EGTA resulted in spatially restricted IP₃-induced spikes and the attenuation of global waves consistent with EGTA inhibiting the positive effect of Ca2+ to facilitate Ca2+ release between spatially separated release sites. In contrast, the fast on-rate buffer BAPTA resulted in larger monotonic Ca2+ release and this was interpreted to reflect the loss of local Ca2+ inhibition of IP₃R (67).

IP₃R 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 (112, 113). The IP₃R1 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 Ca²⁺ signaling systems (116). In pancreatic acinar cells, **PKA** activation results in phosphorylation of IP₃R3 (72, 133). Functionally, phosphorylation of IP₃R in pancreatic acinar cells correlates with IP₃-induced Ca²⁺ release which is decreased in terms of the magnitude and kinetics of Ca²⁺ release (51, 133).

Physiologically relevant concentrations of CCK, but not ACh also result in PKA-dependent phosphorylation of IP₃R (133). This observation is consistent with earlier reports that CCK stimulation leads to an increase in cAMP and PKA activation. CCK-induced phosphorylation of InsP₃R may contribute to the specific characteristics of CCKinduced Ca²⁺ signals as maneuvers which interfere with PKA activation disrupt the pattern of CCKinduced but not ACh-mediated Ca²⁺ signaling. Conversely, raising cAMP converts ACh-induced Ca²⁺ signaling characteristics into signals which resemble CCK stimulation (16, 51). CCKstimulated signaling is not affected by raising cAMP or by stimulation with VIP presumably because PKA activation and phosphorylation of IP₃R has already occurred (51, 153). CCK and bombesin stimulation result in Ca²⁺ signals with similar characteristics and this may be related to the fact that bombesin stimulation also results in phosphorylation of IP₃R in mouse pancreatic acinar cells (90, 133). Paradoxically, although there are numerous examples of similar attenuated Ca²⁺ signaling following PKA phosphorylation in other cell types (175), PKA phosphorylation of individual IP₃R subtypes studied in isolation has only been shown to increase Ca^{2+} release (175). These data raise the possibility that the physiologically relevant phosphorylation event actually occurs on a tightly associated binding partner to inhibit Ca2+ release and not the IP₃R-3 directly. In vitro, IP₃R can also be phosphorylated by protein kinase C, Ca²⁺/calmodulin dependent kinase II and tyrosine kinases of the src family (112, 113). While no direct evidence has been reported regarding phosphorylation of IP₃R by these pathways in pancreatic acinar cells activation of PKC has been shown to inhibit Ca2+ release in permeabilized pancreatic acinar cells and to attenuate Ca²⁺ oscillations stimulated by secretagogues or by direct G-protein activation, without an effect on PI hydrolysis (165, 181). Thus, the possibility exists that the IP₃R is a substrate for other kinases in pancreatic acinar cells.

Cellular levels of ATP also modulate IP₃- induced Ca^{2+} release in permeabilized mouse pancreatic

acinar cells (108). ATP has characteristic distinguishing effects on individual IP₃R subtypes (175) and despite an approximately equal complement of IP₃R2 and IP₃R3 in acinar cells, the modulation has essentially identical properties to IP₃R2 in wild type animals and is indistinguishable from IP₃R3 in IP₃R2 null animals. These data indicate that the specific properties of the IP₃R2 appear to dominate the overall profile of IP₃induced release in pancreatic acinar cells (108) and are consistent with the effects of ATP occurring on heterotetrameric IP₃R in which IP₃R2 is present (1). This hypothesis was directly tested in a heterologous expression system where concatenated IP₃R constructs consisting of tandem cDNAs encoding defined heterotetramers were expressed in IP₃R null cells. The characterisitics of Ca²⁺ release from heterotetrameric IP₃R consisting of at least 2 subunits of IP₃R2 were indistinguishable from homotetramertic IP₃R2 (1, 22).

Introduction of G-protein $\beta\gamma$ subunits into pancreatic acinar cells has also been shown to induce Ca²⁺ release. An initial report attributed this to the activation of PLC β 2 by $\beta\gamma$ and the subsequent production of IP₃ (170). A later report however demonstrated that the G $\beta\gamma$ -induced Ca²⁺ release was independent of InsP₃ production and the result of a direct interaction of G $\beta\gamma$ with IP₃R (183). This association was confirmed by coimmunoprecipitation and shown to increase the open-probability of IP₃R in a manner independent of IP₃. This interaction of G α i linked agonists in pancreatic acinar cells.

