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# Activation of pancreatic acinar cells by very low concentrations of cholecystokinin: mechanism and implications for physiology and pathology

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ABSTRACT

Cholecystokinin (CCK) is one of the three classical gut hormones. CCK is also, in contrast to the other two principal gut hormones (gastrin and secretin), an important neurotransmitter with widespread actions in the brain and in the periphery. Although not signposted by its name, one of the key physiological actions of CCK is to activate the secretion of an enzyme-rich neutral fluid produced by the pancreatic acinar cells. In general, hormones activate their target cells at concentrations that are much lower than those of neurotransmitters but, even in this context, the pancreatic acinar cells are extraordinarily sensitive to extremely low CCK concentrations (low pM). We explore the mechanism underlying this exceptional sensitivity as well as its consequences. The focus is on the intracellular transduction pathways that are activated when acinar cell CCK receptors are excited by the hormone. Uniquely, three different intracellular receptors – all linked to release of $Ca^{2+}$ from intracellular stores – are required for CCK to elicit secretion. The implications of this unusual arrangement for both pancreatic physiology and pathophysiology are discussed.


## 1. Introduction

One key task that our body needs to solve is to transform the energy inherent in the food we consume into a form of energy, namely ATP, that can be utilized to fuel all our vital processes. The chain of events involves digestion, absorption, cellular uptake and mitochondrial metabolism. In digestion, the pancreas plays the most crucial role, as it is the only organ producing the enzymes needed to break down the three key food elements, namely carbohydrates, proteins and fats. The acinar cells in the pancreas are the only cells in the body that have the capacity to synthesize, store and, importantly, secrete all the digestive enzymes upon demand [1].

The mitochondria are the principal producers of ATP but, in spite of a century of ATP research [2], a detailed understanding, based on solid physical, chemical, and biological principles, of how ATP is synthesized, may still not have found its final form [3]. Nevertheless, we do understand many aspects and it is clear that the huge electrical potential difference across the inner mitochondrial membrane (~150 mV with the mitochondrial matrix being negative) is crucial for ATP generation [2]. Pancreatic secretion is energy demanding and ATP production must therefore increase when secretion is stimulated. In general, $Ca^{2+}$ plays a crucial role in linking increased cellular activity to increased ATP production. $Ca^{2+}$ taken up into the mitochondria during signalling activates three $Ca^{2+}$-sensitive dehydrogenases in the Krebs cycle thereby increasing ATP production [4]. This is also the case in both pancreatic acinar and duct cells [5]. In pancreatic pathology, failure of ATP production - mostly due to a reduction, or even collapse, of the electrical potential difference across the inner mitochondrial membrane – is a key problem [5].

The mechanisms controlling exocrine pancreatic secretion have been extensively reviewed [5–10]. However, recent findings [11,12] provide a new context for earlier published data [13–17]. Furthermore, the new hypothesis for the mechanism of action of CCK [12,18] is not in agreement with all published data [19,20]. Reviewing what is currently known about the action of CCK on pancreatic acinar cells, we conclude that the mechanism of action is more complex, flexible and dynamic than hitherto described. This has pathophysiological and translational implications.

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## 2. Cholecystokinin (CCK): hormone and neurotransmitter

In 1943, Harper and Raper [21] published a paper in the *Journal of Physiology* describing experiments showing that a substance extracted from the small intestine of the pig, dog or cat was an effective stimulant of pancreatic enzyme secretion. They named this substance pancreozymin. Chemical analysis of this substance by Mutt and Jorpes [22] disclosed that pancreozymin was identical to a substance that had been discovered much earlier by Ivy and Oldberg [23] to cause gallbladder contraction and therefore had been named cholecystokinin (CCK). Following the discovery by Harper and Raper [21], the hormone was renamed cholecystokinin-pancreozymin (CCK-Pz), reflecting its dual actions [22]. However, the linguistic drive for brevity finally won out and, since the gallbladder contracting effect was discovered long before the action on the pancreas, CCK became the established name of the hormone, although its most important role is to regulate pancreatic acinar enzyme secretion [5].

CCK was discovered as a gut hormone, but it became clear later that it was also present in the brain [24,25] and it is now recognized not only as an important gut hormone controlling pancreatic exocrine secretion, but also as an important neurotransmitter in the brain as well as in the periphery [26,27]. CCK exists in several molecular forms [27] and the most relevant forms for the control of pancreatic secretion are CCK8, CCK33 and CCK58, which all have the same effects on the pancreatic acinar cells with similar potencies [28].

CCK acts, at low pM concentrations, on isolated pancreatic acinar cells from humans, mice, rats, guinea pigs and pigs to elicit secretion by exocytosis, mediated by a rise in the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), occurring as repetitive short-lasting $Ca^{2+}$ spikes (Fig. 1) [5,9]. Each $Ca^{2+}$ spike causes activation of $Cl^-$ channels, exclusively located in the apical membrane (Fig. 1), driving fluid secretion. High-resolution measurements of secretion by exocytosis (capacitance measurements [29]) show that the time course of exocytosis follows closely the time course of individual $Ca^{2+}$ spikes in the apical granular region (Fig. 1). Direct CCK actions on isolated acinar cells have also been observed in pancreatic segments (lobules), in which the normal fine structure of the pancreatic tissue is preserved, as well as in the perfused intact pancreas [5]. Recently, CCK-elicited intracellular $Ca^{2+}$ signaling in pancreatic acinar cells has also been demonstrated *in vivo* [11].

Although CCK can act as a neurotransmitter, its stimulation of pancreatic acinar enzyme secretion *in vitro* is not due to a neurotransmitter effect, as it cannot be inhibited by interfering with nerve action potential generation using pharmacological blockade of voltage-gated $Na^+$ channels in intra-pancreatic nerve cells [30]. There is ample evidence for direct action of CCK on identified acinar cells [5] as well as extensive biochemical and pharmacological evidence for CCK receptors on acinar cells [31]. However, there has been controversy about the existence of the most functionally important CCK1 receptors on *human* acinar cells [32], in spite of the direct demonstration of $Ca^{2+}$ signal generation and amylase secretion from isolated human pancreatic acinar cells in response to physiologically relevant (low pM) CCK concentrations [30]. With the confirmation of the results and conclusions from Murphy et al. [30] by Gaisano's group [33], the issue now seems settled and it is clear that CCK can act directly also on *human* pancreatic acinar cells. This does not of course exclude an additional action on intra-pancreatic nerves. Li & Owyang [34] have proposed that the principal physiological action of CCK *in vivo* is on cholinergic nerves and, therefore, ultimately mediated by acetylcholine (ACh), although Adler et al. [35] have provided strong evidence for direct CCK activation of acinar cells in humans *in vivo*. Recent data from Takano and Yule [11] support the view that the action of CCK on the acinar cells is direct and not mediated via cholinergic nerves as $Ca^{2+}$ signals observed under fasting conditions *in vivo* were abolished by specific CCK1 receptor blockade but were unaffected by local atropine application. In any case, we have detailed molecular data concerning CCK effects on acinar cells, whereas we have no information on the mechanism of action of CCK on cholinergic nerves. To date, there are no published studies directly monitoring intracellular signals generated in intra-pancreatic cholinergic nerves by the action of CCK. Of necessity, the current review will therefore focus on the direct acinar effects.

