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Ca^{2+} signalling and pancreatitis: effects of alcohol, bile and coffee

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Ca^{2+} is a universal intracellular messenger that controls a wide range of cellular processes. In pancreatic acinar cells, acetylcholine and cholecystokinin regulate secretion via generation of repetitive local cytosolic Ca^{2+} signals in the apical pole. Bile acids and non-oxidative alcohol metabolites can elicit abnormal cytosolic Ca^{2+} signals that are global and sustained and result in necrosis. Necrosis results from excessive loss of Ca^{2+} from the endoplasmic reticulum, which is mediated by Ca^{2+} release through specific channels and inhibition of Ca^{2+} pumps in intracellular stores, followed by entry of extracellular Ca^{2+}. Reduction of the cellular ATP level has a major role in this process. These abnormal Ca^{2+} signals, which can be inhibited by caffeine, explain how excessive alcohol intake and biliary disease cause acute pancreatitis, an often-fatal human disease in which the pancreas digests itself and its surroundings.

Pancreatitis lacks specific therapy

Acute pancreatitis is a human disease in which the pancreas digests itself. The incidence of both acute and chronic pancreatitis continues to increase and these disorders cause significant morbidity and mortality [1,2]. However, despite much research and many clinical trials, pancreatitis lacks specific pharmacological therapy, even though there are opportunities for prevention or treatment at various stages of the disease [1]. The central role of Ca^{2+} signalling in controlling normal pancreatic enzyme secretion, the generation of excessive Ca^{2+} signals by hyperstimulation of cells and the importance of premature activation of digestive enzymes in pancreatitis suggest that pancreatic acinar cell injury and its consequences are due to cytosolic Ca^{2+} overload [3]. In this article, we focus on the latest progress in understanding the earliest events in pancreatitis because modification or inhibition of Ca^{2+} signalling mechanisms might improve clinical outcome.

Physiological and pathological Ca^{2+} signals

Intracellular Ca^{2+} is involved in the regulation of virtually all cellular functions. Whereas physiological Ca^{2+} signals are mostly localized and transient, global and sustained elevations of the cytosolic Ca^{2+} concentration {$[Ca^{2+}]_i$} can be fatal [4–6].

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The pancreatic acinar cell is a classic secretion model [7]; in this cell the regulatory Ca^{2+} signals occur as repetitive local spikes that are mostly confined to the granule-containing apical pole [6,8]. Sustained global $[Ca^{2+}]_i$ elevations cause abnormal intracellular enzyme activation, vacuolization and necrosis [9–13], processes that are crucial in the initiation of acute pancreatitis.

Premature digestive enzyme activation

Acinar cells synthesize inactive digestive enzymes, which are stored in apical zymogen granules (ZGs) and secreted into the duodenum where the enzymes are triggered into an activation cascade through conversion of trypsinogen into active trypsin by duodenal enterokinase. In pancreatitis, whatever the precipitant, premature intracellular enzyme activation occurs, contributing to subsequent tissue damage. The identification of mutations in the gene encoding trypsinogen in hereditary pancreatitis [14], which results in mutated trypsinogen that is more readily activated or trypsin that is less readily inactivated, confirms the importance of premature digestive enzyme activation in the pathogenesis of pancreatitis.

Acinar cell injury

Migrating gallstones precipitate acute pancreatitis through biliary reflux into the pancreatic duct and/or pancreatic ductal hypertension [11,15,16]. Bile salts are toxic and induce severe experimental pancreatitis [12].

Ethanol excess precipitates both acute and chronic pancreatitis, although it is the non-oxidative metabolites of ethanol that induce primary acinar cell injury, rather than ethanol itself or acetaldehyde [13]. Acinar cells contain high concentrations of ester synthases, which shuttle ethanol into combination with fatty acids that accumulate as fatty acid ethyl esters; these esters are subsequently hydrolysed and oxidised. Furthermore, fatty acids are likely to mediate acinar cell injury in hyperlipidaemia [13]. In addition to enzyme activation, acinar cell injury is characterized by vacuoles in the apical secretory granular pole [9], and by the colocalization of lysosomes and ZGs [17]. The lysosomal component reduces the pH of these subcellular compartments to less than 5, below which cathepsin B cleaves trypsinogen to release active trypsin [18]. The cytoskeleton is disrupted and secretory polarity is lost, with activation of nuclear factor κB (NF-κB), cytokine expression and/or cell death pathways [19,20].

Paradoxically, neutrophil infiltration and activation increase intrapancreatic digestive enzyme activation and

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exacerbate pancreatitis [21], even though neutrophils are protective in origin. Where acinar cell injury is ongoing with chronic inflammation and fibrosis, chronic pancreatitis ensues, increasing the risk of carcinoma [14].

Cytosolic Ca^{2+} overload and acinar cell injury

The hypothesis that Ca^{2+} overload initiates acute pancreatitis [3] was first tested by cholecystokinin octapeptide hyperstimulation (CCK8) [9,10], an established means of inducing experimental acute and chronic pancreatitis. Subsequent work with bile salts [11,12,22], fatty acids and fatty acid ethyl esters [13] in addition to duct-ligation *in vivo* studies [23] confirmed the crucial role of Ca^{2+} in acinar cell injury. Intracellular Ca^{2+} chelation was found to prevent premature digestive enzyme activation, vacuolization, skeletal disruption and acinar cell necrosis induced by all these toxins. Thus, in this article, we review the mechanisms by which Ca^{2+} overload can occur.