Ryanodine Receptor-Induced Ca²⁺ Release

Evidence exists for Ca²⁺ release initiated through activation of ryanodine receptors (RyR) in pancreatic acinar cells. This family of channels, best studied as the Ca²⁺ release channel in skeletal and cardiac muscle is classified as belonging to the same gene super-family as IP₃R. Indeed, RyR are modulated by similar regulators and share some sequence homology with the IP₃R especially in the putative Ca²⁺ conducting pore region of the cterminus. However, while IP₃R have an absolute requirement for IP₃ with Ca²⁺ as an important coagonist for gating, RyR only require Ca²⁺ to open through a process termed calcium-induced calcium release (CICR). The functional expression of RyR in pancreatic acinar cells has been indicated by a number of studies; for example microinjection of Ca²⁺ in the presence of the IP₃R antagonist heparin results in Ca2+ release in mouse pancreatic acinar cells (66). In addition, treatment of pancreatic acinar cells with high concentrations of ryanodine known to block RyR dampens secretagogueinduced Ca²⁺ signals (101, 134) and one report has shown that low concentrations of ryanodine, which permanently opens the RyR in a sub-conductance state results in Ca²⁺ release (4). In muscle cells, caffeine activates RvR and results in emptying of sarcoplasmic reticulum Ca²⁺ stores. In contrast, the majority of reports from pancreatic acinar cells indicate that caffeine does not elicit Ca²⁺ release and actually inhibits secretagogue induced Ca²⁺ action signaling through an inhibit to phospholipase C and IP₃R-mediated Ca²⁺ release (4, 151). This later effect of caffeine, together with presumably much lower numbers of RyR in pancreatic acinar cells probably explains the absence of caffeine-induced Ca²⁺ 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 (74). 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 (77). A later report however, using and western analvsis sinale cell PCR demonstrated that all three types of RyR were expressed in pancreatic acini (37). A study performed in human acini has reported mRNA for RyR1, but not RyR2/3. Notably, [³H]-ryanodine binding to membrane preparations provides evidence that the RyR protein is indeed expressed

in human pancreatic acini (78). 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 IP₃R 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 (61, 77, 134). As a result of this localization to areas of the cell with low levels of IP₃R it has been suggested that activation of RyR plays an important role in the propagation of Ca2+ signals from the initial release of Ca²⁺ 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 Ca²⁺ waves in mouse pancreatic acinar cells (Figure 5A and B) (101, 134) and also to selectively attenuate the peak [Ca²⁺] in the basal aspects of cells (61).

*Ca*²⁺ *Release Stimulated by cADPR*

In a number of cell types there is evidence that in addition to activation by Ca2+ the RyR activity are modulated by cyclic adenosine diphosphoribose (cADPr). cADPr was first suggested to be a Ca²⁺ releasing second messenger based on experiments performed in sea-urchin eggs where it was shown to release Ca2+ and function as a messenger during fertilization (26). Subsequently, it has been shown to release Ca2+ and satisfy some of the criteria for a second messenger in lymphocytes, mammalian systems including pancreatic β cells and cardiac myocytes (27, 55, 139). Ca²⁺ release following cADPr exposure is inhibited by blocking concentrations of ryanodine and appears to reduce the threshold for CICR through RyR (73). 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 (8, 128).



Figure 5. Contribution of RyR to global Ca^{2+} signals in pancreatic acinar cells. Global Ca^{2+} signals were initiated by photolysis of caged-IP₃. In (**A**) the images and kinetic traces show that global Ca^{2+} signals can be initiated multiple times following exposure to 1,4,5-IP₃. 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-IP₃ (compare (**B**) **I**; absence of ryanodine and (**B**) **IV**; exposure to ryanodine for 15 mins). (From reference (134)).

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 2 photon flash photolysis and by direct application to permeabilized acini (18, 20, 70, 76, 147). In addition cADPr has been reported to release Ca^{2+} from a rat pancreatic microsomal preparation (106). 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 Ca²⁺ release from the apical pole of the cell (147). This Ca²⁺ release was blocked by both ryanodine and interestingly the IP₃R antagonist heparin. These data were interpreted as indicating that Ca²⁺ release mediated by cADPr was dependent on both IP₃R and RyR, presumably as highly localized Ca²⁺ release initially through RyR sensitized neighboring IP₃R to basal levels of IP₃ (147). Consistent with this view it was also shown that low concentrations of ryanodine also resulted in Ca²⁺

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release from the apical pole in a manner apparently dependent on functional IP₃R (4). 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 2-photon photolysis reported that the basal aspect of the cell was more sensitive to cADPr (76). These data are consistent with a report that in permeabilized acini the apical pole exhibited a higher affinity for IP₃ but cADPr released Ca²⁺ exclusively from the basal aspects of the cell (70).