## 3. Actions of CCK and acetylcholine on pancreatic acinar cells: similarities and differences

CCK and ACh are the two principal physiological stimulants of pancreatic acinar enzyme secretion and, at the very least in the mouse and rat, also of neutral fluid formation [36–38]. In contrast, secretin elicits a bicarbonate-rich fluid formation by acting on the pancreatic duct cells [7,39,40].

The mechanisms of action of ACh and CCK, although clearly involving different receptors on the acinar cells – cholinergic M3 receptors in the case of ACh and high-affinity CCK1 receptors for CCK [5,41] – seemed originally very similar. In both cases, release of $Ca^{2+}$ from intracellular stores is an early effect that in turn raises $[Ca^{2+}]_i$, opening $Ca^{2+}$-dependent $Cl^-$ channels in the apical membrane and $K^+$ channels in the basolateral membrane. Opening these channels allows operation of the transport processes forming the neutral acinar fluid secretion [5]. Early findings indicated that the mechanisms underlying the ACh- and CCK-elicited intracellular $Ca^{2+}$ release are identical, mediated by inositol-1,4,5-trisphosphate ($IP_3$) generated by phospholipase C (PLC) activation [42–44]. However, a detailed examination of the spatial-temporal pattern of the intracellular $Ca^{2+}$ signals generated in pancreatic acinar cells by low, and most likely physiologically relevant, concentrations of CCK and ACh, respectively, revealed important differences [13,45,46].

Local $Ca^{2+}$ signals in the apical pole activate secretion by exocytosis of digestive enzymes and pro-enzymes as well as increasing the $Ca^{2+}$-dependent $Cl^-$ current across the apical membrane (Fig. 1). The electrophysiological method of monitoring the time course of the agonist-elicited change in local $[Ca^{2+}]_i$, by exploiting the presence of an endogenous $Ca^{2+}$ sensor (Fig. 1), avoids the problem of increasing the cytosolic $Ca^{2+}$ buffering, which inevitably occurs when introducing exogenous fluorescent $Ca^{2+}$ sensors. It also provides the most sensitive account of the time course of local $[Ca^{2+}]_i$ changes near the apical membrane, precisely where $Ca^{2+}$ control of secretion must occur. In early experiments, it became clear that just supra-threshold concentrations of CCK had a much greater tendency to evoke a mixture of short- and long-lasting $Ca^{2+}$ signals than ACh, which at the lowest effective concentrations only elicited short-lasting spikes (Fig. 1). In later imaging studies, it was shown that the short-lasting $Ca^{2+}$ spikes were always confined to the apical pole whereas the longer-lasting and larger $Ca^{2+}$ transients represented global $[Ca^{2+}]_i$ elevations (Fig. 1) [45].

The electrophysiological approach (Fig. 1) - in addition to its high temporal resolution and focus on assessing $[Ca^{2+}]_i$ changes where it matters in relation to secretory control - has the considerable merit of allowing direct manipulation of the composition of the intracellular fluid. It thereby allows exploration of the direct effects of infusing various signaling molecules into the cell. An inevitable drawback of the method is that the ability to put well-defined solutions into the cell comes at the price of potentially losing some mobile substances from the cell interior by diffusion into the pipette solution [47].

