SYMPOSIUM REVIEW

The role of Ca\textsuperscript{2+} in the pathophysiology of pancreatitis

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Abstract Acute pancreatitis is a human disease in which the pancreatic pro-enzymes, packaged into the zymogen granules of acinar cells, become activated and cause autodigestion. The main causes of pancreatitis are alcohol abuse and biliary disease. A considerable body of evidence indicates that the primary event initiating the disease process is the excessive release of Ca\textsuperscript{2+} from intracellular stores, followed by excessive entry of Ca\textsuperscript{2+} from the interstitial fluid. However, Ca\textsuperscript{2+} release and subsequent entry are also precisely the processes that control the physiological secretion of digestive enzymes in response to stimulation via the vagal nerve or the hormone cholecystokinin. The spatial and temporal Ca\textsuperscript{2+} signal patterns in physiology and pathology, as well as the contributions from different organelles in the different situations, are therefore critical issues. There has recently been significant progress in our understanding of both physiological stimulus–secretion coupling and the pathophysiology of acute pancreatitis. Very recently, a promising potential therapeutic development has occurred with the demonstration that the blockade of Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} currents in pancreatic acinar cells offers remarkable protection against Ca\textsuperscript{2+} overload, intracellular protease activation and necrosis evoked by a combination of alcohol and fatty acids, which is a major trigger of acute pancreatitis.

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Abbreviations 2-APB, 2-aminoethoxydiphenyl borate; ACh, acetylcholine; CaM, calmodulin; CCK, cholecystokinin; CRAC, Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+}; FA, fatty acid; FAEE, fatty acid ethyl ester; IP\textsubscript{3}, inositol 1,4,5-trisphosphate; IP\textsubscript{3}R, inositol 1,4,5-trisphosphate receptor; NMDG, N-methyl-D-glucamine; PMCA, plasma membrane Ca\textsuperscript{2+} ATPase pump; POAEE, palmitoleic acid ethyl ester; SERCA, sarco(endo)plasmic reticulum Ca\textsuperscript{2+} ATPase; TG/Tg, thapsigargin; ZG, zymogen granule.
Introduction

Acute pancreatitis is a human disease, with a significant mortality, in which the pancreas digests itself, causing necrosis and inflammation. Repeated attacks of acute pancreatitis can result in chronic pancreatitis, which increases the risk of developing pancreatic cancer very significantly (10- to 100-fold) (Petersen & Sutton, 2006; Criddle et al. 2007; Petersen et al. 2009, 2011). In 1995, we proposed the hypothesis that an excessive rise in the cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) of pancreatic acinar cells could be the trigger for the initiation of acute pancreatitis (Ward et al. 1995). Much evidence in favour of this hypothesis has since accumulated and major elements of the chain of events initiated by the two major causes of pancreatitis, namely excessive alcohol intake and biliary disease, have been discovered. In what follows, we describe and discuss these cellular and subcellular events. Intracellular Ca\(^{2+}\) is not only a key initiator of pancreatitis, but also a crucial regulator of normal pancreatic acinar cell secretion (Petersen & Tepikin, 2008). It is therefore necessary to consider normal pancreatic acinar Ca\(^{2+}\) homeostasis and the role of Ca\(^{2+}\) in physiological stimulus-secretion coupling in order to fully understand the pathophysiological role of intracellular Ca\(^{2+}\).

Release of Ca\(^{2+}\) from the endoplasmic reticulum and from zymogen granules

Actions of physiological stimulants and their intracellular messengers. Although this review article predominantly deals with pancreatic acinar cells, the earliest mechanistic work on the role of Ca\(^{2+}\) in controlling exocrine secretion was carried out on salivary glands. Douglas & Poisner (1963), in experiments on perfused cat submandibular (submaxillary) glands, discovered that the presence of external Ca\(^{2+}\) was required to sustain acetylcholine (ACh)-evoked salivary secretion, but they also noted that the requirement for external Ca\(^{2+}\) was not as acute as in the case of endocrine glands, such as the adrenal medulla and the neurohypophysis, where hormone secretion is totally and immediately dependent on the presence of Ca\(^{2+}\) in the extracellular solution. Indeed, ACh continues to evoke salivary fluid secretion for quite some time after the removal of Ca\(^{2+}\) from the perfusion fluid. Douglas & Poisner (1963) understood that, 'Calcium has clearly some important role in the stimulant action of ACh on submaxillary salivary secretion. But so little is known of the action of ACh on secretory cells or of the details of the secretory process it initiates that we can only speculate on the nature of this role.'