Crucial regulated Ca^{2+} transport steps in acinar cells

Receptors for acetylcholine and cholecystokinin

Figure 1 illustrates some of the important Ca^{2+}-related processes in the plasma membrane and internal membranes of pancreatic acinar cells. The neurotransmitter acetylcholine (ACh) interacts with muscarinic ACh

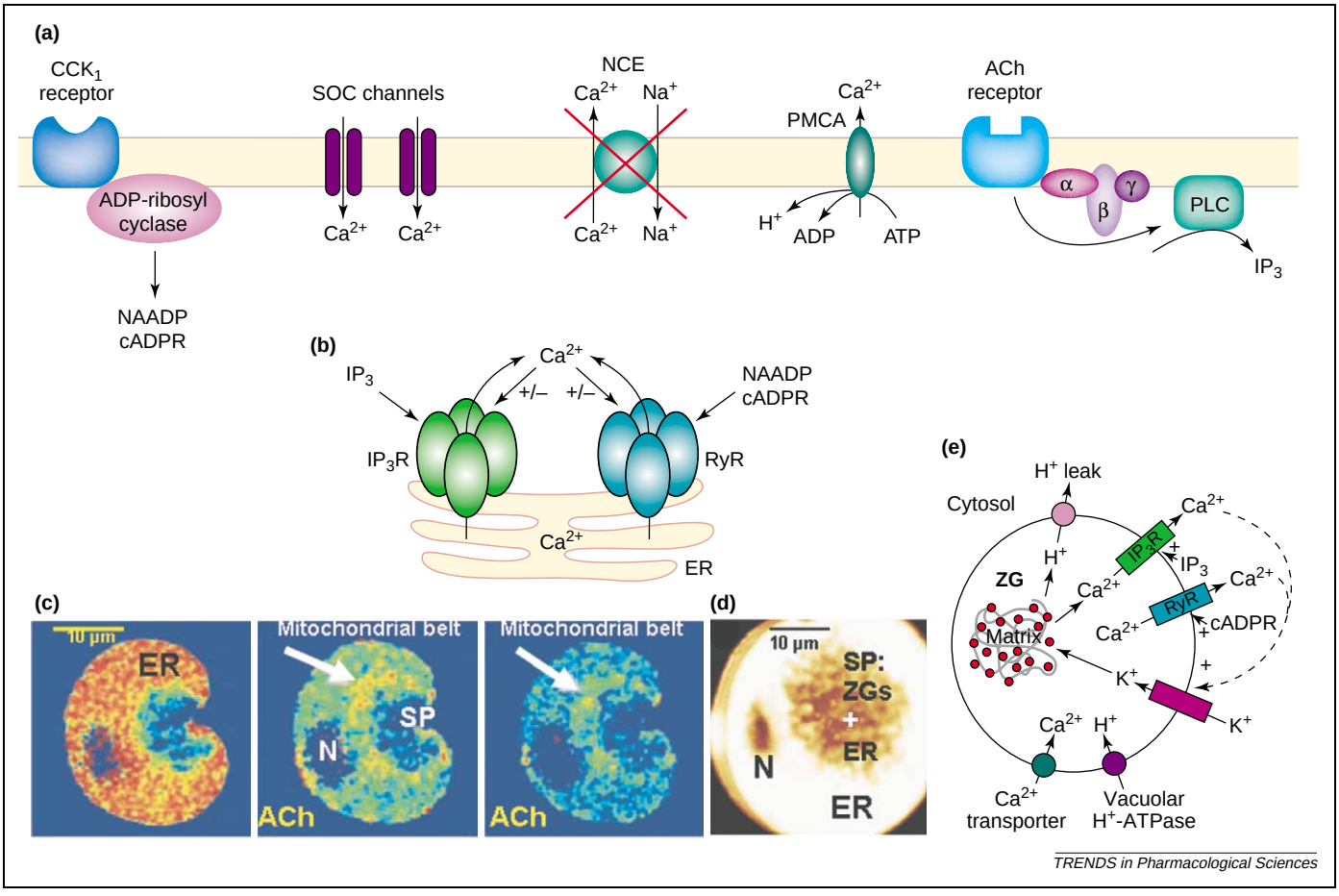

Figure 1. Ca^{2+} signalling events and their control in pancreatic acinar cells. **(a)** Events at the plasma membrane. There are two principal receptor-activated pathways that lead to Ca^{2+} signal generation: one initiated by the binding of cholecystokinin (CCK) to specific CCK_1 receptors and the other mediated by acetylcholine (ACh) binding to muscarinic ACh receptors. CCK receptors are connected, in an unknown manner, to the enzyme ADP-ribosyl cyclase, which leads to the generation of nicotinic acid adenine dinucleotide phosphate (NAADP) from NADP and cyclic ADP-ribose (cADPR) from NAD. High (nanomolar) concentrations of CCK8 lead to the activation of phospholipase C (PLC) and the generation of inositol (1,4,5)-trisphosphate (IP_3). ACh activates PLC, which leads to the generation of IP_3. The plasma membrane also contains store-operated Ca^{2+} (SOC) channels and a Ca^{2+} extrusion pump, the plasma membrane Ca^{2+}-activated ATPase (PMCA). In pancreatic acinar cells, the Na^+–Ca^{2+} exchanger (NCE) is either missing or not functional. **(b)** Events at the endoplasmic reticulum (ER) membrane. There are two regulated Ca^{2+}-release channels, the IP_3 receptor (IP_3R) and the ryanodine receptor (RyR), activated by IP_3 and cADPR or NAADP, respectively. NAADP and cADPR might activate ryanodine receptors via intermediate specific binding proteins [25]. The physiological cytosolic Ca^{2+} spikes in the apical pole are due to Ca^{2+}-mediated positive and negative interactions between the two types of Ca^{2+}-release channels. **(c)** The Ca^{2+} concentration changes in the ER and the mitochondria in response to ACh stimulation, observed by confocal microscopy. The pictures represent differential fluorescence ratio images highlighting exclusively those regions that undergo major ACh-elicited changes in the concentration of Ca^{2+}. In the resting (unstimulated) situation (left image) there is a high Ca^{2+} concentration [warm (red) colour] in the basolateral (left) part of the cell dominated by the ER. Supramaximal ACh stimulation markedly reduces the Ca^{2+} concentration in the ER, as observed in the middle and right images by a gradual change from warm (red) through colder (yellow) to cold (green–blue) colours. A proportion of the Ca^{2+} released from the ER is taken up by mitochondria in the perigranular belt (white arrows pointing to yellow belt). The nucleus (N) and the secretory pole (SP) are marked to help orientation. **(d)** The distribution of the ER in a living acinar cell doublet, visualized by fluorescent thapsigargin binding representing the density of ER Ca^{2+} pumps in different cellular regions. The basolateral parts of the two cells are densely packed with ER, whereas the SP contains mainly zymogen granules (ZGs) and therefore has considerably less staining (ZGs do not have ER-type thapsigargin-sensitive Ca^{2+} pumps). It is functionally important that there are significant ER (light) elements in this part of the cell because these have the highest concentration of IP_3 receptors and therefore are the initial sites of stimulated Ca^{2+} release into the cytosol [see inset in Figure 3(b)]. **(e)** Events in a ZG following strong stimulation with secretagogues or toxic agents. Ca^{2+} release via IP_3 receptors or ryanodine receptors causes a rise in the extragranular Ca^{2+} concentration, which leads to activation of Ca^{2+}-dependent K^+ channels in the ZG membrane. This facilitates K^+ entry into the granule. K^+ displaces Ca^{2+} and H^+ bound in the granular matrix, resulting in acidification of the intragranular solution and matrix disaggregation. This contributes to toxic enzyme activation. (Adapted from [24–27].) The illustration in (e) is based on data and ideas published by Quesada *et al.* [28].

