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REVIEW ARTICLE

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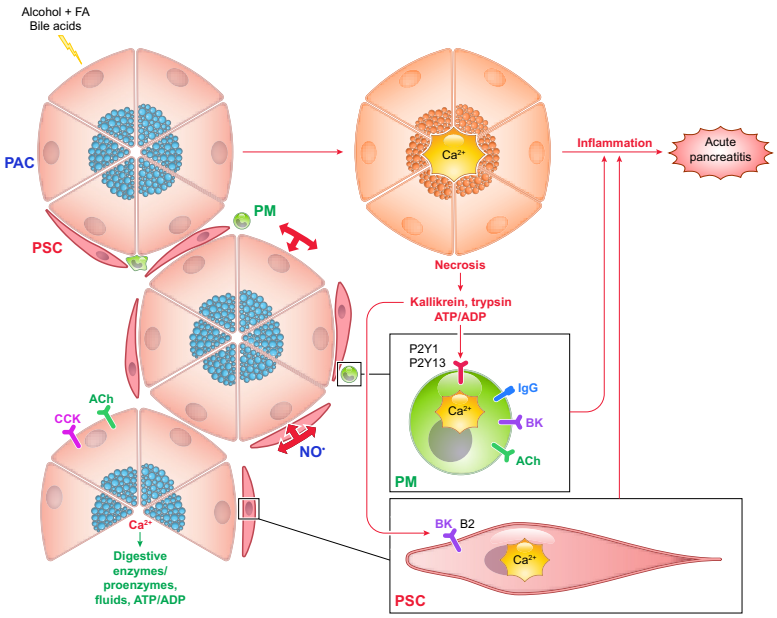

AUTHORS

Ole H. Petersen, Julia V. Gerasimenko, Oleg V. Gerasimenko, Oleksiy Gryshchenko, Shuang Peng

CORRESPONDENCE

PetersenOH@cardiff.ac.uk;
pengshuang@jnu.edu.cn

KEY WORDS

calcium signaling; mitochondrial ATP generation; pancreatic acinar cells; pancreatic duct cells; pancreatic immune cells; pancreatic stellate cells

CLINICAL HIGHLIGHTS

1) The human disease acute pancreatitis is initiated by excessive Ca^{2+} signal generation in the pancreatic acinar cells, mostly due to the action of a combination of alcohol and fatty acids or to a combination of excess physical pressure and high bile acid concentrations in the pancreatic duct. Acute pancreatitis can also occur as a side effect of treating acute lymphoblastic leukemia in children with asparaginase.
2) The excessive Ca^{2+} signal generation in the acinar cells leads to overloading of the mitochondria with Ca^{2+}, which in turn inhibits mitochondrial ATP generation.
3) The excessive Ca^{2+} signal generation also promotes active trypsin formation inside the acinar cells, which together with the reduced ATP level causes necrosis.
4) The necrotic release of ATP, ADP, trypsin, kallikrein, and possibly other substances activates neighboring stellate cells and macrophages via bradykinin receptors on the stellate cells and purinergic receptors on the macrophages.
5) Bradykinin activation of stellate cells, possibly via Ca^{2+}-dependent nitric oxide formation, causes further damage of the acinar cells, creating a necrotic amplification loop. Purinergic activation of the macrophages causes Ca^{2+} signals in these cells that promote release of inflammatory agents.
6) The primary acinar cell Ca^{2+} overloading can be prevented or stopped by inhibition of Ca^{2+} release-activated Ca^{2+} channels. This also prevents excessive Ca^{2+} signal generation in the stellate cells as well as in the macrophages and could therefore be an effective treatment of acute pancreatitis.

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THE ROLES OF CALCIUM AND ATP IN THE PHYSIOLOGY AND PATHOLOGY OF THE EXOCRINE PANCREAS

Ole H. Petersen,[1] Julia V. Gerasimenko,[1] Oleg V. Gerasimenko,[1] Oleksiy Gryshchenko[2], and Shuang Peng[3]

[1]School of Biosciences, Cardiff University, Cardiff, United Kingdom; [2]Bogomoletz Institute of Physiology, Kyiv, Ukraine; and [3]Department of Pathophysiology, Key Laboratory of State Administration of Traditional Chinese Medicine of the People's Republic of China, School of Medicine, Jinan University, Guangzhou, Guangdong, People's Republic of China

Abstract

This review deals with the roles of calcium ions and ATP in the control of the normal functions of the different cell types in the exocrine pancreas as well as the roles of these molecules in the pathophysiology of acute pancreatitis. Repetitive rises in the local cytosolic calcium ion concentration in the apical part of the acinar cells not only activate exocytosis but also, via an increase in the intramitochondrial calcium ion concentration, stimulate the ATP formation that is needed to fuel the energy-requiring secretion process. However, intracellular calcium overload, resulting in a global sustained elevation of the cytosolic calcium ion concentration, has the opposite effect of decreasing mitochondrial ATP production, and this initiates processes that lead to necrosis. In the last few years it has become possible to image calcium signaling events simultaneously in acinar, stellate, and immune cells in intact lobules of the exocrine pancreas. This has disclosed processes by which these cells interact with each other, particularly in relation to the initiation and development of acute pancreatitis. By unraveling the molecular mechanisms underlying this disease, several promising therapeutic intervention sites have been identified. This provides hope that we may soon be able to effectively treat this often fatal disease.

calcium signaling; mitochondrial ATP generation; pancreatic acinar cells; pancreatic duct cells; pancreatic immune cells; pancreatic stellate cells

CLINICAL HIGHLIGHTS

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6) The primary acinar cell Ca^{2+} overloading can be prevented or stopped by inhibition of Ca^{2+} release-activated Ca^{2+} channels. This also prevents excessive Ca^{2+} signal generation in the stellate cells as well as in the macrophages and could therefore be an effective treatment of acute pancreatitis.

1. INTRODUCTION

The exocrine pancreas is an important organ because it is essential for the digestion of the food we consume. Investigations of the function of this organ have also resulted in major discoveries of general importance. Bayliss and Starling (1, 2) discovered that pancreatic fluid secretion could be stimulated by a substance they called secretin, and Starling later introduced the name

"hormone" for this group of chemical messengers of which secretin was the first example (2). The mechanisms by which digestive enzymes are processed and secreted by the acinar cells in the pancreas were investigated by George Palade and James Jamieson, who described the process of exocytosis, which is now recognized as the general mechanism by which substances that do not move easily across membranes are exported from the cells that produce them (3–5).

We have known for more than 50 years that Ca^{2+} plays an important role in the control of fluid and enzyme secretion from exocrine glands, including the pancreas (6–8). In the 1970s it became clear that stimulation of exocrine secretion by neurotransmitters and hormones was mediated by primary release of Ca^{2+} from internal stores, most likely the endoplasmic reticulum (ER), followed by entry of Ca^{2+} from the extracellular (interstitial) fluid (9–12). However, it was only in the 1980s and 1990s that the intracellular Ca^{2+} signaling events, elicited by acetylcholine (ACh) or cholecystokinin (CCK) in the pancreatic acinar cells, were properly characterized and understood. Inositol 1,4,5-trisphosphate (IP_3) was discovered as the intracellular messenger linking ACh activation of its receptor to the release of Ca^{2+} from the ER (13, 14), and the temporal and spatial patterns of the Ca^{2+} signals, as well as their actions on ion channels and exocytosis, were worked out (15–29). This work has been reviewed (30, 31) and is therefore only discussed here to the extent necessary for providing an adequate context for the discussion of more recent findings as well as some continuing controversies.

Whereas work on the role of Ca^{2+} in the physiological regulation of pancreatic acinar secretion has a long history, the recognition that excessive Ca^{2+} signal generation may play an important role in the pathophysiology of acute pancreatitis (AP)—a human disease in which the pancreas digests itself, resulting in a major inflammatory response that can be fatal (32–34)—only began to emerge in the 1990s (35, 36). The role of excessive Ca^{2+} signals in AP then became the subject of intensive investigations from the beginning of the twenty-first century (37–48).

In elegant studies on permeabilized chromaffin cells from the adrenal gland, Baker and Knight (49, 50) established that the two basic requirements for secretion by exocytosis are Ca^{2+} and ATP. It is now clear that ATP and Ca^{2+} link cellular energetics and signaling (51–53), and we understand how local Ca^{2+} spikes in the granular region of the acinar cells activate mitochondrial ATP production and thereby provide the energy powering exocytosis of digestive (pro)enzymes (54–56). It has also become evident that inhibition of mitochondrial ATP generation is a key feature of AP (37, 39, 55, 57). The interplay between Ca^{2+} and ATP has therefore become a crucial topic for the understanding of pancreatic physiology and pathophysiology.

Since the pancreatic acinar cells secrete the enzyme-rich fluid that is essential for the physiological digestion of food in the intestine (58), it is not surprising that Ca^{2+} signaling studies have mostly been confined to these cells. Because the secretion of the bicarbonate-rich fluid from the pancreatic duct cells is principally controlled by the hormone secretin, which activates adenyl cyclase, this secretion process is mainly a cyclic AMP-regulated event (59, 60), and consequently Ca^{2+} signaling studies in the duct cells were for a long time neglected. It is, however, now clear that Ca^{2+} signals evoked by ACh and ATP can occur in duct cells and could be physiologically important for the regulation of ductal bicarbonate secretion (61). Furthermore, there is now also evidence indicating that abnormal Ca^{2+} signaling in the duct cells can play a role in AP (62–65).

Ca^{2+} signaling in the periacinar stellate cells in the pancreas has only been studied in the last few years. These cells are generally regarded as important for the fibrosis that occurs in the pancreas when repeated attacks of AP cause chronic pancreatitis, whereas a role in normal pancreatic physiology is unclear (66). These periacinar cells have generally been regarded as inactive in the normal pancreas, but recent work has shown that these cells generate substantial Ca^{2+} signals in response to stimulation with bradykinin (BK) at concentrations only slightly above those found in the plasma of resting rodents or humans under resting conditions (67). Since BK concentrations in plasma and in tissues increase under various conditions, BK-elicited Ca^{2+} signals will occur in the stellate cells. Ca^{2+} signals are also generated in response to pathophysiological stimulation with bile acids and trypsin (68, 69). There is no evidence for direct (gap junctional) communication between acinar and stellate cells (67), but there are pathophysiologically important interactions between these two cell types, which are explored in this article.

Although it has been recognized for a long time that invasion of immune cells into the pancreas and subsequent production and release of inflammatory cytokines play a crucial role in AP (32–34), it is only very recently that Ca^{2+} signal generation has been examined directly in immune cells inside the exocrine pancreatic tissue (70). These signals are principally generated by the action of ATP on macrophages, and their importance for the development of AP is considered in this review. Thus ATP plays a dual role in both physiology and pathology of the pancreas, as ATP is not only critical inside the cells but also, like in many other systems (71, 72), functions as an important messenger between cells.

There are many different agents that are able to evoke Ca^{2+} signals in the various pancreatic cell types, but these effects are not necessarily occurring in real physiology or pathophysiology. In this article we therefore pay as much attention as possible to examining to what extent there is evidence for the processes identified to actually occur in vivo under realistic conditions. As an increasing number of investigations employ isolated cells, and frequently cultured cells and cell lines, there is a need to relate such findings to what is known, for example, about the concentrations of the various stimulating agents in vivo. For many reasons, there are now decreasing numbers of published investigations based on in vivo studies or even on studies using more intact preparations. In some cases, this means that it is necessary to go back in time to published work on the perfused intact pancreas or studies in vivo, in order to try to clarify the crucial issue of physiological and/or pathophysiological relevance.

Recent work has been done in lobule preparations of the pancreas, in which the normal physical relationships between the various cell types have been preserved, thus enabling Ca^{2+} signaling studies to be carried out under quasi-physiological conditions and also allowing investigations of the functional innervation (67, 69, 70). This recent work provides a new perspective, which is particularly relevant for pancreatic physiology and pathophysiology.

2. NEW ANATOMICAL PERSPECTIVES

The fine structure of the pancreas has been described in numerous textbook chapters including recently in the 2018 edition of the textbook *The Pancreas* (73). These descriptions focus on the "classical" exocrine cells, namely the acinar and ductal cells, and pay relatively little attention to other cell types. They are also based on the morphology of fixed tissues and electron microscopy. However, from a physiological point of view, living morphology is of paramount importance, and given the recent evidence for an important role of stellate and immune cells in pancreatic pathophysiology (66, 70), these cells should not be neglected. Furthermore, the functionally very important and very specific subcellular localizations of the mitochondria, seen in living acinar cells (27, 54) but also in an electron microscopy (EM) study (74), have generally been ignored in anatomical texts. This section therefore deals with these generally neglected aspects of pancreatic morphology, with a focus on those elements that are of physiological or pathophysiological importance.

2.1. Mitochondrial Locations in Acinar Cells

It has been recognized for a long time that the pancreatic acinar cells are highly polarized, with the bulk of the endoplasmic reticulum (ER) surrounding the nucleus at the base of the cell and the zymogen granules containing the digestive (pro)enzymes in the apical part of the cell (73). Nevertheless, there are ER extensions into the granular area, and they come close to the apical membrane (75). Morphologists seem not to have noticed any particular distribution of the mitochondria. However, when mitochondria were visualized by mitochondria-selective fluorescent probes in living acinar cells, it was immediately clear that by far the greatest concentration of these organelles was in a perigranular belt, separating the apical area dominated by the zymogen granules from the basolateral region dominated by the ER (27). In a further study on living cells, it became clear that there were also concentrations of mitochondria just beneath the plasma membrane and around the nucleus (54). An EM study carried out a few years later (74) confirmed these conclusions. The mitochondrial localizations in the acinar cells seem quite stable, probably because there are tethers between the mitochondria and other parts of the cell (76). The close contacts between mitochondria and the ER, which are now regarded as a generally important feature of mammalian cells (77), are undoubtedly also present in the pancreatic acinar cells, because functional evidence shows that Ca^{2+} release from the ER is followed immediately by a rise in the intramitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) (56). The very specific, and stable, positioning of the mitochondria in the perigranular, subplasmalemmal, and perinuclear areas of the acinar cell (54) indicates specific physiological roles, and these arrangements are indeed vitally important for the proper handling of Ca^{2+} movements in and across the acinar cells (31, 54).

2.2. Location of Stellate Cells

Whereas stellate cells ("Sternzellen") in the liver were described in 1876 by Kupffer (78), the existence of such cells in the pancreas was only noticed more than 100 years later (79). The pancreatic stellate cells contain lipid droplets with vitamin A, which can be visualized by multiphoton intrinsic fluorescence (67). The stellate cells express the protein marker desmin, in contrast to fibroblasts, and this can be used for immunocytochemical identification of these cells in fixed tissue (**FIGURE 1**). The stellate cells are particularly easy to identify in the living tissue, as these periacinar cells take up a number of fluorescent probes much more avidly than the acinar cells (**FIGURE 1**). In the normal pancreas, stellate cells have mostly been thought to be inactive, just providing

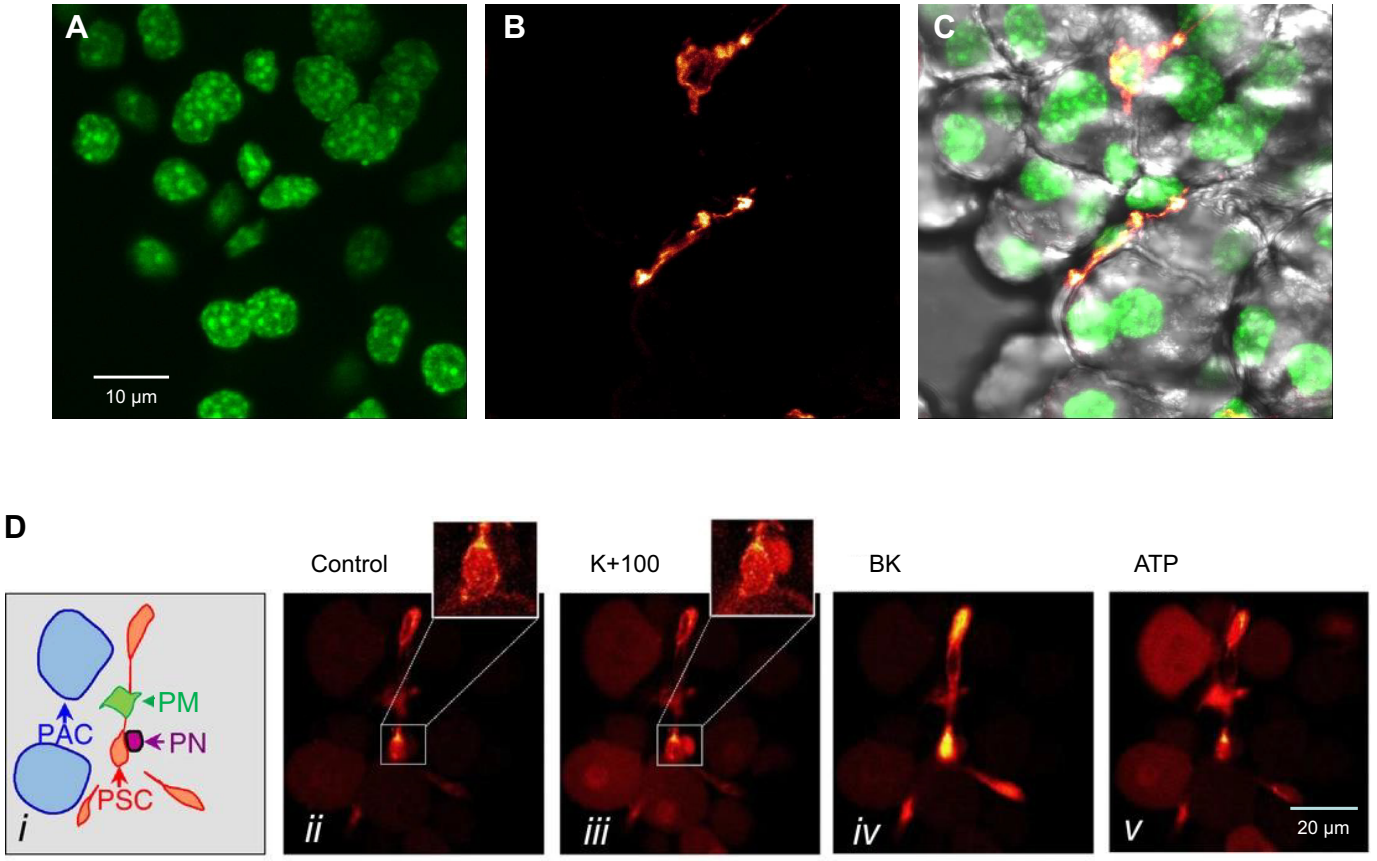

FIGURE 1. The structure of the acinar environment. *A–C* show the same field. *A* shows staining with antibody to 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), visualizing the (acinar) nuclei. *B* shows staining with desmin antibody, demonstrating the stellate cells. *C* is the overlay of the transmitted light image with the antibodies against the nuclei and the stellate cells. *Di* shows an outline of the positions of pancreatic acinar cells (PACs), pancreatic stellate cells (PSCs), a pancreatic neuron (PN), and a pancreatic macrophage (PM) in a lobule. *Dii–Dv* show the same field (live microscopy, Ca^{2+}-sensitive fluorescence, fluo-4). *Dii*: no stimulation. *Diii*: stimulation with high-K^+ solution (100 mM), creating a Ca^{2+} signal in a PN (partly hidden by a PSC) as well as more faint Ca^{2+} signals in PACs. *Div*: stimulation with bradykinin (BK) elicits Ca^{2+} signals exclusively in the PSCs. *Dv*: stimulation with ATP causes a Ca^{2+} signal in a PM and faint Ca^{2+} signals in the PACs. Adapted from Gryshchenko et al. (67, 69) with permission from *Journal of Physiology*.

structural support (66), but recent functional studies (67, 69) indicate that this is misleading, as these cells display remarkable Ca^{2+} signaling properties. Although the stellate cells are a minor component (~5%) of the exocrine tissue (66), the position of these cells in a niche (67) between the acini and the periacinar capillaries may suggest potential functional roles, which are discussed in sect. 5.

2.3. Immune Cells

ATP-sensitive cells have been found in intact pancreatic lobules close to stellate cells (**FIGURE 1**). Immunostaining studies with different antibodies against known surface proteins of macrophages indicate that these immune cells are most likely macrophages (see sect. 7). In the normal pancreas the density of these cells is very low, but in the first days of AP there is a very marked increase in the number of pancreatic macrophages (PMs) (70). The Ca^{2+} signaling characteristics of these cells are discussed in sect. 7.

3. PHYSIOLOGICAL Ca^{2+} SIGNALING IN ACINAR CELLS

The basic characteristics of the physiological Ca^{2+} signals and the mechanisms responsible for their generation were essentially clarified in the 1990s (31), but important new elements have come to light more recently, and there are also some continuing controversies.

The ACh-evoked repetitive Ca^{2+} spikes in the apical granular region (18, 26) are mediated by IP_3 and IP_3 receptors (IP_3Rs) (28) of types 2 and 3 (80), localized in the apical granular region (26, 81, 82). During each of these spikes there is a burst of exocytotic secretion, which can be captured by a transient increase in membrane capacitance (20, 22). Each apical Ca^{2+} spike also causes opening of Ca^{2+}-dependent Cl^- channels (26, 83) specifically located in the apical plasma membrane (84).

The repetitive Ca^{2+} spiking elicited by ACh is not due to fluctuations in the intracellular IP_3 concentration, since

a constant level of a nonmetabolizable IP_3 analog can by itself evoke repetitive Ca^{2+} spiking in the apical region (26, 28, 85, 86). Given that intracellular Ca^{2+} infusion can induce local apical Ca^{2+} spiking (28, 87) and that intracellular infusion of mobile Ca^{2+} buffers dramatically alters the frequency and shape of the cytosolic Ca^{2+} spikes evoked by ACh (88), it is most likely that the repetitive spiking is due to a sequence of Ca^{2+}-induced Ca^{2+} release and Ca^{2+}-induced inhibition of Ca^{2+} release (28, 89, 90). The most likely model accounting for Ca^{2+} oscillations in the pancreatic acinar cells is based on the concept presented by Cancela et al. (85), in which the principal oscillator is the combined set of interacting IP_3Rs and ryanodine receptors (RyRs) in the ER (**FIGURE 2**). The IP_3R is initially opened by a rise in the IP_3 concentration elicited by the ACh-induced phospholipase C (PLC) activation. The release of Ca^{2+} through the IP_3Rs will in turn increase the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in the vicinity of these channels, and, as both IP_3 and an increase in $[Ca^{2+}]_i$ can increase the open state probability, this creates a self-fortifying process (90). The RyRs are also Ca^{2+}-activated channels and will therefore also open, furthering the amplification process. This process is nevertheless self-limiting because Ca^{2+} has dual actions on both IP_3Rs and RyRs (89–91) and, at a certain increased level, the activating effect of Ca^{2+} on these channels changes to become inhibitory, closing the channels. As soon as the release channels close, the Ca^{2+} pumps in the ER will be able to recapture some of the Ca^{2+} lost, thereby decreasing the local $[Ca^{2+}]_i$ (92). $[Ca^{2+}]_i$ will then continue to be reduced until it reaches the level that stimulates the opening of the release channels. A new cycle can then begin.

It was initially difficult to explain how Ca^{2+} signals mediated by release of Ca^{2+} from the ER occur in the apical area, when the ER is mainly located in the basolateral region (73), However, there are significant ER elements in the apical granular region (75), and it is generally accepted that the ER is one luminally continuous organelle (93, 94). The so-called ER Ca^{2+} tunnel experiments clarified the mechanism by which signals were transmitted from the base to the apex. It was shown directly, in studies on living pancreatic acinar cells, that the ER functions as a continuous Ca^{2+} tunnel allowing diffusion of Ca^{2+} from the base of the cell, where the main Ca^{2+} store is located, to the thin ER elements extending into the apical area dominated by zymogen granules (24, 95). Ca^{2+} diffuses more easily through the lumen of the ER than through the cytosol, because the Ca^{2+}-binding capacity in the ER is much lower than in the cytosol (96). Thus, although the main source of Ca^{2+} to be released is in the basal part, where the bulk of the ER is located, the release occurs through IP_3Rs located in the apical area, where the $[Ca^{2+}]_i$ rise is required to activate exocytosis and open the Cl^- channels in the apical membrane required for fluid secretion (30, 97). There are good functional and structural data demonstrating that the acinar IP_3Rs are concentrated in the apical area close to the apical membrane (18, 26, 81, 82).

The Ca^{2+} signals generated in the apical area can be confined to this region because of the perigranular mitochondria (27). These take up Ca^{2+} rapidly during the

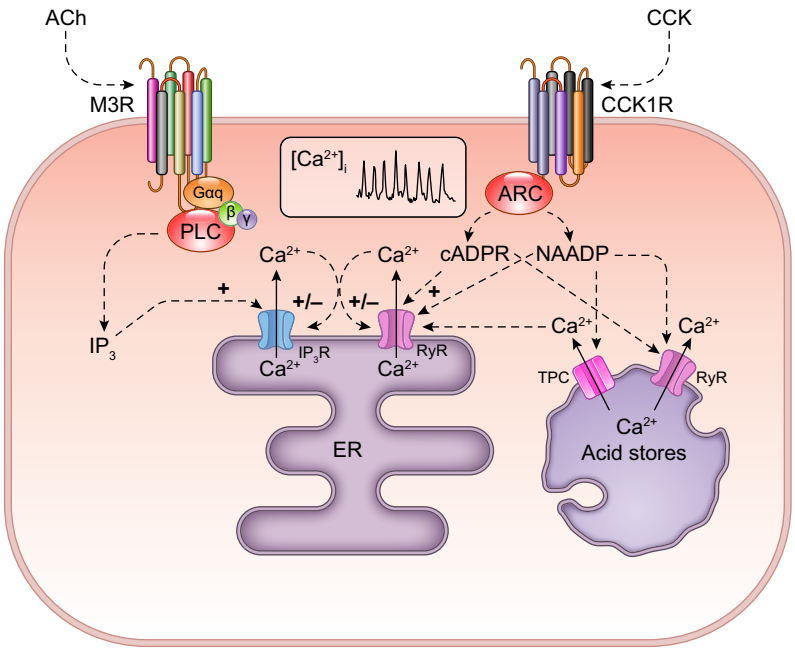

FIGURE 2. Simplified schematic diagram illustrating the different intracellular pathways to local apical Ca^{2+} spiking employed by ACh and CCK, respectively. ARC, ADP-ribosyl cyclase; cADPR, cyclic ADP-ribose; CCK1R, type 1 cholecystokinin receptor; ER, endoplasmic reticulum; IP_3R, inositol trisphosphate receptor; M3R, type 3 muscarinic receptor; PLC, phospholipase C; RyR, ryanodine receptor; TPC, two-pore channel.

apical Ca^{2+} spike and then only slowly release the Ca^{2+} taken up (54, 56), thereby effectively stopping the progression of the cytosolic Ca^{2+} wave toward the base of the cell. Thus Ca^{2+} signal invasion into the basal region, containing the nucleus, is prevented under physiological conditions (27). Normal mitochondrial function is essential for confining the physiological ACh-elicited cytosolic Ca^{2+} spikes to the apical region, because breakdown of the electrical potential difference across the inner mitochondrial membrane, which will prevent Ca^{2+} uptake through the mitochondrial Ca^{2+} uniporter (98, 99), transforms repetitive apical Ca^{2+} spiking into a global sustained elevation of $[Ca^{2+}]_i$ (27).