Important questions to address which are required to establish cADPr as a bona fide Ca²⁺ releasing second-messenger in pancreatic acinar cells are to demonstrate that the molecule is produced following secretagogues stimulation and that it is necessary for Ca2+ release. To these ends it has been reported that CCK and ACh but not bombesin stimulate the activity of a cytosolic ADP-ribosyl cyclase activity resulting in the production of cADPr (132). One such enzyme which possesses this activity is CD38. Ca2+ signaling events in acinar cells prepared from CD38 null mice are dampened and appear reminiscent of RyR blockade (40). In addition, 8-NH2-cADPr, a structural analog of cADPr which antagonizes the effect of cADPr has been reported to block CCK and bombesin but not ACh or IP₃ -induced Ca²⁺ signals in mouse pancreatic acinar cells (13, 19). 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 Ca²⁺ signals reported.

Nicotinic Acid Dinucleotide Phosphate-Induced Ca²⁺ 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 (47) the majority of evidence suggests that the receptor is likely to represent a novel Ca²⁺ release channel since while the activity of IP₃R and RyR exhibits a bell-shaped dependence on Ca2+ the putative NAADP receptor does not support CICR (43). While this property of NAADP is not well suited to play a role in the propagation of Ca²⁺ waves it has been suggested that NAADP is required to initiate Ca²⁺ signals and this initial Ca²⁺ increase subsequently recruits IP₃R and RyR. This idea is supported by the observation that NAADP introduced via the patch pipette into mouse pancreatic acinar cells results in Ca2+ release in the apical pole but that this release is absolutely dependent on both InsP₃R and RyR (18). These data suggest that Ca²⁺ release through NAADP is quantitatively very small but ideally localized to sensitize IP₃R and RyR. While IP₃R and RyR reside in the ER it has been suggested that NAADP primarily acts on a distinct store probably an acidic lysosome related organelle (172). This idea is primarily based on the observation that NAADPinduced Ca²⁺ signaling but not cADPr or IP₃Rinduced Ca²⁺ release is inhibited by experimental maneuvers which either inhibit vacuolar type H⁺ ATPase or result in osmotic disruption of lysosomes (172).

concentration-response The relationship for NAADP-induced Ca²⁺ release also displays unique properties. NAADP-induced Ca2+ release is biphasic; nanomolar levels induce Ca2+ release while micromolar concentrations fail to release Ca²⁺ but render the mechanism refractory to subsequent stimulation (17). 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 Ca²⁺ signals (13, 17). In addition, NED-19 (an inhibitor of NAADP action) attenuates CCK-, but not ACh- induced Ca²⁺ responses (44). These data suggest that CCK and NAADP under these conditions may utilize a common mechanism to induce Ca²⁺ 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 (173). 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 (40). This activity of CD38 occurs at acidic pH and this requirement appears to be fulfilled by the expression of some of the enzyme in an endosomal compartment (40).

The Two Pore Channel (TPC) family of proteins has recently been proposed as a candidate for the cognate receptor for NAADP (14, 43). The TPC gene family is related to the super-family of voltage gated ion channels and consists of three distinct gene products which are represented in both plants and animals (14). The primary supporting evidence for the idea that TPC represent a NAADP receptor is that the expression of TPCs appears to confer Ca²⁺ release activity in the presence of NAADP (14, 43, 124). Additionally, the expression of TPCs also leads to specific binding activity of radiolabeled NAADP and TPCs are primarily expressed in an endo-lysosomal compartment, consistent with the prominent site of NAADP-induced Ca²⁺ release (14, 43). Since this specific binding is much lower affinity than the functional effects of NAADP and specific binding can be observed to coimmunoprecipitated proteins (80, 163), 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- Ca2+ release was reduced in acini prepared from TPC-2 knockout animals (44). Of note NAADP-induced Ca2+ 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 (31). 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 Ca²⁺ release following CCK stimulation.