receptors and generates the classic Ca^{2+}-releasing messenger inositol(1,4,5)-trisphosphate [Ins(1,4,5)P_3] [5,6,8]. The other principal secretagogue in acinar cells is the hormone CCK, which exists in multiple molecular forms. Although the actions of CCK8 have been studied extensively, recent evidence indicates that CCK58 is the physiologically relevant form [29], which is important because CCK8 and CCK58 have been shown to have somewhat different actions [30]. However, the mechanisms of action of these two agents have not yet been compared systematically.

Although CCK is a classic pancreatic secretagogue [8,19], evidence for CCK receptors on human pancreatic acinar cells is lacking and there is so far no evidence to demonstrate direct CCK actions on human cells [31]. This has important implications because hyperstimulation with CCK or CCK analogues in animal experiments is used widely as disease models.

The action of a low (picomolar) concentration of CCK8 is, in contrast to the action of ACh, specifically dependent on functional intracellular receptors for the relatively novel Ca^{2+}-releasing agent nicotinic acid adenine dinucleotide phosphate (NAADP) and can be specifically potentiated by Ins(1,4,5)P_3 [8,32,33]. Low (picomolar) concentrations of CCK8 specifically elicit dose-dependent increases in the intracellular concentration of NAADP [34] but it is not yet known whether the actions of CCK58 depend on functional NAADP receptors or whether low (picomolar) concentrations of this molecular form generate NAADP. The toxic effects of high (nanomolar) concentrations of CCK8 [which generate Ins(1,4,5)P_3 and are used to elicit pancreatitis-like changes] might depend on interactions between Ins(1,4,5)P_3 and NAADP in addition to another Ca^{2+}-releasing messenger, cyclic ADP-ribose (cADPR) [33]. Therefore, it will be important to determine whether the more physiologically relevant CCK58 also mediates its effects through NAADP. Low (picomolar) concentrations of CCK8 also generate cADPR (Figure 1) [34]. ACh does not increase NAADP levels but elicits a modest increase in the concentration of cADPR [34].

Ca^{2+} entry

ACh and CCK do not, primarily, open Ca^{2+} channels in the surface cell membrane but instead release Ca^{2+} from the endoplasmic reticulum (ER) [8]. There is, however, delayed activation of Ca^{2+} influx [8] following agonist stimulation in a process known as store-operated Ca^{2+} entry [35]. Store-operated Ca^{2+} entry is required because cellular Ca^{2+} loss occurs as a result of Ca^{2+} extrusion from the cell following release of Ca^{2+} from the ER and elevation of $[Ca^{2+}]_i$ (Figure 1). Extracellular Ca^{2+} entry is therefore compensatory, rather than a primary signalling step. The molecular nature of the pancreatic Ca^{2+}-entry channels is unclear because, to date, there are no single-channel current measurements relevant to store-operated Ca^{2+} entry in the pancreas. Interest is currently focused on channels that belong to the so-called transient receptor potential (TRP) family. Some of these channels might be controlled by changes in the Ca^{2+} concentration in the ER lumen {$[Ca^{2+}]_{ER}$} [36]. Changes in $[Ca^{2+}]_{ER}$ can be sensed and relayed to regulate Ca^{2+} entry but the mechanism is still unclear [35]. Recent evidence indicates a role for proteins belonging to the STIM (stromal interaction molecule) family, which concentrate in certain parts of the ER when $[Ca^{2+}]_{ER}$ is reduced [37,38].