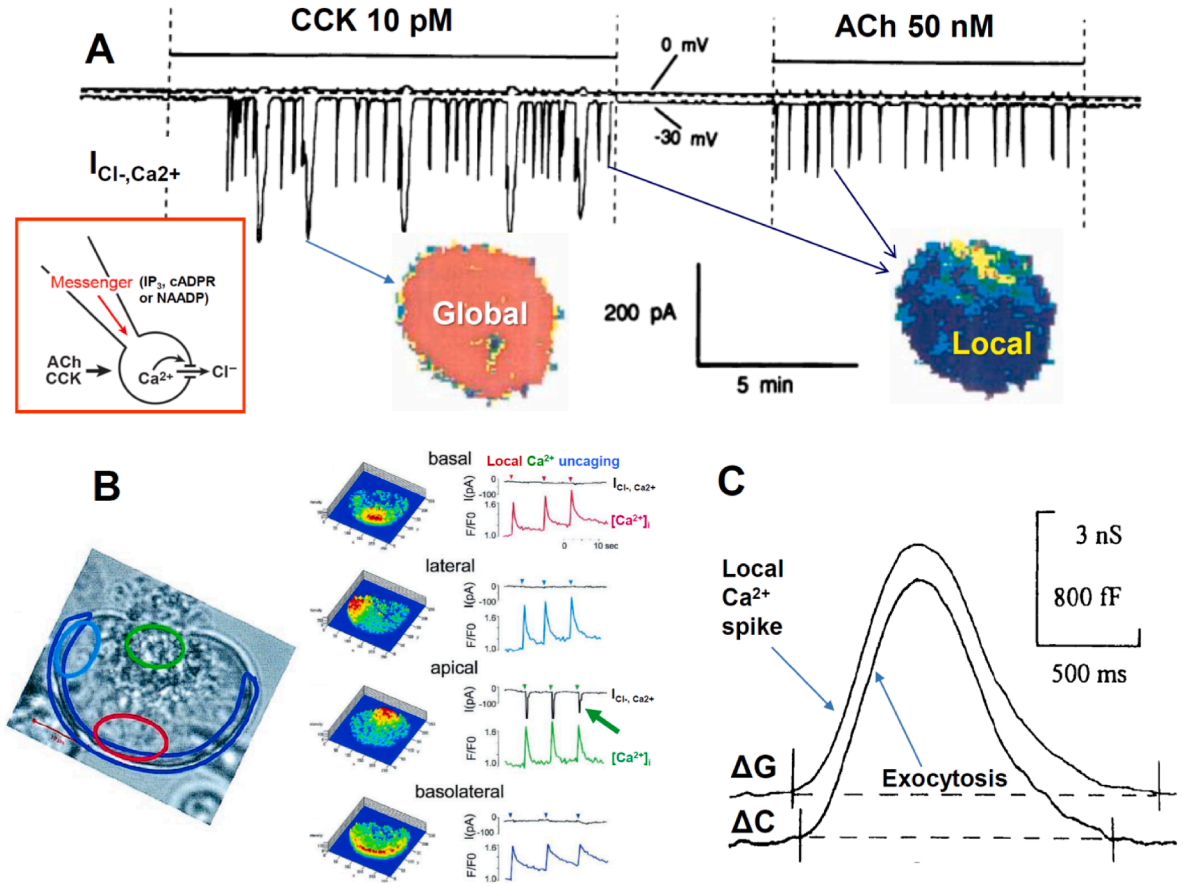


**Fig. 1.** Cytosolic $Ca^{2+}$ signals activate $Cl^-$ channels and exocytosis in the apical membrane of pancreatic acinar cells. **A** Continuous electrophysiological trace showing CCK- and ACh-elicited repetitive spikes of $Ca^{2+}$-dependent Cl current ($I_{Cl-, Ca2+}$). In this experiment, there is no transmembrane $Cl^-$ gradient at a membrane potential of 0 mV, because the $Cl^-$ concentration is the same on both sides of the membrane, but at −30 mV there is a gradient favouring $Cl^-$ outflow (inward current – downward deflection). Every time $[Ca^{2+}]_i$ rises, the $Cl^-$ channels open, shown by a downward deflection. The repetitive spikes of $I_{Cl-, Ca2+}$, therefore represent repetitive cytosolic $Ca^{2+}$ spikes and each of the short-lasting current spikes turns out to represent a local apical $[Ca^{2+}]_i$ rise, whereas the longer and larger current spikes - seen during CCK stimulation - represent global $[Ca^{2+}]_i$ elevations. (Adapted from Petersen et al. [13] and Thorn et al. [45]). **B** Local uncaging of caged $Ca^{2+}$ activates $I_{Cl-, Ca2+}$, but this only occurs when $Ca^{2+}$ is uncaged in the apical pole of an isolated pancreatic acinar cell (field outlined in green in transmitted light picture). This shows that the $Cl^-$ channels are exclusively present in the apical membrane. (Adapted from Park et al. [103]). **C** A single local apical $Ca^{2+}$ spike, represented here by a transient rise in the $Ca^{2+}$-dependent $Cl^-$ conductance, triggers a short burst of secretion (exocytosis), captured here by a transient increase in surface membrane area (capacitance). The fusion of zymogen granules (compound exocytosis) with the apical membrane starts shortly after the $[Ca^{2+}]_i$ rise and is followed by endocytic membrane retrieval. Secretion follows changes in $[Ca^{2+}]_i$ faithfully. (Adapted from Maruyama & Petersen [104]).

The different $Ca^{2+}$ 'signatures' elicited by low concentrations of ACh and CCK can also be observed in intact cells in experiments where changes in $[Ca^{2+}]_i$ are monitored by $Ca^{2+}$-sensitive fluorescent probes trapped in the cytosol (Fig. 2). All $Ca^{2+}$-sensitive probes are by definition $Ca^{2+}$-binding substances and will therefore, to a larger or smaller extent, act as $Ca^{2+}$ buffers. Small transient $Ca^{2+}$ signals are therefore in danger of being lost in such experiments. In the experiments shown in Fig. 2, the finer elements of the $Ca^{2+}$ signals observed electrophysiologically (Fig. 1) have indeed disappeared (Fig. 2). In the CCK experiment only the longer and larger $Ca^{2+}$ spikes can be seen and in the ACh experiment short-lasting spikes can only be observed on top of an elevated base-line (Fig. 2).

The original concept that both CCK and ACh primarily activate PLC, generating $IP_3$, which in turn binds to $IP_3$ receptors ($IP_3$Rs) on the endoplasmic reticulum (ER), thereby opening $Ca^{2+}$ permeable channels [42–44] turned out to be too simplistic, because $IP_3$ is not the only $Ca^{2+}$-releasing messenger. Hon Cheung Lee discovered two new messengers, cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP), both shown to be potent liberators of stored $Ca^{2+}$ in sea urchin egg cells [48]. There may be several additional $Ca^{2+}$-releasing messengers, whose potential importance remains to be established. It has, for example, been proposed that ADPR and 2-deoxy-ADPR may function as liberators of stored $Ca^{2+}$ [49], but we are not aware of evidence for a role of these agents in pancreatic acinar cells.

Cancela & Petersen [50] showed that cADPR, added directly to the interior of pancreatic acinar cells, elicits a rise in $[Ca^{2+}]$i that can be blocked by the cADPR antagonist 8-$NH_2$-cADPR. Importantly, the effect of a low CCK concentration (10 pM) was also blocked by 8-$NH_2$-cADPR, whereas the effects of ACh were undiminished [50]. Shortly thereafter, it was shown that NAADP can also release $Ca^{2+}$ from stores in pancreatic acinar cells [51] and that inactivating intracellular NAADP receptors by a high desensitizing concentration of NAADP abolishes the response to CCK but has no effect on the action of ACh in the same cells [52].

The original finding by Streb et al. [44], showing CCK-elicited production of $IP_3$, turned out to have been observed at excessively high, and completely un-physiological, levels of CCK (3 μM). In a detailed study from John Williams' group, even stimulation with the slightly supra-physiological CCK concentration of 10 pM did not produce any observable increase in $IP_3$ formation [53].

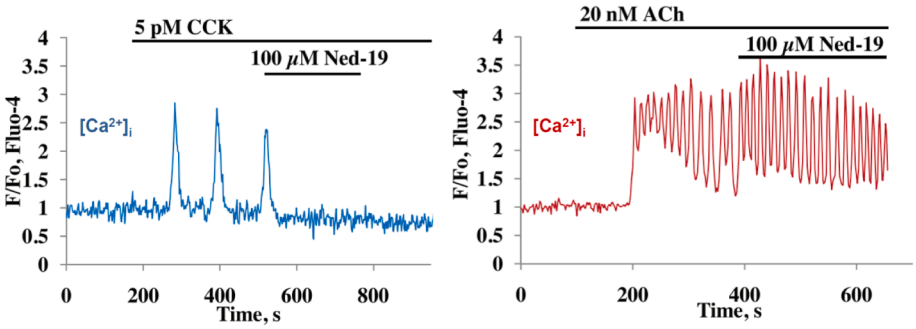


**Fig. 2.** $[Ca^{2+}]_i$ changes in intact pancreatic acinar cells measured by a $Ca^{2+}$-sensitive fluorescent dye (Fluo-4). Note the different $Ca^{2+}$ spike patterns evoked by CCK and ACh. Whereas the NAADP antagonist Ned-19 abolishes $Ca^{2+}$ spiking elicited by CCK it has no effect on $Ca^{2+}$ signaling evoked by ACh. (From Gerasimenko et al. [105]).