The Ca^{2+} uptake into the mitochondria not only is important for shaping the Ca^{2+} signals but also serves the crucially important purpose of matching energy production to the need for fueling the secretion process. Both fluid and enzyme secretion are energy-requiring processes, and when ACh- or CCK-elicited Ca^{2+} signals activate secretion there is a need for a rapid increase in ATP production. The fast increase in $[Ca^{2+}]_m$ in the perigranular mitochondria, in response to an increase in the local $[Ca^{2+}]_i$, is in fact rapidly followed by an increase in the NADH concentration, signaling increased Krebs cycle activity (54, 56). Furthermore, direct monitoring of changes in both the mitochondrial and cytosolic ATP concentrations has shown that physiological stimulation with ACh or CCK increases the intracellular ATP level despite the increased ATP utilization by the secretory process (55). This precise coupling of Ca^{2+} signaling to metabolism, stimulus-metabolism coupling, is a prerequisite for proper stimulus-secretion coupling.

There are still some controversies about the mechanisms underlying normal Ca^{2+} signaling in the acinar cells. These relate to the initiation mechanism and the mechanism for replenishing the intracellular Ca^{2+} pool. There is also still no definitive understanding of the mechanism by which CCK evokes Ca^{2+} signals and even controversy about the existence of CCK receptors on human pancreatic acinar cells. Recording of the Ca^{2+}-activated Cl^- current has been an important tool for assessing changes in the local $[Ca^{2+}]_i$ at the inner aspect of the apical membrane, where the critical regulation of secretion must occur, but it is now also becoming apparent that there are important functional and structural interrelationships between the Ca^{2+}-activated Cl^- channels and the Ca^{2+} release process, and this is therefore discussed first.

3.1. The Ca^{2+}-Activated Cl^- Channel

Experimental evidence for the existence of Ca^{2+}-activated Cl^- channels was first obtained in an electrophysiological study on insect salivary glands by Michael Berridge and collaborators in 1975 (100). A few years later it was shown that the ACh-elicited membrane depolarization of pancreatic acinar was due to opening of a Cl^- conductance pathway (101) and furthermore shown that the action of ACh on the outside of the cell could be mimicked by intracellular Ca^{2+} injection (16). In 1978 it was shown directly that Ca^{2+} injection into pancreatic acinar cells evoked opening of Cl^- channels in the acinar membrane (83). The anion selectivity of the pancreatic Cl^- channel was investigated a few years later, and the following permeability sequence (in descending order of permeability) was found: $NO_3^- > I^- > Br^- > Cl^- > HCO_3^-$ (102). This is the same sequence found many years later in studies on the Ca^{2+}-activated Cl^- channel in *Xenopus* oocytes (103, 104). It corresponds to the Eisenman 1 series (105) and is different from the permeability sequence in another major Cl^- channel, namely the cyclic AMP- activated CFTR (cystic fibrosis transmembrane regulator) (106). Ca^{2+}-activated Cl^- channels were rediscovered in the 1980s in studies on *Xenopus* oocytes, without reference to the extensive original work in exocrine glands (103). These channels have now been found in very many different tissues, where they are important for many different cellular functions (103).

In the pancreatic acinar cells, it was assumed—correctly as it turned out—that the Ca^{2+}-activated Cl^- channels would be localized exclusively to the apical membrane (107). The intracellular $[Cl^-]$ is held above equilibrium (108), because of the operation of the basolateral Na^+-K^+-$2Cl^-$ cotransporter (30), and opening of Cl^- channels in the apical membrane would therefore allow outflow of Cl^- into the acinar lumen, forming an essential component of the acinar fluid secretion mechanism (30). The direct proof that the Ca^{2+}-activated Cl^- channels in the pancreatic acinar cells are exclusively localized at the apical membrane was delivered by using a combination of whole cell recording of the Cl^- current with intracellular Ca^{2+} imaging and controlled localized uncaging of caged Ca^{2+} (84). In these experiments, it could be shown that only intracellular Ca^{2+} uncaging near the apical membrane, and not at the basal or lateral membranes, could activate the Cl^- current (84). The Ca^{2+}-activated Cl^- channels in the pancreatic acinar cells are therefore located very close to the primary site of intracellular Ca^{2+} release via IP_3Rs in the apical part of the ER (97).

We now know that TMEM16A (transmembrane protein 16), also known as ANO1 (anoctamin 1), forms the Ca^{2+}-activated Cl^- channel (109–112). The anion permeability sequence of TMEM16A (113) is the same as the one established in the pancreatic acinar cells long before the nature of the channel protein was known (102). There is some controversy about the mechanism by

which Ca^{2+} activates the channel. The majority view is that Ca^{2+} binds directly to TMEM16A. When residues in the Ca^{2+}-binding site are mutated, there are marked changes in the Ca^{2+} sensitivity of the Cl^- current (109, 110, 112). However, there is a study providing evidence for Ca^{2+} activation via the Ca^{2+}-binding protein calmodulin (114).

As already mentioned, there is good evidence showing that a local rise in $[Ca^{2+}]_i$ near the apical Cl^- channels in the pancreatic acinar cells can elicit channel opening, whereas $[Ca^{2+}]_i$ rises further away, for example at the base, fail to do so (84). The IP_3Rs are located very close to the apical membrane (81, 82, 97, 115), and the ACh- and IP_3-elicited $[Ca^{2+}]_i$ rise is maximal close to the apical membrane (15, 26). In nociceptive neurons, the TMEM16A channels can only be activated by the local IP_3-mediated Ca^{2+} release and not by Ca^{2+} influx through voltage-gated Ca^{2+} channels (116). IP_3Rs and TMEM16A are tethered together, and this seems functionally important, as disruption of microdomains, preventing interaction between these two molecules, inhibited Cl^- channel activation mediated by the IP_3Rs (116). It would appear that there may be a two-way interaction between IP_3Rs and TMEM16A. In addition to Ca^{2+} release from the IP_3Rs affecting the Cl^- channel function, TMEM16A can have an effect on the Ca^{2+} release. Overexpression of TMEM16A enhances Ca^{2+} release, whereas knockdown of TMEM16A inhibits Ca^{2+} liberation (117). A very recent study reinforces the idea of functional bidirectional interactions between TMEM16 proteins and intracellular Ca^{2+} release, as TMEM16 activation by SARS-CoV-2 Spike increased the amplitude of spontaneous Ca^{2+} signals and indicated a role for these interactions in SARS-CoV-2 Spike-induced syncytia formation (118). At this point in time it is difficult to judge to what extent these potentially important interactions, which have mostly been studied in cell lines, are relevant for pancreatic acinar physiology, but they could influence pathophysiological events (119), and this is discussed in sect. 10.

3.2. Ca^{2+} Signal Initiation Mechanism

When the spiking apical Ca^{2+} signals in the pancreatic acinar cells were discovered (18, 26), it was assumed that ACh would act on muscarinic receptors located at the base and generate IP_3 there. IP_3 would then diffuse across the cell and bind to IP_3Rs located in the apical area, eliciting the local apical Ca^{2+} signal (26). The general hypothesis advanced by Kasai and Petersen (120) was that the primary site of intracellular Ca^{2+} release is determined not by the localization of the receptors for the relevant neurotransmitter or hormone on the plasma membrane but by the localization inside the cell of the IP_3Rs. Shmuel Muallem and his collaborators (121) argued against this idea, on the basis that the rapid diffusion of IP_3 required for the fast initiation (within milliseconds, but see below) of the Ca^{2+} signal in the apical region was unrealistic. Instead, they proposed that the physiologically important muscarinic receptors were located at or very close to the tight junctions, which would mean that the muscarinic receptors would be located at the apical end of the cells close to the site of the initial Ca^{2+} release. This arrangement would obviate the need for long-distance intracellular IP_3 diffusion (121). Furthermore, this group performed experiments with micropipette application of the muscarinic agonist carbachol, CCK, or the frog peptide bombesin at either the apical or basal poles of isolated acinar cell doublets (122). They found that it was much easier to evoke Ca^{2+} signals when the stimulants were applied apically rather than basally and reported that, at physiological agonist concentrations, Ca^{2+} signals could only be evoked by stimulation at the apical pole (122). The view advanced by Muallem and his collaborators, namely that the primary site of intracellular Ca^{2+} release is determined by the localization of the receptors for the relevant neurotransmitter or hormone on the plasma membrane (121–123), was thus exactly the opposite of what was proposed by Kasai and Petersen (120).

However, there are problems with both the general argument against the IP_3 diffusion hypothesis and the experimental demonstration of the apparent inability to elicit Ca^{2+} signals by stimulation at the basal pole. There is no evidence for the view expressed by Shin et al. (121) that Ca^{2+} release in the apical pole starts within a few milliseconds of cell stimulation. The most accurate and sensitive method to assess the latency between stimulation and response is by electrophysiology. This can be done by measurement of the time lag between very close microionophoretic ACh application to the plasma membrane and the resulting membrane depolarization (30), now known to be due to the rise of the Ca^{2+}-activated Cl^- current (84). The shortest latency ever measured is several hundred milliseconds (124). Furthermore, the latency for the membrane depolarization in response to maximal electrical field stimulation (parasympathetic nerve stimulation) is ~1 s (30, 125). There is also a question about the physiological levels of stimulation. There are no data about the ACh concentrations attained near the muscarinic receptor sites during low-frequency (physiological) nerve stimulation, but we do know the physiological concentration range of CCK (1–10 pM) (126–128). At these low concentrations, the latency for activation of the Ca^{2+}-dependent Cl^- current is very variable and can be very long (minutes) (88). This is understandable, as only a few CCK molecules would be present in the immediate surroundings of an acinar cell at these very low concentrations.

Under real physiological conditions, in vivo, both neurotransmitters and hormones would inevitably approach the acinar units at the base. Furthermore, the parasympathetic nerves do not come close to the plasma membrane, as is the case in the neuromuscular junctions, but rather pass by in the neighborhood (so-called "en passant" innervation). It would also appear that not every single acinar cell receives a nervous input (125). However, because acinar cells are electrically and chemically coupled via a high density of gap junctions, creating effectively a fully connected acinar network of up to 500 cells (129, 130), each acinar cell seems to respond to electrical nerve stimulation (125). Clearly, with such an arrangement the highest ACh concentration attained with nerve stimulation would occur near the base of the cell, rather than at the apical end.

The hypothesis that ACh released from nerve endings, interacting predominantly with muscarinic receptors at the base of the acinar cells, can evoke primary cytosolic Ca^{2+} signals in the apical pole has been tested directly. Muscarinic receptors within a very small area of the basal plasma membrane, isolated by a gigaseal attached patch pipette, were activated by uncaging of caged carbachol inside the pipette by ultraviolet (UV) laser light (131). Furthermore, after the gigaseal between the pipette and the basal plasma membrane had been established, the whole lobule was superfused with an atropine-containing solution, precluding any activation of muscarinic receptors outside the isolated patch area. These experiments showed that activation of muscarinic receptors within a very small area of the basal plasma membrane generated Ca^{2+} signals that invariably started in the apical area within 1–2 s after the UV light flash. At low levels of stimulation the Ca^{2+} signals were confined to the apical granular area, but at higher stimulation intensities Ca^{2+} waves progressing throughout the cell were observed, but these invariably started in the apical area (131). These experiments demonstrate directly the capability of the acinar cells to transmit internally a signal generated near the base to the Ca^{2+} release sites in the apical area >10 μm away (131). The hypothesis proposed by Kasai and Petersen (120), namely that IP_3 generated at the base in response to muscarinic receptor can diffuse across the cell and release Ca^{2+} in the apical region where the IP_3Rs are located, therefore offers the most plausible explanation for the signal initiation, particularly as IP_3 diffuses much more easily in the cytosol than Ca^{2+} (120, 132).

3.3. Store-Operated Ca^{2+} Influx

In the pancreatic acinar cells, the principal role of store-operated Ca^{2+} influx is to replenish intracellular Ca^{2+} stores that have been fully or partially emptied by agonist-elicited release. Cells cannot survive without mechanisms that keep the cytosolic Ca^{2+} concentration at a very low level, and they therefore restore this low level after any challenge (133). All cells depend on an effective mechanism extruding Ca^{2+}. The most important Ca^{2+} export mechanism is the plasma membrane Ca^{2+}-activated ATPase (134). In the acinar cells, this pump is distributed all over the plasma membrane but with the highest concentration in the apical membrane (135, 136). It has been shown directly that Ca^{2+} extrusion from the acinar cells is accelerated whenever a stimulus evokes an increase in $[Ca^{2+}]_i$ (137, 138). This means that any type of Ca^{2+} signaling inevitably results in a loss of cellular Ca^{2+}. In fact, the whole content of the intracellular Ca^{2+} stores can be exported from the acinar cells within a few minutes after maximal ACh or CCK stimulation (137, 138). It follows from these observations that Ca^{2+} signaling by release of Ca^{2+} from intracellular stores depends on a mechanism that allows the stores to be replenished and that this can only be achieved by allowing Ca^{2+} entry from the extracellular fluid (25).

The general mechanism by which store-operated Ca^{2+} entry occurs is now well understood (139, 140) and is therefore not reviewed in detail here. Very briefly, the now generally accepted concept is that a reduction in the Ca^{2+} concentration of the ER ($[Ca^{2+}]_{ER}$) causes translocation of a Ca^{2+} sensor in the ER membrane (STIM) to points (puncta) at which the ER membrane comes so close to the plasma membrane that molecular interaction between STIM and Ca^{2+} entry channels can occur. This results in opening of the so-called Ca^{2+} release-activated Ca^{2+} (CRAC) channels. The debate on how this mechanism operates in the acinar cells centers on the location of the STIM-Ca^{2+} channel interaction as well as the nature of the Ca^{2+} entry channels.

3.3.1. Nature of the Ca^{2+} entry channels.

The CRAC current was discovered by Hoth and Penner (141) in mast cells and has since been intensely studied in several different cell types (139, 140, 142). The CRAC channels are extremely Ca^{2+} selective, can only carry monovalent ions in the absence of divalent ions, have a very low single-channel conductance, and display an inwardly rectifying current-voltage relationship (139, 140). A CRAC current with these properties has been identified in pancreatic acinar cells (**FIGURE 3**). The Orai1 protein has been identified as an essential component of CRAC channels (140), which are now often referred to as Orai1 channels. In acinar cells, Muallem and collaborators (143) provided indirect evidence for Orai1 channels as the main carrier of store-operated Ca^{2+} entry by showing that an Orai1 inhibitory peptide markedly reduced the magnitude of the apparent

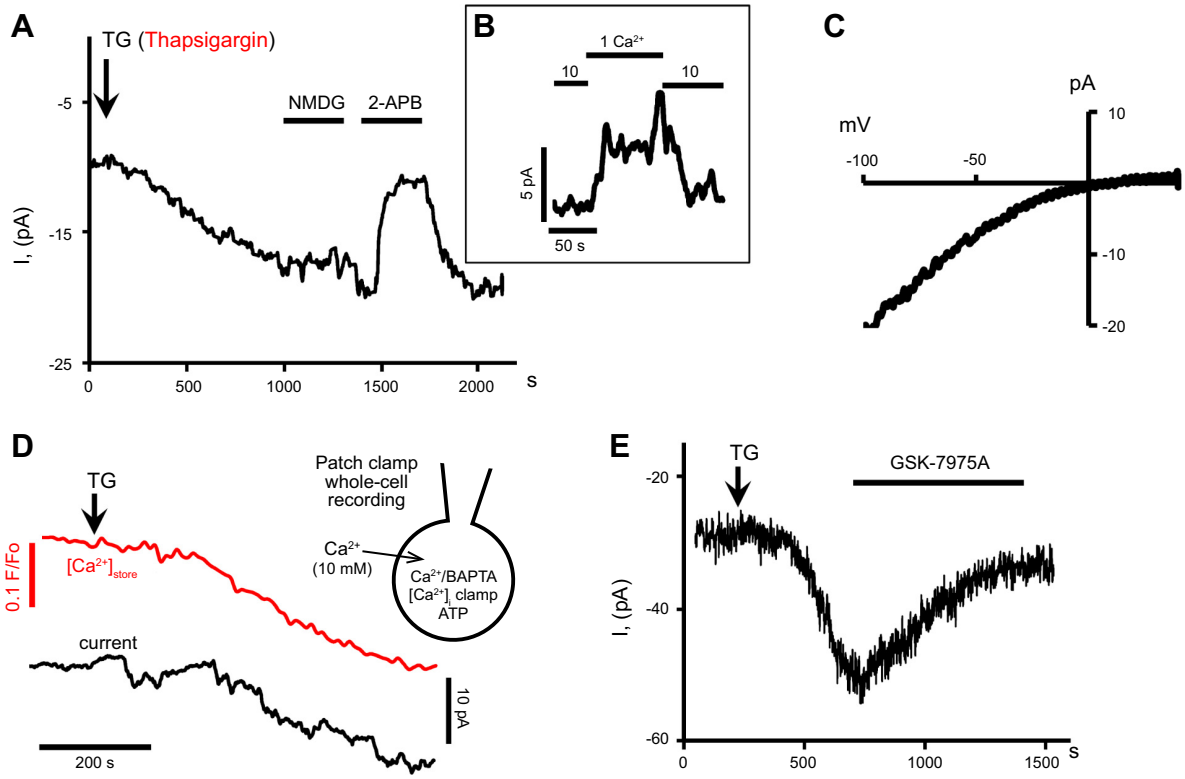

FIGURE 3. Recording of CRAC current in pancreatic acinar cells. The sketch in *center* shows the patch-clamp whole cell recording configuration and the most essential details about the solutions inside and outside the cell. *A*: thapsigargin (TG)-elicited inward current (*I*). When all Na^+ in the external solution was replaced by *N*-methyl-D-glucamine$^+$ ($NMDG^+$), there was no reduction in the inward current, demonstrating that it is not carried by Na^+. A well-known (but nonspecific) blocker of CRAC current [2-aminoethoxydiphenyl borate (2-APB)] markedly and reversibly reduces the inward current. *B*: at the plateau of the TG-evoked inward current, the extracellular Ca^{2+} concentration was reversibly reduced from 10 to 1 mM, causing a marked and reversible reduction in the inward current. *C*: the current-voltage relationship of the TG-induced current shows the inward rectification characteristic of CRAC currents. *D*: simultaneous recordings of TG-elicited inward current and reduction in store Ca^{2+} concentration ($[Ca^{2+}]_{store}$). F/F_0, change in fluorescence intensity from baseline. *E*: the TG-elicited inward current is markedly reduced by the CRAC channel inhibitor GSK-7975A. Adapted from Gerasimenko et al. (40) with permission from *Proceedings of the National Academy of Sciences of the United States of America*.

plateau of elevated cytosolic Ca^{2+} concentration during supramaximal stimulation of the muscarinic receptors. However, another type of Ca^{2+} entry channel, which may also serve as a store-operated entry pathway, namely a TRPC channel, also exists in the acinar cells (143). TRPCs are a family of nonselective cation channels, permeable to Ca^{2+} (144). Opening of such channels would cause not only Ca^{2+} influx but also inflow of Na^+, whereas opening of Orai1 channels would selectively increase Ca^{2+} influx. Kim et al. (44, 45) proposed that TRPC3 is responsible for a major part of the store-operated Ca^{2+} entry. A supramaximal agonist concentration typically evokes a substantial transient rise in $[Ca^{2+}]_i$, due to intracellular Ca^{2+} release, followed by a sustained elevated $[Ca^{2+}]_i$ plateau, due to store-operated Ca^{2+} entry (25). In experiments from Muallem's group (44) comparing carbachol-elicited Ca^{2+} concentration changes in acinar cells from wild-type (WT) and TRPC3−/− mice, this was also the case, but in the TRPC3−/− cells the magnitude of the elevated $[Ca^{2+}]_i$ plateau was markedly reduced compared with the WT cells. Furthermore, after termination of carbachol stimulation, and addition of atropine, the $[Ca^{2+}]_i$ rise evoked by CCK was very markedly reduced in the TRPC3−/− cells compared with control. These data were interpreted to indicate that a major part of the store-operated Ca^{2+} entry occurred through TRPC3 channels rather than Orai1 channels. However, the current density, of the apparently store-operated cation entry (measured under the rather special Ca^{2+}-free condition), was only modestly reduced in the TRPC3−/− cells compared with the control cells (44). Furthermore, it was clearly shown that the initial intracellular Ca^{2+} release elicited by supramaximal carbachol stimulation was severely reduced by the knockout of the TRPC3 channels. Interpretation of these data (44) is therefore difficult. In a further study (45), an inhibitor of TRPC3, Pyr3, was used to show that both the CCK-evoked sustained elevation of $[Ca^{2+}]_i$ and the apparent store-operated cation entry current (but again measured under divalent-free conditions) were reduced.

A more direct approach to identifying the current pathway responsible for store-operated Ca^{2+} entry in the pancreatic acinar cells has been to measure both

the depletion of the ER Ca^{2+} store and the simultaneously developing CRAC current. To avoid potential complications that could arise from direct channel effects of muscarinic receptor activation, one can employ thapsigargin, a very specific inhibitor of the ER Ca^{2+} pump (145), to elicit a slow depletion of the ER Ca^{2+} store. As $[Ca^{2+}]_{ER}$ declined, an inward Ca^{2+} selective current with clear CRAC characteristics was observed (**FIGURE 3**). Importantly, there was no Na^+ component in this inward current, as replacement of all extracellular Na^+ with $NMDG^+$ failed to cause any reduction in the magnitude of the inward current (**FIGURE 3**). These data indicate that, as is the case in immune cells (139, 140), the store-operated channels in the acinar cells are predominantly of the Orai type. Orai1 and Orai2 are expressed in both mouse and rat pancreatic acinar cells (146).

Potent inhibitors of Orai channel opening have been developed (147, 148), and the selectivity of one of these, GSK-7975, has been tested in some detail (40, 147). It is particularly important, with regard to assessing potential contributions to Ca^{2+} entry from other channels in the acinar cells, to emphasize that GSK-7975A does not inhibit opening of TRP4 and TRPC3 channels (147). Both GSK-7975A (**FIGURE 3**) and another Orai inhibitor, CM4620, have been shown to markedly inhibit Ca^{2+} entry stimulated by ER Ca^{2+} store depletion, evoked either by thapsigargin blockade of Ca^{2+} pumps in the ER (40) or by activation of CCK receptors (146).

3.3.2. Location of Ca^{2+} entry.

The original Ca^{2+} tunnel experiments (24) showed that the ER could be refilled with Ca^{2+}, after ACh-elicited loss, by Ca^{2+} entry occurring exclusively through a small isolated area of basal plasma membrane, covered by a gigaseal attached patch pipette. It is therefore indisputable that store-operated Ca^{2+} entry in pancreatic acinar cells occurs through the basal plasma membrane (97). This does not, however, exclude that Ca^{2+} entry could also occur across the apical (luminal) membrane. Muallem and collaborators (123, 143) have proposed that Ca^{2+} entry mainly occurs at the apical end of the acinar cells because of their findings that all Ca^{2+} signaling proteins are located in close proximity of the tight junction, very close to the apical plasma membrane. With regard to the Ca^{2+} entry channel Orai1, which is generally accepted as the most important store-operated channel (140), Hong et al. (143) found that it is localized in the apical area, where the Ca^{2+}-activated Cl^- channels (ANO1/TMEM16A) are localized (84, 97). There is no doubt that the apical granular area contains a high concentration of Orai1, but the functional importance of this finding remains unclear (115). The key point from a functional perspective is that emptying the ER Ca^{2+} stores in the acinar cells causes translocation of STIM1 specifically to areas very close to the basolateral membrane, where Orai1 is also present (115), and far away from the IP_3Rs localized in the apical pole. Thus it is only at the basolateral membrane that STIM1 and Orai1 come close together, and therefore this must be the site where Ca^{2+} entry channels would be activated. This might appear problematic in the case of pancreatic acinar cells, as the ER in the basolateral areas of these cells is of the rough, rather than smooth, type because of the presence of relatively large ribosomes. These are sufficiently large to prevent the close interaction between ER and plasma membrane that is needed for CRAC channel activation. However, it has been shown, by EM studies, that the ER membrane is cleared of ribosomes at points where the ER and plasma membranes come very close together, and it is undoubtedly at these contact sites that store-operated Ca^{2+} entry occurs (115). The conclusion from these studies (115), that store-operated Ca^{2+} entry occurs at the base of the cells, is in complete agreement with the functional imaging studies directly demonstrating store-operated Ca^{2+} entry into peripheral mitochondria situated very close to the basolateral membrane (54).

A study of Orai1-mediated Ca^{2+} entry in HEK cells indicates that Anoctamin 8 (ANO8), which—unlike ANO1—does not appear to function as a Cl^- channel, may play an important role in assembling key Ca^{2+} signaling proteins, such as Orai1, STIM1, the ER Ca^{2+} pump, and IP_3Rs at the junctions between the ER and the plasma membrane involved in controlling Ca^{2+} entry (149). It remains to be determined whether, or to what extent, these findings are relevant for the highly polarized acinar cells, in which IP_3 causes Ca^{2+} release specifically at the apical pole activating the strictly localized Ca^{2+}-sensitive Cl^- channels (ANO1), whereas Orai1-mediated Ca^{2+} entry is spread over the whole of the basolateral membrane (24, 54, 97).

3.3.3. Requirements for coupling intracellular Ca^{2+} release to Ca^{2+} entry.

Pancreatic acinar cells, because of their highly polarized structure, have been particularly useful for dissection of certain key aspects of the control of store-operated Ca^{2+} entry. Some aspects may be special to the acinar cells, but others have been helpful for exploring some general concepts. Two issues require examination here: Is there a special compartment of the ER that is specifically controlling Ca^{2+} entry? and Is IP_3 directly involved locally in regulating opening of the CRAC channels?