Ca^{2+} entry occurs across the basolateral membrane (which covers ~95% of the acinar cell surface) because ER Ca^{2+} stores can be refilled, after agonist-elicited depletion, from any point source at the base of an isolated acinar cell [6,8]. The wide channel distribution enables sustained Ca^{2+} entry without any concentration of Ca^{2+} locally but can result in a global $[Ca^{2+}]_i$ elevation.

Store-operated Ca^{2+} channels might be crucial in the development of acute pancreatitis because agents such as high toxic concentrations of CCK8, non-oxidative alcohol metabolites and bile acids all elicit prolonged $[Ca^{2+}]_i$ elevations, which are mostly dependent on the presence of external Ca^{2+} (Figure 2). Indeed, protection against trypsinogen activation and vacuolization occurs following removal of external Ca^{2+} [9]. The demonstration that arachidonic acid is generated in response, specifically, to store-operated Ca^{2+} entry in the RBL cell line might be important because arachidonic acid subsequently forms the potent pro-inflammatory leukotriene LTC_4 [39]. Ca^{2+}-entry channel blockers might be an attractive avenue for eventual therapy of acute pancreatitis.

Ca^{2+} extrusion

Net Ca^{2+} influx can be elicited not only by opening Ca^{2+} channels in the plasma membrane but also by inhibiting Ca^{2+} extrusion. There are two major pathways that export Ca^{2+}, namely the plasma membrane Ca^{2+} pump [also known as plasma membrane Ca^{2+}-activated ATPase (PMCA)] and Na^+–Ca^{2+} exchange [40]. In the pancreatic acinar cell, Na^+–Ca^{2+} exchange is of little quantitative importance (Figure 1) [6,8], which explains why Ca^{2+} overloading is particularly dangerous in these cells. In other cell types that possess both extrusion pumps the Na^+–Ca^{2+} exchanger can dispose of Ca^{2+} at a much higher rate than the PMCA but is only activated at higher levels of $[Ca^{2+}]_i$ [40]. In neurons, activation of the Na^+–Ca^{2+} exchanger prevents excitotoxic Ca^{2+} overload and cell death, whereas inactivation of the transporter causes neuronal death [41].

ATP-dependent PMCA-mediated Ca^{2+} extrusion occurs at a low level during resting, unstimulated conditions and any rise in $[Ca^{2+}]_i$ from the resting level of ~100 nM rapidly activates the Ca^{2+} extrusion pump. During toxic stimulation by, for example, bile acids and alcohol metabolites when mitochondria are depolarized [13,22], the lack of ATP prevents Ca^{2+} extrusion and increases the cytosolic Ca^{2+} overload.

In contrast to Ca^{2+} entry, Ca^{2+} extrusion is polarized, with the PMCA concentrated in the small apical membrane area [6,8]. This makes physiological sense because the principal Ca^{2+} release sites are located in the apical ER [6,8] but it is also of pathophysiological importance. The PMCA is a Ca^{2+}–calmodulin (CaM)-stimulated enzyme and CaM translocates from the base to the apical pole in the initial phase of an agonist-elicited $[Ca^{2+}]_i$ rise. This apical concentration of CaM is short-lived and during a sustained $[Ca^{2+}]_i$ elevation it is