When it was subsequently discovered that CCK (at 10 pM), but not ACh, evoked a rapid increase in the formation of NAADP [54], the simplest hypothesis indicated that whereas ACh would elicit $Ca^{2+}$ signals via $IP_3$ formation, CCK would primarily act via generation of NAADP [52]. This was in agreement with the finding that the effect of CCK was blocked by the NAADP receptor antagonist Ned-19, whereas this inhibitor had no effect on the response to ACh (Fig. 2). However, there was a complication: the effects of CCK, NAADP and cADPR were all inhibited by blocking the $IP_3$Rs [19,52]. As already mentioned, the action of CCK was also abolished by blocking the intracellular cADPR receptors (Fig. 3). Furthermore, both CCK and ACh could increase production of cADPR [54]. Because only CCK can activate NAADP production [54], this messenger became specifically associated with the generation of the typical CCK $Ca^{2+}$ 'signature', although it remained puzzling why the effect of CCK - at physiologically relevant concentrations – should depend on functional receptors for all the three $Ca^{2+}$-releasing messengers [5,18–20]. It is still not clear why the apparent mechanism of action of CCK should have evolved to be so complicated.

## 4. New evidence: the CCK1 receptor is linked to PLC via the G-protein $G\alpha_q$

Given, that the stimulating action of physiologically relevant CCK concentrations depends on functional $IP_3$Rs, it was of some importance to test whether the CCK1 receptor on the pancreatic acinar cells was linked to a G-protein that would be expected to activate PLC. Takano et al. [12] have recently provided evidence indicating that excitation of both muscarinic M3 and CCK1 receptors activates $G_{q/11}$. It is generally accepted that this G-protein is linked to PLC activation and therefore to formation of $IP_3$ and diacylglycerol. Takano et al. [12] also showed that the stimulating actions of CCK and ACh on pancreatic $Ca^{2+}$ signal generation, $Cl^-$ channel opening and amylase secretion were all blocked by YM-254890, a well-known specific $G_{q/11}$ antagonist. This lead to the conclusion that CCK primarily, like ACh, acts to generate $IP_3$ and that this is the principal mechanism by which secretion is activated [12,18]. However, this conclusion does not seem compatible with the key experimental finding that blocking or inactivating intracellular NAADP receptors specifically abolishes $Ca^{2+}$ signal generation elicited by CCK, but not by ACh (Fig. 2) [52]. If $IP_3$ formation were a significant feature of stimulation with a low pM CCK concentration - as proposed by Takano et al. [12] but not confirmed by the actual measurements published by Matozaki et al. [53] - blockade of NAADP receptors should not prevent CCK from releasing $Ca^{2+}$ from intracellular stores [19,20]. The fact that CCK-elicited $Ca^{2+}$ signaling depends on $G_{q/11}$ activation might indicate a requirement for at least some $IP_3$ production but, taking into account all the available data, this potential $IP_3$ production must be relatively minor and, if it occurs, clearly insufficient to initiate $Ca^{2+}$ signal generation. This suggests that CCK binding to CCK1 receptors must have effects other than just PLC activation. Given that CCK-elicited $Ca^{2+}$ signaling depends on functional NAADP receptors [52], the most obvious additional action would be initiation of NAADP production and there is indeed direct evidence for this [54]. How this NAADP production is activated remains uncertain [55], but could be related to receptor-specific accessory RGS proteins [56].

## 5. $Ca^{2+}$ signaling patterns evoked by direct intracellular infusion of different $Ca^{2+}$-releasing messengers

It has been proposed that whereas CCK principally initiates $Ca^{2+}$ signaling, and therefore secretion, via $IP_3$-generated intracellular $Ca^{2+}$ release, the special 'CCK $Ca^{2+}$ signal pattern' is likely to be imposed by NAADP production [12,18], which is specifically elicited by CCK but not by ACh [54]. This would seem to require NAADP to act in a way different from $IP_3$, and perhaps even at a different site. In the first study of the effect of intracellular NAADP application in pancreatic acini [51] it was reported that NAADP could elicit several different $Ca^{2+}$-signaling patterns. In some cases, the repetitive simple short-lasting $Ca^{2+}$ spikes were similar to those typically observed in response to intracellular $IP_3$ infusion [57,58], but other patterns that looked similar to those evoked by CCK were also observed. However, these traces, displaying broader $Ca^{2+}$ transients [51], were from recordings with patch-clamp seals that were not optimally tight and therefore less reliable. Further work [52,59] showed that the $Ca^{2+}$ signaling patterns evoked by all the three $Ca^{2+}$-liberating messengers are remarkably similar. Because high NAADP concentrations inactivate the NAADP receptors, it is difficult to record concentration-effect relationships over a broad concentration range, but this has been done for $IP_3$ and, even better, for a stable (non-metabolizable) $IP_3$ analogue, namely inositol-1,4,5-trisphophorothioate ($IPS_3$). As seen in Fig. 4, $IPS_3$ can produce an ACh-like $Ca^{2+}$ signal pattern at a low concentration and a CCK-like pattern at a higher concentration. However, the experimental fact that $IP_3$, at relatively high concentrations, can produce global $Ca^{2+}$ signals does not explain the difference in signal patterns evoked by CCK and ACh, as it would require CCK to produce more $IP_3$ than ACh. This is clearly not the case as there is no measurable $IP_3$ production in response to physiological stimulation with the hormone [53]. Intriguingly, $Ca^{2+}$ is itself a $Ca^{2+}$ releasing messenger and can elicit repetitive $Ca^{2+}$ spiking via a process known as $Ca^{2+}$-induced $Ca^{2+}$ release (CICR). In electrophysiological patch clamp whole cell recording experiments, it has been shown that intracellular $Ca^{2+}$ infusion can trigger repetitive $Ca^{2+}$ spikes that are unaffected by blockade of $IP_3$Rs and most likely due to activation of ryanodine