In most cell types, Ca^{2+} entry occurs across all aspects of the cell membrane, and, given that Ca^{2+} entry has to take place at points where the ER and plasma membranes come close together, it has often been assumed that Ca^{2+}

entry would be specifically regulated by the ER elements close to the plasma membrane. IP_3 generated at the plasma membrane, via action on IP_3Rs, could therefore have a direct role in locally regulating Ca^{2+} inflow (150). However, in the acinar cells the site of IP_3-elicited release of Ca^{2+} from the ER occurs in the apical granular area, whereas the store-operated Ca^{2+} entry can occur selectively at the base of the cell, >15 μm away from the intracellular Ca^{2+} release site (24). In the basal area of the acinar cells the sensitivity of the ER Ca^{2+} release mechanism to IP_3 is extremely low (18, 26, 151), and local ACh stimulation, at the base of the cell, causes primary intracellular Ca^{2+} release in the apical part of the cell, far away from the site of IP_3 generation (131). It is also possible to directly visualize that the IP_3Rs are concentrated in the apical area, whereas the location of STIM1, after stimulant-elicited translocation, is at the basolateral membrane (115). Certainly, at least as far as the pancreatic acinar cells are concerned, the proposition that local functional IP_3Rs very close to the store-operated Ca^{2+} entry channels control the opening of these pores (150) seems very unlikely. Furthermore, given that store-operated Ca^{2+} entry, through CRAC channels, functions perfectly well in the acinar cells (40), the generality of the concept that IP_3Rs could play an important role in controlling CRAC channel opening (150) is questionable.

The idea that it is a special subcompartment of the ER that is involved in the control of store-operated Ca^{2+} entry was advanced by Parekh et al. (152) and continues to be supported (149). The experiments by Parekh et al. (152) indicated that Ca^{2+} release and Ca^{2+} influx could be dissociated, as they apparently possess differential sensitivities to IP_3. In a study on rat basophilic leukemia (RBL) cells, low IP_3 concentrations could induce substantial intracellular Ca^{2+} release without any CRAC current, which needed much higher IP_3 levels to become activated (152). In contrast, the first study that directly monitored changes in the store Ca^{2+} concentration and CRAC current, also conducted in RBL cells (153), showed that store-operated Ca^{2+} entry was closely linked to the filling state of the internal store. Specifically, Hofer et al. (153) showed that the CRAC current was already activated after very small drops in the store Ca^{2+} concentration. This finding is in agreement with what has been shown in the pancreatic acinar cells, where the time course of CRAC current development follows closely the gradual reduction in $[Ca^{2+}]_{ER}$ after blockade of the ER Ca^{2+} pumps by thapsigargin (**FIGURE 3**). Even a relatively small drop in $[Ca^{2+}]_{ER}$ was associated with CRAC channel opening. It would appear that this is a functional necessity, because the repetitive release of small amounts of Ca^{2+} in the apical area, which controls physiological secretion in the acinar cells, can only be sustained when $[Ca^{2+}]_{ER}$ is kept at a high level (95). The high mobility of Ca^{2+} in the lumen of the fully connected ER, which allows full reequilibration of the free Ca^{2+} concentration throughout the ER within ~6 s after a local perturbation (95), obviates the need for postulating that different compartments of the ER control Ca^{2+} release and CRAC current. Although Ca^{2+} release occurs in the apical area whereas CRAC channels are at the base, there is time for the Ca^{2+} release at the apex to influence the Ca^{2+} concentration also in the basal part of the ER before opening of CRAC channels occurs, as there is a delay of ~7 s from even maximal ER Ca^{2+} release until the start of Ca^{2+} entry (154).

3.4. CCK and Its Intracellular Signaling Pathway

CCK is one of the earliest discovered gastrointestinal peptide hormones and is structurally related to the even earlier-characterized hormone gastrin (128). Its principal actions are contraction of the gallbladder and secretion of an enzyme-rich fluid from the exocrine pancreas (155). There is a striking similarity between CCK and the frog skin decapeptide caerulein, in which the carboxy-terminal 8 amino acid residues, with the exception of no. 6 (from the carboxy terminus), are identical to those of CCK (128, 155). The actions of caerulein on the pancreas are indistinguishable from those of CCK (156), and caerulein was therefore much used as a CCK analog in the early years of investigations, when pure preparations of CCK were in short supply. Another frog peptide, bombesin, has very similar actions on the pancreas (157, 158) but, unlike caerulein, acts on receptors that are distinct from those for CCK (159, 160). Early work on the actions of CCK on Ca^{2+} movements in the pancreas was conducted on several species, including mouse, rat, and guinea pig, essentially with similar results (10, 12, 156, 161). It is undisputed that, in all these species, CCK—in all its active forms, CCK8, CCK33, CCK58—acts on specific high-affinity receptors (CCK1 receptors) on pancreatic acinar cells (155, 162). However, there has been, and to some extent still is, a dispute about the existence of functional CCK receptors on human pancreatic acinar cells and therefore whether the undisputed fact that CCK stimulates pancreatic enzyme secretion in vivo is due to direct action of CCK on acinar cells or is indirect via stimulation of vagal nerves (127). Furthermore, the intracellular signaling pathway(s) activated by CCK is much less clear than it is for activation of the muscarinic receptors. Here we therefore focus on these two issues.

3.4.1. Are there functional CCK receptors on human pancreatic acinar cells?

Given the well-established direct actions of CCK on acinar cells isolated from several different species,

including the pig (163), it was a surprise when Logsdon and collaborators (164, 165) published data indicating that isolated human pancreatic acinar cells lack functional responses to CCK (and gastrin). Concentrations of CCK8 from 10 pM to 100 nM failed to elicit any increase in amylase secretion, whereas carbachol (1 μM to 1 mM) evoked a concentration-dependent increase in amylase output. Furthermore, 100 nM CCK8 failed to elicit any increase in $[Ca^{2+}]_i$, whereas in the same cells a subsequent challenge with 1 mM carbachol produced a clear $[Ca^{2+}]_i$ rise. Only low levels of CCK receptor expression were found in the human cells (164).

However, a later study (166) concluded that functional CCK receptors do exist on isolated human pancreatic acinar cells. In that study (166), even very low, and physiologically relevant, CCK8 and CCK58 concentrations (2–10 pM) evoked clear increases in $[Ca^{2+}]_i$ also in the presence of atropine (1 μM) and the nerve-blocking agent tetrodotoxin (TTX; 100 nM). These $[Ca^{2+}]_i$ increases had exactly the same characteristics as those previously described for mouse acinar cells. At the lowest CCK concentration (2 pM CCK58) tested, the Ca^{2+} signals were observed as repetitive short-lasting spikes localized to the apical pole, whereas at higher concentrations they could spread out from the initiation point in the apical area and invade the whole cell. CCK8 (10 pM) also evoked a clear increase in amylase secretion, assessed by three different methods, in the presence of both atropine and TTX (166).

There is a clear discrepancy between the data from the groups of Logsdon (164, 165) and Petersen (166). What could explain this? Given that preparation of isolated acinar cells inevitably involves a step of digesting small pieces of pancreatic tissue with collagenase, as well as some mechanical agitation, and that any damaged acinar cells would release trypsin that could potentially damage receptors on the acinar surface membrane, there will always, in such studies, be concerns about the state of the isolated cells. The best test is obviously functional, i.e., whether the acinar cells respond acutely to physiological levels of hormones or neurotransmitters. The living acinar cells employed by Murphy et al. (166) displayed typical polarized morphology and responded to a low ACh concentration (50 nM) with typical Ca^{2+} spiking, which was completely reversible upon application of atropine, whereas in the study of Ji et al. (164) only a sustained rise of $[Ca^{2+}]_i$ was shown in response to stimulation with a very high (1 mM) carbachol concentration (equivalent to 100 μM ACh).

Clearly, not every group has found it easy to observe CCK responses in human pancreatic acinar cells. In an article from Pandol's group (167), it is mentioned that in pilot experiments only a very high CCK concentration (10 nM) was able to elicit amylase secretion or $[Ca^{2+}]_i$ changes. However, these data were reported as "not shown." On the other hand, a study from Gaisano's group (168) has confirmed the positive data from Petersen's group (166) and shown clear evidence of CCK-elicited Ca^{2+} signal generation and exocytosis in the presence of both atropine and TTX. The study from Gaisano's group (168) employed a human pancreatic slice preparation in which nerves or nerve elements would be present, but the clear effects of CCK could not have been mediated by cholinergic nerves, as high concentrations of atropine were used. There could in theory be nerves secreting transmitters other than ACh, but the presence of TTX would have prevented action potential generation and therefore secretion of any neurotransmitter in these studies (168). From experiments on mouse and guinea pig pancreatic tissues, we know that all effects of nerve stimulation on $[Ca^{2+}]_i$ in the acinar cells are mediated by ACh, as atropine abolishes Ca^{2+} signal generation in response to nerve stimulation (69, 169, 170). In the guinea pig, nerve stimulation does result in additional release of a noncholinergic neurotransmitter [most likely vasoactive intestinal polypeptide(VIP)]. This does not cause Ca^{2+} signals but generates cyclic AMP and induces secretion (169, 170). So far, we have no such data for the human pancreas.

At this stage of our knowledge, it would appear that there are functional CCK receptors on human pancreatic acinar cells and that, at least in this respect, the human pancreas is not different from the pancreas of the mouse, rat, guinea pig, and pig. It is difficult to find arguments against the positive and clear results from two independent groups (166, 168), but it is worth noting that human pancreatic preparations, usually obtained from surgery on old cancer patients, often are in a poor state as also noted by Murphy et al. (166), and this may well account for the negative results with regard to CCK actions reported by two other groups (164, 167).

3.4.2. Does CCK activate pancreatic acinar secretion in vivo by direct action on acinar cells?

Although the studies of CCK action on isolated pancreatic acinar cells, as well as on acinar cells in the more intact lobule or slice preparations, would appear to provide evidence indicating that CCK stimulates pancreatic enzyme and fluid secretion by directly activating CCK1 receptors on the acinar plasma membrane, this is not a view universally shared (127). Even in the rat, where it is undisputed that CCK can act directly on isolated acinar cells to generate Ca^{2+} signals and secretion, it has been proposed that this action is not physiologically relevant and that the effect of CCK in vivo is mediated via cholinergic nerves. The opposing views are clearly articulated

in the most recent (3rd) edition of *The Pancreas* in the chapters by Petersen (58) and Owyang (127). The principal argument in favor of the hypothesis that the CCK action on the pancreas is indirect rests on important in vivo experiments conducted by Li and Owyang (171). In these experiments on anesthetized rats, intravenous infusions of CCK8 caused significant elevations of the plasma CCK8 levels, which were measured together with the secretory responses from the pancreas. The plasma CCK8 levels obtained by the exogenous CCK8 infusions were compared to those seen after a casein meal. The pancreatic protein secretion in response to CCK8 was assessed in the presence or absence of the muscarinic antagonist atropine, as well as in the presence or absence of the nicotinic antagonist hexamethonium. The results reported by Li and Owyang (171) showed that the CCK8-elicited protein secretion at plasma levels up to 8 pM, the level obtained after a casein meal, was essentially abolished by atropine as well as by hexamethonium. However, at only a slightly higher CCK8 concentration in the plasma (12 pM), although the secretion was reduced both by atropine and hexamethonium, a major part of the secretory response was retained in the presence of the receptor blockers. The conclusion drawn by Owyang (127) that CCK, even in the rat, activates secretion indirectly via the vagal nerve seems to rest on the assumption that CCK plasma levels up to 8 pM are physiological whereas a plasma level of 12 pM is not. This seems to be a tiny difference in concentration, certainly in relation to normal considerations of concentration-response curves. However, available data indicate that physiological plasma levels of CCK in the human body after a meal do not exceed 10 pM (126). This dispute shows the extraordinary difficulty of determining what is real physiology and what is not! The problem is further exacerbated by the fact that CCK58 and not CCK8 would appear to be the dominant form of CCK (172, 173).

An important study in humans by Guido Adler and collaborators (174) on the interaction between the cholinergic system and CCK in the control of pancreatic enzyme secretion indicates that although cholinergic nerves may be the most important regulators of secretion, CCK does play an important role and acts directly on the acinar cells. The most convincing evidence comes from experiments with atropine in which CCK levels well within the physiological range evoked a clear increase in trypsin output (174). Adler et al. (174) concluded that this can only be explained by CCK having a direct effect on the acinar cells. However, Adler et al. (174) also point out that there are clearly interesting and potentially complex interactions between the cholinergic system and CCK. Although both the muscarinic antagonist atropine and the CCK1 receptor antagonist loxiglumide markedly reduced enzyme output in response to test meals, the effect of atropine was significantly stronger, with atropine able to abolish secretion whereas loxiglumide could not fully suppress the effect of test meals (174). This suggests that a degree of cholinergic stimulation is needed in order for CCK to exert its effect (174). It is not easy to reconcile these findings with the evidence from studies on isolated human pancreatic acini showing that a physiological CCK8 concentration (10 pM) evokes a clear increase in exocytosis and amylase output in the combined presence of atropine and TTX (166). If it turns out that CCK58 is indeed the real physiological CCK form (172, 173), it would also be important to note that in isolated human acini CCK58, in as low a concentration as 2 pM, evoked clear Ca^{2+} signals (166). The discrepancy between the in vivo and in vitro data indicate that we still have an incomplete picture of the many potential interactions between different nervous pathways and hormones in the intact body.

3.4.3. The signal transduction mechanism activated by physiological CCK concentrations.

Early work indicated that ACh and CCK elicited much the same changes in cellular Ca^{2+} movements (9, 10, 161, 175). However, a more detailed electrophysiological analysis of the pattern of the ACh- or CCK-elicited increase in the Ca^{2+}-activated Cl^- current, monitoring faithfully $[Ca^{2+}]_i$ changes near the apical membrane (26, 84), revealed significant differences (88). At the very lowest agonist concentrations eliciting signals (low nM for ACh; low pM for CCK), repetitive short-lasting spikes were evoked by both agents. At increasing agonist concentrations, the evoked responses became oscillations on top of a sustained elevated current plateau in the case of ACh, whereas the CCK-induced changes became a mixture of short-lasting spikes and much longer, but still transient increases (88). At maximal agonist concentrations, a sustained elevation of the Ca^{2+}-dependent Cl^- current was seen (88). By using low to intermediate agonist concentrations and matching the levels of CCK and ACh in such a way that the frequency of the short-lasting spikes was the same, it could be seen that in the case of CCK stimulation the short-lasting spikes were from time to time followed by a much longer-lasting transient, whereas this was not the case for ACh stimulation (88). In other words, the short-lasting spikes elicited by CCK have a greater tendency to elicit a further larger and longer response than is the case for ACh stimulation. Combining electrophysiology and fluorescent $[Ca^{2+}]$ recordings, and taking great care to minimize the Ca^{2+}-buffering effect of fluorescent Ca^{2+} probes, it became clear that the short-lasting spikes of

Ca^{2+}-dependent Cl^- current correspond to local apical rises in $[Ca^{2+}]_i$, whereas the longer transients represent global elevations of $[Ca^{2+}]_i$ (26). Nevertheless, with standard fluorescence measurements of agonist-elicited $[Ca^{2+}]_i$ changes, it is mostly difficult to resolve the short-lasting spikes, because of the inevitable Ca^{2+} buffering by the Ca^{2+}-binding dye, so the response to a low CCK concentration often just appears as repetitive broad transients from the baseline at a very low frequency, whereas the response to a low ACh concentration can be seen as relatively high-frequency oscillations on top of a sustained slightly elevated $[Ca^{2+}]_i$. The remarkable differences in the pattern of $[Ca^{2+}]_i$ changes evoked by the two physiologically important stimulants of acinar fluid and enzyme secretion are puzzling, and the physiological implications of these differences are still not clear. There is, however, at least one interesting consequence of the different Ca^{2+} spike patterns evoked by ACh and CCK. The local short-lasting Ca^{2+} spikes that are elicited by low-level physiological stimulation with ACh do not spread to neighboring coupled cells, whereas the global longer-lasting Ca^{2+} transients, which are some times triggered by local spikes in the case of physiological CCK stimulation, do spread to neighboring cells in communicating acinar cell clusters (176).

There is a substantial amount of evidence demonstrating that whereas ACh binding to muscarinic receptors on pancreatic acinar cells activates PLC, this is not the case for CCK binding to CCK1 receptors at physiological hormone levels (**FIGURE 2**). In the case of ACh stimulation, it is clear that IP_3 is the intracellular messenger releasing Ca^{2+} from the ER (31, 177). What has been confusing is that CCK at high (nM) nonphysiological concentrations also activates PLC but this does not happen at the low physiologically relevant pM concentrations (31, 178). At these low CCK concentrations, another Ca^{2+}-releasing messenger, nicotinic acid adenine dinucleotide phosphate (NAADP), is generated (179). Intriguingly, this intracellular messenger for CCK is effective at concentrations significantly lower than the IP_3 levels required for this classic messenger's Ca^{2+}-releasing action (85, 86).

There are strong arguments indicating that NAADP acts as an intracellular messenger for CCK-elicited Ca^{2+} release from stores inside pancreatic acinar cells (**FIGURE 2**). Intracellular NAADP infusion can mimic the effect of extracellular CCK application, and a high desensitizing NAADP concentration inside the acinar cells abolishes the ability of CCK to elicit Ca^{2+} signals (180) but, in the same cells, fails to reduce the ACh-evoked Ca^{2+} signal generation (85, 86). Furthermore, CCK, but not ACh, evokes a rapid rise in the intracellular NAADP concentration (179). This rise is mediated by ADP-ribosyl cyclase (ARC) ectoenzyme CD38 (181), which is also expressed in the endosomes (182). This is a NAADP synthase that couples CCK receptor activation to the primary Ca^{2+} mobilization from endosomes and lysosomes (182). In addition to NAADP, cyclic ADP-ribose (cADPR) is also produced in response to CCK stimulation, but more slowly (179). It would appear that both NAADP and cADPR are important intracellular mediators, because CCK-elicited Ca^{2+} signaling can be abolished by the NAADP antagonist Ned-19 as well as by the cADPR antagonist 8-NH_2-cADPR (85).

From these data it would appear that ACh activation of muscarinic receptors generates IP_3, whereas CCK interaction with CCK1 receptors primarily generates NAADP and cADPR (**FIGURE 2**). However, the situation is complicated by the fact that the physiological repetitive Ca^{2+} spiking elicited not only by CCK but also by ACh depends on functional intracellular receptors for both IP_3 and ryanodine (85, 86). So even in the relatively simple case of ACh stimulation, it is clear that more than one intracellular Ca^{2+} release channel is involved (**FIGURE 2**). In the case of ACh, the initial trigger Ca^{2+} comes from IP_3-mediated primary release via IP_3Rs, which then via Ca^{2+}-induced Ca^{2+} release from RyRs elicits further Ca^{2+} release. When the local $[Ca^{2+}]_i$ reaches a certain level, Ca^{2+}-induced Ca^{2+} release changes into Ca^{2+}-induced inhibition of Ca^{2+} release from both types of receptors (89, 183, 184). $[Ca^{2+}]_i$ then falls, and the cycle repeats itself. In the case of CCK stimulation, the original suggestion was that the primary action of NAADP is to open RyRs in the ER to release Ca^{2+} via these channels (85, 185), which then via Ca^{2+}-induced Ca^{2+} release would elicit further Ca^{2+} liberation via both receptor types until $[Ca^{2+}]_i$ would reach the level at which Ca^{2+} inhibition of Ca^{2+} release would take over. Again the cycle would repeat itself. The strongest evidence for NAADP acting on RyRs in the ER came from experiments on a preparation of isolated nuclei from pancreatic acinar cells with surrounding ER in which NAADP, like IP_3 (and cADPR) could evoke Ca^{2+} release (185). Since there is no evidence, in general, for a NAADP binding site on RyRs, it was suggested that there might be an intermediary NAADP-binding protein (185). There is now evidence indicating that Jupiter microtubule-associated homolog 2 (JPT2) is a NAADP-binding protein that could link NAADP to opening of RyRs (186), and the hypothesis of Gerasimenko et al (185) has been revived in a more generalized form by Guse and Diercks (187). There is ample evidence that all the intracellular Ca^{2+}-releasing messengers can liberate Ca^{2+} not only from the ER but also from acidic stores (188–190). Menteyne et al. (190) proposed that the primary action of NAADP would be on acidic stores in acid endosomes and lysosomes, which would then via Ca^{2+}-induced Ca^{2+} release from the ER activate the main oscillator (**FIGURE 2**).

Although there is evidence for NAADP-elicited Ca^{2+} release via RyRs from pancreatic acinar cells and immune cells (187), work from other groups has invoked primary NAADP action on two-pore channels (TPCs) (191–193). The question about the possible involvement of TPCs in CCK-elicited Ca^{2+} signaling in pancreatic acinar cells has therefore been investigated in some detail in a study on permeabilized acinar cells employing specific antibodies against RyRs of types 1, 2, and 3 as well as TPCs of types 1 and 2, combined with knockout of RyR3 and TPC2 (194). When Ca^{2+} release was studied specifically from acidic stores, both functional RyRs and TPCs were required for NAADP to liberate Ca^{2+}. In the presence of antibodies to both TPC1 and TPC2, NAADP hardly elicited any Ca^{2+} release, and the same result was obtained in the presence of antibodies to both RyR1 and RyR3. However, when all stores were included, there was still a small Ca^{2+}-releasing effect of NAADP when antibodies to TPC1 and TPC2 were present or when acinar cells from TPC2−/− mice were investigated in the presence of an antibody against TPC1. In contrast, NAADP failed completely to release Ca^{2+} in the presence of ryanodine or antibodies to all RyRs. The results of these investigations indicated that TPC1 was of relatively minor importance for NAADP-elicited Ca^{2+} release compared with TPC2 and that RyR2, which was shown to be very important for cADPR-evoked Ca^{2+} release, was of minor importance for the ability of NAADP to liberate Ca^{2+} compared with RyR1 and RyR3 (194). NAADP activation of TPCs, like the activation of RyRs, is likely to occur via the NAADP-binding protein JPT2 (195).

A possible interpretation of these results would be that NAADP primarily triggers the release of a very small amount (nondetectable with current technology) of Ca^{2+} from an acidic store via opening of TPC2s and that the rise of $[Ca^{2+}]_i$ in a microdomain around the acidic store is sufficient, via Ca^{2+}-induced Ca^{2+} release, to activate RyR1s and RyR3s on both other acidic stores and the ER (**FIGURE 2**). Although this is in agreement with currently available data, and plausible, direct evidence for this hypothesis is still missing.

3.5. *Do the ACh- and CCK-Elicited $[Ca^{2+}]_i$ Elevations Occur in Real Physiology and Are They Functionally Relevant?*

In an age when an increasing number of investigations are carried out on cultured cells or cell lines, which of course have many advantages with regard to molecular manipulations, the question about real physiological relevance is often overlooked. The majority of Ca^{2+} signaling work on pancreatic acinar cells has been carried out on freshly isolated mouse acinar cells or small cell clusters with low, and therefore physiologically relevant, concentrations of ACh and CCK, but it has not so far been possible to monitor $[Ca^{2+}]_i$ changes in pancreatic acinar cells in vivo. However, experiments on intact segments (lobules) of pancreas have shown that the Ca^{2+} signaling patterns seen in isolated acinar cells are also observed in undissociated preparations (131, 168).

In an intact animal or human being, ACh is released onto the surface of pancreatic acinar cells from vagal nerve endings in the pancreatic tissue. Induction of action potential in these nerves would therefore seem to be the most physiological type of stimulation. Indeed, stimulation of intrinsic nerves in mouse and rat pancreatic tissue by electrical field stimulation has demonstrated opening of Ca^{2+}-activated Cl^- channels depolarizing the acinar membrane (30, 125). Such effects were observed at stimulation frequencies as low as 5 Hz, which are in the real physiological range (125). These effects were blocked by atropine, which of course also blocked the effects of direct ACh application, as well as by the neurotoxin TTX, which—as expected—did not block the effect of direct ACh stimulation (125). In fact, electrical stimulation of the cervical vagal nerve was also shown to evoke acinar depolarization (ion channel opening) in pancreatic acinar cells in the living, anesthetized mouse (196). Given the considerable body of evidence showing that Cl^- channel opening in pancreatic acinar cells is mediated by a rise in $[Ca^{2+}]_i$ (16, 26, 29, 84, 87, 197), the early work employing low and physiological levels of electrical nerve stimulation in the intact pancreas (125, 196) indicates that Ca^{2+} signals in pancreatic acinar cells are real physiological phenomena.

More recently, functional innervation has been studied in pancreatic lobule preparations in which intrinsic nerves were stimulated by K^+ depolarization (69). This elicited Ca^{2+} signals in the nerve cells and then in the acinar cells. These are the first simultaneous recordings of Ca^{2+} signal generation in a nerve and the resulting Ca^{2+} signal in an adjacent acinar cell (**FIGURE 4**). Whereas the Ca^{2+} signals in the nerve cells persisted in the presence of atropine, the Ca^{2+} signals in the acinar cells disappeared, as expected (**FIGURE 4**). The Ca^{2+} signal in the nerve cell, evoked by membrane depolarization opening voltage-gated Ca^{2+} channels (198), caused release of ACh, and the Ca^{2+} signal in the acinar cell is then mediated via the action of the neurotransmitter on muscarinic receptors (69).