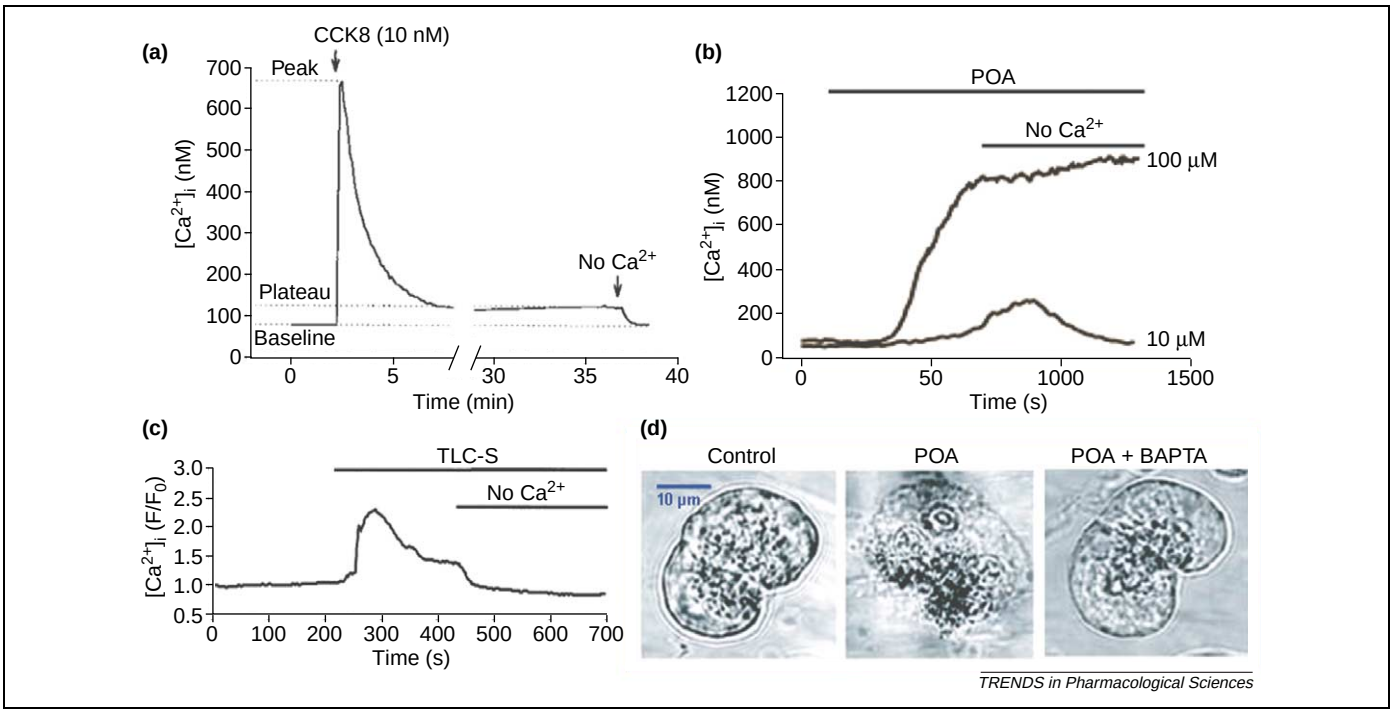

Figure 2. Ca^{2+} signals generated by toxic stimulation with cholecystokinin (CCK), the fatty acid palmitoleic acid (POA) and the bile acid taurolithocholic acid sulfate (TLC-S) cause Ca^{2+}-dependent necrosis. **(a)** Hyperstimulation with 10 nM CCK8 (the physiological level is 1–10 pM) evokes a large initial transient rise in the cytosolic Ca^{2+} concentration {$[Ca^{2+}]_i$} followed by a smaller sustained plateau, which is acutely dependent on the presence of external Ca^{2+}. **(b)** The fatty acid POA evokes a dose-dependent $[Ca^{2+}]_i$ rise, which is reversed by external Ca^{2+} removal at the lower POA concentration of 10 μM but not at 100 μM. **(c)** The bile acid TLC-S (500 μM) evokes a sustained $[Ca^{2+}]_i$ rise, which is acutely dependent on the presence of external Ca^{2+}. **(d)** All these prolonged $[Ca^{2+}]_i$ rises cause acinar cell necrosis and as an example the effect induced by 100 μM POA is shown. The total destruction of the plasma membrane is visible. Pre-incubation with a Ca^{2+} chelator (BAPTA), in a membrane-permeant form, preserves normal morphology during stimulation with 100 μM POA. (Adapted from [9,11,13].)

followed by translocation into the nucleus [6,42], which possibly limits the rate of Ca^{2+} extrusion during prolonged stimulation. Furthermore, because Ca^{2+} entry occurs all over the basolateral surface, whereas the Ca^{2+} extrusion pumps are concentrated in the apical membrane, there is also a spatial mismatch, which might prevent effective disposal of a cellular Ca^{2+} overload.

The endoplasmic reticulum

In the ER membrane there are two types of regulated Ca^{2+}-release channels, namely Ins(1,4,5)P_3 receptors and ryanodine receptors (Figure 1). Both participate in the apical cytosolic Ca^{2+} spiking response to stimulation with low (quasi-physiological) concentrations of ACh or CCK [6,8].

All agents that induce acinar cell necrosis (toxic agonist concentrations, bile acids or non-oxidative alcohol metabolites) release Ca^{2+} from the ER. In externally Ca^{2+}-free conditions, stimulation with, for example, the fatty acid palmitoleic acid (POA) causes a major transient $[Ca^{2+}]_i$ elevation and thereafter even supramaximal agonist stimulation fails to cause Ca^{2+} release because the ER is empty (Figure 3). Ca^{2+} release evoked by the bile acid taurolithocholic acid sulfate (TLC-S) is blocked by inhibiting the Ins(1,4,5)P_3 receptors with caffeine (Figure 3). Hyperstimulation with CCK (in contrast to physiological stimulation) activates phospholipase C (PLC), and thus many of the different agents that elicit pancreatitis-like cellular changes might do so by releasing Ca^{2+} from the ER via activation of Ins(1,4,5)P_3 receptors by Ins(1,4,5)P_3, which is produced by PLC. Caffeine might be a useful Ins(1,4,5)P_3 receptor antagonist *in vivo* because it is extremely membrane permeable [8,43], which also means that its inhibitory effect is rapidly reversible (Figure 3). Indeed, coffee consumption has recently been shown to reduce the risk of alcoholic pancreatitis [44].

Ins(1,4,5)P_3 receptors are regulated by many factors other than Ins(1,4,5)P_3, including Ca^{2+} itself and ATP, and the different receptor subtypes have different affinities for Ins(1,4,5)P_3 and other modulating agents [45]. All three subtypes of Ins(1,4,5)P_3 receptors are present in acinar cells [8] but knockout and double-knockout experiments show that $IP_3$2 and $IP_3$3 receptors are by far the most important for agonist-elicited Ca^{2+} signals and secretion and that these two subtypes can substitute for each other [46]. At present, there are no pharmacological tools available to differentiate between the involvement of different Ins(1,4,5)P_3 receptor subtypes. The other type of ER Ca^{2+}-release channel, the ryanodine receptor, has also been implicated in the generation of pancreatitis or pancreatitis-like cellular changes, specifically those elicited by hyperstimulation with CCK or carbachol [47].