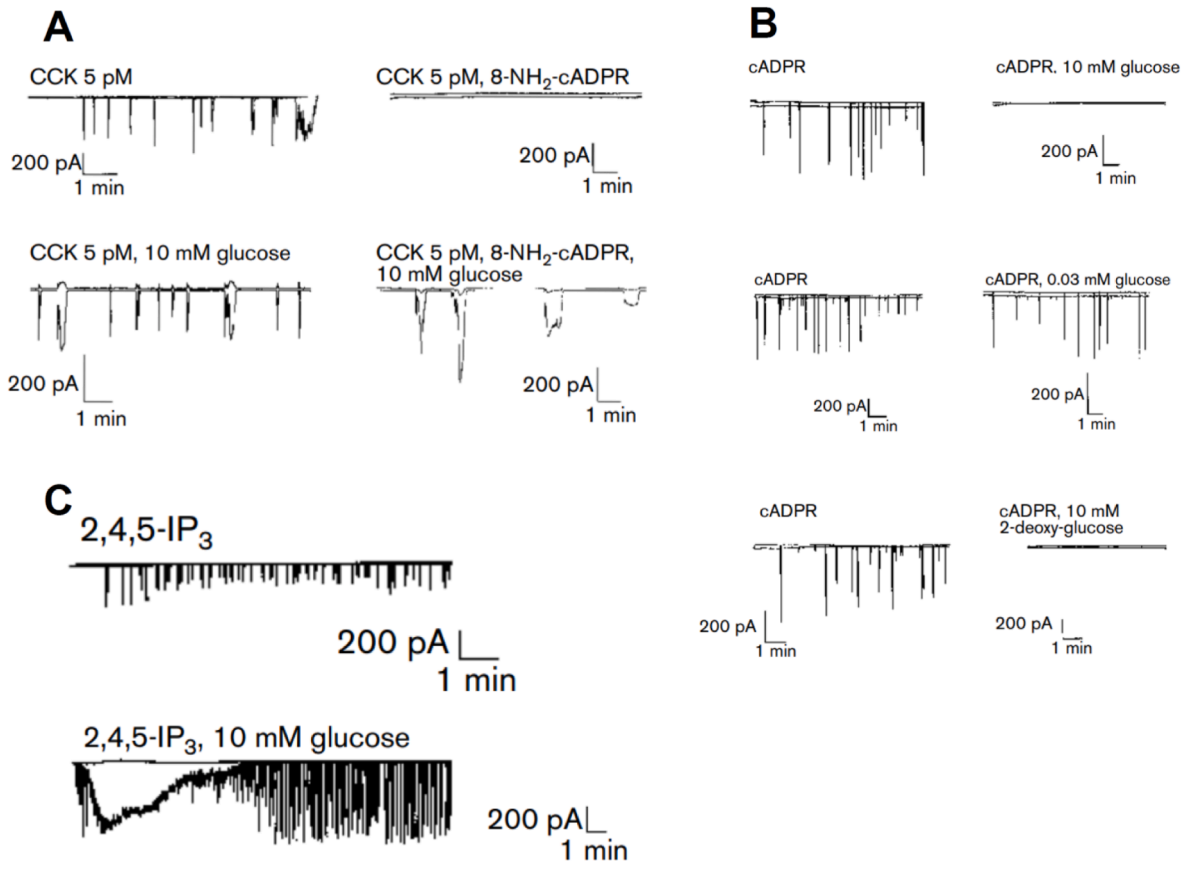


**Fig. 3.** $Ca^{2+}$-dependent $Cl^-$ current spikes evoked by CCK or intracellular infusion of cADPR or the stable $IP_3$ analogue 2,4,5-$IP_3$ under various intracellular conditions. **A** CCK elicits $Ca^{2+}$ spiking irrespective of whether the intracellular fluid contains glucose or not. However, the cADPR antagonist 8-$NH_2$-cADPR only blocks the CCK-elicited signal in the absence of intracellular glucose. **B** cADPR-elicited $Ca^{2+}$ spiking is blocked by a high (10 mM) intracellular glucose (or 2-deoxy-glucose) concentration. **C** $Ca^{2+}$ spiking elicited by 2,4,5-$IP_3$ is markedly enhanced when the intracellular solution contains 10 mM glucose. (Adapted from Cancela et al. [15]).

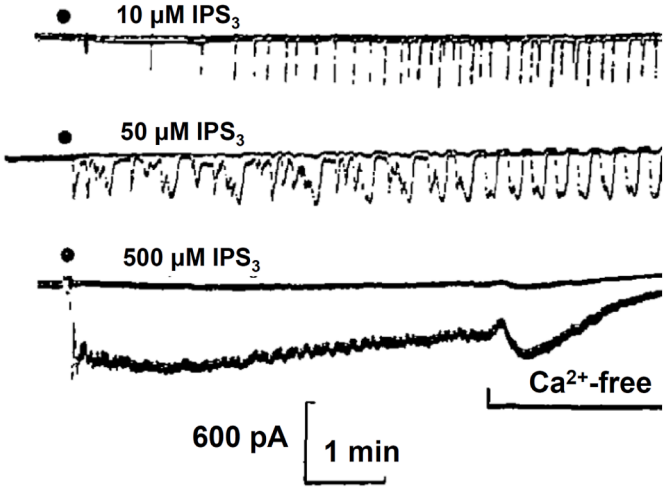


**Fig. 4.** $Ca^{2+}$-dependent $Cl^-$ current spikes elicited by different intracellular concentrations of the stable $IP_3$ analogue $IPS_3$. Whereas local repetitive $Ca^{2+}$ spiking can continue for a long time in the absence of external $Ca^{2+}$ (Wakui et al. [57]), a sustained $[Ca^{2+}]_i$ elevation requires the continued presence of extracellular $Ca^{2+}$, as seen in the lowest trace. (Adapted from Petersen et al. [14]).

receptors [58]. Ryanodine receptors, like $IP_3$Rs, can be opened by a rise in $[Ca^{2+}]_i$, but can also be inhibited at high levels of $[Ca^{2+}]_i$ [5]. As shown in Fig. 5, a sharp rise in $[Ca^{2+}]$i, by local uncaging of caged $Ca^{2+}$ in the apical pole, can trigger $Ca^{2+}$ waves that spread across the whole cell by CICR, but in many cases this requires an additional stimulus, for example, by a subthreshold CCK concentration [17].

## 6. Is NAADP required for the generation of a CCK-like $Ca^{2+}$ signal pattern? Evidence from experiments with bombesin

Early evidence, obtained from studies of the action of bombesin on pancreatic acinar cells, long since forgotten, turns out to be relevant to the question of whether NAADP production and action are requirements for generation of the so-called CCK-like $Ca^{2+}$ signal pattern. Bombesin is a frog peptide but structurally similar to the mammalian gastrin releasing peptide that, in addition to the function implied by its name, plays important roles in the nervous system [60–63]. In pancreatic acinar cells, bombesin evokes intracellular $Ca^{2+}$ release, membrane conductance changes and amylase secretion very much like CCK and at similar low concentrations [64,65]. Bombesin acts on receptor sites distinct from those binding CCK [66]. Later, it was shown that bombesin evokes $Ca^{2+}$ signal patterns in the pancreatic acinar cells that are very similar to those elicited by CCK, at equally low (pM) concentrations, and this signal generation was abolished by blockade of cADPR receptors [67]. However, the effects of bombesin, unlike those of CCK, were completely unaffected by inactivation of NAADP receptors and are therefore not mediated by NAADP [16]. This is a strong argument against the hypothesis that CCK-like $Ca^{2+}$ signal patterns depend on NAADP production and activation of NAADP receptors.