With regard to CCK, we know the physiological levels in the plasma, which, as already mentioned, are in the range of 1–10 pM (126), and it has been repeatedly shown that such very low levels of CCK induce opening of Ca^{2+}-activated Cl^- channels and directly measured $[Ca^{2+}]_i$ elevations in the acinar cells (26, 85, 88)

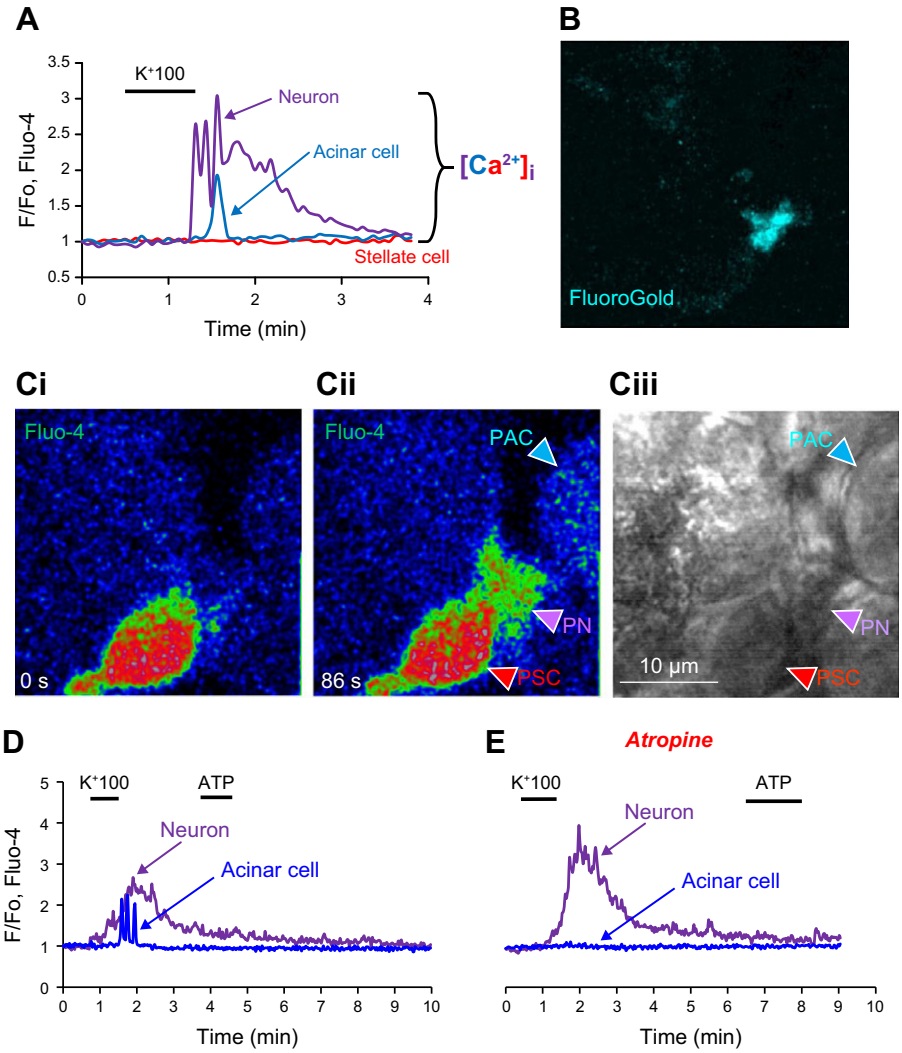

FIGURE 4. Neuroglandular transmission. A high extracellular K^+ concentration (100 mM), via membrane depolarization, evokes a $[Ca^{2+}]_i$ rise in a pancreatic neuron and subsequently in a neighboring acinar cell but not in a neighboring stellate cell. *A*: simultaneous Ca^{2+}-sensitive fluorescence recordings (fluo-4) from a neuron, a stellate cell, and an acinar cell. F/F_0, change in fluorescence intensity from baseline. *B*: localization of the pancreatic neuron by Fluoro-Gold labeling. *Ci*: fluorescence image at *time 0* (before high-K^+ stimulation). *Cii*: fluorescence image at time 86 s [during transient $[Ca^{2+}]_i$ rises in both the neuron (PN) and the acinar cell (PAC) but without any change in the stellate cell (PSC)]. *Ciii*: transmitted light image with arrowheads showing the localization of the 3 cells from which the recordings in *A* were obtained. *D*: traces similar to those in *A*. K^+ depolarization evokes Ca^{2+} signals in a neuron and then in a neighboring acinar cell. *E*: same experiment as in *D*, but now in the presence of the muscarinic antagonist atropine. In this case, K^+ depolarization only evokes a Ca^{2+} signal in the neuron but not in the acinar cell. From Gryshchenko et al. (69) with permission from *Journal of Physiology*.

including, as already described in sect. 3.4.1, in the human pancreas (166). Taking all this together, there is a strong case for concluding that Ca^{2+} signals in pancreatic acinar cells play a critical role in physiological control of enzyme secretion in the intact body.

4. PATHOLOGICAL Ca^{2+} SIGNALING IN ACINAR CELLS

In 1995, Ward et al. (36) proposed that abnormal excessive Ca^{2+} signal generation could be central to the initiation and development of AP. This has turned out to be a fruitful hypothesis, as pathological Ca^{2+} signals are typically evoked by stimuli that have been shown to initiate changes in the pancreatic acinar cells similar to those seen in AP, a human disease in which the pancreas digests itself, resulting in an initially nonbacterial inflammatory response, often with a fatal outcome (32–34, 39, 201). Early studies generating AP-like features in isolated pancreatic preparations (202, 203) employed hyperstimulation of CCK receptors, with nanomolar rather than the physiological picomolar concentrations, often using the CCK analog caerulein rather than CCK itself. This approach has provided important evidence regarding the disease mechanism, and the most important results of this type are therefore considered first. However, the real and major causes of AP are complications from gallstones and alcohol abuse, and the main emphasis in what follows is therefore on the effects of bile acids and combinations of alcohol and fatty acids.

4.1. *Effects of Supramaximal CCK Receptor Activation*

Early studies by Babkin et al. (204) indicated that prolonged stimulation of the pancreas could lead to the appearance of large vacuoles in the acinar cells and eventually to their destruction. A careful study by Lampel and Kern (205) confirmed this and drew attention to the fact that hyperstimulation of the pancreas with caerulein

caused morphological changes in the pancreas, namely fusion of condensing vacuoles with zymogen granules leading to formation of large vacuoles in the cytoplasm of the acinar cells. Furthermore, caerulein hyperstimulation markedly increased the serum amylase levels. It was therefore suggested that hyperstimulation with caerulein could be used as an experimental model of AP (205).

As outlined in the previous section on physiological Ca^{2+} signaling (sect. 3), low and physiologically relevant concentrations of CCK (low pM) evoke repetitive rises in $[Ca^{2+}]_i$ in the acinar cells. In contrast, high CCK levels, at nanomolar concentrations, evoke sustained elevations of $[Ca^{2+}]_i$. The sustained elevated $[Ca^{2+}]_i$ plateau is acutely dependent on the presence of Ca^{2+} in the extracellular solution (47). As discussed in sect. 3, the primary agonist-elicited release of Ca^{2+} from internal stores is followed by activation of Ca^{2+} entry pathways of the Orai1 type in the plasma membrane, and it is this Ca^{2+} entry that sustains the elevated $[Ca^{2+}]_i$, irrespective of whether the Ca^{2+} signal has been evoked by CCK or ACh. In pancreatic acinar cells, the principal physiological function of Ca^{2+} entry through Orai1 channels is to refill the ER after the IP_3-mediated release of Ca^{2+}. Under physiological conditions, the pump-mediated Ca^{2+} uptake into the ER prevents any significant increase in $[Ca^{2+}]_i$ in the basal part of the cell (97), because of the close apposition of Orai1 channels in the plasma membrane and the Ca^{2+} pumps in the ER (97, 115, 149). However, under pathological conditions, where mitochondrial function is inhibited, there may be insufficient ATP to power Ca^{2+} pump-mediated Ca^{2+} uptake into the ER. Furthermore, the mitochondria close to the basolateral membrane will be depolarized and therefore also unable to take up Ca^{2+}. Because of lack of ATP, the plasma membrane Ca^{2+} pumps might also be unable to extrude the excess Ca^{2+} from the cytosol. In such cases, an elevated global $[Ca^{2+}]_i$ will be sustained. Although there are mechanisms that should guard against toxic $[Ca^{2+}]_i$ elevations due to Ca^{2+} entry, namely the channel inactivator SARAF (store-operated calcium entry-associated regulatory factor) (206, 207) as well as fast and slow Ca^{2+}-dependent inactivation of the Orai1 channels (208, 209, 283), this may not be sufficient to overcome the problem caused by lack of ATP production.

Following the proposal that a sustained elevated $[Ca^{2+}]_i$ in the acinar cells could be the trigger for AP (36), a study on isolated cells (35) showed that the intracellular trypsinogen activation, a hallmark of AP, induced by hyperstimulation with either caerulein or a muscarinic agonist was absolutely dependent on the presence of Ca^{2+} in the suspending medium. Thereafter, imaging studies demonstrated that the Ca^{2+}-dependent hyperstimulation-induced trypsinogen activation occurred in the granule-rich apical region (46, 47). Furthermore, intracellular vacuole formation was shown to be induced by CCK hyperstimulation. In a study employing both light and electron microscopy, it was shown that high (nM) CCK concentrations failed to induce vacuole formation when the acinar cells were preloaded with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) or when the extracellular solution did not contain Ca^{2+} (47). A sustained elevation of $[Ca^{2+}]_i$ brought about by poisoning the ER Ca^{2+} pumps with the very specific inhibitor thapsigargin also induced extensive vacuole formation, as well as trypsinogen activation (47).

Although it is clear that intracellular protease activation requires a sustained $[Ca^{2+}]_i$ elevation, which in turn depends on Ca^{2+} influx (35, 47), this does not mean that intracellular Ca^{2+} release is unimportant. First of all, Ca^{2+} influx is, as already described, a consequence of the reduced $[Ca^{2+}]$ in the ER following the primary liberation of Ca^{2+} from this store (40). Furthermore, IP_3-mediated Ca^{2+} release from intracellular stores in permeabilized acinar cells has been shown to activate trypsinogen (41, 42). There are also data suggesting that RyRs play a special role in mediating early zymogen activation in pancreatitis (210), although some of the data in this study (210) are contradicted by other investigations. The finding that the carbachol-elicited rise in $[Ca^{2+}]_i$ is much larger in the basolateral part of the acinar cells than in the apical region (210) differs from results obtained in two other laboratories showing that the $[Ca^{2+}]_i$ rise starts earlier in the apical pole and is either markedly larger in this region than in the basolateral pole (15) or of similar magnitude (211). The suggestion that RyRs play a particularly important role in zymogen activation (210) is based on data indicating that inhibitors of RyR opening (ryanodine and dantrolene) markedly reduced caerulein- or carbachol-elicited zymogen activation (210, 212) as well as on data showing that pretreatment with caffeine, a well-known activator of RyRs, reduced caerulein-induced chymotrypsinogen activation (210). The caffeine data, indicating specific release of Ca^{2+} in the basolateral region, were interpreted to indicate that when Ca^{2+} had been liberated from the ryanodine-sensitive Ca^{2+} stores zymogen activation could no longer be induced (210). However, in other studies on intact pancreatic acinar cells, caffeine did not release any Ca^{2+} from intracellular stores (28, 213, 214), and its principal action in these cells was to inhibit opening of IP_3 receptors (28) as well as ACh-elicited IP_3 formation (213, 214). The action of caffeine is almost instantly reversible, as cell membranes are extremely permeable to caffeine (214). The effect of pretreatment with caffeine in the experiments of Husain et al. (210) may therefore have been very short lasting. Furthermore, evidence from two different groups shows that

hyperstimulation-elicited protease activation starts in the apical granule-rich region and thereafter spreads out to the whole cell (42, 46, 47) rather than being initiated in the basolateral region as suggested by Husain et al. (210). Given that trypsinogen activation evoked by fatty acid ethyl esters (FAEEs) in permeabilized pancreatic acinar cells can be virtually abolished by knockout of both type 2 and 3 IP_3Rs, it seems unlikely that there is a specific role for RyRs in the initiation of AP. Because zymogen activation in intact cells depends on Ca^{2+} release-activated Ca^{2+} influx (40, 215), any inhibition of the opening of RyRs or IP_3Rs will tend to reduce intracellular protease activation evoked by AP-inducing agents.

Intracellular trypsinogen activation and vacuole formation occur within the same time frame (47), indicating that these two events might be linked mechanistically. This question was investigated in dynamic imaging studies on isolated acinar cell clusters (48). EM pictures indicated that the intracellular vacuoles formed in response to CCK hyperstimulation might be endocytic. The pancreatic acini were therefore incubated together with an extracellular fluorescent tracer, Texas Red dextran, and the uptake of this tracer was monitored continuously by two-photon microscopy during prolonged stimulation with a high CCK concentration (10 nM). These studies demonstrated the endocytic nature of the vacuole formation. The large vacuoles are formed complete, by disconnection of postexocytotic structures, and not assembled by fusion of many small endosomes. The process of endocytosis, observed directly, showed the formation of an extended thin channel between the acinar lumen and the postexocytotic structure. This channel became gradually longer and thinner and finally broke, leaving large vacuoles containing extracellular markers inside the cell (48). Importantly, these studies also demonstrated that it is exactly in these endocytic vacuoles that trypsinogen activation takes place (48). The vacuoles are acidic, because of operation of a bafilomycin-sensitive proton pump, and they are also very permeable to Ca^{2+}, as seen in experiments in which the vacuoles were loaded with a caged Ca^{2+} compound. Uncaging Ca^{2+} by UV laser light caused a marked increase in $[Ca^{2+}]$ inside the vacuoles, but this was only very transient, as Ca^{2+} leaked out quickly. As already mentioned, CCK stimulation causes NAADP formation, and a major part of the NAADP-elicited Ca^{2+} release from acidic stores, including endocytic structures, occurs through TPCs (194). It is therefore possible that a major part of the Ca^{2+} outflow from the vacuoles occurs through TPCs. In this context it is interesting that cellular uptake of SARS-CoV-2 is abolished by bafilomycin and can also be markedly reduced by inhibition of TPCs (216). The mechanism that is responsible for intracellular trypsin activation causing AP may therefore also be important for SARS-CoV-2 infection (216).

Recently, the pancreatic vacuoles have been studied further by Tepikin and his collaborators (217), who demonstrated the rupture of such vacuoles inside pancreatic acinar cells and also fusion of the vacuoles with both the apical (luminal) and basal plasma membrane. Thus active trypsin will be delivered into the cytosol and also into both the acinar lumen and the interstitial fluid (**FIGURE 5**). This work (217) provides evidence for the mechanism by which active trypsin formed in the postexocytotic endocytic vacuoles can induce damage not only to the cells in which the vacuoles have been formed but also to other cells in its environment (see also sect. 9).

Although studies employing hyperstimulation of CCK receptors have provided important insights into the mechanism by which AP is initiated, real AP is not caused by high circulating concentrations of CCK. To the best of our knowledge, high (nM) plasma concentrations of CCK have never been reported. However, a related hormone, gastrin, is produced in excess in Zollinger–Ellison syndrome, resulting in high plasma levels of gastrin (218). Gastrin can activate CCK receptors on pancreatic acinar cells, but only at concentrations much higher than those required for activation by CCK itself (219). Although there have been sporadic reports of a few cases of AP associated with Zollinger–Ellison syndrome, this does seem to be extremely rare (220). It is clear that hyperstimulation of CCK receptors is not an important cause of AP. It was therefore of interest to determine whether the excessive and prolonged elevations of $[Ca^{2+}]_i$, which have been shown to be crucial for the development of AP-like features in the hyperstimulation models (46, 47), also occur in, and are critical to the development of, real AP induced as a consequence of gallstones or alcohol abuse.

4.2. Ca^{2+} Signals Elicited by Bile Acids

In 2002, two groups independently discovered that certain bile acids evoked Ca^{2+} signals in pancreatic acinar cells (43, 221). One study focused on the effects of monohydroxy bile acids, in particular taurolithocholic acid 3-sulfate (TLC-S) (221), whereas the other mainly employed a bile acid mixture (43). In both studies bile acids evoked rises in $[Ca^{2+}]_i$, but the mechanisms proposed were different.

With regard to which bile acids are able to cause $[Ca^{2+}]_i$ rises in acinar cells, there is general agreement about the actions of one but discrepancies with regard to some others. TLC-S, at concentrations between 100 and 500 μM, clearly evokes rises in $[Ca^{2+}]_i$. Depending on the concentration, it can evoke short-lasting repetitive local spikes in the apical region dominated by zymogen granules or prolonged global $[Ca^{2+}]_i$ elevations

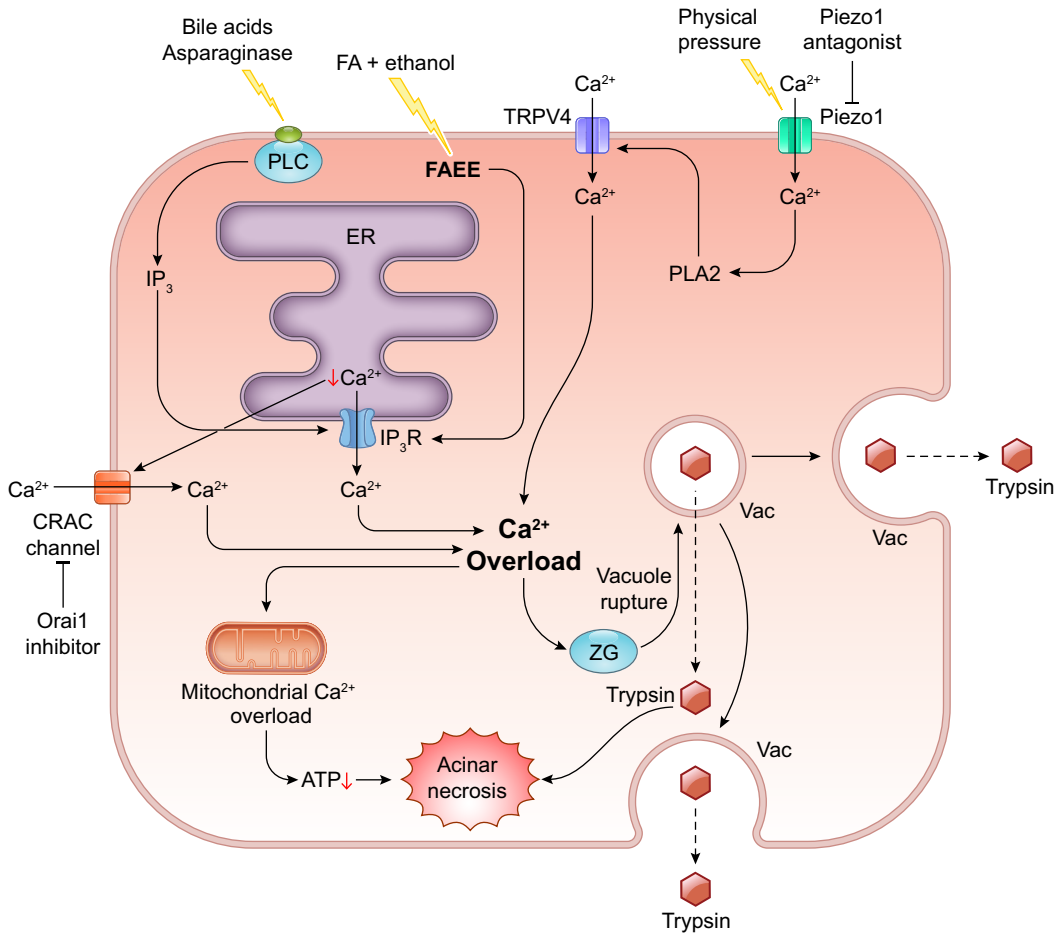

FIGURE 5. Schematic diagram illustrating the processes involved in the pathological Ca^{2+} overloading of pancreatic acinar cells induced by a combination of fatty acids (FA) and alcohol, bile acids, asparaginase, or physical pressure. The diagram also shows the consequences of Ca^{2+} overloading, namely reduced ATP formation, vacuole (Vac) formation, trypsinogen activation, and release of trypsin into the cytosol as well as into the extracellular solution, leading to acinar necrosis. Orai1 inhibitors can be used to reduce opening of CRAC channels, whereas Piezo1 antagonists can reduce Ca^{2+} inflow, not only through Piezo1 channels but, indirectly, also through TRPV4 channels. ZG, zymogen granule.

(221, 222). The situation is quite different for taurocholate (TC). In one study, 5 mM TC only evoked short-lasting $[Ca^{2+}]_i$ spikes (221), whereas in another study a much lower concentration (300 μM) evoked a small and very slowly rising $[Ca^{2+}]_i$ elevation (43). In an earlier study TC failed to evoke any rise in $[Ca^{2+}]_i$ (223), and in more recent work (68) TC (5 mM) also had no effect on $[Ca^{2+}]_i$ in the acinar cells but evoked substantial Na^+-dependent rises in $[Ca^{2+}]_i$ in the immediately neighboring stellate cells (see sect. 5). TC is supposed to be taken up into cells via the Na^+-taurocholate cotransporting polypeptide, which was reported to be present in rat pancreatic acinar cells (43) but was not found in these cells in a later study on mouse pancreatic lobules in which it was, however, clearly identified in the stellate cells (68). Overall, there must be doubts about the ability of TC to induce pathologically important Ca^{2+} signals in pancreatic acinar cells. The situation is clearly complicated by the fact that TC causes major Ca^{2+} signals in the stellate cells and in these cells causes nitric oxide (NO) production via Ca^{2+} activation of nitric oxide synthase (NOS) (68, 224) (see sect. 5). Pathological effects of TC on the acinar cells may therefore be indirect via primary action on stellate cells (see sect. 9). The actions of other bile acids on acinar cells have only been studied sporadically. Taurodeoxycholate and taurochenodeoxycholate were reported to evoke sustained $[Ca^{2+}]_i$ elevations in acinar cells (43, 223), whereas deoxycholate had no effect (225). Cholate had no effect on acinar cells but, in the same experiments, evoked substantial sustained $[Ca^{2+}]_i$ elevations in neighboring stellate cells (68).

The mechanism of action of TLC-S has been investigated in some detail and in many ways seems to conform to the mechanism of action established for muscarinic receptor activation (221). Thus the initial $[Ca^{2+}]_i$ rise evoked by TLC-S is independent of the presence of external Ca^{2+}, whereas the following sustained elevation is acutely dependent on the presence of Ca^{2+} in the extracellular fluid. The Ca^{2+} signal generation depends on functional IP_3Rs, as it is immediately

blocked by caffeine (213, 221), which is also the case for the Ca^{2+} signals evoked by ACh (28). Although caffeine is best known as an activator of RyRs, it is also an effective, but low-affinity, inhibitor of the opening of IP_3Rs (28, 91, 201). In a study of permeabilized acinar cells, it was shown that TLC-S evokes release of Ca^{2+} from both the ER and acidic stores through both IP_3Rs and RyRs (222).

What is the primary step in the action of TLC-S? Following the discovery of the cell surface G protein-coupled bile acid receptor Gpbar1 (226), Perides et al. (227) tested the hypothesis that the action of TLC-S on pancreatic acinar cells is mediated by this pathway. They found that Gpbar1 is expressed at the apical pole of mouse pancreatic acinar cells and that deletion of this receptor markedly reduced pathological Ca^{2+} signal generation and intracellular zymogen activation induced by TLC-S but not by caerulein (227). Furthermore, they found that Gpbar1 deletion also reduced all parameters associated with bile acid-induced AP in vivo but, in contrast, had no protective effect on caerulein-induced AP (227). These data indicate that bile acids, which under pathological conditions would approach the acinar cells from the lumen acting on the apical membrane, initiate excessive Ca^{2+} signal generation by binding to the bile acid receptor Gpbar1, also known as TGR5 (228). Our knowledge about bile acid interaction with Gpbar1 has mostly been obtained from studies on liver cells and various cell lines. In general, it would appear that Gpbar1 can activate a number of different G proteins including Gq, which is coupled to PLC and thereby can generate IP_3 (228).

A different model of bile acid action on pancreatic acinar cells was presented by Kim et al. (43). They proposed that bile acids are taken up into the acinar cells via the Na^+-taurocholate cotransporting polypeptide and inside the cells inhibit the ER Ca^{2+} pump, causing gradual loss of Ca^{2+} from the ER, which would then in turn open up store-operated Ca^{2+} channels in the plasma membrane, resulting in Ca^{2+} influx. Later work from the same group proposed that TC evoked Ca^{2+} influx through TRPC3 channels (44). However, as mentioned above, recent evidence indicates that the Na^+-taurocholate cotransporting polypeptide may not be present in pancreatic acinar cells (68). On the basis of all the evidence currently available, it seems most likely that bile acids exert their effect on the pancreatic acinar cells by binding to the bile acid receptor Gpbar1 and thereby initiate the excessive Ca^{2+} signal generation that causes necrosis.

Although the initial step evoked by bile acids is release of Ca^{2+} from intracellular stores, it is the subsequent opening of CRAC channels that leads to the sustained Ca^{2+} overload that ultimately destroys the cell (**FIGURE 5**). Gallstone migration is generally accepted as the most frequent cause of AP (229), and injection of TLC-S retrogradely into the bile duct causes experimental AP (57, 213), whereas control injections of saline retrogradely into the pancreatic duct did not induce damage (57). A common duct connecting the bile duct and the pancreatic duct—which would allow direct passage of bile acids from the liver and the gallbladder into the pancreatic duct and therefore into the pancreatic tissue—is found in about two-thirds of the population but is found more frequently in patients with AP than in the general population (229). These arguments would seem to lead to the conclusion that direct effects of bile acids on pancreatic acinar cells are causing the human disease AP. However, there is an opposing view. The strongest argument comes from experiments carried out on the American opossum, in which the severities of AP induced by ligation at different sites of the pancreatic-biliary duct system were compared (230). Three experimental situations were compared: separate ligations of the bile duct and the pancreatic duct, ligation of the pancreatic duct alone, and ligation of the common duct. Only the last ligation would have allowed bile acids to enter the pancreatic duct. The result was that the severity of the induced AP was the same in all three cases. This led to the conclusion that flow of bile acids into the pancreatic tissue is not important for the development of gallstone-related AP but that it is the obstruction of fluid flow from the pancreas, generating a high pressure in the pancreatic duct, that is the principal cause of the disease (230). It is now clear that pressure itself can indeed induce pancreatitis, via a rise in $[Ca^{2+}]_i$, and a plausible molecular mechanism for this effect has recently been proposed (**FIGURE 5**) (see also sect. 4.5). This does not, however, mean that the importance of the direct effects of bile acids on the acinar cells can be dismissed. In the study from Robert Sutton's group (57), retrograde injections of fluid into the pancreatic duct only induced AP when a bile acid was included. Bile that has been stored in the gallbladder can have very high bile acid concentrations, due to the fluid reabsorption by the gallbladder epithelium (231, 232), and if bile with such high bile acid levels reaches the pancreatic cells, significant effects would be expected (68).

Is AP caused by gallstone complications mostly due to pressure on the acinar cells or direct effects of bile acids on these cells? At this point in time a definitive answer to this question cannot be provided. There is very clear evidence for the existence of both mechanisms, and there is no logical reason for excluding one of them. It is entirely possible that AP is caused by a mixture of pressure and direct effects of bile acids, and it cannot be excluded that the relative importance of the two mechanisms may vary depending on the specific circumstances.