There must be active uptake of Ca^{2+} into the ER lumen to compensate for the resting leak of Ca^{2+} from the ER into the cytosol. This is demonstrated by the observation that thapsigargin, a specific inhibitor of the ER Ca^{2+} pump [sarco(endo)plasmic reticulum Ca^{2+}-activated ATPase (SERCA)], elicits net Ca^{2+} release from the ER (Figure 3). Thus, the normally passive loss of Ca^{2+} is

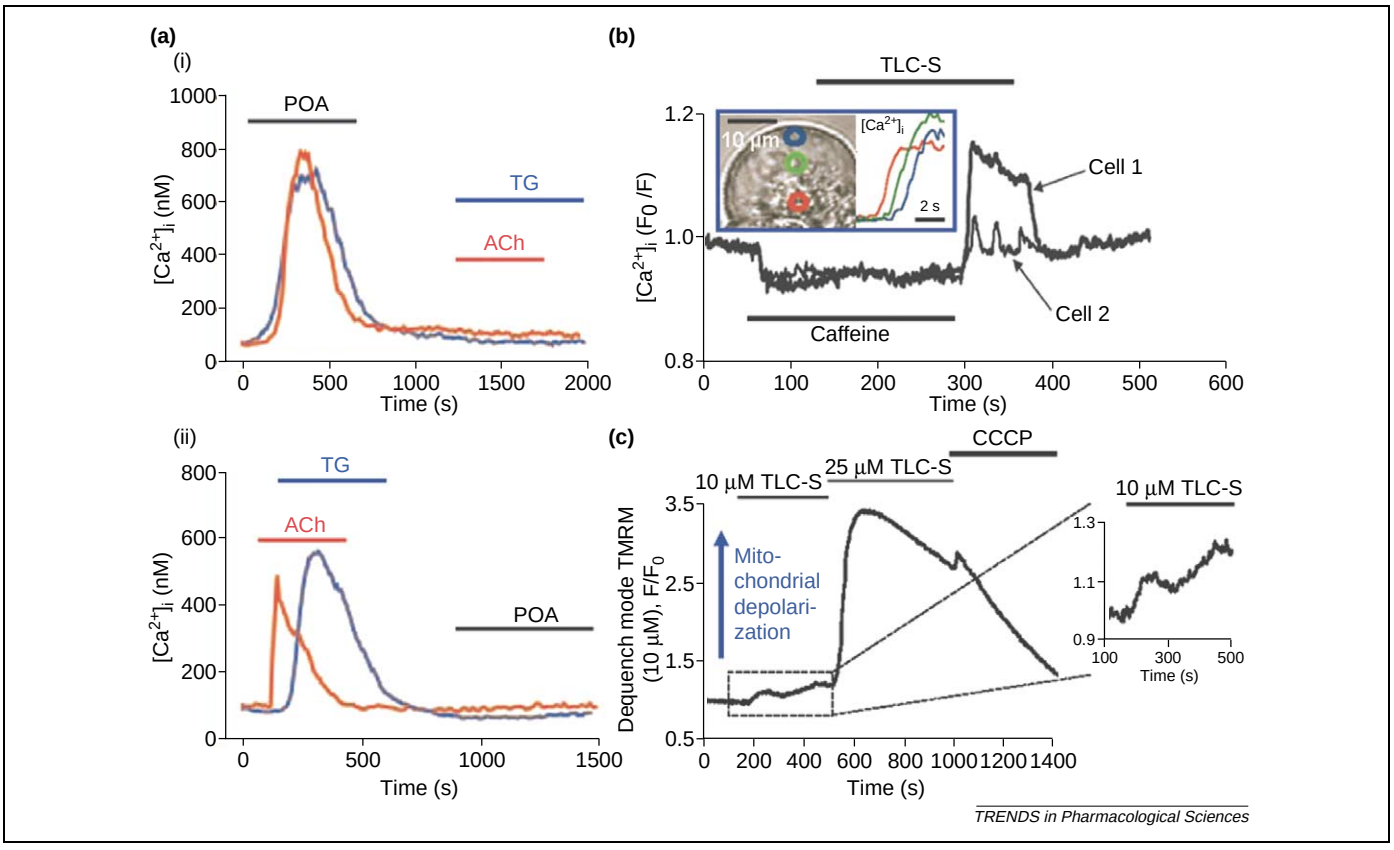

Figure 3. Mechanism of action of fatty acids and bile acids. **(a)** (i) Under externally Ca^{2+}-free conditions, the fatty acid palmitoleic acid (POA) (50 μM) evokes a substantial $[Ca^{2+}]_i$ rise. Thereafter, neither the endoplasmic reticulum Ca^{2+} pump inhibitor thapsigargin (TG) nor acetylcholine (ACh) can evoke any further Ca^{2+} signal. (ii) In the reverse experiment, ACh or thapsigargin evoke clear Ca^{2+} signals first but subsequent stimulation with POA (50 μM) fails to elicit any effect. **(b)** In the presence of caffeine (20 mM), used as an Ins(1,4,5)P_3 receptor antagonist, the bile acid taurolithocholic acid sulfate (TLC-S) (200 μM) fails to elicit any cytosolic Ca^{2+} signal but immediately after wash-out of caffeine from the bath solution the $[Ca^{2+}]_i$ rises, albeit with different patterns in two neighbouring cells. The inset shows the initial rise in $[Ca^{2+}]_i$ in response to 200 μM TLC-S (in the absence of caffeine in a separate experiment) in three different subcellular regions: red, apical pole; green, perigranular area; and blue, basal sub-plasmalemmal region. The TLC-S-elicited cytosolic Ca^{2+} signal starts in the apical pole near the apical membrane where the Ins(1,4,5)P_3 receptors are concentrated and then spreads (within ~2 s) to the base. **(c)** TLC-S depolarizes the inner mitochondrial membrane as observed by dequench mode TMRM (tetramethyl rhodamine methyl ester) fluorescence assessment [22] of changes in the mitochondrial membrane potential. A low concentration of TLC-S causes minor depolarization whereas a slightly higher, but still low, concentration causes virtually complete depolarization, as observed by the failure of a subsequent application of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to elicit any further change. (Adapted from [11,13,22].)