B. Cellular Mechanisms Underlying Ca²⁺ 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 (152). This influx is not blocked by antagonists of voltage-gated Ca^{2+} channels, but is attenuated by Lanthanides (107, 152). Ca^{2+} influx is also sensitive to changes in extracellular pH, being enhanced by alkaline and inhibited in acidic conditions (107, 152).

Store-Operated Ca²⁺ Entry and its Molecular Components

Functionally, Ca²⁺ influx can be initiated by substantial depletion of intracellular Ca²⁺ pools, the so called "store-operated" or "capacitative" Ca2+ entry (SOCE) pathway (119). This pathway can be readily demonstrated by inhibition of ER Ca2+ pumps with the plant sesquiterpene lactone thapsigargin, which results in Ca2+ influx independent of receptor activation and PI hydrolysis (92, 119, 152, 178). Recently, significant progress has been made regarding the molecular candidates responsible for the SOCE Using siRNA screens pathway. the first breakthrough was the identification of stromal interaction molecule 1 (STIM-1) as the ER Ca2+ sensor responsible for coupling intracellular Ca²⁺ store depletion to the opening of the Ca2+ permeable conductance in the plasma membrane (81, 122, 188). STIM-1 is an integral ER membrane protein, which harbors an EF hand type Ca2+ binding domain localized in the ER lumen. Following depletion of ER stores, Ca²⁺ dissociates from the EF 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 Ca²⁺ influx (84, 169). 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_{CRAC}). The channel has been extensively characterized in lymphocytes as a highly selective Ca2+ channel (35, 59, 154). In particular, homomultimers of Orai1 form channels with the biophysical characteristics of I_{CRAC} (57). Both STIM-1 and Orai-1 are expressed in mouse pancreatic acinar cells and thus represent good candidates for mediating SOCE (56, 59, 86). Lower levels of secretagogue stimulation result in only modest ER Ca²⁺ 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 [Ca²⁺] can activate Orai channels following physiological stimulation (12). It is, however, clear that marked global depletion of ER Ca2+ occurs upon stimulation with maximal concentrations of secretagogues and upon exposure to pancreatic toxins, such as bile acids and fatty acid esters of ethanol (45, 46, 111). Thus, the SOCE pathway may be important during strong stimulation and during pathological Ca²⁺ 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 (71, 97, 98, 120). Consistent with this idea, inhibition of Ca²⁺ influx with pharmacology that targets SOCE has been shown to reduce the severity of pancreatitis in several experimental animal disease models (48, 162, 164) as well as mitigating the detrimental cellular hallmarks of pancreatitis ex vivo (155). Further, maneuvers which inhibit Ca²⁺ release, including blockade of IP₃R with caffeine which prevent depletion of the ER and thus activation of SOCE, also are protective in experimental pancreatitis (60).

Contribution of TRP Channels

In exocrine cells there is also evidence that members of the canonical Transient Receptor

Potential channel family (TRPC) contribute to Ca²⁺ influx following store depletion (59, 68, 82). This channel, while Ca²⁺ permeable is a non-selective cation channel, exhibiting significant Na⁺ permeability. STIM-1 has been reported to also function as the ER calcium sensor for this class of proteins (168). 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 (59, 102). TRPC 3 and 6 are expressed in pancreatic acinar cells (68). In TRPC 3 null transgenic animals, the magnitude of secretagogue- stimulated Ca2+ influx is reduced and this is associated with reduced agonist stimulated amylase secretion (68). In addition, the frequency of agonist-stimulated Ca2+ oscillations was reduced in TRPC 3 null animals supporting the view that Ca²⁺ influx through this channel may also serve to maintain Ca²⁺ oscillations (68). Interestingly the absence of TRPC 3 is also protective in animal models of pancreatitis indicating that SOCE may be deleterious under these conditions (68).

While the spatial properties of the Ca²⁺ signal can be largely attributed to the localization of the Ca²⁺ release machinery, recent data has also suggested that the distribution of the components of the SOCE Ca²⁺ 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 (56, 58, 68, 86). 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 Ca2+ influx following store depletion could be detected earlier in the apical region when compared to more basal aspects of the cell (59). Of note, a majority of STIM-1 does not co-localize with Orai-1 following store depletion (59, 86). Indeed, a study monitoring the localization of adenovirally expressed fluorescently tagged STIM-1 reported little co-localization with Orai-1 and that the vast majority of STIM-1 translocated to the basolateral aspects of cells following stimulation (86). Further work will be

necessary to unequivocally confirm the cellular localization of this influx pathway in acinar cells.