4.3. Ca^{2+} Signals Evoked by Alcohol and Fatty Acids

Since alcohol abuse has long been recognized as one of the main causes of AP and since sustained elevations of $[Ca^{2+}]_i$ in pancreatic acinar cells have been shown to initiate events leading to AP, as discussed in sects. 4.1 and 4.2, it was not unreasonable to expect that exposure of isolated acinar cells to ethanol would cause major Ca^{2+} signaling events. However, when the appropriate experiments were done, it turned out that the acinar cells were surprisingly insensitive to even very high (>500 mM) ethanol concentrations (38). In most cases even 850 mM ethanol only evoked a minor, but sustained, elevation of $[Ca^{2+}]_i$. In a minority of the cells investigated, this extraordinarily high ethanol concentration evoked a substantial, but only transient, increase in $[Ca^{2+}]_i$ followed by a tiny sustained elevated $[Ca^{2+}]_i$ level (38). The possibility that it was not ethanol itself, but a metabolite, that exerted the toxic effect on the pancreas was therefore investigated. Ethanol is oxidized by a process, catalyzed by alcohol dehydrogenase, that generates acetaldehyde. However, acetaldehyde turned out not to generate any Ca^{2+} signals in the acinar cells, even at a high (5 mM) concentration (38).

In a study completely unrelated to Ca^{2+} signaling, Laposata and Lange (233) had much earlier presented evidence indicating that alcohol-related organ damage, particularly in the pancreas, was caused not by ethanol itself but rather by the nonoxidative combination of ethanol with long-chain fatty acids (FAs), generating fatty acid ethyl esters (FAEEs). They also demonstrated that the pancreas produced FAEEs more efficiently than other organs (233). The early findings of Laposata and Lange (233) were brought into the context of Ca^{2+} signaling studies when it was discovered that FAEEs very effectively induced substantial increases in $[Ca^{2+}]_i$ (38). The actions of palmitoleic acid ethyl ester (POAEE) were investigated in particular detail. When POAEE was added on top of a high ethanol concentration, which in itself had only induced a very minor $[Ca^{2+}]_i$ elevation, there was a substantial increase in $[Ca^{2+}]_i$ (38). This happened at pathophysiologically relevant POAEE concentrations (38) that had been found in the pancreas from patients who had been admitted to hospital in an emergency, following excessive alcohol intake, and died as a result of severe AP (233). The effect of POAEE could be mimicked by other long-chain FAEEs, both saturated and unsaturated, namely palmitic acid ethyl ester, arachidonic acid ethyl ester, and arachidic acid ethyl ester (38).

The POAEE-elicited rise in $[Ca^{2+}]_i$ is primarily due to release of Ca^{2+} from intracellular stores (37) and is then followed by Ca^{2+} entry (37) due to opening of store-operated Ca^{2+}-selective CRAC (Orai1) channels (**FIGURE 5**). The prolonged elevation of $[Ca^{2+}]_i$ causes intracellular protease activation and necrotic cell death (37, 38, 40). FAs can also induce $[Ca^{2+}]_i$ elevations, but these develop more slowly than those elicited by FAEEs (37). The mechanism by which POAEE elicits intracellular Ca^{2+} release has been investigated in studies on permeabilized acinar cells. Pancreatic acinar cells were isolated from wild-type mice, as well as from mice in which IP_3 receptors of type 2, or both types 2 and 3, had been deleted. The data obtained showed that the POAEE-elicited intracellular Ca^{2+} release was markedly reduced but not abolished by inhibition or deletion of IP_3Rs. There was also a relatively smaller reduction in the POAEE-elicited Ca^{2+} release by inhibition of RyRs. When both IP_3Rs and RyRs were inhibited, POAEE hardly evoked any release of Ca^{2+} from the intracellular stores. POAEE liberated Ca^{2+} from both the ER and the acidic stores. The release from the acidic stores was markedly reduced when type 2 IP_3 receptors were knocked out and almost abolished when both types 2 and 3 had been deleted (42). In these experiments it was also shown that, in parallel with the reduced Ca^{2+} release evoked by POAEE, when IP_3Rs were either inhibited pharmacologically or knocked out there was a quantitatively similar reduction in the degree of trypsinogen activation (42). It would therefore appear that the major release of Ca^{2+} from the intracellular stores elicited by POAEE occurs through IP_3Rs, qualitatively exactly like the primary event induced by ACh stimulation, but only to a much larger extent. The release of Ca^{2+} from the acidic stores would appear to be related to the intracellular trypsinogen activation (42), but, unfortunately, we still do not understand the mechanism by which this happens. There is direct evidence showing that IP_3 can release Ca^{2+} from isolated zymogen granules and that this is sufficient to cause a significant rise in the local $[Ca^{2+}]_i$ surrounding the granules (188). This may be important, since clamping $[Ca^{2+}]_i$ at the normal resting level in the permeabilized cells used by Gerasimenko et al. (42) prevented trypsinogen activation. Mechanisms have been proposed for how Ca^{2+} release from zymogen granules could lead to trypsinogen activation, based on ion exchange theories from other systems (201, 234, 235), but they are speculative. Detailed studies of ion transport mechanisms across the membrane of the zymogen granules combined with analysis of changes in intragranular ionic concentrations will be needed before we can reach any valid conclusions. We also lack an understanding of the mechanism by which FAEEs open IP_3Rs. The fact that the action of FAEEs depends on functional IP_3Rs does not by itself imply that there needs to be activation of PLC and therefore additional generation of IP_3. In the study by

Gerasimenko et al. (42), PLC inhibition did not prevent the POAEE-elicited trypsinogen activation. Possibly, FAEEs in some way sensitize IP_3Rs to the resting level of IP_3.

How do FAs and ethanol elicit the excessive Ca^{2+} signals that can destroy the acinar cells? FAs can be taken up into cells by FA transporters, and in the presence of ethanol, which moves easily across plasma membranes, FAs can combine with ethanol to form FAEEs inside the acinar cells. This process is catalyzed by carboxyl ester lipase (236). This enzyme is mainly concentrated in the apical zymogen granule-containing region (236), and this is therefore the site where the FAEEs would be primarily generated and where they would primarily act to allow opening of IP_3Rs in both the apical ER extensions as well as the acidic stores, including the zymogen granules. After the Ca^{2+} stores in the ER have been emptied, store-operated Ca^{2+} entry across the whole of the basolateral membrane would be triggered as already explained (see sect. 3).

As is the case with the $[Ca^{2+}]_i$ elevations caused by hyperstimulation of CCK receptors or by exposing acinar cells to bile acids, the prolonged high levels of $[Ca^{2+}]_i$ elicited by FAEEs have important consequences. The overloading of the mitochondria with Ca^{2+} causes opening of the mitochondrial permeability transition pore (237), depolarizing the inner mitochondrial membrane (37), and this reduces mitochondrial ATP production (55). The combination of metabolic failure and intracellular trypsinogen activation is responsible for the necrotic cell death (**FIGURE 5**).

As already mentioned, FAs alone can also evoke an increase in $[Ca^{2+}]_i$, but the mechanism of action seems somewhat different from that of FAEEs. It would appear that FAs do not primarily act to release Ca^{2+} from intracellular stores but essentially work by inhibiting ATP production. This could be shown in experiments in which the effect of direct intracellular ATP infusion, via a patch-clamp pipette (whole cell recording configuration), was tested. In the case of stimulation with POAEE the sustained rise in $[Ca^{2+}]_i$ was transformed into repetitive spiking by the ATP supplementation, whereas in the case of palmitoleic acid (POA) stimulation the infusion of ATP prevented POA from evoking any change in $[Ca^{2+}]_i$ (37).

An attack on the pancreas by the combination of ethanol and FAs creates a vicious circle in which the primary intracellular Ca^{2+} release, followed by Ca^{2+} entry, leads to overloading of the cell with Ca^{2+}, which is worsened by the reduction of mitochondrial ATP production because this prevents the Ca^{2+} pumps in both the plasma membrane and the ER from disposing of the excess Ca^{2+} in the cytosol. This leads to further overloading of the mitochondria with Ca^{2+}, further exacerbating the metabolic situation. The excessive and sustained $[Ca^{2+}]_i$ elevation induced by FAEEs can be prevented or made reversible by pharmacological inhibition of the opening of the store-operated Orai1 channels (**FIGURES 3 and 5**). This will also prevent the intracellular protease activation and the subsequent necrotic cell death (40, 146, 215).

It is clear from all the data reviewed in this section that alcohol-induced damage of pancreatic acinar cells can be mediated by FAEEs and that the toxic effects are due to excessive and sustained elevations of $[Ca^{2+}]_i$. However, this does not prove that alcohol-related AP is caused by FAEEs. The key question is whether alcohol intake in humans can generate FAAEs in concentrations that are sufficiently high to be able to cause the damage seen in the experiments on isolated acinar cells. In alcohol drinking experiments in healthy subjects blood ethanol levels of ~30 mM were measured, whereas blood ethanol concentrations of ~70 mM have been measured in alcoholic subjects (62). At a 30 mM blood alcohol concentration, the peak FAEE concentration in serum reached levels of ~2 mM (238). The FAEE concentration declined quickly without further alcohol intake, but elevated serum FAEE levels (~50 μM) persisted for 100 h (238). As shown by Laposata and Lange (233), the pancreas has a particularly high capacity for FAEE production and after alcohol intake the human pancreas can contain both saturated and unsaturated FAEEs at concentrations in excess of 100 μM. It would therefore appear that the 10–100 μM concentrations of FAEEs that have been demonstrated to evoke substantial $[Ca^{2+}]_i$ elevations causing necrosis in isolated acinar cells (38) are easily attained in humans in vivo.

4.4. Asparaginase-Induced $[Ca^{2+}]_i$ Elevations

Although AP is mainly caused by gallstone complications and alcohol abuse, there is another toxic agent for the pancreas that is significant for children undergoing treatment for acute lymphoblastic leukemia. For many years now this disease has been treated very successfully by L-asparaginase (240). Asparaginase acts by depriving cancer cells of asparagine, which they, in contrast to normal cells, cannot produce themselves. However, in ~5–10% of the cases there is a side effect resulting in AP (241). If the attack of AP is severe, the asparaginase treatment has to be discontinued, with the risk of inadequate treatment of the leukemia. Given that other toxic agents inducing AP do so by primarily overloading the pancreatic acinar cells with Ca^{2+}, as already described, it was of interest to investigate whether asparaginase would also have such an effect. Asparaginase does indeed elicit Ca^{2+} signals in the acinar cells, and, depending on the concentration

used, these can be either repetitive short-lasting spikes or sustained $[Ca^{2+}]_i$ elevations (242). These effects of asparaginase are independent of the presence or absence of asparagine (242). Therefore, the side effect of asparaginase leading to AP is caused by a mechanism that is completely different from the mechanism that kills the cancer cells.

The mechanism by which asparaginase elicits Ca^{2+} signals in pancreatic acinar cells has been investigated, and it turns out that it acts in much the same way as ACh. The PLC inhibitor U73122 abolishes asparaginase-elicited Ca^{2+} signaling, and caffeine, inhibiting the opening of IP_3Rs, also markedly reduces the effect. There is also involvement of RyRs, as ryanodine reduces the asparaginase-induced $[Ca^{2+}]_i$ elevation. As with other stimuli described above, Ca^{2+} signals can be initiated in the absence of external Ca^{2+}, but the sustained $[Ca^{2+}]_i$ elevation depends acutely on the presence of Ca^{2+} in the extracellular fluid (242). It would appear that asparaginase acts by binding to protease-activated receptors on the plasma membrane, as Ca^{2+} signal generation is abolished by a blocker of the type 2 protease-activated receptor (242). Asparaginase destroys the acinar cells in much the same way as bile acids and FAEEs, by primarily evoking excessive intracellular Ca^{2+} release, which then opens CRAC channels, overloading the cytosol with Ca^{2+}. This will inevitably cause all the deleterious downstream effects already described (242) (**FIGURE 5**).

Asparaginase markedly reduces the rate of active Ca^{2+} extrusion across the plasma membrane, which almost certainly is due to the reduction in ATP production caused by mitochondrial Ca^{2+} overloading leading to depolarization of the inner mitochondrial membrane (243). ATP production is thus markedly reduced by asparaginase, as it is with other AP-inducing agents (**FIGURE 5**). However, there is also another element. It appears that glucose utilization is inhibited by AP-inducing agents including asparaginase. This may be due to inhibition of hexokinases (243), but the requirement for the initial and crucial physiological formation of glucose-6-phosphate can be bypassed by using galactose (**FIGURE 6**). A marked protection of ATP formation can therefore be achieved in the early stage of AP by galactose feeding (**FIGURE 6**).

It is challenging to determine whether the acute $[Ca^{2+}]_i$-elevating effects of asparaginase seen in isolated acinar cells, and their consequences, are processes that actually occur in clinical cases of AP induced by the asparaginase-based treatment of acute lymphoblastic leukemia. In the case of alcohol-related AP, the disease process can develop very quickly, within hours after an alcoholic binge, but AP occurring as a result of asparaginase treatment develops much more slowly, typically after several injections of the enzyme over a number of weeks. The discrepancy between the time course required for the acute laboratory experiments on isolated acinar cells and the actual disease development in patients is therefore much more problematic than in the case of, for example, alcohol-related AP. In the isolated cell experiments, particularly with regard to $[Ca^{2+}]_i$ measurements, only acute effects can be properly examined, which necessitates the use of asparaginase concentrations that are well above those attained during clinical use. Although it is a plausible hypothesis that the

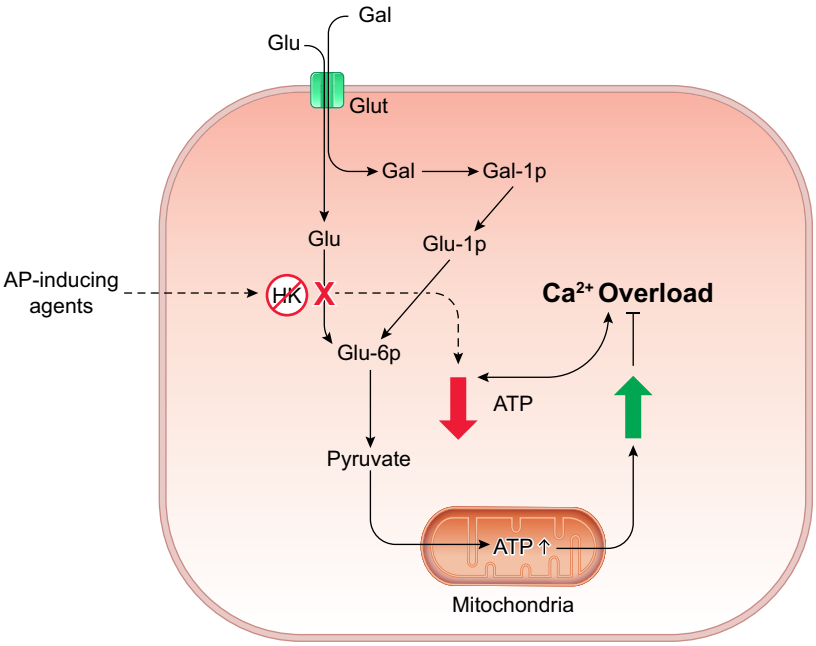

FIGURE 6. Schematic diagram illustrating how galactose, by circumventing the blocked hexokinase (HK) step in AP, can restore ATP formation and thereby prevent Ca^{2+} overloading. Gal, galactose; Gal-1p, galactose-1-phosphate; Glu, glucose; Glu-1p, glucose-1-phosphate; Glu-6p, glucose-6-phosphate; Glut, glucose transporter.

laboratory experiments reviewed here have revealed data relevant to real AP in children treated with asparaginase, this has not yet been proven. However, the relevance of the in vitro experiments has been strengthened by in vivo data showing that pretreatment with galactose for 24 h before asparaginase administration, and continued galactose feeding during the following 4-day period before the mice were euthanized, markedly reduced the degree of pancreatic inflammation and necrosis (243).

4.5. Pressure-Induced Ca^{2+} Signals

Pancreatic duct ligation, which inevitably increases the intraductal pressure, induces a slow and gradual increase in $[Ca^{2+}]_i$ in pancreatic acinar cells and leads to AP (244). It has also been known for some time that duct obstruction causes colocalization of zymogen granules and lysosomes, a process that is important in the initiation of AP (245). Furthermore, acinar cells isolated from a pancreas with an obstructed duct have a high level of intracellular trypsin activity (166). Physical pressure on the pancreas, during surgery or as a consequence of a blow to the abdomen, can also induce AP (200). As already mentioned, gallstone complications (obstruction of duct) are frequent causes of AP. Although bile acid reflux into the pancreatic tissue is a plausible hypothesis for the development of AP, given that bile acids can induce excessive Ca^{2+} signal generation, it is possible, and even likely, that pressure itself could be an important component of the disease process. Recently, it has been shown that the mechanically activated cation channel Piezo1 is expressed in pancreatic acinar cells and mediates pressure-induced AP (**FIGURE 5**) (246). Piezo1 is a nonselective cation channel, and Na^+, K^+, Ca^{2+}, and Mg^{2+} can all pass through the pore (247). Piezo1 can be directly activated to open by physical pressure, but a small synthetic molecule named Yoda1 can also open Piezo1 without any exertion of physical pressure, and this has become a useful experimental tool (248). Opening of Piezo1 can be inhibited very specifically by the spider toxin GsMTx4 (247). In pancreatic acinar cells, it has been shown that Yoda1 evokes a rise in $[Ca^{2+}]_i$, which can be blocked by GsMTx4. When Piezo1 was specifically ablated in the acinar cells, Yoda1 failed to evoke any increase in $[Ca^{2+}]_i$, whereas CCK, as usual, induced Ca^{2+} signals. Yoda1 also induced leakage of lactate dehydrogenase from the acinar cells and necrosis (246). Furthermore, it was shown that Yoda1 could by itself, in the absence of any exertion of pressure, induce AP in vivo (246). In contrast to all the other pathological Ca^{2+} signal generation mechanisms described in this section, activation of Piezo1 produces an elevation of $[Ca^{2+}]_i$ purely by causing Ca^{2+} influx without any need for an initial liberation of intracellularly stored Ca^{2+} (246).

More recently, it has become clear that the situation is more complicated than initially thought. It turns out that activation of Piezo1 is the initial and essential, but not sufficient, element in the process of generating a sustained pressure-induced $[Ca^{2+}]_i$ elevation causing AP (249). The major and sustained part of the Ca^{2+} influx occurs through opening of TRPV4 channels (**FIGURE 5**). Mice in which the TRPV4 gene had been deleted were protected from Piezo1 agonist- and pressure-induced pancreatitis (249). In the absence of TRPV4 channel opening, activation of Piezo1 only evoked a transient rise in $[Ca^{2+}]_i$, which was insufficient to evoke mitochondrial depolarization, trypsinogen activation, and necrosis. Thus, the dangerous Ca^{2+} overloading of the acinar cells that triggers the processes that cause necrotic cell death is due to Ca^{2+} influx mediated by TRPV4 channels (249). Further analysis indicates that Piezo1 opening leads to phospholipase A_2 activation, which in turn is responsible for the opening of the TRPV4 channels (**FIGURE 5**). The role of TRPV4 channels in the development of AP may be limited to pressure-induced AP, because pharmacological blockade of these channels did not prevent the sustained elevation of $[Ca^{2+}]_i$ elicited by a high CCK concentration (249). In contrast, inhibition of the opening of Orai1 channels by CM4620, which markedly reduced the magnitude of the elevated $[Ca^{2+}]_i$ plateau induced by CCK hyperstimulation, had no effect on the Ca^{2+} entry evoked by the Piezo1 activator Yoda1 (249). Although a minor role of TRP4 in the development of AP induced by bile acids, alcohol and fatty acids, or asparaginase cannot be completely excluded, there is currently no evidence for it. On the basis of the available data reviewed in this section, it would appear that AP induced by ethanol and FAs, bile acids, CCK hyperstimulation, or asparaginase is due to intra-acinar Ca^{2+} overloading due to Ca^{2+} influx via CRAC channels of the Orai1 type, whereas pressure-induced AP is mediated by Ca^{2+} influx via TRPV4 channels (**FIGURE 5**).

4.6. Ca^{2+}-Mediated Impairment of Mitochondrial Function

As discussed in sect. 3, physiological Ca^{2+} spikes in the cytosol give rise to transient rises in the mitochondrial $[Ca^{2+}]$, stimulating the Krebs cycle and thereby generating ATP in a process that can be described as stimulus-metabolism coupling. The mitochondrial Ca^{2+} uptake, mediated by the mitochondrial Ca^{2+} uniporter (51, 53, 99), inevitably causes a small depolarization of the inner mitochondrial membrane. However, whereas physiological stimulation of the pancreatic acinar cells with ACh

and CCK gives rise to small mitochondrial depolarizations and rises in the NAD(P)H levels, pathological agents, in concentrations that can induce AP, such as bile acids, ethanol plus FAs, physical pressure, and asparaginase, evoke larger mitochondrial depolarizations and reduce the levels of NAD(P)H, indicating loss of ATP formation (37, 57, 242, 243, 249) Direct monitoring of changes in ATP levels, employing cytosolic- or mitochondrial-targeted luciferases, showed that pathological stimuli evoked an acute reduction in both mitochondrial and cytosolic ATP levels (55). The larger mitochondrial depolarization evoked by pathological stimulation appears to be due to opening of the mitochondrial permeability transition pore (57, 250), and mitochondrial depolarization occurring as a consequence of the opening of this large channel is known to prevent ATP generation (237). Specific genetic or pharmacological deletion or inhibition of the mitochondrial permeability transition pore (251–253) prevented the mitochondrial depolarization in acinar cells evoked by different pathological stimuli and also prevented the loss of ATP generation and the subsequent necrosis (57, 250). Indeed, all biochemical, immunological, and histopathological parameters were dramatically improved by abolishing the operation of the permeability transition pore in in vivo mouse models of AP (57).

The conclusion that reduced ATP production is central to the development of AP has been reinforced by experiments, already described, in which it was shown that boosting metabolism by administration of galactose (or directly by pyruvate) markedly reduced the loss of ATP production and thereby prevented necrosis (**FIGURE 6**).

4.7. Effector Mechanisms of Ca^{2+} Toxicity: the Roles of Calmodulin and Calcineurin

The overloading of the cytosol with Ca^{2+}, which has been described in sects. 4.1 to 4.5 in relation to a number of pathological challenges, gives rise to what can be termed Ca^{2+} toxicity. As detailed above, all agents inducing AP elicit abnormal sustained global increases in $[Ca^{2+}]_i$, and the result of this Ca^{2+} toxicity is acinar necrosis. This, in turn, triggers the inflammatory response that often has a fatal outcome. In this section we focus on the initial steps by which an abnormally high and global $[Ca^{2+}]_i$ elevation starts the destructive process. The initial event must be binding of Ca^{2+} to effector proteins. The effector proteins are Ca^{2+}-binding proteins, and one of the most important is calmodulin. Binding of Ca^{2+} to calmodulin results in a conformational change of the protein allowing interaction of the Ca^{2+}-calmodulin complex with numerous other proteins (254). A well-known physiological example of such a process is the activation of the plasma membrane Ca^{2+} pump that occurs in response to a rise in $[Ca^{2+}]_i$, which is mediated by Ca^{2+}/calmodulin interaction with the pump protein (134). Here, we are focusing on the role of calmodulin in mediating the destruction of the acinar cells in AP. In the exocrine pancreas there is direct evidence showing that both ACh and CCK can induce translocation of calmodulin. Short pulses of ACh stimulation cause movement of calmodulin into the secretory granule region, whereas prolonged stimulation with supramaximal secretagogue concentrations evokes translocation into the nucleus (255).

One of the most important molecules activated by Ca^{2+}/calmodulin is the serine/threonine phosphatase calcineurin (256). Husain et al. (257) provided the first evidence indicating that the intracellular activation of proteases induced by excessive rises in $[Ca^{2+}]_i$ was mediated by calcineurin. In a study on isolated rat pancreatic acini, they showed that the Ca^{2+}-dependent intracellular chymotrypsin activity induced by supramaximal stimulation of CCK receptors was markedly reduced by the calcineurin inhibitors FK506 and calcineurin inhibitory peptide, whereas these inhibitors did not have any effect on enzyme secretion. The calcineurin inhibitors had no effect on the cytosolic Ca^{2+} signals evoked by CCK receptor activation, indicating that they were acting at a step after this initial signal generation (257). Further evidence for the importance of calcineurin for AP induction was obtained in studies with hyperstimulation of the muscarinic receptors (258). An important in vivo study on mice confirmed the importance of calcineurin activation for the initiation of AP (259). In this investigation, FK506 markedly reduced pancreatic trypsin activity induced by CCK hyperstimulation as well as many other markers of AP, including acinar cell vacuolization, inflammation, and serum amylase levels (259).

Hyperstimulation of CCK or muscarinic receptors is not the cause of clinical AP, so it is important that evidence for the role of calcineurin has also been obtained in relation to bile-induced AP (260). In this study the effect of three well-known calcineurin inhibitors, FK506, calcineurin inhibitory peptide, and cyclosporine, were tested, and it was shown that they markedly reduced leakage of lactate dehydrogenase from acini as well as uptake of propidium iodide (260).

One of the most important roles of calcineurin in the immune system is to regulate the activity of NFAT (nuclear factor of activated T cells) proteins. Calcineurin controls the transport of NFAT into the nucleus, where it plays an important role in cytokine gene transcription (261). There is also evidence that in the pancreas one form of NFAT, NFATc3, is mediating the calcineurin-induced damage to pancreatic acinar cells in AP, as

pharmacological NFAT inhibition or lack of NFATc3 prevented trypsinogen activation (262). A more recent study from Husain and collaborators (263) confirms NFAT activation in two in vivo AP models as well as in isolated mouse and human pancreatic acinar cells. However, pharmacological blockade of calcineurin-NFAT interaction failed to have any effect on acinar necrosis, raising some doubts about the pathophysiological role of NFAT in AP (263). Although the evidence for a critical role of calcineurin in AP induction seems compelling, the precise molecular steps influenced by calcineurin in this process need further clarification.

5. Ca^{2+} SIGNALING IN STELLATE CELLS

Pancreatic stellate cells have many similarities with hepatic stellate cells and have long been thought to play an important role in the production of the fibrotic matrix that is characteristic of chronic pancreatitis and pancreatic cancer (66, 264, 265). The stellate cells are supposed to exist in two states, quiescent and activated. In the normal healthy pancreas they are expected to be quiescent but may be important for the turnover of the extracellular matrix (266), whereas under disease conditions, most notably pancreatic cancer, they change to an activated myofibroblast-like phenotype, which leads to proliferation and migration (66).