compensated for by active reuptake of Ca^{2+}. In general, non-compensated loss of Ca^{2+} from the ER causes disruption of protein handling with potentially disastrous consequences [24]. Indeed, thapsigargin causes trypsinogen activation and vacuolization in pancreatic acinar cells [9].

Although bile acid-induced Ca^{2+} release from the ER is sensitive to inhibition of Ins(1,4,5)P_3 receptors [11], bile acids can also release stored Ca^{2+} by inhibiting SERCA pumps [12]. A third potential site of action for bile acids and other agents, such as non-oxidative alcohol metabolites, is Ca^{2+} leak channels. Little is known about the resting Ca^{2+} leakage from the ER but translocons might be important [48]. The electrical potential difference across the ER membrane is an important force that influences Ca^{2+} leakage and activated release. There are no data regarding the ER membrane potential but theoretical considerations indicate that it must be below 100 mV (lumen negative) [24]. The ionic conductances responsible for this potential and their possible regulation are unknown. Ca^{2+} transport across the ER membrane might be influenced by changes in conductances to ions other than Ca^{2+}.

The rate of SERCA-mediated reuptake is determined by $[Ca^{2+}]_{ER}$ so that a decrease activates the pumps whereas an increase causes inhibition. Under normal physiological conditions, stimulant-elicited Ca^{2+} release is followed by Ca^{2+} reuptake, and the rate of uptake decreases as $[Ca^{2+}]_{ER}$ increases until the uptake rate equals the resting leak rate. The ER luminal Ca^{2+} sensor is most probably the ERp57–calreticulin complex, which binds in a Ca^{2+}-dependent manner to SERCA and inhibits its activity [24]. The SERCA pump is also activated by a rise in $[Ca^{2+}]_i$ [6,8,24]. Pathological changes in these regulatory mechanisms might have major implications for Ca^{2+} signalling.

The mitochondria

Ca^{2+} uptake into the mitochondrial matrix occurs via the Ca^{2+} uniporter, a Ca^{2+}-selective ion channel [49]. The driving force is the electrical potential difference across the inner mitochondrial membrane (~150 mV; matrix negative), which enables the concentration of Ca^{2+} in the matrix [50]. The Na^+–Ca^{2+} exchanger provides an essential Ca^{2+} exit pathway [51]. The mitochondria are organized principally in a perigranular belt that separates

the ZGs from the basolateral part of the acinar cell [6,8] and is in close proximity to the Golgi [52]. There are also concentrations of mitochondria just beneath the plasma membrane and around the nuclei [6,8].

Mitochondria sense $[Ca^{2+}]_i$ in their immediate environment and take up Ca^{2+}, via the uniporter, when $[Ca^{2+}]_i$ increases [50]. Thus, ACh-elicited release of Ca^{2+} from the ER causes uptake of Ca^{2+} into the perigranular mitochondria because of the concentration of Ins(1,4,5)P_3 receptors in the ER elements invading the apical pole. The transient shift of Ca^{2+} from the ER into the perigranular mitochondria immediately after the start of supra-maximal ACh stimulation can be visualized directly (Figure 1). The perigranular mitochondria function as a Ca^{2+} buffer barrier, limiting the penetration of Ca^{2+} signals into the basolateral part of the cell, which contains the nucleus. Mitochondrial Ca^{2+} uptake activates several Krebs' cycle enzymes, which results in ATP production fuelling secretion, SERCA-mediated Ca^{2+} reuptake into the ER and Ca^{2+} extrusion [8].

Depolarization of the inner mitochondrial membrane, as observed following stimulation with bile acids (Figure 3) or fatty acids [13], prevents mitochondrial Ca^{2+} uptake by removing the necessary electrical gradient. This causes spreading of the local Ca^{2+} signal to the whole of the cell [8] and reduces ATP generation. Lack of ATP reduces the ability of the cell to take Ca^{2+} back into the ER and also prevents Ca^{2+} extrusion. This is the most likely explanation for the inability of the $[Ca^{2+}]_i$ rise, induced by a high concentration of the fatty acid POA, to be reversed when external Ca^{2+} is subsequently removed (Figure 2).

The ZGs

The total granular Ca^{2+} content in acinar cells is extremely high ($\sim$15 mM). The major proportion of this ZG content is not in free solution but is tightly bound in the granular matrix. The free $[Ca^{2+}]$ inside the ZGs is $\sim$50 μM, which is much higher than $[Ca^{2+}]_i$. Both Ins(1,4,5)P_3 and cADPR elicit the release of Ca^{2+} from isolated ZGs [53]. These findings have been controversial but are now supported by a substantial body of evidence from different types of secretory granules [28,54]. Work from Verdugo's group [28,54] indicates that Ca^{2+} and H^+ are tightly bound in the granular matrix. Ins(1,4,5)P_3-induced Ca^{2+} release causes a local extragranular $[Ca^{2+}]_i$ rise, which would open Ca^{2+}-activated K^+ channels in the granular membrane, enabling K^+ to enter the granule interior. The matrix behaves like an ion exchanger replacing Ca^{2+} and H^+ with K^+ (Figure 1). The loss of Ca^{2+} from the matrix would cause disaggregation and this, in addition to the decrease in the intragranular pH, in the case of the ZGs, would help convert trypsinogen to trypsin [55]. These steps have not all been identified for ZGs but X-ray microanalysis of pancreatic sections shows that stimulation with a high (nanomolar) CCK concentration causes the ZGs to lose Ca^{2+} and gain K^+ [56], in agreement with the analysis of mast cell secretory granules [28].