Selinger et al. (1970) were the first to demonstrate the existence of ATP-dependent Ca\(^{2+}\) uptake into a microsomal fraction from parotid and submaxillary glands. A few years later, we showed that ACh and adrenaline evoked a marked increase in the rate of release of 45Ca\(^{2+}\) from intracellular stores in preloaded perfused cat submandibular glands, and proposed – correctly as it turned out – that ACh (and adrenaline) acts by releasing Ca\(^{2+}\) from the endoplasmic reticulum (ER) (Nielson & Petersen, 1972). Shortly thereafter, similar results were obtained in studies on superfused mouse and rat pancreatic fragments (Case & Clausen, 1973; Matthews et al. 1973). For many years thereafter, it was a major discussion point at all meetings in the field how interaction between a neurotransmitter and a hormone with a receptor site on the outside of the plasma membrane (Iwatsuki & Petersen, 1977; Philpott & Petersen, 1979) could evoke Ca\(^{2+}\) release from an intracellular source. This key question was finally answered by experiments on permeabilized pancreatic acinar cells and isolated microsomal vesicles, in which it was shown that inositol 1,4,5-trisphosphate (IP\(_3\)) released Ca\(^{2+}\) from the ER (Streb et al. 1983, 1984).

The key experimental evidence that led to the now well-known concept of hormone- or neurotransmitter-evoked intracellular Ca\(^{2+}\) signalling by the release of Ca\(^{2+}\) from the ER came from experiments on exocrine gland cells, but there were also some important complicating issues that specifically arose from further work on these cells. In the earliest imaging studies, it was shown that the cytosolic Ca\(^{2+}\) signals evoked by ACh, in both pancreatic and lacrimal acinar cells, always started in the apical (granular) part of the cells (Kasai & Augustine, 1990; Toesca et al. 1992). More importantly, it then became clear that, during sustained stimulation with a low (and therefore most probably physiological) concentration of ACh, or intracellular perfusion with IP\(_3\), the cytosolic Ca\(^{2+}\) signals were confined to the apical granular area and did not spread out towards the base (Kasai et al. 1993; Thorn et al. 1993; Gerasimenko et al. 1996b). Moreover, application of ACh specifically at the base of the cell initiated a cytosolic Ca\(^{2+}\) signal at the opposite end of the cell, at the apical pole (Thorn et al. 1993; Ashby et al. 2003). A detailed study of the distribution of organelles in living pancreatic acinar cells, confirming the general notion from many electron microscopic studies (Bolender, 1974), showed that the bulk of the ER was localized in the basolateral area, whereas the apical part of the cells was dominated by the secretory (zymogen) granules (ZGs). Nevertheless, the apical granular-rich area contained thin elements of ER that penetrated all the way to the apical membrane (Gerasimenko et al. 2002). The conclusion from the early imaging studies (Kasai et al. 1993; Thorn et al. 1993), namely that the apical area of the acinar cells contained the highest concentration or the most sensitive IP\(_3\) receptors (IP\(_3\)Rs), was confirmed by Nathanson et al. (1994) and Lee et al. (1997), who showed by immunocytochemistry that the IP\(_3\)Rs were indeed concentrated in the apical region. The apical Ca\(^{2+}\) signals are physiologically
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important as they activate Ca$^{2+}$-sensitive Cl$^{-}$ channels, which are exclusively present in the apical membrane and are crucial for acinar fluid secretion (Park et al. 2001), as well as the exocytotic enzyme secretion, which can be monitored by capacitance measurements (Maruyama et al. 1993; Maruyama & Petersen, 1994).

Given that it was well established that IP$_3$ elicits Ca$^{2+}$ release from the ER (Berridge, 1993), it might be regarded as surprising that the physiological cytosolic Ca$^{2+}$ signals should occur in an area of the acinar cells that contains relatively little ER. With the discovery that IP$_3$ could release Ca$^{2+}$ from single isolated ZGs (Gerasimenko et al. 1996a), it became necessary to consider the possibility that the physiological apical Ca$^{2+}$ signals could have arisen from release from ZGs rather than from the ER. Although the finding that IP$_3$ could release Ca$^{2+}$ from ZGs was regarded initially with great suspicion, similar results were obtained from studies of isolated secretory granules from tracheal goblet cells (Nguyen et al. 1998) and mast cells (Queseda et al. 2003). There is now no longer any doubt that Ca$^{2+}$ can be released via IP$_3$Rs from an acid non-ER store, dominated by ZGs, as this has been documented in great detail (Gerasimenko et al. 2006a, 2009), but this does not necessarily mean that Ca$^{2+}$ release from such stores plays an important role in normal stimulus–secretion coupling.