Pancreatic stellate cells in the normal pancreas can display considerable Ca^{2+} signaling activity in response to a variety of stimuli (67–69), but they are not electrically excitable, because depolarization of the plasma membrane by exposure of the cells to a solution with a high (100 mM) [K^+] failed to elicit Ca^{2+} signals (**FIGURE 4**). Unlike the situation for the acinar cells, there is no evidence for functional innervation of the stellate cells. Whereas stimulation of nerve cells within the pancreatic tissue (by K^+ depolarization) evoked Ca^{2+} signals in the acinar cells, which could be blocked by atropine, no Ca^{2+} signals were detected in the stellate cells (**FIGURE 4**).

5.1. The Effects of Bradykinin

The nonapeptide bradykinin (BK), which is produced by the proteolytic action of the enzyme kallikrein on kininogen, is best known as a proinflammatory vasodilator (267, 268). It was shown many years ago that BK evokes Ca^{2+} signals in astrocytes (269). These are not directly transmitted to neurons, but the Ca^{2+} signals cause glutamate release from the astrocytes, which then, in turn, can elicit Ca^{2+} signals in neurons (269). Studies on isolated pancreatic segments or lobules, in which the normal microscopic structure of the tissue is preserved, have shown that BK elicits elevations of $[Ca^{2+}]_i$ in the stellate cells (**FIGURE 7**) but never in the neighboring acinar cells (67). The concentration-response curve is relatively steep, with a threshold for Ca^{2+} signal generation at a BK concentration of ~50 pM and maximal effects at 1 nM (**FIGURE 7**). Since the normal BK concentration in plasma is ~40 pM at rest and can increase to ~80 pM after exercise (270), it is likely that Ca^{2+} signal generation occurs under physiological conditions in normal pancreatic stellate cells. Furthermore, by far the highest concentration of kallikrein is found in glandular tissues, including the pancreas (267, 268), and since BK has a short half-life in the circulation local generation of BK at much higher concentrations than found in the circulation is likely to occur (271). The effect of BK on the stellate cells is mediated solely by type 2 BK receptors and results in a biphasic Ca^{2+} signal (**FIGURE 7**). The first short-lasting rise in $[Ca^{2+}]_i$ is due to release from internal stores, as it is independent of external Ca^{2+}, whereas the following more prolonged elevation of $[Ca^{2+}]_i$ is acutely dependent on the presence of external Ca^{2+}, indicating that it is caused by Ca^{2+} influx (67). BK-elicited Ca^{2+} signal generation is blocked by the IP_3R inhibitors

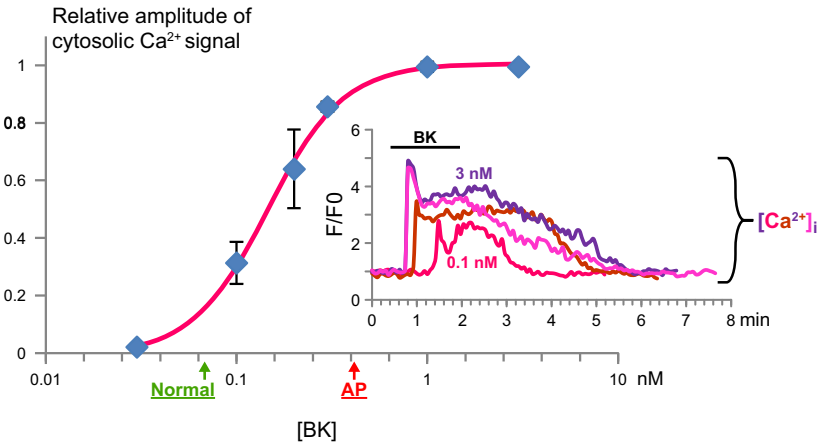

FIGURE 7. Bradykinin (BK)-elicited rise in $[Ca^{2+}]_i$ in pancreatic stellate cells: the concentration-response relationship. The normal BK concentration in plasma as well as the BK concentration in plasma from rats with experimentally induced AP are shown. *Inset*, typical examples of the time courses of $[Ca^{2+}]_i$ changes in response to stimulation with different BK concentrations. F/F_0, change in fluorescence intensity from baseline. Adapted from Gryshchenko et al. (67) with permission from *Journal of Physiology*.

caffeine and 2-aminoethoxydiphenyl borate (2-APB) as well as by the PLC inhibitor U73122, indicating that the primary BK-elicited Ca^{2+} release is mediated by IP_3 acting on IP_3Rs. The prolonged $[Ca^{2+}]_i$ elevation following the initial intracellular release can be abrogated by the Orai1 channel inhibitor GSK-7975A, whereas this inhibitor has no effect on the initial BK-elicited Ca^{2+} release (67). Orai1, Orai2, and STIM1 are all expressed in mouse pancreatic stellate cells (146). The mechanism underlying the Ca^{2+} signal generation evoked by BK is therefore "classical," conforming entirely to the standard model by which activation of G protein-coupled receptors causes release of Ca^{2+} from internal stores followed by opening of store-operated Ca^{2+} channels in the plasma membrane (177).

Compared to the detailed knowledge of Ca^{2+} handling in the pancreatic acinar cells, the information currently available about many issues concerning Ca^{2+} transport inside the stellate cells, as well as into and out of these cells, is rudimentary. We know little about the intracellular Ca^{2+} stores. The initial BK-elicited intracellular Ca^{2+} rise is very short lasting, which may indicate that the intracellular Ca^{2+} store is small compared with the store in the acinar cells. Given that pancreatic acinar cells have a particularly large and well-developed ER, which is needed for their large-scale protein synthesis, this may not be very surprising. On the other hand, even after a short-lasting BK stimulus, the stellate cells display a very pronounced and prolonged phase of $[Ca^{2+}]_i$ elevation (**FIGURE 7**), which is completely dependent on the presence of external Ca^{2+} and therefore must be due to Ca^{2+} influx (67, 69). It seems likely that the initial internal Ca^{2+} release from the small store is rather complete and therefore allows substantial opening of CRAC channels resulting in a dominant Ca^{2+} influx phase. The stellate cells possess Ca^{2+}-activated K^+ channels (272), and opening of such channels, as a result of the initial release of Ca^{2+} stored intracellularly, would favor Ca^{2+} influx through the store-operated Ca^{2+} channels by hyperpolarizing the stellate cell membrane and thereby creating a large electrochemical gradient for Ca^{2+} movement into the cells. We are still at the very start of Ca^{2+} signaling studies in the pancreatic stellate cells, and many key parameters have not yet been evaluated. Measurements of $[Ca^{2+}]$ inside the stores and their changes upon stimulation are needed, as well as proper characterization of the natures of Ca^{2+} entry and extrusion.

What are the consequences of the BK-elicited Ca^{2+} signals in the stellate cells? At this stage, the information about possible Ca^{2+}-mediated actions in these cells is very limited. The main effect so far described is the production of NO, due to activation of the Ca^{2+}-sensitive enzyme nitric oxide synthase (NOS) (224). BK elicited a rise in the NO concentration in the stellate cells, immediately following the rise in $[Ca^{2+}]_i$ (**FIGURE 8**), that was blocked by the general NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), as well as by aminoguanidine, the irreversible inhibitor of NOS2. Immunochemical studies showed that NOS was present in the stellate but not in the acinar cells and was colocalized with type 2 BK receptors (224).

There is no evidence, at this stage, for a physiological role of BK-elicited Ca^{2+} signaling and NO formation in the stellate cells. NO formation has only been detected in response to maximal or supramaximal BK stimulation, but this does not necessarily mean that there is no NO formation at physiological levels of BK stimulation, as the sensitivity of the fluorescence measurements for NO is far inferior to that of the Ca^{2+} measurements. Given the localization of the stellate cells in the space between the acinar cells and the periacinar capillaries (311), a role in the vasodilatation that occurs upon stimulation of secretion would seem possible.

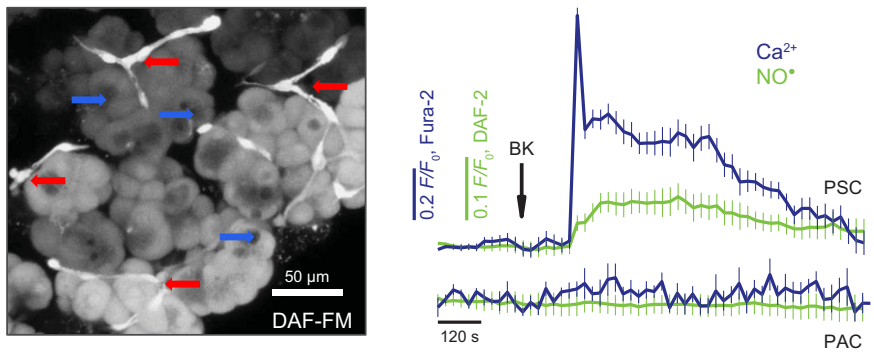

FIGURE 8. Nitric oxide (NO) formation induced by a $[Ca^{2+}]_i$ rise in pancreatic stellate cells. The image shows stellate cells (white, signposted by red arrows) in a live pancreatic lobule loaded with the NO-sensitive probe DAF-FM. The lobule has been stimulated by 1 mM H_2O_2, which caused a marked elevation of $[Ca^{2+}]_i$. The blue arrows point to acinar cells (PACs). The NO and Ca^{2+} traces on *right* show the time courses of the BK (20 nM)-elicited increase in $[Ca^{2+}]_i$ and in the intracellular NO concentration in the stellate cells (PSCs). It can also be seen that BK fails to cause any changes in $[NO]_i$ or $[Ca^{2+}]_i$ in the acinar cells. From Jakubowska et al. (224) with permission from *Open Biology*.

There is evidence for a pathophysiological role of BK in AP. It was suggested already many years ago that BK could play an important role in AP (273), and, well before anything was known about the action of BK on stellate cells, it was shown that the plasma level of BK is markedly increased in AP (274) (**FIGURE 7**). Furthermore, several studies have shown that pharmacological blockade of type 2 BK receptors inhibits the development of the cellular changes that are characteristic for AP in different disease models (274–278). The potentially important interactions between stellate and acinar cells are discussed in sect. 9.

As mentioned in the introduction to this section, the stellate cells undergo a transformation to what has been termed an activated state during AP, but, until recently, it was not known whether this would involve a change in the Ca^{2+} signaling response to BK. When Ca^{2+} signaling responses in stellate cells to BK in pancreatic lobules from normal mice were compared to those in pancreatic lobules from mice in which alcohol-related AP had been induced by a combination of ethanol and FA, it turned out that there were substantial differences. Whereas the threshold for BK-elicited Ca^{2+} signaling in the stellate cells from the normal pancreas was at a BK concentration of 100 pM and near-maximal effects were observed at 1 nM, the threshold in stellate cells from AP mice was at a BK concentration of 1 nM and maximal effects were only seen at a concentration of 30 nM. In the case of alcohol-related AP, the concentration-response curve was thus moved very significantly to the right (69). It seems plausible, also in view of other considerations (see sect. 9), that this desensitization is due to a prolonged exposure to high BK concentrations in the early stages of AP development (69).

As already mentioned, all effects of BK in stellate cells from normal pancreatic lobules are mediated by type 2 BK receptors, but in lobules from mice in which alcohol-related AP was induced, at a time when only high BK concentrations could evoke effects on $[Ca^{2+}]_i$, the stellate cells had developed a degree of sensitivity to a BK analog, Sar-Des-BK, that selectively activates type 1 BK receptors. This agent had no effect on the stellate cells in normal lobules but could, albeit at a rather high concentration (1 μM), evoke $[Ca^{2+}]_i$ elevations in stellate cells from AP mice (69). This shift, from type 2 to type 1 BK receptors, is a phenomenon known to occur generally in inflammatory diseases (279). Given that the BK concentrations required to elicit Ca^{2+} signals via type 1 receptors in stellate cells from AP mice are very high, the pathophysiological significance of this pathway is currently unclear.

5.2. Effects of Proteases

Given that any damage of pancreatic acinar cells would cause leakage of proteases into the periacinar environment, actions of these enzymes on the stellate cells could be of considerable pathophysiological importance. In the normal pancreas neither trypsin or another protease, thrombin, elicit any changes in $[Ca^{2+}]_i$ in the stellate cells (67, 69), but after the transformation that occurs during the development of AP, induced by alcohol and FA, the situation changes. As the stellate cells lose sensitivity to BK (see sect. 5.1) they develop sensitivity to trypsin and, to some extent, also to thrombin (69) so that these proteases now evoke substantial Ca^{2+} signals. The mechanism underlying this signal generation has not yet been established, but the biphasic Ca^{2+} signals evoked by trypsin have a pattern very similar to those elicited by BK, so it would not be a great surprise if it should turn out that the protease-evoked signals are generated in much the same way as those induced by BK.

5.3. Effect of Bile Acids

As described in sect. 4, some bile acids evoke Ca^{2+} signals in pancreatic acinar cells, whereas there are doubts about the action of others. Recent studies have shown that acinar and stellate cells respond with Ca^{2+} signals to different bile acids. TLC-S, which consistently evokes rises in $[Ca^{2+}]_i$ in the acinar cells, had no effect on the stellate cells, whereas cholate and taurocholate evoked $[Ca^{2+}]_i$ elevations in the stellate cells but, in the same experiments, failed to evoke any response in neighboring acinar cells (68, 224).

The effects of cholate and taurocholate have been investigated in some detail. The $[Ca^{2+}]_i$ elevations induced by these two bile acids are totally dependent on the presence of Ca^{2+} in the extracellular solution, which is different from what was observed with regard to the effects of BK (see above). This is difficult to understand fully at this stage, as the bile acid-induced Ca^{2+} signals, like those evoked by BK, are abolished by caffeine. This would seem to indicate that the bile acid effects, like the BK-induced actions, are due to activation of IP_3Rs. However, if this were the case one would expect that an initial release of Ca^{2+} from intracellular stores should be independent of external Ca^{2+} and that therefore the bile acid-elicited Ca^{2+} signal should have an initial transient component that would be manifest also in the absence of external Ca^{2+}. This issue requires further investigation.

The Ca^{2+} signal generation evoked by the bile acids is not due to a detergent effect, as the $[Ca^{2+}]_i$ rise is abolished by reducing the external Ca^{2+} concentration from the usual 1 mM to 100 μM (68). If the bile acids merely permeabilized the plasma membrane, then the bile acids should still evoke a major $[Ca^{2+}]_i$ rise—in fact to 100 μM—in that condition. A nonspecific detergent effect could also not explain why cholate and taurocholate evoke Ca^{2+} signals in the stellate but not in the acinar cells. The

strongest argument against a detergent effect is that the Ca^{2+} signals induced by bile acids are totally Na^+ dependent (68). The simplest explanation for the Na^+ dependence is that the bile acids need to be transported into the stellate cells by Na^+ cotransporters in order to exert their effect. It turns out that the Na^+-taurocholate cotransporting polypeptide is present in the stellate but not the acinar cells. In normal pancreatic lobules, the Na^+-taurocholate cotransporting polypeptide is clearly colocalized with the type 2 BK receptors (68).

It has been instructive to compare the actions of three bile acids, namely cholate, taurocholate, and TLC-S, on Ca^{2+} signaling and NO production. As already mentioned, cholate and taurocholate evoke Ca^{2+} signals in stellate but not acinar cells, whereas TLC-S elicits Ca^{2+} signals in acinar but not stellate cells (68, 224). When changes in $[Ca^{2+}]_i$ and $[NO]_i$ evoked by these bile acids were monitored simultaneously, in isolated superfused pancreatic lobules, it could be seen that NO was generated solely in response to those bile acids, cholate and taurocholate, that evoked a rise in $[Ca^{2+}]_i$ in the stellate cells, whereas the $[Ca^{2+}]_i$ elevation in the acinar cells, evoked by TLC-S, did not result in any NO production (224). The rise in $[NO]_i$ elicited by cholate or taurocholate was reduced and finally abolished, in a concentration-dependent manner, by two different inhibitors of the Ca^{2+}-sensitive NOS, namely L-NAME (N^G-nitro-L-arginine methyl ester) and aminoguanidine, indicating production of NO due to NOS activation by the Ca^{2+} signals (224). Cholate and taurocholate caused necrosis in both acinar and stellate cells, and this was markedly reduced by NOS inhibition. Although the mechanism by which NO contributes to the killing of the cells is unknown, the finding that certain bile acids can induce a particularly extensive necrosis of stellate cells [5 mM cholate killed 80% of these cells (224)] is interesting because it may provide an explanation for the well-established fact that bile-related AP seldom leads to chronic pancreatitis, whereas alcohol-related AP frequently does (280).

The concentrations of cholate and taurocholate required for Ca^{2+} signal generation in the pancreatic stellate cells (1–5 mM) are rather high, and it could be questioned whether such high concentrations of these bile acids could occur in the ducts inside the pancreas during attacks of gallstone-related AP. Considerations similar to those made in the section on bile acid effects on the acinar cells (sect. 4) are relevant here. Very high bile acid concentrations (>50 mM) have been found in bile from the gallbladder (231, 232), so the bile acid concentrations of 1–5 mM employed in the experiments evaluating effects on the stellate cells (68) might well be pathophysiologically relevant. However, there is currently no proof that such concentrations actually occur inside the pancreas under in vivo conditions.

5.4. Effects of Other Transmitters

The only transmitter that has been shown to reliably evoke Ca^{2+} signals in every stellate cell tested in normal pancreatic lobules is BK (67). However, there are other transmitters that can induce Ca^{2+} signals in many, but not all, stellate cells. ATP evoked $[Ca^{2+}]_i$ elevations in a substantial minority of stellate cells (~40%). VIP could also evoke Ca^{2+} signals in a similar proportion of these cells, and so could the frog peptide bombesin (69). However, the physiologically important acinar stimulants ACh and CCK have never been shown to have any effect on $[Ca^{2+}]_i$ in stellate cells (67). The clearly documented absence of any effect of CCK on $[Ca^{2+}]_i$ in normal mouse pancreatic stellate cells (67) is worth noting, as it has been proposed that CCK could elicit Ca^{2+} signals in human acinar cells by stimulating ACh secretion from human stellate cells, which would then act on acinar cells (281). No acute effects of CCK on human stellate cells have been shown so far, but a very low CCK concentration (1 pM) was shown to cause a modest increase (less than a doubling) of ACh output (over a 15-min period) from a preparation of human stellate cells, whereas a 20 pM CCK concentration hardly had any effect (281). It cannot of course be excluded that human pancreatic stellate cells have properties somewhat different from those in mice, but—as discussed in sect. 3—the claim that human pancreatic acinar cells have properties different from those in mice, and many other species, with regard to CCK receptors (164) has not been supported by more recent work (166, 168).

6. Ca^{2+} SIGNALING IN DUCT CELLS

The principal function of the pancreatic duct cells is to produce an alkaline bicarbonate-rich fluid that, when entering the duodenum, will neutralize the acid secretion from the stomach and thereby create an optimal pH for the activation of the digestive proenzymes secreted by the acinar cells (123). Although the acinar cells, as mentioned in sect. 3, also secrete fluid themselves, the additional fluid secretion in the ducts will help to carry the substantial mass of secreted protein into the gut. The principal stimulant of ductal fluid secretion is secretin, which, as mentioned in sect. 1, was the first hormone discovered (1). For a long time, Ca^{2+} signaling was not thought to be particularly important for the function of the duct cells, as the bicarbonate-rich secretion produced in the duct system by secretin stimulation (282) is independent of the presence or absence of Ca^{2+} in the perfusion solution (108, 284) but acutely dependent on the presence of CO_2/HCO_3^- (297). In contrast, and in the same experiments, the CCK-elicited fluid and enzyme

secretion was acutely abolished when Ca^{2+} was omitted from the perfusion fluid (108, 284). It has indeed been clear for many years that the action of secretin, stimulating ductal HCO_3^- secretion, is mediated by cAMP (59, 60), rather than—as is the case for ACh and CCK in acinar cells—by Ca^{2+} (9, 10, 12).

In pancreatic acinar cells, the discovery of Ca^{2+}-activated Cl^- channels (16, 83) and Ca^{2+}-activated K^+ channels (23, 285, 286) turned out to be very helpful for the characterization of Ca^{2+} signaling events. Similarly, in the duct cells, the discovery of Ca^{2+}-activated K^+ channels (287) and Ca^{2+}-activated Cl^- channels (288) began to increase interest in the possibility that Ca^{2+} signaling could also play a role in these cells. In the duct, as in the acinar cells (84), the Ca^{2+}-activated Cl^- channels are located in the apical (luminal) membrane (289, 290), whereas the Ca^{2+}-activated K^+ channels were originally thought to be in the basolateral membrane (287) but are now also thought to be present in the luminal membrane (291).

6.1. Potential for Physiological Ca^{2+} Signaling in Duct Cells

It turns out that ACh can evoke Ca^{2+} signals not only in the acinar but also in the duct cells, although relatively high concentrations are needed. Furthermore, ACh does evoke ductal fluid secretion, and the concentration relationships for Ca^{2+} signaling and fluid secretion are very similar (292). Although there is much less detail available about the Ca^{2+} signaling properties of the duct cells than is the case for the acinar cells, it is clear that similar events occur. Thus the initial rise in $[Ca^{2+}]_i$ is due to release from internal stores, via IP_3Rs, whereas a further prolonged elevation of $[Ca^{2+}]_i$ depends on Ca^{2+} influx from the extracellular fluid (292). A number of other substances, including ATP, angiotensin II, histamine, bombesin, and CCK, have also been reported to elicit Ca^{2+} signals in duct cells (63, 64, 293, 294). However, not all of these findings may be relevant to pancreatic physiology or pathology. The case of the CCK-elicited $[Ca^{2+}]_i$ elevations in the duct cells (293, 294) illustrates the difficulties of assigning physiological relevance to such findings. For many agents, the relevant agonist concentrations under physiological conditions are unknown, but in the case of CCK we know that the physiological range is ~1–10 pM (126). In one of the studies of CCK effects on duct cells, all the records shown are responses to 400 pM CCK, which is way outside the physiological range, although bar charts indicate that very small responses were obtained at a CCK concentration of 4 pM (293). In another study, a single response to the colossal CCK concentration of 10 nM is shown, and these authors dismiss the relevance of CCK-elicited $[Ca^{2+}]_i$ changes in the duct cells (294).

It would appear that duct cells can be stimulated to generate Ca^{2+} signals from both sides of the epithelium. The muscarinic ACh receptors are on the basolateral membrane, whereas the purinergic ATP receptors are predominantly on the apical membrane (63, 64, 290). Ca^{2+}-sensing receptors (CaRs) have been identified on the apical membrane of pancreatic duct cells in rat and human duct cells (295, 296), and in experiments on the CaR-expressing adenocarcinoma cell line Capan-1, it was shown that activation of these receptors resulted in intracellular Ca^{2+} release (296). Most likely, Ca^{2+} at concentrations >1 mM can therefore induce Ca^{2+} signals in pancreatic duct cells and thereby contribute to stimulation of bicarbonate and fluid secretion.

Overall, it is clear that an effective Ca^{2+} signaling mechanism does exist in the duct cells, which can be activated by a number of physiologically relevant transmitters, and that such Ca^{2+} signals can stimulate ductal bicarbonate secretion. However, the existence of such a mechanism does not necessarily prove that it functions under physiological conditions. The case of ACh may be of particular relevance. For ACh to increase ductal fluid secretion in the rat pancreas, it must be present at a concentration of 1 μM, and maximal secretion requires 10 μM (292), whereas in the same species ACh can evoke maximal acinar fluid and enzyme secretion at a concentration of 0.1 μM (297). The ACh concentrations attained at the muscarinic receptor sites on acinar or duct cells during nerve stimulation are unknown, and it is theoretically possible that the functional cholinergic innervation of the duct cells is much better than for the acinar cells, although there is no evidence for this. What is still lacking is information about the ability of nerve stimulation, at physiologically relevant low frequencies (~2–5 Hz), to stimulate ductal secretion. As described in sect. 3, such information is available for the acinar cells. We can therefore be reasonably sure about the importance of ACh as a physiologically relevant transmitter controlling acinar cell function but do not have such evidence for the duct cells. It certainly cannot be excluded that Ca^{2+} signaling in duct cells has physiological importance, but it would appear that the principal function of the duct cells, namely the secretion of a bicarbonate-rich fluid, is mainly controlled by secretin via the intracellular messenger cAMP and that Ca^{2+} signals, unlike in the acinar cells, only play a relatively minor role (60).

6.2. Pathophysiological Ca^{2+} Signaling in Duct Cells

The same type of agents that cause pathological $[Ca^{2+}]_i$ changes in acinar and stellate cells also work on the pancreatic duct cells (62, 64, 65, 298, 299). At a low concentration (0.1 mM) the bile acid chenodeoxycholate

evoked repetitive small oscillatory $[Ca^{2+}]_i$ rises, but at a higher concentration (1 mM) sustained $[Ca^{2+}]_i$ elevations were observed. These were particularly pronounced when the bile acid was applied from the luminal side in studies on isolated perfused ducts. The conjugated bile acid glycochenodeoxycholate is much less effective (300). At a low concentration it stimulated secretion, whereas at a high concentration it inhibited ATP formation and secretion (298, 300). With regard to the effects of ethanol and FAs, it was observed that a high concentration of ethanol (100 mM) together with a FA, presumably forming FAEEs inside the cells, caused a sustained elevation of $[Ca^{2+}]_i$ and inhibited secretion (63). Although the mechanisms involved have not been studied in nearly as much detail as in the acinar cells, it would appear that IP_3Rs are involved and that an initial release of Ca^{2+} from internal stores is followed by opening of store-operated Ca^{2+} channels (63).

7. Ca^{2+} SIGNALING IN IMMUNE CELLS

There is a huge literature on Ca^{2+} signaling in immune cells, essentially based on isolated cells in vitro and dominated by work on CRAC channels in T cells (301, 302), which is outside the scope of this article. Until very recently there were no reports of Ca^{2+} signaling studies on immune cells in the pancreatic tissue. Now, with two-photon confocal microscopy in live mouse pancreatic lobules, complemented by immunocytochemistry to test the identity of monitored cells, there is for the first time information about Ca^{2+} signal generation in immune cells in the exocrine pancreatic tissue (70).

During investigations of the Ca^{2+} signaling properties of stellate cells in the quasi-intact pancreatic tissue, a new and unknown cell type was identified. These cells were initially named X-cells and shown to generate large Ca^{2+} signals in response to stimulation with ATP (69). In a separate study of these X-cells (70), immunostaining with different antibodies against surface proteins of immune cells, labeled with the fluorescent indicator Alexa Fluor 647, enabled poststaining at the end of functional experiments in which cells responding to ATP had been identified.