Contribution of ARC Channels

As stated earlier, during secretagogue stimulation only relatively minor depletion of the ER pool as a whole occurs (111). This observation raises the question whether SOCE operates under physiological conditions. If global ER Ca²⁺ 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 (49) which are replete with IP₃R (176) must occur allowing the activation of STIM1/2. A contrary view is that a different mechanism largely controls Ca²⁺ 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 Ca²⁺ influx (93). This conductance has been termed I_{ARC} and is present in many non-excitable cell types (127, 145). Notably, this channel also requires STIM-1 for activation and the pore is formed from multimers of Orai1 and Orai3 (146).

C. Cellular Mechanisms Underlying Ca²⁺ Clearance from the Cytosol

Following $[Ca^{2+}]_i$ elevation, mechanisms must be present to reduce $[Ca^{2+}]_i$ rapidly and efficiently during the falling phase of Ca^{2+} oscillations and ultimately to terminate Ca^{2+} signals following secretagogue removal. Moreover, the resting $[Ca^{2+}]_i$ 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^{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. Tepikin and colleagues employing a technique where the extracellular [Ca²⁺] is monitored by an indicator in a small volume of extracellular fluid demonstrated that Ca2+ is extruded across the plasma membrane following agonist stimulation (142, 144). This in all probability occurs by Ca²⁺ pumps of the plasma membrane Ca2+-ATPase gene family (PMCA). Ca2+-ATPase activity is present in plasma membrane preparations isolated form pancreatic acinar cells and immunoblotting demonstrates the expression of PMCA family members (2, 6, 74). Upon supermaximal stimulation with CCK or ACh the amount of Ca2+ lost from the cell approximates the entire agonistreleasable pool (143). Ca²⁺ extrusion also occurs during more physiological stimulation with CCK and the activity follows the $[Ca^{2+}]_{l}$ (144). PMCA is also important for maintaining resting [Ca²⁺], since it is activity can be demonstrated at basal [Ca²⁺]_i. Indeed the rate of pumping has been shown to have a steep dependence on [Ca²⁺]₁ (Hill coefficient of ~3) and is effectively saturated at [Ca2+] above 400 nM (15). The activity of the PMCA can also be increased by agonist stimulation in a manner independent of Ca²⁺ and this may reflect of modulation the pumps activity bv phosphorylation or association with regulatory proteins (186). PMCA is not homogeneously distributed over the entire plasma membrane and appears to be most abundant in the luminal and lateral plasma membrane (74). The localization of PMCA correlates with the site of most apparent Ca²⁺ pumping activity since Ca²⁺ extrusion occurs preferentially from the apical aspects of mouse pancreatic acinar cells when monitored using an indicator with limited diffusional mobility (7).

Ca²⁺ Uptake into the ER

 Ca^{2+} pumps are also expressed on ER membranes (117, 160). Indeed, the ER in pancreatic acinar cells has been shown to effectively function as a single continuous Ca^{2+} store (95, 111). 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 Ca²⁺ signals can be initiated at the apical pole (95). ER Ca²⁺-ATPases belong to the sarcoplasmic and endoplasmic reticulum Ca²⁺ ATPase gene family (SERCA). Both SERCA 2A and SERCA 2B have been reported to be expressed in pancreatic acinar cells and specific sub-cellular distributions of specific SERCA pumps have been reported (74). SERCA 2A appears to be distributed very similar to InsP₃R 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 Ca²⁺ leak from the ER store. In pancreatic acinar cells, thapsigargin treatment results in a uniform rise in [Ca2+]i and abolishes the characteristic secretagogue-stimulated apically initiated Ca²⁺ wave even before the ER is fully depleted (74). The later observation indicates that the microenvironment created by SERCA pumps is crucial for the initiation of Ca²⁺ signals in the apical pole during Ca²⁺ oscillations. This could occur as a function of the SERCA pump controlling either the cytosolic [Ca2+] or local luminal ER [Ca2+] in the apical pole as both SERCA pumps and IP₃R are markedly influenced by the [Ca2+] on both faces of the ER membrane (96, 174).