The so-called Ca$^{2+}$ tunnel experiments (Mogami et al. 1997) showed that the ER could be refilled, after ACh-elicited emptying, from a point source at the base of an isolated acinar cell by a thapsigargin-sensitive process, and that re-stimulation with ACh would again cause a primary [Ca$^{2+}$]$_i$ rise in the apical pole, more than 10 μm away from the Ca$^{2+}$ entry point at the base, and without any discernible rise in [Ca$^{2+}$]$_i$ during the refilling period. A few years later, we were able to demonstrate directly that a high ACh concentration caused a major reduction in [Ca$^{2+}$]$_i$ in the intracellular stores in the basal part of the cells (dominated by ER), but not in the apical part (dominated by ZGs), in spite of the fact that [Ca$^{2+}$]$_i$ rose primarily in the apical pole (Park et al. 2000; Petersen et al. 2001). It was also shown that the whole of the ER, including the fine extensions and terminals in the apical pole, is functionally connected, and that Ca$^{2+}$ diffuses easily inside the lumen of the ER (Park et al. 2000; Petersen et al. 2001). These studies indicated that physiological stimuli, such as ACh, primarily release Ca$^{2+}$ from the ER, and that the bulk of the Ca$^{2+}$ comes from basal stores. However, the primary Ca$^{2+}$ release into the cytosol occurs in the apical area because this is where the IP$_3$Rs are concentrated. Ca$^{2+}$ tunnelling through the ER works because, in the pancreatic acinar cells, the Ca$^{2+}$ binding capacity of the cytosol (∼3000) is much higher than that of the ER (∼20) (Mogami et al. 1999).

The principal physiological stimulants of pancreatic acinar secretion are ACh, released from parasympathetic nerve endings in the pancreatic tissue, acting predominantly on muscarinic M3 receptors (Nakamura et al. 2013), and the circulating hormone cholecystokinin (CCK), acting on CCK1 receptors. There is no doubt that the primary intracellular mediator of the action of ACh is IP$_3$. Intracellular infusion of IP$_3$, like ACh, evokes repetitive local cytosolic Ca$^{2+}$ spikes in the apical region, and the ACh-evoked spikes are blocked by the intracellular infusion of the IP$_3$R antagonists heparin and caffeine (Wakui et al. 1989, 1990). Furthermore, deletion of type 2 and 3 IP$_3$Rs abolished ACh-evoked Ca$^{2+}$ signal generation (Futatsugi et al. 2005). CCK also evokes Ca$^{2+}$ spiking, but with a somewhat different pattern from that generated by ACh (Petersen et al. 1991). The CCK action is also inhibited by the IP$_3$R blocker caffeine but, unlike the action of ACh, that of CCK can be inhibited by intracellular infusion of a solution with a very high concentration of NAADP, known to inactivate NAADP receptors (Cancela et al. 2000). Although all Ca$^{2+}$ spiking, irrespective of whether it is evoked by ACh or CCK, can be blocked by IP$_3$R antagonists or ryanodine receptor antagonists, it would appear that the action of ACh is initiated by phospholipase C activation via IP$_3$ generation, whereas the action of CCK is initiated by a rise in the intracellular NAADP concentration. In both cases, the Ca$^{2+}$ spiking is caused by concerted interactions of IP$_3$Rs and ryanodine receptors via Ca$^{2+}$-induced Ca$^{2+}$ release (Cancela et al. 2002; Gerasimenko et al. 2003).

**Physiology and pharmacology.** Following the discovery of local and global cytosolic Ca$^{2+}$ oscillations in pancreatic acinar cells (Kasai et al. 1993; Thorn et al. 1993), it is now generally recognized that physiological Ca$^{2+}$ signals are not only oscillating (Berridge, 1993), but that the spatial extent of the signal is of great functional importance (Kasai & Petersen, 1994; Petersen et al. 1994; Parekh, 2011). Although the physiological stimulants, ACh and CCK, can liberate most of the Ca$^{2+}$ stored in the ER in pancreatic acinar cells, they only do so at high concentrations that are unlikely to occur under physiological conditions. At low (physiological) concentrations, the cytosolic Ca$^{2+}$ signals consist mostly of local apical spikes that are caused by the release of only very small quantities of Ca$^{2+}$ that do not result in a large reduction in the [Ca$^{2+}$]$_{ER}$ ([Ca$^{2+}$]$_{ER}$) (Petersen & Tepikin, 2008). The smallest and shortest cytosolic Ca$^{2+}$ spikes, evoked by what are likely to be the most physiological levels of neurotransmitter or hormone, are caused by such small amounts of Ca$^{2+}$ release that it has proven to be impossible to resolve the reduction in [Ca$^{2+}$]$_{ER}$ during each spike (Park et al. 2000). At a slightly higher level of stimulation, it is possible to see small dips in [Ca$^{2+}$]$_{ER}$ during each spike and also to see that, following the reduction, there is a slightly longer
lasting recharging of the ER before the next spike occurs. The important point is that physiological Ca\(^{2+}\) spiking occurs from the resting baseline and that therefore, under physiological conditions, there is no sustained elevation of [Ca\(^{2+}\)]; and, perhaps most importantly, [Ca\(^{2+}\)]\(_{ER}\) remains at all times very close to its resting level.