These studies revealed clear staining of X-cells with the antibodies F4/80 as well as CD11b, revealing that X-cells were actually macrophages (70). This was also in agreement with the typical ear-shaped nuclei of these cells, which was very different from the classical round nuclei observed in other pancreatic cells when stained with Hoechst 33342 (**FIGURE 9**). In the normal unstimulated pancreas, only a very small number of macrophages were found. However, 2 days after initiation of experimental AP by injecting mice with a mixture of ethanol and FA the number of macrophages increased markedly, and after 3 days there had been an almost sevenfold increase in density (**FIGURE 9**). Whereas the control antibodies IgG are known to induce Ca^{2+} signals in activated immune cells (303), there was hardly any effect of IgG on macrophages in the normal pancreas. However, after induction of AP they became responsive and displayed repetitive Ca^{2+} spiking, after a delay of several minutes, when challenged with IgG (**FIGURE 9**).

Although there have been suggestions that immune cells may possess voltage-gated Ca^{2+} channels (301), the functional studies of pancreatic macrophages indicated that these cells are not electrically excitable. Whereas depolarization of cells in pancreatic lobules by exposure to solutions with a high $[K^+]$ caused elevation of $[Ca^{2+}]_i$ in acinar cells and neurons, there was no effect on the macrophages (70). As already noted (see sect. 3) the $[Ca^{2+}]_i$ elevation in the acinar cells in such experiments is not due to the depolarization of the acinar cell membrane but is mediated by ACh release from depolarized periacinar nerve endings, which do have voltage-gated Ca^{2+} channels (198) (**FIGURE 4**). In addition to the conclusion that the pancreatic macrophages are electrically nonexcitable, these data also indicate that the macrophages are not in close contact with nerve endings and/or are relatively insensitive to transmitters released from nerve endings in their environment (70).

The ATP- and ADP-elicited Ca^{2+} signals in macrophages in the normal pancreatic tissue are primarily due to release from intracellular stores via IP_3Rs, but this is quickly followed by entry of Ca^{2+} from the interstitial fluid through CRAC channels of the Orai1 type. Pharmacological experiments employing specific agonists and antagonists indicate that the functional ATP/ADP receptors on the macrophages are of the P2Y1 and P2Y13 types. Some of the macrophages also responded to ACh and to concentrations of BK that are higher than those required to elicit Ca^{2+} signals in neighboring stellate cells (70).

This recent study of pancreatic macrophages (70) has revealed that Ca^{2+} signals are important in yet another pancreatic cell type and that, most likely, such signals generated by ATP and ADP leaking out of damaged acinar cells in the initial stages of AP would play an important role in the further progression of this disease (304). Potential effects of Ca^{2+} signals in the pancreatic macrophages could be mediated via Ca^{2+}-calmodulin activation of calcineurin, which in turn would translocate the transcription factor NFAT into the nucleus (305).

8. Ca^{2+} SIGNALING IN OTHER CELLS

In addition to acinar, stellate, immune, and duct cells, there are nerves and blood vessels in the pancreas. So far, there have been no publications reporting $[Ca^{2+}]_i$ measurements in pancreatic blood vessels inside the

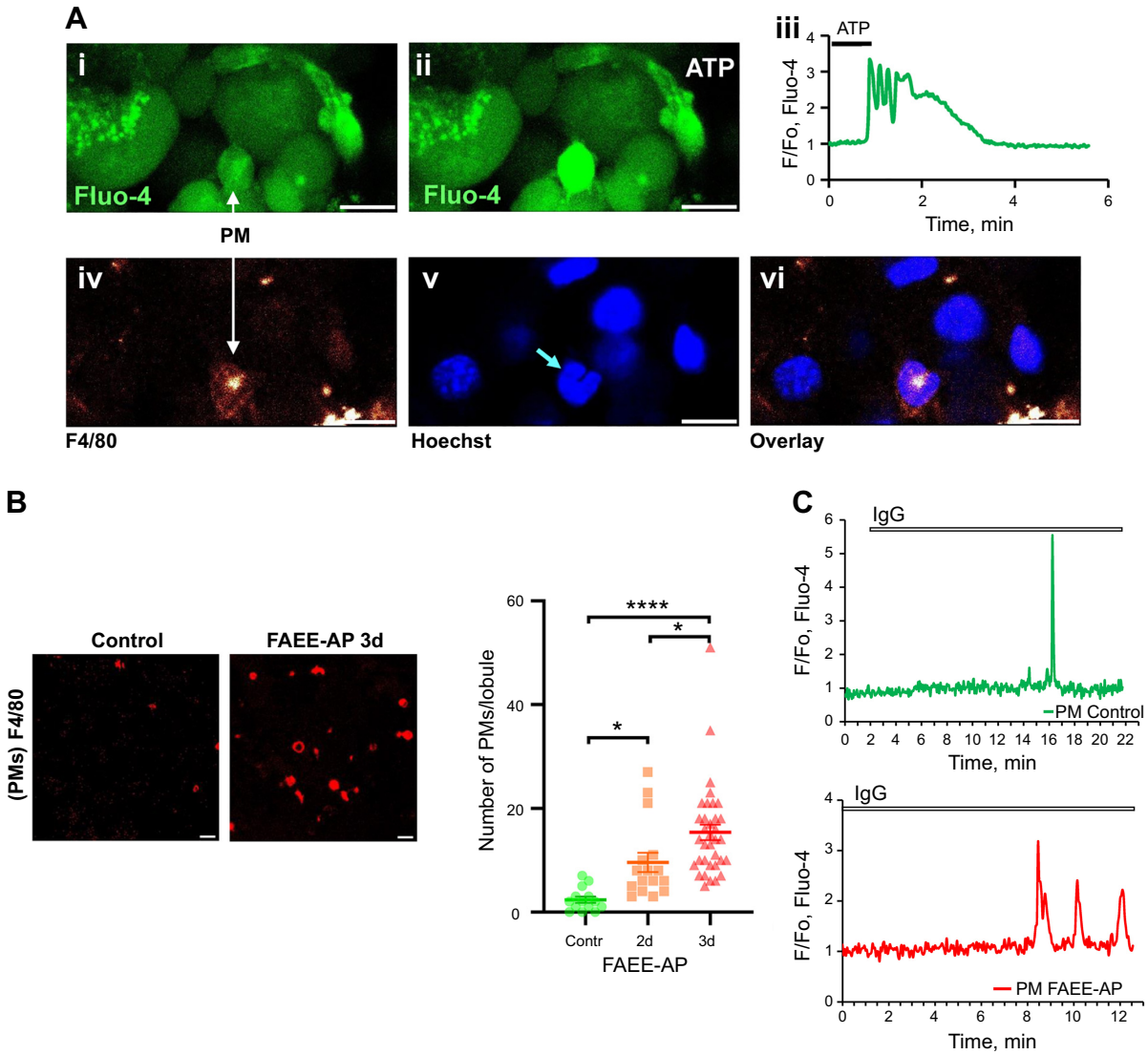

FIGURE 9. ATP- and IgG-elicited rises in $[Ca^{2+}]_i$ in macrophages in situ in live pancreatic lobules. *A*: immunostaining with antibody against macrophages (F4/80) reveals that the ATP-induced $[Ca^{2+}]_i$ rise in the lobule occurs in a pancreatic macrophage (PM). *Ai* is the fluo-4 fluorescence image before stimulation. *Aii* is taken at the height of the ATP-elicited $[Ca^{2+}]_i$ rise. *Aiii* shows the time course of the ATP-elicited $[Ca^{2+}]_i$ change. F/F_0, change in fluorescence intensity from baseline. *Aiv* shows F4/80 immunostaining and *Av* the staining of the nuclei in the lobule with Hoechst 33342. It can be seen that the PM (white arrow points toward the PM) displays the typical kidney-shaped nucleus. *Avi* is the overlay of *Aiv* and *Av*. White bars represent 10 μm. *B* shows the marked increase in the density of PMs in pancreatic lobules 2 and, even more so, 3 days (d) after induction of AP in mice by a combination of fatty acid and alcohol (FAEE-AP). The white scale bars in the 2 images represent 20 μm. *C*: IgG induces hardly any Ca^{2+} signal in a macrophage from a control lobule, but—after a substantial delay—IgG elicits regular Ca^{2+} spiking in a macrophage from a lobule taken from a mouse 48 h after AP had been induced. Adapted from Gryshchenko et al. (70) with permission from *Function*.

pancreas, but Ca^{2+} signals have been recorded from pancreatic neurons in response to membrane depolarization evoked by a high extracellular K^+ concentration (**FIGURE 4**). Unlike the $[Ca^{2+}]_i$ elevations in acinar cells evoked by K^+ depolarization, the Ca^{2+} signals in the neurons were not blocked by atropine (**FIGURE 4**). The nerve cells could be identified as such by Fluoro-Gold labeling or by specifically targeting the Ca^{2+} sensor GCaMP6 to neurons. The nerve cells identified were located close to stellate cells, and in some cases neuronal Ca^{2+} signal propagation could be followed along bodies and elongations of the stellate cells (69). Several transmitters have been shown to evoke Ca^{2+} signals in pancreatic neurons, namely epinephrine (via alpha receptors), substance P, and bombesin (69), but the potential physiological importance of these actions is unknown.

9. PHYSIOLOGICAL AND PATHOLOGICAL INTERCELLULAR SIGNALING

Here we deal with interactions between cells of the same type as well as between different cell types. In the exocrine pancreas, gap junctional communication would

appear only to occur between cells of the same type, so interactions between, for example, acinar and duct cells or acinar and stellate cells can only occur by chemical transmission.

9.1. Gap Junctional Communication

It has been known for a long time that acinar cells within one acinar unit, separated from other acinar units by a short segment composed of duct cells, are extremely well coupled—via large areas of tightly packed gap junctions—both electrically and chemically (12, 16, 129, 130, 306–310). The gap junctions in the acinar cells are mainly composed of connexons C26 and C32, in contrast to the endocrine beta cells, in which C36 dominates (310). There are no gap junctions between acinar and duct cells (309), which means that the whole of an acinar unit, composed of up to a few hundred cells (129, 307), functions as one big cell, at least from an electrophysiological point of view (129). Small molecules, up to a molecular weight of ~800, move easily from cell to cell, and by continuous injection of, for example, Lucifer yellow into one acinar cell in a quasi-intact live pancreatic preparation it is possible gradually to fill up an entire acinar unit with the fluorescent dye, thereby outlining the entire functional unit. In this way one can estimate the number of connected cells in an acinar unit, which is up to several hundreds (307). The lateral plasma membranes of the acinar cells are dominated by gap junctions, as seen in freeze fracture electron microscopic images (309), but despite this high density of channels connecting the cytosol of all acinar cells within a functional secretory unit, the physiological importance of these channels is still not fully understood. Gap junctional communication between acinar cells allows all cells in a unit to share the relatively small number of large Ca^{2+}- and voltage-activated K^+ channels [there are only ~50–100 channels per cell (23)], thereby maintaining a steady membrane potential (130). This membrane potential is important as a driving force for the fluid secretion that is required for the transport of secreted proenzymes from the acinar lumen into the duct system, as well as for the uptake of amino acids via Na^+-coupled transport (107, 124).

At high concentrations of ACh or CCK, the acinar cells uncouple from each other by closing the gap junctions, an action that is completely reversible and mediated by a high $[Ca^{2+}]_i$ and/or a low intracellular pH (16, 129, 308). This would appear to be a protection mechanism that serves the purpose of preventing a dying acinar cell, in which $[Ca^{2+}]_i$ inevitably rises and the intracellular pH falls, from endangering the survival of neighboring cells (130).

In contrast to the rich information about gap junctional communication between acinar cells, little is known about such channels in the duct cells. These cells are coupled by gap junctions, but there is little detail and the functional implications are unknown (239).

9.2. Interaction between Acinar and Stellate Cells

As described in the sections on Ca^{2+} signaling in acinar (sects. 3 and 4) and stellate (sect. 5) cells, these two types of cells, which are placed immediately next to each other in the intact pancreatic tissue (**FIGURE 1**), appear to generate Ca^{2+} signals in response to entirely different agents. Substantial Ca^{2+} signals can be generated in acinar cells that are not transmitted to neighboring stellate cells and vice versa (67). As already described in the section on stellate cell signaling (sect. 5), these cells can, however, react to the activated protease trypsin that will be released from dying acinar cells in AP and, in particular, to the product of the action of the protease kallikrein, namely BK, which will also be liberated from damaged acinar cells during an AP attack (**FIGURE 10**). Under pathophysiological conditions, evidence is thus available indicating that products released from dying acinar cells can acutely induce Ca^{2+} signals in stellate cells (**FIGURE 10**). There is, however, no direct evidence demonstrating that the stellate cells can acutely influence signaling in the acinar cells. Despite this, several pieces of indirect evidence suggest that signals generated in stellate cells may have significant effects on the acinar cells (67, 224, 311).

The first indication that stellate cell function could influence events in the acinar cells came from studies on isolated pancreatic fragments in which the extent of acinar necrosis after treatment with AP-inducing agents (bile acid mixture or FAEEs) was monitored. It turned out that the percentage of dead cells was very markedly reduced in the presence of the type 2 BK receptor blocker WIN64338 (67). Since BK2 receptors are only expressed in stellate and not in acinar cells (68), the simplest interpretation of these results would indicate that BK, generated by release of kallikrein from dying acinar cells in response to AP-inducing agents (268, 273, 277, 312) via action on the BK2 receptors, could promote a process in the stellate cells causing damage to the acinar cells. Further evidence for acinar cell injury caused by events in the stellate cells emerged from studies on pancreatic fragments examining the effects of the bile acid TLC-S and BK, acting separately or together. Whereas BK alone did not evoke any significant necrosis of either acinar or stellate cells and TLC-S alone only caused a degree of necrosis selectively in the acini, the combination of TLC-S and BK caused extensive necrosis

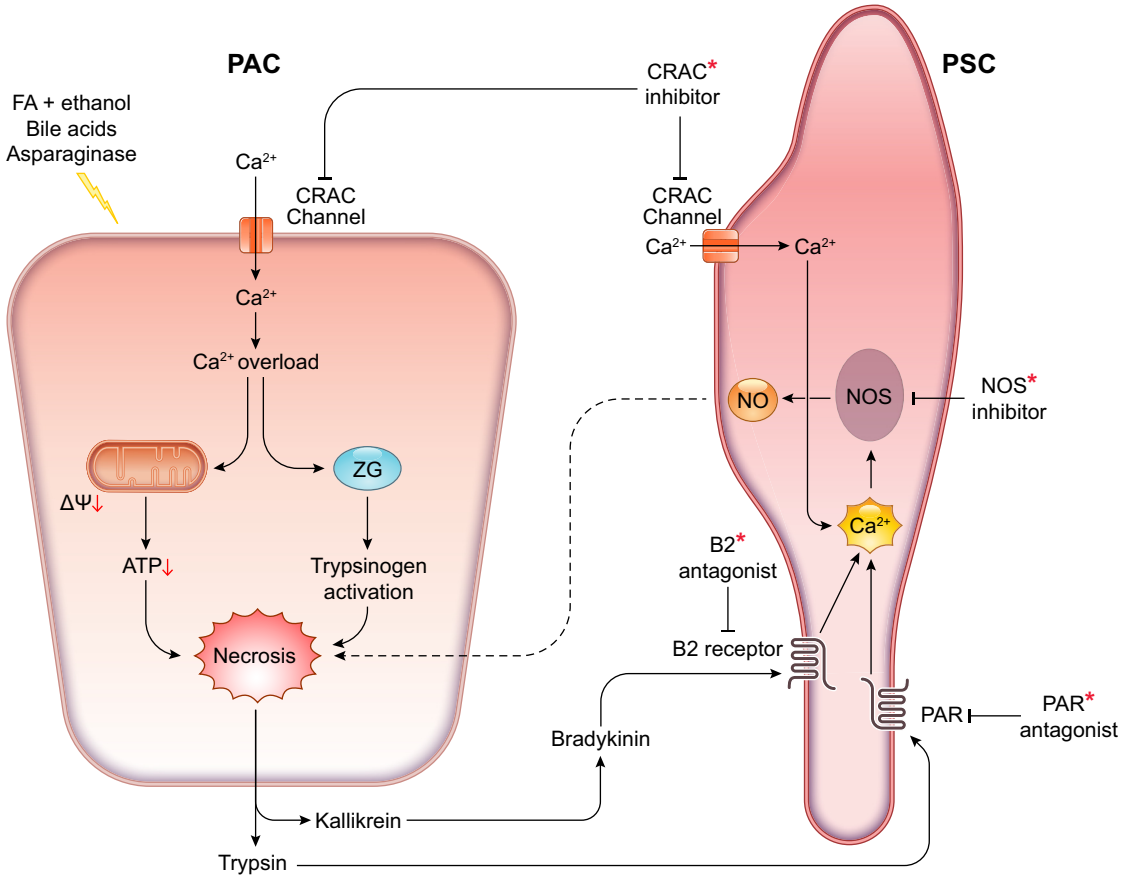

FIGURE 10. Schematic diagram illustrating the processes underlying the necrotic amplification loop between acinar (PAC) and stellate (PSC) cells. When some acinar cells die, due to Ca^{2+} overload elicited by pathological stimuli, many intracellular substances will be released into the interstitial fluid surrounding the stellate cells. Among these are agents that will activate these cells, including the proteases kallikrein [generating bradykinin (BK)] and trypsin. BK induces Ca^{2+} signals in the stellate cells, which in turn activates the Ca^{2+}-dependent nitric oxide synthase (NOS) catalyzing the formation of nitric oxide (NO). NO could diffuse into adjacent acinar cells and there boost the necrotic process, as pharmacological inhibition of NOS markedly reduced the degree of acinar necrosis evoked by AP-inducing agents. After desensitization of the BK2 (B2) receptors, the stellate cells become sensitive to trypsin. This agent can then elicit Ca^{2+} signals in the stellate cells with the same result as BK stimulation. Sustained Ca^{2+} signal generation in both acinar and stellate cells depends on Ca^{2+} influx through CRAC channels and can therefore be reduced by CRAC channel inhibitors. Antagonists against B2 and protease-activated receptors (PARs) can also put a brake on the necrotic cycle. ZG, zymogen granule.

of both acinar and stellate cells (68). Given that TLC-S only causes Ca^{2+} signals in the acinar cells and BK only in the stellate cells (68), the fact that adding BK to a TLC-S stimulus dramatically increases the level of acinar necrosis suggests that Ca^{2+} signals in the stellate cells have caused damage to the acini.

The mechanism by which stellate cell Ca^{2+} signals could damage the acinar cells has not been fully clarified. At this point in time the only known consequence of Ca^{2+} signal generation in the stellate cells is NO production (**FIGURE 8**). Pharmacological inhibition of the Ca^{2+}-sensitive enzyme NOS in the stellate cells markedly reduced the extent of necrosis in both acini and stellate cells caused by exposure of pancreatic fragments to cholate, a bile acid that only evokes Ca^{2+} signals in the stellate cells. This could be explained by postulating that NO generated in the stellate cells after exposure to cholate (224) would diffuse into neighboring acini and there, in some way, act to promote necrosis of these cells (**FIGURE 10**). Although the rise in NO concentration following exposure of the pancreatic lobule to agents acting on the stellate cells has so far been observed exclusively in these cells (**FIGURE 8**), this should not be taken as proof that NO does not diffuse into neighboring acinar cells. The NO concentration attained in the acini would inevitably be significantly lower than in the stellate cells (3-dimensional diffusion from the small stellate cell source into the much larger acinar compartments) and might not be detected because of the relatively low sensitivity of the NO-sensitive probe (as compared, for example, to the Ca^{2+}-sensitive fluorescent probes). The generally accepted pattern for NO action in other tissues is that it diffuses from its production site to neighboring target cells, where it activates soluble guanylate cyclase to generate cyclic GMP, which in turn activates cyclic GMP-dependent kinases (313). This signaling machinery is present in the exocrine pancreatic tissue (314, 315), although it is now clear that the NO-

forming enzyme NOS is preferentially, and perhaps even exclusively, located in the stellate cells (224). However, the potential role of NO in AP is controversial, as both protective and toxic effects have been described (66).

Although it cannot be excluded that NO concentrations in the acinar cells could become high enough to exert pathological effects, it may well be the case that NO itself is not the toxic messenger. There is a substantial amount of evidence indicating that NO is not a particularly toxic substance but plays a physiological regulatory role in many different systems (279, 313). In general, toxic NO effects are often due to peroxynitrite formation (reaction of NO with superoxide) (313). If this were true also for stellate-acinar cell interactions, NO toxicity might depend critically on the simultaneous production of superoxide. Superoxide could, for example, come from immune cells invading the pancreatic tissue in the early phases of AP (**FIGURE 9**). It would be valuable to test this hypothesis using fluorescent or luminescent biosensors for intracellular detection of superoxide (316) and peroxynitrite (317, 318) and assess the action of these substances in the exocrine pancreatic tissue. So far, such experiments have not been reported.

9.3. *Interaction between Acinar and Ductal Cells*

Unlike the situation for stellate and acinar cells, which have large parts of their surfaces close to each other, there is for the vast majority of acinar and duct cells no direct contact between the two cell types. Given that the primary pancreatic juice is produced in the acini and then enters the lumen of the duct system, the principal means by which acinar products can affect ductal function is via action of substances secreted by the acinar cells on the luminal (apical) membranes of the duct cells. Although the most important products of the exocrine pancreas clearly are the digestive (pro)enzymes from the acinar cells, the ductal secretion is essential, as this alkaline bicarbonate-rich fluid will prevent premature activation of the proenzymes, which can then only become active enzymes at the neutral pH in the duodenum, as a result of combining the acid fluid from the stomach with the alkaline fluid from the pancreas (61).

It would seem advantageous if there were some coordination of the acinar and ductal secretion processes. One well-established way in which this happens is by the combined action of the hormones CCK and secretin on acinar and duct cells, respectively (58, 60). Both hormones are released into the bloodstream when food products and acid enter the duodenum from the stomach (127), but more recently it has become clear that there are also internal coordination mechanisms within the pancreas itself. Thus ATP and Ca^{2+}, which are packaged into the acinar zymogen granules together with the digestive proenzymes, can activate purinergic and Ca^{2+}-sensing receptors on the luminal (apical) membranes of the duct cells, as already described in sect. 6. ATP has been detected at nanomolar levels in human pancreatic juice (319) and could act on P2Y and P2X receptors on the duct apical membrane (320, 321). This would elicit Ca^{2+} signals in these cells, which could then stimulate bicarbonate and fluid secretion. The Ca^{2+} concentration in pancreatic juice can reach 1–3 mM (322), which would be sufficient to activate Ca^{2+}-sensing receptors (323). In fact, Ca^{2+} will enter the acinar, and therefore also the duct, lumen via three separate routes, namely by exocytosis from Ca^{2+}-containing zymogen granules, by extrusion of Ca^{2+} from the acinar cytosol by Ca^{2+} pumps concentrated in the luminal (apical) acinar plasma membrane (135), and by diffusion of Ca^{2+} across the acinar epithelium via the leaky tight junctions (324). Protons are another component of the acinar secretion that could have an effect on the duct cells. The primary acinar fluid secretion is slightly acid. Behrendorff et al. (325) have shown that a quasi-physiological CCK concentration (<20 pM), acting exclusively on the acinar cells, causes a pH drop in the acinar lumen of ~1 unit. Acid-sensing receptors on the apical duct membranes could therefore potentially play a role in ductal function (61).

10. THE ROLES OF Ca^{2+} AND ATP IN PANCREATIC DISEASE

The major disorders of the exocrine pancreas are pancreatitis and pancreatic cancer. Although there is no straightforward progression from acute to chronic pancreatitis and then to pancreatic cancer, there are clearly links. Thus repeated attacks of AP caused by alcohol abuse will frequently end up as chronic pancreatitis, whereas AP caused by gallstone complications rarely gives rise to this chronic condition (280). AP itself, and therefore not only chronic pancreatitis, is now recognized as a clear risk factor for pancreatic cancer (326). As described in sects. 4–7, excessive Ca^{2+} signals in acinar, duct, and stellate cells evoke changes in the pancreas characteristic of those seen in AP, and blockade of such Ca^{2+} signal generation prevents the development of AP-like cellular changes and, in vivo, protects effectively against experimental AP. It is therefore undisputed that Ca^{2+} plays a critical role in the initiation of AP. In contrast, there is at this point in time little detail about possible roles of Ca^{2+} in the development of pancreatic cancer, but recent findings may indicate that CRAC channel blockade could be a potentially helpful addition to chemotherapy (327).

AP is a very painful autodigestive and inflammatory disease. The incidence worldwide is increasing (currently 34 per 100,000 person-years) (328), and although the overall AP-related mortality is relatively low (just below 1%), the consequences of AP are frequently severe and long lasting, including new-onset diabetes and exocrine pancreatic insufficiency. The cost to the health care system is also substantial (329). In the United States alone, the expenditure is estimated to be more than U.S. $9 billion annually (33). AP is characterized by edema, necrosis, invasion of immune cells into the pancreas, and reduced secretion (32). Although the primary disorder arises in the acinar cells, it is becoming increasingly clear that processes in the duct, stellate, and immune cells play critical roles even in the initial stages of the disease and, to a large extent, determine the severity of the disease.

CRAC channel inhibition in isolated acinar cells markedly, and in a concentration-dependent manner, reduces the extent of necrosis subsequently elicited by FAEEs (40) or bile acids or hyperstimulation of CCK receptors (215). That this inhibition is specifically related to Ca^{2+} entry was shown by demonstrating that elevation of the external Ca^{2+} concentration, increasing the gradient for Ca^{2+} entry through CRAC channels, counteracted the effect of the CRAC channel inhibitor (40). These results provided strong evidence for the now generally accepted view that a toxic Ca^{2+} signal in the acinar cells is the critical initiator of AP (32, 33).

10.1. Toxic Ca^{2+} Signals in Pancreatic Acinar Cells Initiate AP

Section 4 dealt with the pathological Ca^{2+} signals in acinar cells initiated by various agents known to induce AP and how such Ca^{2+} signals caused intracellular protease activation and necrosis. However, there is also another feature of AP that is functionally important, and that is reduced secretion of both fluid and enzymes.