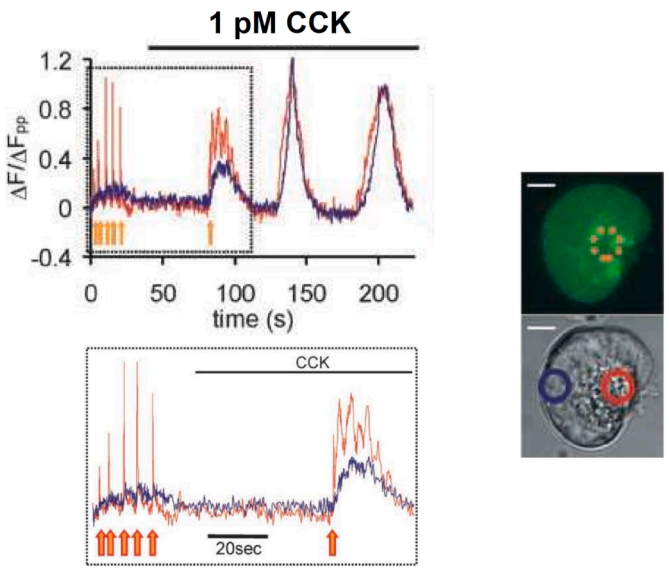


**Fig. 5.** Uncaging of caged $Ca^{2+}$ (yellow arrows) evokes $Ca^{2+}$ spiking, but only in the presence of sub-threshold CCK stimulation (1 pM). The very short-lasting $Ca^{2+}$ spikes seen in the first part of the recording simply represent the rise of $[Ca^{2+}]_i$ that results directly from the physical uncaging event itself. During CCK stimulation this initial physically induced $[Ca^{2+}]_i$ rise triggers a much larger and longer 'real' $Ca^{2+}$ spike that spreads throughout the cell. Orange/Red trace represents $[Ca^{2+}]_i$ in apical pole whereas blue trace represents $[Ca^{2+}]_i$ in the basal area. White bars in the fluorescent and transmitted light pictures represent 10 μm. (From Ashby et al. [17]).

## 7. Intracellular $Ca^{2+}$ stores that can be mobilized by intracellular messengers

As the principal difference between the $Ca^{2+}$ signaling patterns evoked by CCK and ACh relates to the issue of spreading or lack of spreading of the $Ca^{2+}$ signal initiated in the apical granular area, it is necessary to consider the localizations of the intracellular $Ca^{2+}$ stores and the mechanisms by which they can release $Ca^{2+}$. To gain direct access to the intracellular $Ca^{2+}$ stores, two-photon permeabilization of the pancreatic acinar plasma membrane [68] turned out to be a valuable experimental approach. In this preparation, it could be shown that all the three $Ca^{2+}$-mobilizing messengers, $IP_3$, cADPR and NAADP, release $Ca^{2+}$ from both the ER and from acid stores located in the apical granular area, comprising zymogen granules, endosomes and lysosomes [68]. The only important difference between the action of $IP_3$ and the actions of cADPR or NAADP was that the $Ca^{2+}$ releasing effects of cADPR or NAADP were blocked by ryanodine or ruthenium red (both well-known inhibitors of the opening of ryanodine receptors) whereas the action of $IP_3$ was unaffected [68]. The two functionally important intracellular $Ca^{2+}$ release channels would therefore appear to be present on both the ER and the acid stores.

In intact cells, flooding the cytosol with $IP_3$ evokes $Ca^{2+}$ spiking exclusively in the apical granular region, indicating that this is where the $IP_3Rs$ are concentrated [45]. This was confirmed by immunochemical localization of the intracellular $IP_3Rs$ [69,70]. Furthermore, Kasai et al. [71] showed that localized injections of $IP_3$ at a relatively low concentration easily elicited $Ca^{2+}$ spikes in the apical pole, but that such an effect could only be achieved in the basal part of the cells at much higher $IP_3$ concentrations. The situation is even more extreme concerning the effect of local $[Ca^{2+}]_i$ elevations and can be tested by highly localized uncaging of caged $Ca^{2+}$. Such experiments showed that CICR could only be activated in the apical granular pole, but not in the basal area (Fig. 5). However, when successfully initiated in the apical pole (Fig. 6), the $Ca^{2+}$ signal could then, under certain conditions, propagate into the basal part of the cell (Fig. 7), spreading throughout the cell. This may require additional sensitizing factors (see also section 11).

## 8. Different routes to the same $Ca^{2+}$ oscillator

In the intact cell, $Ca^{2+}$ spiking - irrespective of whether it is activated by CCK or ACh, or by intracellular infusion of $IP_3$, cADPR or NAADP - is blocked by inhibition of the opening of $IP_3Rs$ by heparin or by inhibition of the opening of ryanodine receptors by ryanodine or ruthenium red [5]. High-resolution imaging has shown that the time course of the evolution of the primary $[Ca^{2+}]_i$ rise in the apical granular pole is identical for signals generated by CCK and ACh [52]. These findings suggest that although CCK and ACh act via distinct pathways, these converge on one and the same regenerative $Ca^{2+}$ release mechanism, consisting of $IP_3Rs$ and ryanodine receptors in close proximity of each other [5,52]. Given, that ACh elicits measurable $IP_3$ production, whereas CCK at physiologically relevant concentrations does not, and that CCK is the only agonist that evokes NAADP production, the model that has prevailed since 2000 of one common $Ca^{2+}$ oscillator activated via two separate pathways still has merit [52]. However, this model does not explain the special CCK $Ca^{2+}$ signature.

There is an intriguing interaction between the ACh and CCK pathways. Stimulation with CCK markedly enhances the magnitude of a subsequent response to a low ACh concentration and this also happens when the CCK response itself has been abolished by inactivation of the NAADP receptor [52]. In other words, CCK markedly potentiates ACh-elicited $Ca^{2+}$ release, but this effect is not mediated by NAADP. However, the potentiating effect of CCK can be prevented by 8-$NH_2$-cADPR and may therefore be mediated by cADPR [52].

The notion that the action of CCK is primarily mediated via cADPR and/or NAADP receptors, whereas the ACh action is exclusively mediated via $IP_3Rs$, may be too simplistic. Although CCK-elicited $Ca^{2+}$ signals are specifically inhibited by blocking cADPR receptors, whereas $Ca^{2+}$ signals evoked by ACh are not, this difference is not hard-wired, but depends on the cell's metabolic state. In most patch-clamp whole cell recording experiments on isolated pancreatic acinar cells, there was no glucose in the patch pipette solution dialyzing the cytosol, but Mg-ATP was always present. In this situation, cADPR elicited regular $Ca^{2+}$ spiking and the CCK-evoked $Ca^{2+}$ signal generation was abolished when the cADPR receptors were blocked (Fig. 3). However, when 10 mM glucose was included in the patch pipette solution, cADPR could no longer elicit $Ca^{2+}$ signals and the CCK-evoked $Ca^{2+}$ signals were not abolished by blockade of the cADPR receptors. On the other hand, in this situation $IP_3$-elicited $Ca^{2+}$ signal generation was remarkably enhanced (Fig. 3). Thus, an elevated cytosolic glucose concentration strikingly reduces the sensitivity to cADPR but markedly sensitizes the $IP_3Rs$ to $IP_3$. Unfortunately, the mechanism underlying this intriguing glucose dependent switch is unknown.