The actions of pathological stimulants and their mediators. Acute pancreatitis is mainly caused by alcohol abuse or biliary disease, and the principal mediators of the toxic effect on acinar cells are non-oxidative products of alcohol and long-chain fatty acids (fatty acid ethyl esters – FAEEs) and bile acids, respectively. These agents, in concentrations that are pathophysiologically relevant, evoke massive Ca\(^{2+}\) release from both the ER and acid stores, principally activating IP\(_3\)Rs, but also ryanodine receptors (Criddle et al. 2006; Gerasimenko et al. 2006a, 2009; Petersen et al. 2009, 2011). It is the release of Ca\(^{2+}\) from the acid stores, via operational IP\(_3\)Rs, that is most closely associated with the trypsinogen activation that causes autodigestion of the pancreas and leads to necrosis. Knock-out of IP\(_3\)Rs of types 2 and 3 dramatically reduces both the intracellular Ca\(^{2+}\) release and the intracellular trypsinogen activation evoked by FAEEs (Gerasimenko et al. 2009; Petersen et al. 2009, 2011). The combination of ethanol and fatty acids (FAs) is particularly lethal, as FAs markedly reduce mitochondrial ATP production. Therefore, the massive Ca\(^{2+}\) release induced by FAEEs cannot be disposed of by the Ca\(^{2+}\) extrusion following a major rise in extracellular Na\(^{+}\) (Voronina et al. 2010).

It is very important to realize that the most widely used pancreatitis model, based on the hyperstimulation of the CCK receptors (which does not mimic the actual human disease process), is not a good model from the point of view of understanding severe pancreatitis. The main reason is that CCK (or caerulein) hyperstimulation does not lead to a reduction in mitochondrial ATP production, whereas this is the case for the pathophysiologically much more relevant stimulation with products of FAs and ethanol (Voronina et al. 2010).

It is both interesting and important that the pancreatic acinar cells possess an intrinsic protective mechanism against excessive intracellular Ca\(^{2+}\) release, in the form of calmodulin (CaM). Whereas, for example, ethanol alone only has a very modest effect on intracellular Ca\(^{2+}\) release in intact acinar cells, it has a very much stronger effect in permeabilized cells, where CaM would have been washed out of the cytosol. When CaM is added to the solution surrounding permeabilized acinar cells, in a concentration corresponding to that found in intact cells, the effect of ethanol is reduced to that seen in intact cells (Gerasimenko et al. 2011). Given the crucial importance of functional IP\(_3\)Rs for ethanol- and FAEE-induced Ca\(^{2+}\) release, the simplest hypothesis for the mechanisms of action of CaM would be the inhibition of the opening of IP\(_3\)Rs, but this has not yet been proven (Gerasimenko et al. 2011).

Overall Ca\(^{2+}\) homeostasis: transport events at the plasma membrane

Ca\(^{2+}\) extrusion. The steady state [Ca\(^{2+}\)], is determined by Ca\(^{2+}\) transport processes across the plasma membrane. Like all other cell types (Brini & Carafoli, 2009), pancreatic acinar cells possess plasma membrane Ca\(^{2+}\) ATPase pumps (PMCA) and these transporters are responsible for maintaining a low [Ca\(^{2+}\)]. The first measurements of [Ca\(^{2+}\)] in exocrine gland cells were made in insect salivary gland cells, using Ca\(^{2+}\)-selective microelectrodes, and gave values of 100–300 nM (Berridge, 1980; O’Doherty et al. 1980). A few years later, we used a different approach by employing Ca\(^{2+}\)-activated K\(^{+}\) channels in pig pancreatic acinar cells as endogenous Ca\(^{2+}\) sensors. By comparing the voltage–activation curves for these channels in excised inside-out membrane patches, at different [Ca\(^{2+}\)] in the solution in contact with the inside of the membrane, with the voltage–activation curve in the intact acinar cell, we came to the conclusion that [Ca\(^{2+}\)], was between 10 and 100 nM (Maruyama et al. 1983), in good agreement with many measurements made later using fluorescent Ca\(^{2+}\)-sensitive probes (Petersen, 1992).