Ca²⁺ Uptake by Mitochondria

A further Ca²⁺ uptake mechanism that plays a significant role in spatially shaping Ca²⁺ signals in acinar cells pancreatic is through Ca²⁺ sequestration into mitochondria. Mitochondrial Ca²⁺ uptake occurs as a function of the large electrical potential across the inner mitochondrial membrane via a ruthenium red sensitive Ca2+ uniporter (21). Because the Ca²⁺ uniporter is a relatively low affinity, high capacity transporter, it was generally believed that mitochondrial Ca²⁺ uptake was only relevant under pathological conditions when the [Ca2+] 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 Ca²⁺ signaling in many cell types (121). 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 (30). 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 long sought after molecular components of the uniporter have recently been mitochondrial extensive elucidated. Initially, comparative proteomics for inner mitochondrial membrane proteins present in vertebrates, kinetoplastids (protists containing flagella) but not yeast (which do not have a uniporter) identified MICU1 as essential for mitochondrial uptake (114). Second, with the knowledge that the uniporter complex contained a highly Ca²⁺ selective channel (69), two groups identified an integral membrane protein, MCU which had co-evolved with MICU1 (5, 29). Together these proteins form the core of a complex. which reconstitutes ruthenium-red sensitive mitochondrial Ca²⁺ uptake. A recent study utilizing cells prepared from MCU knockout animals clearly demonstrated has that mitochondrial Ca2+ uptake is disrupted, thus providing strong evidence for its role in acini (25). Interestingly, disruption of mitochondrial Ca²⁺ 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^{2+} release sites since stimulation with physiological concentrations of both CCK and ACh which raise global $[Ca^{2+}]_i$ only to sub-micromolar levels lead to mitochondrial Ca^{2+} uptake (54, 109, 156). Furthermore, mitochondrial Ca^{2+} uptake in pancreatic acinar cells is coupled to the conversion of NAD to NADH (156). Further, stimulation of pancreatic acinar cells with Ca^{2+} mobilizing secretagogues has been shown to result in the net generation of ATP as reported by adenovirally expressed mitochondrially targeted luciferase constructs (158). In common with other Ca²⁺ clearance mechanisms in acini, energized mitochondria also have specific sub-cellular localization (**Figure 6A**) (157). The majority of studies have reported that mitochondria are concentrated in a perigranular "belt" (134, 149), together with additional further sub-populations surrounding the nucleus and immediately below the basolateral plasma membrane (109). Strikingly, It appears that these perigranular mitochondria play a role in limiting the spread of Ca²⁺ signals upon stimulation with threshold concentrations of agonist as disruption of mitochondrial Ca²⁺ uptake facilitates the spread of normally spatially confined Ca²⁺ transients (**Figure 6B**) (134, 149).





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 peri-granular belt surrounding the zymogen granules. (**B**) photolysis of threshold concentrations of 1,4,5-IP₃ result in apically limited Ca²⁺ 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 Ca²⁺ uptake, an identical exposure to 1,4,5 IP₃ results in a global Ca²⁺ signal. These data suggest that functional mitochondria are required to constrain Ca²⁺ signals in the apical portion of the acinar cell. (From reference (134)).

IV. Summary

A cartoon summarizing the molecular components currently believed to be involved in the processes

of Ca²⁺ release, Ca²⁺ influx and Ca²⁺ clearance from the cytosol of pancreatic acinar cells following secretagogue stimulation is shown in **Figure 7**.



Figure 7. Intracellular Ca²⁺ 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 Gag and increased activity of phospholipase C-B (PLC) which cleaves the membrane phospholipid phosphatidylinositol,4,5,bisphosphate (PIP2) into diacylglycerol and inositol 1,4,5-trisphosphate (IP₃). IP₃ diffuses through the cytoplasm and interacts with InsP3 receptors (IP3R), largely type-2 and -3 present predominantly on the apical endoplasmic reticulum (ER) resulting in Ca²⁺ release into the cytoplasm. Ca²⁺ release from IP₃R acts a co-agonist to increase further IP₃R activity and also acts on Ryanodine receptors (RyR) to induce Ca²⁺ release. Depletion of Ca²⁺ within the ER results in Ca²⁺ influx from the extracellular space. The ER Ca²⁺ sensor has been identified as stromal interaction molecule-1 (stim-1). Following ER depletion, Ca²⁺ 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 Ca2+ channels and leads to Ca2+ 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 Ca²⁺ 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, Ca²⁺ can also be released from a store, which accumulates Ca²⁺ 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. Ca²⁺ is removed from the ccytoplasm by the concerted action of SERCA (ER Ca²⁺ ATPase), PMCA (PM Ca²⁺-ATPase) and the action of MCU (mitochondrial uniporter).

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