10.1.1. Reduced fluid secretion.

The basic mechanisms underlying ACh- or CCK-elicited acinar fluid secretion have been known for a long time (30, 107) and are therefore only outlined briefly in order to provide the necessary background required to understanding how excessive Ca^{2+} signals can inhibit the process. Repetitive short-lasting rises in $[Ca^{2+}]_i$ in the apical, granule-containing region open Cl^- channels exclusively present in the apical membrane (84), allowing Cl^- exit into the acinar lumen, which is a major driving force for the fluid secretion. The Cl^- efflux into the lumen is assisted by Ca^{2+} activation of K^+ channels in the apical part of the lateral membranes, which maintains the intracellular negativity that is an important part of the driving force for the Cl^- exit. The reduced intracellular Cl^- concentration, together with the initial shrinkage of the acinar cells due to the loss of Cl^- and K^+, contribute to an increased turnover of the Na^+-K^+-$2Cl^-$ cotransporter (330) in the basolateral membrane, increasing the intracellular Na^+ concentration, which in turn activates the Na^+-K^+-ATPase pump in the basolateral membrane, thereby effectively recirculating Na^+. Ca^{2+} activation of the high-conductance K^+ channels allows increased turnover of the Na^+-K^+-$2Cl^-$ cotransporter by providing the crucial recirculation pathway (286). In contrast, Cl^- moves across the acinar epithelium, taken up into the cell by the Na^+-K^+-$2Cl^-$ cotransporter in the basolateral membrane and exiting into the lumen via the Ca^{2+}-activated Cl^- channel in the apical membrane. The importance of the Na^+-K^+-$2Cl^-$ cotransporter for exocrine secretion has been confirmed in humans, since patients born with defective Na^+-K^+-$2Cl^-$ cotransporters suffer from pancreatic exocrine dysfunction (331). The transepithelial Cl^- flux into the acinar lumen makes the acinar lumen negative, attracting Na^+ from the extracellular compartment to move into the lumen via the paracellular pathway through the leaky tight junctions. Water will follow, partly via the paracellular pathways and partly through the acinar cells via aquaporins in the acinar membranes. Ultimately, the fluid secretion process is powered by the Na^+-K^+-ATPase pump, generating the critical Na^+ and K^+ transmembrane gradients (30, 107). These gradients are also essential for the amino acid uptake into the acinar cells, which is required for the synthesis of the digestive proenzymes (107).

Given the functional importance of the transmembrane Na^+ and K^+ gradients, it may seem surprising that the acinar cells also possess a channel that is capable of dissipating these gradients. However, a Ca^{2+}-activated nonselective cation channel was the very first ion channel identified in the pancreatic acinar cells (21). These channels, now known to belong to the TRPM4 family, have since been found in many different cell types, where they may play various roles (332–334). Opening of the nonselective cation channels lowers the membrane potential and reduces the transmembrane Na^+ and K^+ gradients, thereby reducing the driving force for Na^+-linked substrate uptake and increasing ATP expenditure on Na^+-K^+ pumping (334). However, despite the fact that opening of these channels would seem to be deleterious to one of the principal functions of the acinar cells, namely fluid secretion, it has been shown that the nonselective cation channels can be activated in the intact cells by physiological levels of CCK (335). However, the overall magnitude of the cationic current is small compared with the physiologically much more

important Ca^{2+}-activated Cl^- current (30). Opening of the Ca^{2+}-activated nonselective cation channels is inhibited by intracellular ATP as first shown in guinea pig pancreatic acinar cells (336). It has now been established that in the presence of ATP there are four bound ATP molecules in the channel tetramer, i.e., one per subunit (337). In healthy cells the normal ATP level would severely limit the degree of cation channel opening that could be evoked by a rise in $[Ca^{2+}]_i$. As described in sect. 4, stimuli that initiate pathological changes in acinar cells generate global and sustained elevations of $[Ca^{2+}]_i$. Whereas a local apical Ca^{2+} signal can activate all available Cl^- channels in the apical membrane, it will only reach a small proportion of the lateral membranes and therefore only be able to open a small subsection of the Ca^{2+}-activated nonselective cation channels, which are distributed all over the basolateral membrane. A global Ca^{2+} signal, however, will be able to potentially activate all the cation channels, generating a relatively large current, which will then markedly reduce fluid secretion. As described in sect. 4, a global sustained $[Ca^{2+}]_i$ elevation will also reduce the ATP level and thereby further contribute to opening of the Ca^{2+}-activated nonselective cation channels. Overall, it is therefore likely that opening of these channels is a major contributor to the reduced fluid secretion in AP, which in turn will limit the enzyme secretion. By preventing the washout of the secreted enzymes, there will also be further damage of the pancreatic tissue, as the digestive enzymes will have enhanced opportunities to act on the pancreatic tissue itself. The reduced ATP level inside the acinar cells that, as already described (sect. 4), is an important feature of pancreatic pathology will also contribute to the reduced fluid secretion by limiting the energy supply to the Na^+-K^+ pump.

Although the Ca^{2+}-activated nonselective cation channels have been found in many different cell types (332, 337), it is of course not certain that such channels would be present in pancreatic acinar cells from all species. These channels have been directly demonstrated by single-channel current recording in acinar cells from the mouse, rat, and guinea pig pancreas but have not, so far, been described in the limited patch-clamp studies carried out on pig (23, 163) and human acinar cells (285).

10.1.2. Reduced enzyme secretion.

As already mentioned in sect. 10.1.1, a markedly reduced fluid secretion will itself cause a reduction in enzyme secretion. This was already demonstrated many years ago, when it was shown that in the absence of extracellular Na^+ ACh did not evoke any increase in amylase output from small superfused segments of mouse pancreatic segments. However, when subsequently Na^+ was reintroduced into the external bathing solution, in the absence of ACh, there was a secretory response. It turned out that this did not amount to a specific requirement for Na^+. In fact, exactly the same observations were made in the absence of external Cl^- and upon Cl^- readmission (12). Given that Na^+ and Cl^- are the main electrolytes in the primary acinar fluid and are both absolutely required for acinar fluid secretion (58), the simplest conclusion from these experiments is that acinar fluid secretion is required to "wash out" the enzymes secreted by exocytosis.

In AP there is also a more direct inhibitory effect on exocytotic enzyme secretion. Unlike the stimulation of ATP production observed in response to physiological local Ca^{2+} spiking in the acinar granule-rich region, a sustained global elevation of $[Ca^{2+}]_i$ reduces the intracellular ATP concentration (55), partly because excess Ca^{2+} uptake into the mitochondria opens the mitochondrial permeability transition pore, thereby reducing the electrical potential difference across the inner mitochondrial membrane, and thus reducing ATP production (57), and partly because ATP consumption is increased because of the enhanced demands for pumping Ca^{2+} and Na^+ out of the cytosol. Since exocytosis is an energy-requiring process (50), this will inevitably limit the rate of enzyme secretion.

10.2. Necrotic Amplification Loops Damaging Acinar Cells and Ultimately Causing Multiple Organ Failure

10.2.1. Interactions between acinar and stellate cells.

As shown in **FIGURE 10**, Ca^{2+} signals elicited by BK can damage the acinar cells. We have also, in sect. 4, described how various AP-inducing agents including ethanol and FAs, bile acids, physical pressure, and the drug asparaginase can cause acinar necrosis. In an initial attack by one or more of these agents, some acinar cells will die in necrosis, which is an explosive event in which intracellular components will be released into the interstitial fluid. Among these components will be a number of different enzymes including kallikrein and trypsin. Kallikrein is an enzyme that will split off the nonapeptide BK from the larger molecule bradykininogen. BK can then elicit Ca^{2+} signals in the stellate cells, which via NO formation can damage additional acinar cells, causing further necrosis, which in turn will release more kallikrein, generating more BK. Thus we have a self-fortifying circle of events that could lead to very high degree of acinar necrosis (**FIGURE 10**). The principal pieces of

evidence for this chain of events are that *1*) BK levels in plasma are increased in AP (274), *2*) a specific antagonist of BK type 2 receptors reduces acinar necrosis evoked by ethanol, FAEEs, or bile acids (67), *3*) stellate cells are desensitized to BK during the first days of AP (69), most likely because of the sustained high plasma levels of BK, and *4*) inhibitors of NOS, which is present in stellate but not acinar cells, markedly reduce acinar necrosis evoked by various bile acids (224).

As the effect of BK on the stellate cells is gradually diminished by desensitization, these cells enter a state in which they become sensitive to trypsin. In the normal pancreas this enzyme fails to evoke Ca^{2+} signals in the stellate cells, but a few days after AP induction it now generates clear $[Ca^{2+}]_i$ elevations in ~20% of these cells. Some stellate cells also become sensitive to yet another enzyme, namely thrombin (69).

10.2.2. Potential interactions between acinar cells and macrophages.

Macrophages that invade the exocrine pancreatic tissue during AP (**FIGURE 9**) generate Ca^{2+} signals in response to both ADP and ATP (70), which are among the substances released from dying acinar cells into the interstitial fluid surrounding the pancreatic macrophages. At this point in time we have no concrete information about the specific processes in the pancreatic macrophages that may be triggered by these Ca^{2+} signals, but, based on general knowledge of macrophage function, some possibilities can be suggested.

Macrophages serve multiple functions in the body. They are phagocytosing cells, important for host defense and wound healing, but also play a very important role in immune regulation (338). Ca^{2+} signals mediated by purinergic receptors are important for chemotactic and phagocytic responses as well as for proinflammatory cytokine secretion (339). The severity of AP is to a large extent determined by the strength of the inflammatory response to the initial injury of the acinar cells, and it is an excessive immune response, generating what is now often termed a cytokine storm, that is responsible for the multiple organ failure that is characteristic of the final stage of severe AP (34, 340). Extracellular ATP also plays a potentially important role by spreading the activation among macrophages, as Ca^{2+} signals in macrophages can cause secretion of ATP from these cells, which in turn will activate neighboring macrophages via purinergic receptors (341). We could therefore envisage a possible chain of events in which ATP and/or ADP released from necrotic acinar cells activate immediately neighboring macrophages, which then via ATP secretion generate Ca^{2+} signals in additional macrophages, amplifying the overall secretion of cytokines. Further support for the idea that extracellular ATP plays a significant role in the development of AP and systemic inflammation has come from experiments in which it has been shown that extracellular ATP levels are elevated in experimental AP and that blocking the effect of ATP by suramin reduced the levels of cytokines in the plasma, including interleukin-6 (IL-6), as well as pancreatic injury (342).

10.2.3. Cytokine and bradykinin storms.

There are some interesting similarities between severe cases of AP and COVID-19, since in both these diseases there is multiple organ failure due to cytokine storms (32, 199). A recent study comparing the patterns of cytokine elevations in the two diseases indicates remarkable similarities (340). In both diseases acute respiratory distress syndrome (ARDS) is a significant factor in the often fatal outcome (32, 34). In addition to the cytokine storm, there is now evidence indicating that a BK storm contributes significantly to ARDS in COVID-19 (343). BK provokes vasodilatation and increased capillary permeability and also inhibits the amiloride-sensitive epithelial Na^+ channel (ENaC), which plays an important role in clearing mucus and alveolar fluid in the respiratory tract (344). In COVID-19, the increased BK level seems to arise from an imbalance in several components of the renin-angiotensin system, including increased kallikrein levels (343). Pancreatic acinar cells are one of the main sources of kallikrein, and acinar necrosis in AP should therefore increase the level of BK and, as already mentioned, such an increase has been observed (274). BK, by inducing Ca^{2+} signals in pancreatic macrophages (70), may contribute to the cytokine storm, causing further damage. The potential interactions between the three key cell types in the acinar environment are shown schematically in **FIGURE 11**.

A further potential link between COVID-19 and AP has recently been proposed, based on a study of the link between Ca^{2+}-activated Cl^- channels (TMEM16A) and intracellular Ca^{2+} release from the ER (119) (see also sect. 3). As previously mentioned, there are data indicating that TMEM16A can enhance IP_3-elicited Ca^{2+} release. A potential vicious cycle has been identified in which Ca^{2+} activation of TMEM16A causes further enhancement of IP_3-mediated Ca^{2+} release stimulating nuclear factor-κB (NF-κB) translocation into the nucleus, leading to IL-6 formation. This, in turn, would not only be an important part of the cytokine storm but also, by action on IL-6 receptors on the pancreatic acinar cells, stimulate additional expression of TMEM16A. Although most of the data are based on experiments on the pancreatic AR42J cell line, it was also shown that pharmacological TMEM16A inhibition could ameliorate caerulein-

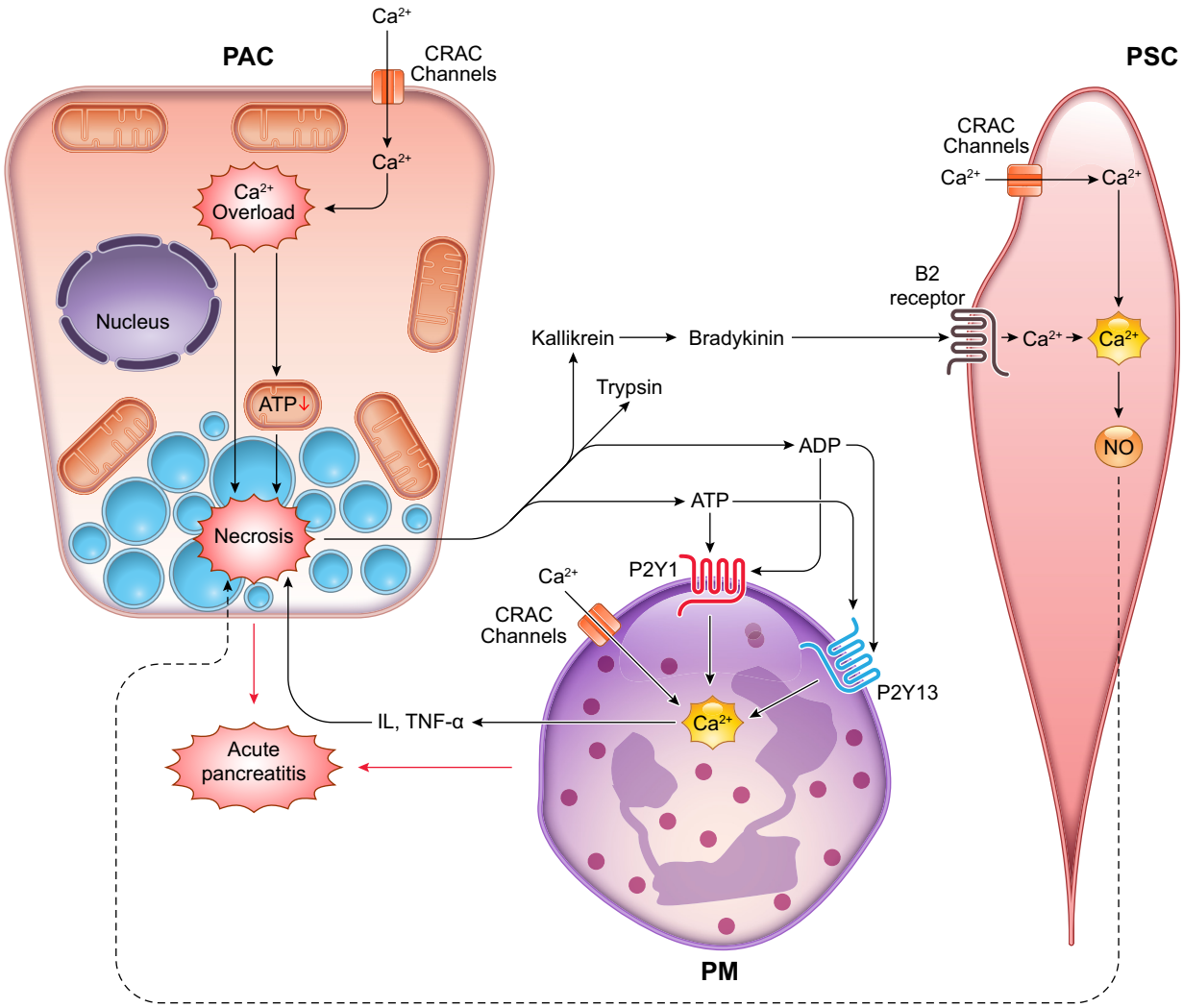

FIGURE 11. Schematic diagram highlighting some of the overall most important processes driving the development of AP. AP is almost certainly initiated in the acinar cells (PACs) by pathological stimulation causing excessive Ca^{2+} influx, overloading the cell. Some acinar cells will die, releasing many active agents including proteases, ATP, and ADP. These substances will have effects on both neighboring stellate cells (PSCs) and macrophages (PMs). The effects on the stellate cells have already been signposted in **FIGURE 10**. ATP and ADP will mainly affect the macrophages, inducing Ca^{2+} signals in these cells that will both lead to proliferation of these cells and to production/secretion of inflammatory cytokines including interleukins and tumor necrosis factor (TNF)-α. These will not only act on the neighboring cells but also enter the circulation, acting on distant targets potentially causing multiple organ failure. Importantly, the excessive Ca^{2+} signals in all 3 cell types, driving the destructive processes, depend on Ca^{2+} influx through CRAC channels of the Orai1 type. Inhibition of the opening of these channels therefore presents a rational option for effective therapeutic intervention.

induced experimental AP in mice (119). In this context it is interesting that it has very recently been proposed that pharmacological inhibition of TMEM16 proteins, which reduced Ca^{2+} signaling evoked by SARS-CoV-2-Spike and thereby blocked syncytia formation, could be a potential therapy for COVID-19 (118).

11. POTENTIAL THERAPEUTIC INTERVENTION SITES

As our knowledge of the pathophysiology of AP has increased, a number of potential new avenues for therapy against this disease have come into focus. Some of the intervention points could be useful for combating AP irrespective of the cause; others may only be useful in cases initiated by specific agents.

11.1. Inhibition of Ca^{2+} Entry through the Plasma Membrane

Because AP is generally initiated by excessive release of Ca^{2+} from internal stores, it might seem natural to prevent or treat AP by using pharmacological agents that could reduce the opening of channels through which Ca^{2+} can be liberated from intracellular organelles. As described in sect. 4, the principal Ca^{2+} release channel is the IP_3R. As already discussed, there is an

effective, but low-affinity, inhibitor of the opening of IP_3Rs, namely caffeine (28, 91), and there is clinical evidence that coffee drinking, in a dose-dependent manner, offers a degree of protection against alcohol-related AP (345). In a detailed study of experimental AP in mice, it was shown that caffeine protected against both bile-related and alcohol-related AP by inhibition of Ca^{2+} release mediated by IP_3Rs (213). However, the caffeine concentrations required for this protective effect were rather high, and because caffeine is also an activator of RyRs and has other pharmacological effects, there are dangers, particularly arising from neuroexcitatory effects and possible induction of cardiac arrhythmias, which severely limit the usefulness of caffeine in the clinic (213).

The most important consequence of the intracellular Ca^{2+} liberation is the opening of the CRAC (Orai1) channels in the plasma membrane through which Ca^{2+} can continue to flow into the cells, thereby sustaining an elevated $[Ca^{2+}]_i$. It should therefore be possible to treat all forms of AP by blocking or markedly reducing the opening of Orai1 channels. The proof of principle, showing that this approach could work, was first provided in 2013, in a study on isolated acinar cells or small clusters of acinar cells in which it was shown that GSK-7975A, an agent that had been shown to block Orai1 channels in some immune cells (142), could markedly reduce the sustained global $[Ca^{2+}]_i$ elevation evoked by FAEEs as well as the subsequent intracellular protease activation and necrosis (40). Two years later, this was confirmed in a study that also showed that GSK-7975A could be successfully used in vivo to treat experimental AP induced in mice by FAs and ethanol, bile acids, or hyperstimulation of CCK receptors (215). Further confirmation of the rationale for this treatment came from experiments on isolated human acinar cells and from the use of another Orai1 channel blocker (CM4620/Auxora) that was shown to have exactly the same effects as GSK-7975A (146, 215).

CRAC channel inhibitors clearly act on the acinar cells (40), but this is not the only target in the exocrine pancreas. The store-operated Ca^{2+} entry in stellate cells and pancreatic macrophages, which follows the initial intracellular Ca^{2+} release evoked, respectively, by BK and ATP or ADP, is also mediated by Orai1 CRAC channels and has been shown to be inhibited by GSK-7975A (67, 70). The impressive protective and curative effects of CRAC channel inhibition observed in mice in vivo in three different models of experimental pancreatitis (215) are therefore most likely due to reduced Ca^{2+} overload in all three cell types. The inhibition of Ca^{2+} signal generation in macrophages (70) and other immune cells should reduce the cytokine storm, and in this context it is interesting that evidence is beginning to emerge that CRAC channel inhibition by CM4620 (Auxora) may also be helpful in the treatment of severe COVID-19 (346).

Although CRAC channel inhibition was shown to be effective in the treatment of both alcohol- and bile-related experimental AP, there are reasons to believe that it might not fully prevent AP due to gallstone complications, as this involves an element of pressure-induced AP (see sect. 4). In such cases the toxic Ca^{2+} signals are due to activation of the pressure-sensitive Piezo1 channels followed by opening of TRPV4 channels (246, 249). There are specific inhibitors of both these channels, and they do indeed prevent the AP-like effects induced in acinar cells by shear stress (246, 249). Since gallstone-related AP is likely to be due to effects of bile acids on the acinar cells as well as pressure on these cells due to blockage of the pancreatic duct, it seems likely that a combination of CRAC channel inhibition and inhibition of either Piezo1 channels or TRPV4 channels may turn out to be the best treatment. Some of the potential intervention sites, specifically in relation to the interactions between PACs and PSCs, are shown in **FIGURE 10**.

11.2. Preventing Loss of ATP Production

As described in sect. 4, cytosolic Ca^{2+} overloading causes mitochondrial Ca^{2+} overload, which in turn opens the mitochondrial permeability transition pore, depolarizing the inner mitochondrial membrane. The result is inhibition of mitochondrial ATP production (55). Our current knowledge indicates that all the different agents causing AP induce acinar Ca^{2+} overloading and in this way reduce mitochondrial ATP production. Therefore, in addition to trying to prevent the cytosolic Ca^{2+} overloading by CRAC channel inhibition, there could also be merit in specifically preventing the consequences of the subsequent mitochondrial Ca^{2+} overload, thereby preserving mitochondrial function. Cyclophilin D is a key modulator of the mitochondrial permeability transition pore (252), and pharmacological cyclophilin D inhibition by two different agents had significant beneficial effects in four different mouse AP models, markedly improving all biochemical, immunological, and histopathological parameters (57). A more recent study has confirmed this, using a novel cyclosporin A derivative (NIM811), which is likely to be more useful clinically, as a cyclophilin D inhibitor (250).

As already mentioned, there is evidence indicating that glucose utilization is impaired in AP and that this may be due to hexokinase inhibition (243). Metabolism could therefore be boosted bypassing this step. It turned out that galactose, which can be converted to glucose-6-phosphate without the involvement of hexokinases by using the Leloir pathway (**FIGURE 6**),

prevented the rundown of ATP formation seen in experimental AP in mice and markedly improved all histopathological parameters (243). Galactose administration is likely to be particularly helpful as a preventive measure. When using asparaginase to treat children with acute lymphoblastic leukemia, there is a very significant risk of AP development (241), and in such cases it may be sensible to offer protection by preventive galactose consumption.

11.3. *Interfering with the Necrotic Amplification Loops*

As discussed in sect. 10.2, stellate and acinar cells are part of a necrotic amplification loop, whereby injured acinar cells liberate the enzyme kallikrein, which splits the small nonapeptide BK from the larger kininogen molecule (267, 268). BK, in turn, evokes Ca^{2+} signals in the stellate cells. activating NOS and thereby generating NO (**FIGURE 10**). It would appear that NO, either directly or more likely indirectly, can further damage neighboring acinar cells because pharmacological inhibition of NOS markedly reduces the extent of acinar necrosis caused by AP-inducing agents (224). One potential consequence of the NO formation would then be further release of kallikrein, creating a vicious circle (69, 347). BK acts principally via BK2 receptors, which are specifically located on the stellate cells (68), and treatment with BK2 receptor antagonists would therefore be a rational step (67). There is evidence that such treatment can be effective against experimental AP (274).

12. CONCLUSIONS

There has been remarkable progress over the last three decades in understanding both the molecular mechanisms responsible for the control of normal exocrine secretion and the processes that cause the often fatal disease AP. It is now abundantly clear that both Ca^{2+} and ATP play crucial roles in the physiology and pathology of the exocrine pancreas and that the interactions between these two agents are critical. We now know many of the most important properties of the principal molecules that govern the handling of Ca^{2+} in the pancreatic cells and in many cases also know how to interfere with their functions. An important feature of pancreatic research in the last decade, in particular, has been the ability to monitor dynamic changes in the cytosolic Ca^{2+} concentration simultaneously in different adjacent cell types in the acinar cell environment. We are therefore now beginning to acquire a detailed picture of the interactions between the different cell types, which has been helpful, particularly for a better understanding of the pathophysiology of AP. There is still, at this point in time, no authorized specific treatment of this disease, but the last decade has finally seen the identification of several very promising molecular targets. Although many important details still have to be worked out, it seems likely that the next decade will see clinical benefits from the basic science work reviewed in this article. Specifically, it seems possible that clinical trials based on interfering with the molecular targets identified (348) will finally result in one or more rational treatments of AP.

GLOSSARY

ACh	Acetylcholine
AP	Acute pancreatitis
ARC	ADP-ribosyl cyclase
ARDS	Acute respiratory distress syndrome
BK	Bradykinin
cADPR	Cyclic ADP-ribose
CCK	Cholecystokinin
$[Ca^{2+}]_{ER}$	$[Ca^{2+}]$ in the endoplasmic reticulum
$[Ca^{2+}]_i$	$[Ca^{2+}]$ in the cytosol
CaR	Ca^{2+}-sensing receptor
CRAC channel	Ca^{2+} release-activated Ca^{2+} channel
ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
FA	Fatty acid
FAEE	Fatty acid ethyl ester
IL-6	Interleukin-6
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
L-NAME	N^{ω}-nitro-L-arginine methyl ester
NAADP	Nicotinic acid adenine dinucleotide phosphate
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NFAT	Nuclear factor of activated T cells
NFKB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
PLC	Phospholipase C
POAEE	Palmitoleic acid ethyl ester
RBL	Rat basophilic leukemia
RyR	Ryanodine receptor
TC	Taurocholate
TLC-S	Taurolithocholic acid sulfate
TMEM16A	Transmembrane protein 16
TPC	Two-pore channel
TRP	Transient receptor potential
VIP	Vasoactive intestinal polypeptide

CORRESPONDENCE

Correspondence: O. H. Petersen (PetersenOH@cardiff.ac.uk); S. Peng (pengshuang@jnu.edu.cn).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

O.H.P. J.V.G., O.V.G., and S.P. prepared figures; O.H.P., J.V.G., O.V.G., O.G., and S.P. drafted manuscript; O.H.P., J.V.G., O.V.G., O.G., and S.P. edited and revised manuscript; O.H.P., J.V.G., O.V.G., O.G, and S.P. approved final version of manuscript.

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