The importance of the PMCA in maintaining a low [Ca\(^{2+}\)], and in restoring the low [Ca\(^{2+}\)] after a challenge which increases [Ca\(^{2+}\)], is illustrated by the experimental result shown in Fig. 1. A modest inhibition of the PMCA results in an increase in [Ca\(^{2+}\)] and, after a major challenge by a supramaximal ACh concentration in the presence of a thapsigargin concentration that abolishes Ca\(^{2+}\) pump function in the ER, the restoration of the prestimulation [Ca\(^{2+}\)], is markedly slower than under control conditions (Fig. 1). The slightly elevated [Ca\(^{2+}\)], seen when the PMCA is partially inhibited has relatively little consequence in itself but, if the cells are challenged with, for example, an oxidant such as menadione, the level of necrosis is markedly enhanced when compared with the control situation without PMCA inhibition (Fig. 1). This shows that even a slightly elevated [Ca\(^{2+}\)], carries a significant risk for the cell (Ferdeki et al. 2012).

In many cell types, Na\(^{+}/Ca\(^{2+}\) exchange plays an important role in restoring a low [Ca\(^{2+}\)] after a rise. In cardiac cells, for example, Na\(^{+}/Ca\(^{2+}\) exchange is the main process extruding Ca\(^{2+}\) from the cytosol to the extracellular environment following an action potential (Berberian et al. 2012). However, in pancreatic acinar cells, the rate of Ca\(^{2+}\) extrusion following a major rise in [Ca\(^{2+}\)], is completely unaffected by the removal of extracellular Na\(^{+}\) (Fig. 2), indicating that the only process responsible for maintaining a low [Ca\(^{2+}\)], and restoring

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a low \([\text{Ca}^{2+}]_i\) after a challenge is the PMCA. As the \(\text{Na}^+/\text{Ca}^{2+}\) exchange system generally has a much larger capacity than the PMCA system for moving \(\text{Ca}^{2+}\) out of cells, this has consequences for pathological situations in which pancreatic acinar cells have become overloaded with \(\text{Ca}^{2+}\), and have to rely solely on the PMCA to extrude the excess \(\text{Ca}^{2+}\).

\(\text{Ca}^{2+}\) extrusion has been studied directly by measuring the \(\text{ACh}\)-evoked increase in extracellular \([\text{Ca}^{2+}]\) in a small saline droplet in which an isolated acinar cell is immersed (Tepikin et al. 1992a,b). The rate of \(\text{Ca}^{2+}\) extrusion depends on \([\text{Ca}^{2+}]_i\), in the range 70–400 nM, but is flat above 500 nM (Camello et al. 1996). This means that any increase in \([\text{Ca}^{2+}]_i\) from the physiological resting level will result in activation of the PMCA, but that increases in \([\text{Ca}^{2+}]_i\) from an already elevated level will fail to trigger any further \(\text{Ca}^{2+}\) extrusion. In other words, pancreatic acinar cells have a well-functioning mechanism for maintaining and restoring \([\text{Ca}^{2+}]_i\) in the physiological range, but are ill equipped to deal with substantial \(\text{Ca}^{2+}\) overloading.

\(\text{Ca}^{2+}\) entry Given that there is a constant low level of \(\text{Ca}^{2+}\) extrusion in the absence of any stimulation (Tepikin et al. 1992a), it is clear that, even in the resting unstimulated situation, there must be a constant leak of \(\text{Ca}^{2+}\) into the cytosol through the plasma membrane. However, the nature of that leak is unknown. \(\text{Ca}^{2+}\) influx is markedly increased after various kinds of stimulation, occurring – for example – during sustained stimulation of the acinar cells with \(\text{ACh}\) or \(\text{CCK}\). Although the initial phase of stimulant-evoked pancreatic enzyme secretion is completely independent of the presence of \(\text{Ca}^{2+}\) in the external solution, secretion will stop after several minutes unless \(\text{Ca}^{2+}\) is readmitted to the perfusion solution (Petersen & Ueda, 1976).

It is instructive to compare the control of \(\text{Ca}^{2+}\) entry into pancreatic acinar cells and the neighbouring insulin-secreting \(\beta\)-cells. In nerve and endocrine cells, the principal \(\text{Ca}^{2+}\) entry pathway is provided by voltage-gated \(\text{Ca}^{2+}\) channels. The initiating event is membrane depolarization, opening up these channels.