## 9. Factors promoting $Ca^{2+}$ signal spreading

One important barrier to the spreading of $Ca^{2+}$ signals initiated in the apical granular region to the basal area, is the so-called mitochondrial belt surrounding the granular region [72]. The mitochondria take up $Ca^{2+}$ rapidly through the mitochondrial $Ca^{2+}$ uniporter (MCU) [4,73], a process driven by the high intracellular negativity in the intra-mitochondrial space [2,74]. Because release of $Ca^{2+}$ from the mitochondria, via $Na^+/Ca^{2+}$ exchange, is much slower than the uptake [4,74], these organelles provide an important brake on the progression of $Ca^{2+}$ signals from the apical

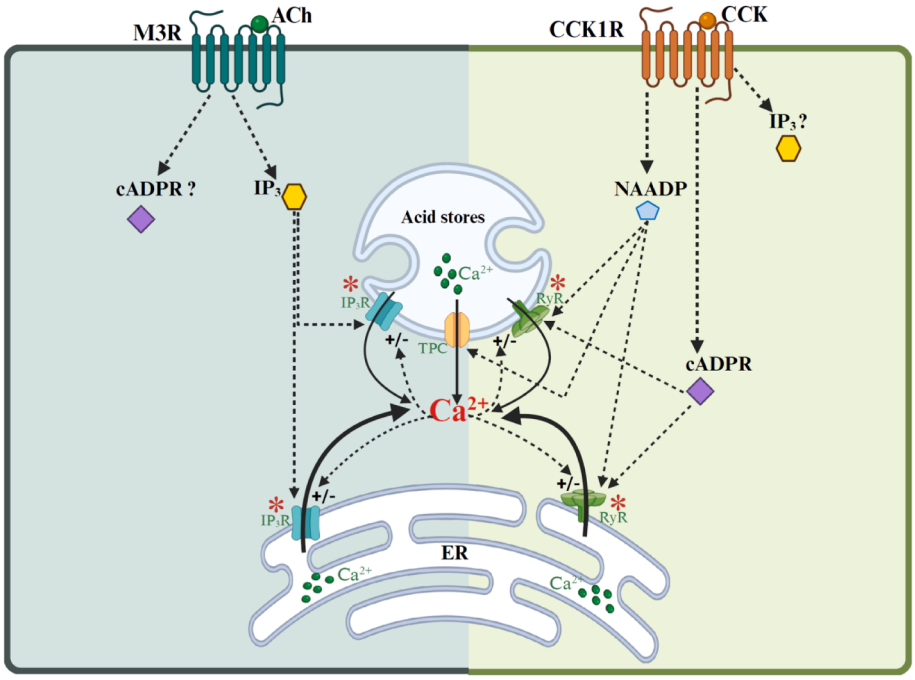


**Fig. 6.** Schematic diagram illustrating the different intracellular pathways employed by stimulation with ACh and CCK, respectively, to generate $Ca^{2+}$ spiking. The red asterisks represent input from various agents that potentially control the sensitivity of the ion channels to $Ca^{2+}$ and the various intracellular messengers. The±signs indicate that $Ca^{2+}$ feedback to the channels is positive (increasing the open state probability) when $[Ca^{2+}]_i$ starts rising, but turns negative (reducing the open state probability) when $[Ca^{2+}]_i$ reaches a high level. The diagram only deals with the initial processes controlling the release of $Ca^{2+}$ from intracellular stores. The subsequent and obligatory opening of CRAC channels in the plasma membrane is not represented. For further explanation, see text.

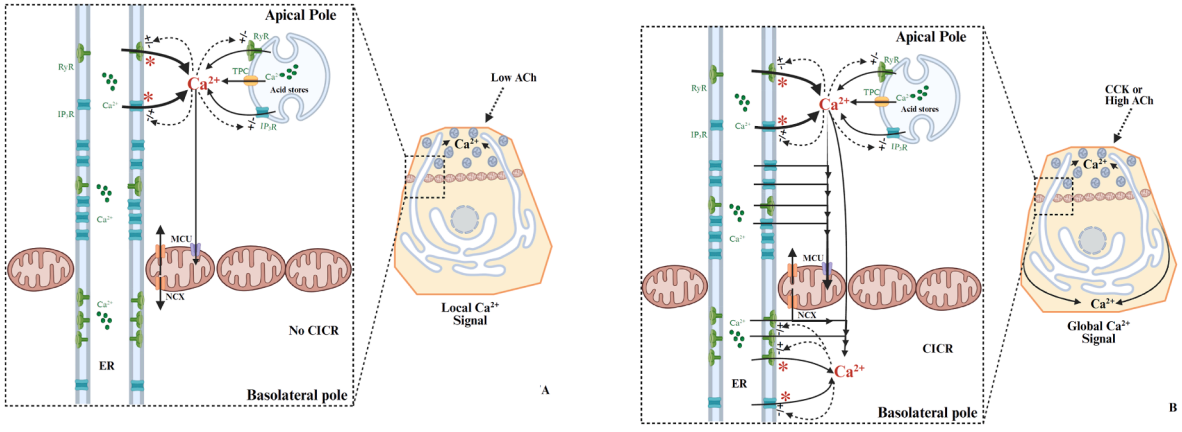


**Fig. 7.** Schematic diagram illustrating the concept of $Ca^{2+}$ signal globalisation and the potential underlying mechanism. **A** Local (apical) $Ca^{2+}$ signal that does not penetrate the peri-granular mitochondrial belt. **B** Larger $Ca^{2+}$ signal, initiated in the apical pole, that overwhelms the mitochondrial barrier and spreads throughout the cell due to $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) involving both $IP_3$Rs and ryanodine receptors. As in Fig. 6, the red asterisks represent the influence of potential sensitizing agents. For further explanation, see text.

into the basal area [72]. Nevertheless, the mitochondrial barrier can be overwhelmed by larger $Ca^{2+}$ signals, and this is what is happening at higher concentrations of the $Ca^{2+}$ releasing messengers. The peri-granular mitochondrial barrier can also become functionally ineffective if the electrical potential across the inner mitochondrial membrane collapses. This happens, for example, when the mitochondria become overloaded with $Ca^{2+}$, triggering the opening of the large permeability transition pores [5].