Protein kinase C and other signalling mechanisms

The serine/threonine kinases include the large protein kinase C (PKC) family, which contributes to a range of cellular responses, including those occurring during inflammation [57]. Many family members are Ca^{2+} dependent, and Ca^{2+}-activated PKC translocation and substrate phosphorylation has been visualized [58]. PKC isoforms activate NF-κB cytokine expression and phospholipase A_2 (PLA_2) and regulate the synthesis of nitric oxide and reactive oxygen species. Examination of NF-κB activation in pancreatic acinar cells shows that ethanol differentially affects Ca^{2+}–calcineurin- and PKC-mediated activation [59]. A significant and specific role for the novel isoforms PKC-δ and PKC-ε (which lack the C2 domain but can nevertheless be activated via Ca^{2+}-dependent diacyl glycerol formation [58]) in mediating NF-κB activation emerged from recent experiments on hyperstimulated dispersed rat pancreatic acini [60,61]. Although these data indicate specific pathways through which acinar cells respond to injury, the overall importance of many PKC isoforms in pancreatitis remains to be determined.

The role of phosphoinositides (PIs) in cell signalling is complex [62]. PIs are membrane lipids that bind to and regulate many proteins following receptor-mediated activation of a range of PtdIns kinases, phosphatases and lipases. PIs modulate Ins(1,4,5)P_3 receptor- and ryanodine receptor-mediated Ca^{2+} release [63] and SERCA pump activity [64], and both pharmacological and genetic inhibition of PtdIns kinase activity reduces trypsinogen activation and the severity of secretagogue-, diet- and taurocholate duct injection-induced pancreatitis [65–67]. Both class I and III PtdIns 3-kinases have been implicated but the complexity of their effects, including their effects on cell death pathways [62,67] and on NADPH oxidase [68] that is important in neutrophil activation in pancreatitis [21], makes identification of the specific mechanism difficult, although this area is under pharmacological development [69].

Apoptosis or necrosis

The regulatory mechanisms of the two principal death pathways leading to apoptosis or necrosis are of great importance. In pancreatitis, a significant degree of necrosis is associated with increased mortality [1,70]. One factor that determines whether cell death occurs by apoptosis or necrosis is mitochondrial function [71]. Thus, hyperstimulation of glutamate receptors causes collapse of the mitochondrial potential in a subpopulation of neurons and these die by necrosis. In other neurons the mitochondrial potential and the energy level recovers and they later undergo apoptosis [71]. In acinar cells, the oxidant menadione induces only moderate and transient mitochondrial depolarization and apoptosis occurs [72]. By contrast, bile acids and non-oxidative alcohol metabolites, in concentrations that elicit necrosis, cause complete collapse of the mitochondrial membrane potential, as observed by the inability of a subsequent protonophore application to elicit further depolarization (Figure 3). Thus, the mitochondrial function is a crucial factor in determining the type of acinar cell injury. Apoptosis requires ATP and in its absence the only cell death pathway available is necrosis [73]. The mitochondrial depolarization evoked by toxic stimulation is, at least

Box 1. Potential therapeutic targets

- Plasma membrane Ca^{2+}-entry channel
- Ca^{2+}-release receptors
- Vacuolar ATPase
- Granular ion exchange channels
- Esterases
- Phosphoinositide 3-kinase
- Protein kinase C
- Endothelin and nitric oxide production

in part, Ca^{2+} dependent [22] and might be due to Ca^{2+} overloading of mitochondria and subsequent opening of the so-called permeability transition pore [51].

Targets for pharmacological therapy

To date, there is no specific pharmacological therapy for pancreatitis. Enzyme inhibitors have a modest preventative role [74] but are of no therapeutic value. New approaches are required based on recent advances in our understanding of Ca^{2+} transport in pancreatic acinar cells (Box 1). Because cytosolic Ca^{2+} overload is a crucial feature, inhibition of Ca^{2+} entry, enhancement of Ca^{2+} extrusion or inhibition of the primary Ca^{2+} release from the ER are all attractive targets. Caffeine, an inhibitor of Ins(1,4,5)P_3 receptor opening [43] that also inhibits PLC and therefore Ins(1,4,5)P_3 generation [75], has already proven to be of some value [44] but has limited potential because of its rather low affinity for the Ins(1,4,5)P_3 receptor [43]. However, more-useful analogues could be developed, and combined inhibition of ER Ca^{2+} release and store-operated Ca^{2+} entry might be particularly effective. The membrane-permeant agent 2-amino-ethoxydiphenylborate (2-ABP), originally thought to be a specific inhibitor of Ins(1,4,5)P_3 receptors, also inhibits store-operated Ca^{2+} channels but has several other effects [35]. Modified compounds might have therapeutic potential. The recent advances in our knowledge of pancreatic acinar Ca^{2+} transport mechanisms and their regulation and deregulation now provide opportunities for the development of pharmacological tools for clinical therapy.

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