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**Figure 1.** Submaximal inhibition of the plasma membrane \(\text{Ca}^{2+}\) pump (PMCA) increases \([\text{Ca}^{2+}]_i\) and markedly increases the probability of necrosis induced by oxidative stress

The two traces represent typical \([\text{Ca}^{2+}]_i\) changes in response to 10 \(\mu\text{M}\) thapsigargin (Tg) and 10 \(\mu\text{M}\) acetylcholine (Ach) in a control cell (blue trace) and a cell treated with 1 \(\text{mM}\) caloxin 3A1 (red trace; arrow indicates time point of caloxin 3A1 addition). Inset: comparison of necrosis levels in control pancreatic acinar cells and cells treated with 1 \(\text{mM}\) caloxin 3A1, 30 \(\mu\text{M}\) menadione or both 1 \(\text{mM}\) caloxin 3A1 and 30 \(\mu\text{M}\) menadione. Modified from Ferdek et al. (2012).
Ca\textsuperscript{2+} enters and the secretory (exocytotic) sites are very close to the Ca\textsuperscript{2+} entry points, so that high local cytosolic Ca\textsuperscript{2+} concentrations can be attained by activating the exocytotic machinery (Boquist et al. 1995). The principal stimulus for insulin secretion is an increase in the plasma glucose level following a meal, which evokes membrane depolarization, causing firing of action potentials (Dean & Matthews, 1968, 1970). In the insulin-secreting cells, the resting potential is largely a result of ATP/ADP-sensitive K\textsuperscript{+} channels, and the depolarization evoked by glucose uptake into the cells is principally caused by K\textsuperscript{+} channel closure evoked by the increase in the cytosolic ATP/ADP ratio, which is a consequence of the mitochondrial processes occurring during sugar metabolism (Petersen & Findlay, 1987). The Ca\textsuperscript{2+} entry, principally through L-type Ca\textsuperscript{2+} channels, is totally controlled by the membrane potential, and both action potentials and cytosolic Ca\textsuperscript{2+} signals are quickly abolished when the ATP/ADP-sensitive K\textsuperscript{+} channels are activated pharmacologically (for example by diazoxide), causing hyperpolarization. Glucose-evoked insulin secretion, as a consequence of this arrangement, is totally and acutely dependent on the presence of Ca\textsuperscript{2+} in the extracellular solution (Wollheim & Sharp, 1981). In sharp contrast, the pancreatic acinar cells work in a completely different manner. These cells do not possess voltage-gated Ca\textsuperscript{2+} channels, and the cytosolic Ca\textsuperscript{2+} signals that activate exocytotic enzyme secretion are primarily caused by release from intracellular stores, as already described. If all the Ca\textsuperscript{2+} that was released from the ER in response to stimulation were taken up again into the ER, there would be no need for Ca\textsuperscript{2+} entry, but the plasma membrane Ca\textsuperscript{2+} pumps, as already mentioned, will be stimulated to extrude more Ca\textsuperscript{2+} whenever [Ca\textsuperscript{2+}]\textsubscript{i} rises. All cytosolic Ca\textsuperscript{2+} signals are therefore inevitably associated with a loss of Ca\textsuperscript{2+} from the cells. In order for the cell not to run out of Ca\textsuperscript{2+} within the stores, there is a need for compensatory Ca\textsuperscript{2+} entry from the extracellular solution. The main purpose of controlled Ca\textsuperscript{2+} entry in the pancreatic acinar cells is therefore not acute regulation of exocytotic enzyme or fluid secretion, but rather refilling of the intracellular Ca\textsuperscript{2+} stores after release. Under physiological conditions, when low levels of ACh or CCK evoke repetitive short-lasting Ca\textsuperscript{2+} spikes largely confined to the apical granular area, Ca\textsuperscript{2+} entry would appear not to make any direct contribution to the acute control of secretion, as it has no effect on [Ca\textsuperscript{2+}]\textsubscript{i} in the apical pole, but simply feeds the ER with Ca\textsuperscript{2+} from the basal side, via sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA)-mediated Ca\textsuperscript{2+} pumping. This Ca\textsuperscript{2+} entry occurs without any measurable increase in [Ca\textsuperscript{2+}]\textsubscript{i} (Mogami et al. 1997, 1998; Park et al. 2000).

There has been a great deal of confusion about the nature, control and even the precise role of Ca\textsuperscript{2+} entry in pancreatic acinar cells. From the earliest days of investigating exocrine gland Ca\textsuperscript{2+} transport, it was clear that physiological stimulants, such as ACh, did not primarily evoke Ca\textsuperscript{2+} entry into the acinar cells, but rather a delayed opening of Ca\textsuperscript{2+} entry pathways following the primary release of Ca\textsuperscript{2+} from internal stores (Nielsen & Petersen, 1972; Muallem & Verkhratsky, 2013). Following the concept of store-operated Ca\textsuperscript{2+} entry (Putney, 1986; Parekh & Putney, 2005), the focus – in the case of epithelial cells and, in particular, all the exocrine glands – has therefore rightly been on this type of Ca\textsuperscript{2+} entry. Two aspects have been of major interest: the mechanism by which store depletion causes opening of Ca\textsuperscript{2+} channels in the plasma membrane and the biophysical nature of the Ca\textsuperscript{2+} entry pathways.

With regard to the coupling of Ca\textsuperscript{2+} store depletion to Ca\textsuperscript{2+} entry, it would appear that the pancreatic acinar cells conform to the now generally accepted model in which ER Ca\textsuperscript{2+} store depletion causes the ER Ca\textsuperscript{2+} sensor STIM1 to

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**Figure 2. Plasma membrane Ca\textsuperscript{2+} ATPase pump (PMCA) is the main mechanism of Ca\textsuperscript{2+} extrusion in pancreatic acinar cells**

A and B, average traces showing changes in [Ca\textsuperscript{2+}]\textsubscript{i} elicited by the application of thapsigargin (Tg) in the absence of external Ca\textsuperscript{2+} followed by a period of exposure to 5 mM Ca\textsuperscript{2+} in the presence of Na\textsuperscript{+} (A) or in the absence of Na\textsuperscript{+} (substituted by N-methyl-D-glucamine\textsuperscript{+} (NMDG\textsuperscript{+}) 200 s before exposure to 5 mM Ca\textsuperscript{2+}) (B). Replacing Na\textsuperscript{+} with NMDG\textsuperscript{+} does not affect the rate of Ca\textsuperscript{2+} extrusion. From Ferdek et al. (2012).
translocate and become concentrated in certain puncta in the ER membrane, where it comes close to the plasma membrane, and where STIM1 therefore can physically interact with the appropriate Ca\(^{2+}\) channel protein (Liou et al. 2005; Roos et al. 2005). This has been demonstrated directly in normal mouse pancreatic acinar cells, where emptying of the ER Ca\(^{2+}\) store has been shown to cause translocation of STIM1 to puncta very close to the basal acinar plasma membrane, where Orai1 [the molecule responsible for Ca\(^{2+}\)-selective Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channel currents] is present (Lur et al. 2009). This might then suggest that the major Ca\(^{2+}\) entry channel belongs to the Orai1 type (Feske et al. 2006), but other groups have suggested that non-selective cation channel types provide the molecular basis for store-operated Ca\(^{2+}\) entry (Krause et al. 1996; Kim et al. 2009, 2011).

We have recently revisited this problem to assess what is the dominant store-operated inward current in the pancreatic acinar cells. Using the classical store-operated Ca\(^{2+}\) entry protocol, the ER store was emptied of Ca\(^{2+}\) by application of the very specific SERCA pump inhibitor thapsigargin, in the absence of external Ca\(^{2+}\), and Ca\(^{2+}\) entry then occurred when Ca\(^{2+}\) was readmitted to the external solution. Ca\(^{2+}\)-selective CRAC channels are very permeable to Ba\(^{2+}\), but Ba\(^{2+}\) cannot be extruded by the PMCA. In order to assess unilateral divalent cation inflow through CRAC channels, it is therefore useful to employ Ba\(^{2+}\). As shown in Fig. 3, the inward flow of Ba\(^{2+}\) is almost abolished by the relatively specific CRAC channel blocker GSK-7975A (Derler et al. 2012), which also blocks the elevated [Ca\(^{2+}\)]\(_{\text{pl}}\), plateau caused by store-operated Ca\(^{2+}\) entry.

The evolution of the inward Ca\(^{2+}\) current, after blockade of the SERCA pumps, follows closely the time course of the reduction in [Ca\(^{2+}\)]\(_{\text{ER}}\) (Fig. 4). The store-operated inward current is insensitive to the removal of external Na\(^{+}\), but is markedly diminished by reduction of the external Ca\(^{2+}\) concentration and is blocked by 2-aminoethoxydiphenyl borate (2-APB), a well-known, but not particularly selective, CRAC channel blocker. The current–voltage relationship displays strong inward rectification, typical for CRAC channel currents, and the inward current is markedly inhibited by the CRAC channel blocker GSK-7975A (Fig. 4). These recently published data (Gerasimenko et al. 2013) indicate that the dominant store-operated current is of the Ca\(^{2+}\)-selective CRAC channel type and is therefore most probably carried by Orai1 channels.

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**Figure 3.** Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channel blocker GSK-7975A inhibits Ba\(^{2+}\) and Ca\(^{2+}\) entry induced by Ca\(^{2+}\) store depletion

A and B, representative traces of Fura-2 measurements of Ba\(^{2+}\) influx into cells treated with GSK-7975A (10 μM) for 10 min (B) when compared with control cells (A). C and D, average traces of store-operated Ba\(^{2+}\) influx in the presence or absence of 10 μM GSK-7975A. E, acute inhibitory effect of GSK-7975A (10 μM) on the elevated [Ca\(^{2+}\)]\(_{\text{pl}}\) plateau following re-admission of external Ca\(^{2+}\) (5 mM) after thapsigargin (TG) treatment in nominally Ca\(^{2+}\)-free solution. Modified from Gerasimenko et al. (2013).
These data (Fig. 4) are particularly relevant to pathological conditions. Bile acids and FAEEs, in pathophysiologically relevant concentrations, evoke massive release of Ca$^{2+}$ stored in both the ER and acid pools (Gerasimenko et al. 2009), and this, in turn, elicits the opening of store-operated Ca$^{2+}$ channels, which causes and maintains an elevated [Ca$^{2+}$]i. Indeed, palmitoleic acid ethyl ester (POAEE) induces a marked and sustained elevation of [Ca$^{2+}$]i, which can be dramatically reduced by the CRAC channel blocker GSK-7975A (Gerasimenko et al. 2013). The POAEE-elicited [Ca$^{2+}$]i elevation is of great importance as it causes intracellular trypsinogen activation and necrosis. CRAC channel blockade markedly inhibits both protease activation and necrosis, indicating that the cell destruction caused by POAEE depends on Ca$^{2+}$ entry through CRAC channels (Gerasimenko et al. 2013).

Towards a rational therapy for acute pancreatitis

The two phases of Ca$^{2+}$ involvement in stimulus–secretion coupling, intracellular Ca$^{2+}$ release followed by Ca$^{2+}$ entry from the external solution (Petersen & Ueda, 1976), also govern the pathological processes that lead to acute pancreatitis. In experiments on permeabilized acinar cells, intracellular protease activation occurs as a consequence of massive release of Ca$^{2+}$ from both the ER and the acid store, mediated mainly by IP$_3$Rs, although, under these conditions, it is the release from the acid stores that is of particular significance (Gerasimenko et al. 2009). However, in intact acinar cells, intracellular protease activation depends on store-operated Ca$^{2+}$ entry mediated by CRAC channels following Ca$^{2+}$ depletion of the ER (Gerasimenko et al. 2013). CRAC channel blockade would also inhibit the function of immune cells (Parekh, 2010; DiCapite et al. 2011), but this would actually be
advantageous in the acute stage of severe pancreatitis, as the inflammatory response triggered by the acinar necrosis contributes significantly to the severity of the disease.

It is likely that the activation of proteases inside the ZGs depends on both a reduction in the intragranular \([\text{Ca}^{2+}]\) as well as an elevation of \([\text{Ca}^{2+}]\). We have shown that FAEE-induced release of \(\text{Ca}^{2+}\) from the intracellular stores can be markedly inhibited by a synthetic peptide activator of CaM, CALP-3, which also markedly inhibits trypsinogen activation (Gerasimenko et al. 2011). More recently, we have shown that the CRAC channel blocker GSK-7975A markedly inhibits FAEE-induced trypsinogen and general protease activation, as well as the very dangerous necrosis leading to severe acute pancreatitis (Gerasimenko et al. 2013). It is likely that a combination of CaM activation and CRAC channel blockade could be an effective therapy against the life-threatening condition of severe acute pancreatitis (Fig. 5). The proof of principle for such treatment will hopefully soon lead to in vivo studies and thereafter clinical trials, as there is currently no specific therapy for this important disease.

Figure 5. Schematic diagram illustrating the two major drug targets: inositol 1,4,5-trisphosphate receptor (IP$_3$R) Ca$^{2+}$ release channels in the endoplasmic reticulum (ER) and zymogen granules (ZGs) and Ca$^{2+}$ release-activated Ca$^{2+}$ (CRAC) channels in the plasma membrane (SERCA, sarco(endo)plasmic reticulum Ca$^{2+}$ ATPase).

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Additional information

**Competing interests**

None.