High concentrations of mobile low-affinity $Ca^{2+}$ buffers can also assist the spreading of intracellular $Ca^{2+}$ signals. The presence of such agents will blunt $Ca^{2+}$ signals by binding $Ca^{2+}$ and the $Ca^{2+}$-buffer complex can then diffuse away from the initial generation site, thereby spreading the attenuated $Ca^{2+}$ elevation [13]. The level of the resting $[Ca^{2+}]_i$ in the acinar cells also influences the agonist-elicited $Ca^{2+}$ signal pattern. In a study exploiting the fact that there is substantial variation in the resting $[Ca^{2+}]_i$, it was shown that the normal ACh signal pattern was mostly elicited at $[Ca^{2+}]_i$ ~100 nM, whereas at ~200 nM the same ACh concentration would often evoke $Ca^{2+}$ signals with a CCK-like signature [75]. Unfortunately, the mechanism underlying this phenomenon has not yet been identified, but the finding further emphasizes that the generation of a CCK-like signature is a complex phenomenon that cannot be explained by a simple single-molecule theory.

## 10. Models for control of $Ca^{2+}$ signal generation and globalization

The mechanism(s) of $Ca^{2+}$ signal initiation in the pancreatic acinar cells, as well as control of $Ca^{2+}$ signal globalization, are complex and we do not currently have sufficiently complete datasets that would allow us to construct definitive models. The simple notion that basic $Ca^{2+}$ signaling is always initiated by $IP_3$ formation and that CCK-elicited formation of NAADP would provide a modifying influence facilitating globalization [12,18], is not in agreement with substantial amounts of critical data already discussed above and must therefore be abandoned. What emerges, from an analysis of all available data, is a much more dynamic and flexible system than hitherto described, in which certain critical pathways can be switched on and off, dependent on the metabolic state of the cell and the concentration of the stimulating hormone or neurotransmitter. It is also clear that the mechanism controlling the initiation of $Ca^{2+}$ signaling may not necessarily determine whether the signal becomes global. It would appear that these are two separate issues.

It is generally accepted that ACh initiates $Ca^{2+}$ signals via $IP_3$ formation [5]. However, ACh also activates cADPR formation [54]. It is currently not clear whether this plays any functional role, because blockade of cADPR receptors by 8-$NH_2$-cADPR does not inhibit ACh-initiated $Ca^{2+}$ signaling [50]. CCK, at physiologically relevant concentrations, evokes production of both NAADP and cADPR, but no measurable $IP_3$ formation [53,54]. Nevertheless, CCK-elicited $Ca^{2+}$ signal generation is abolished by blockade of any of the receptors for the three messengers [52]. However, this is not so under all conditions. As shown in Fig. 3, an increase in the intracellular glucose concentration can markedly reduce the sensitivity of the $Ca^{2+}$ release mechanism to cADPR and create a situation in which the CCK response is no longer dependent on functional cADPR receptors. We do not know whether the same might apply to NAADP receptors, as this has not yet been tested. High intracellular glucose sensitizes the $IP_3$Rs to $IP_3$ and, in this situation, a small amount of $IP_3$ (not measurable with current technology) that might be produced by CCK stimulation could be sufficient to initiate $Ca^{2+}$ signal generation. In certain circumstances, it is therefore conceivable that CCK may initiate $Ca^{2+}$ signals via $IP_3$. $Ca^{2+}$ signal globalization is a complex phenomenon and there are many, very different, ways of influencing this process. Every one of the three $Ca^{2+}$-releasing messengers can, under the right circumstances, produce global $Ca^{2+}$ signals.

Production of intracellular $Ca^{2+}$ releasing messengers is not the only way to initiate $Ca^{2+}$ signaling. The sulphydryl-group-oxidising agent thimerosal generates substantial $Ca^{2+}$ signals in pancreatic acinar cells with a clear CCK-like pattern, but there is no indication of $IP_3$ production [76]. Most likely, thimerosal acts to sensitize $IP_3$Rs to the resting $IP_3$ level as well as activating ryanodine receptors [76]. There is evidence, also from work on hepatocytes, of such a mechanism [77]. Control of the excitability of the basal area is an important factor in $Ca^{2+}$ signal globalization. Everything else being equal, combination of several intracellular messengers favors $Ca^{2+}$ signal globalization (Fig. 7).

The many different ways of controlling $Ca^{2+}$ signal initiation, as well as $Ca^{2+}$ signal expansion, testify to the physiological importance of these signals that regulate pancreatic exocrine secretion. The redundancy in the system should guarantee that even if one regulatory branch fails, others provide back up. The short-lasting local $Ca^{2+}$ signals in the apical pole are sufficient for the control of exocytosis, which – under physiological conditions - can only occur across the apical acinar membrane, but $Ca^{2+}$ signals invading the nuclear region may be important for mitosis [78] and there is evidence indicating that CCK, rather than ACh, can promote pancreatic growth [79].

## 11. Translational aspects

The physiological $Ca^{2+}$ signaling system in the pancreatic acinar cells possesses remarkable precision, sensitivity and safety. It is precise because it specifically and primarily directs $Ca^{2+}$ signals to the apical pole near the apical membrane where the exocytosis process exclusively occurs. It responds to very low CCK concentrations and is sensitive to tiny changes in this concentration. It is safe because the repetitive and relatively short-lasting $Ca^{2+}$ spikes, in contrast to the effects of a sustained rise in $[Ca^{2+}]_i$, prevent inappropriate activation of processes that could lead to cell death.

The very low concentrations of CCK in the blood that occur physiologically [80] are sufficient to stimulate pancreatic secretion. This safeguards the pancreatic acinar cells from desensitization and may also be important for preventing activation of CCK receptors in the brain as the relatively small CCK molecules may, at least to some extent, be capable of penetrating the blood-brain barrier [27]. Although hyperstimulation of CCK receptors, by high doses of CCK, or the frog peptide analogue caerulein, has been, and continues to be, used experimentally to induce AP by many investigators, there is no evidence for the existence of high CCK levels in the blood of humans [80] and no evidence for CCK-elicited pancreatitis. Pharmacological inhibition of CCK receptors is therefore not important for AP therapy.

The extreme sensitivity of the pancreatic acinar cells to CCK may be physiologically advantageous, but also carries risks. Even in the fasting state, the CCK level in the blood is sufficient to generate small $Ca^{2+}$ signals in a significant minority of acinar cells *in vivo*, although the majority are 'silent' [11]. However, as shown in Fig. 5, a modest $Ca^{2+}$ challenge that is in itself insufficient to generate $Ca^{2+}$ spiking may do so in the presence of a subthreshold CCK concentration. This has implications for pathophysiology. In addition to the $Ca^{2+}$ signaling systems so far described in this article, the acinar cells possess the ability to open $Ca^{2+}$ influx channels in the plasma membrane in response to physical pressure, which is relevant to conditions under, for example, pancreatic surgery and blockage of the pancreatic duct. Physical pressure opens Piezo-1 channels in the acinar cell membrane, allowing a small $Ca^{2+}$ influx sufficient to activate phospholipase $A_2$ which, in turn, opens TRPV channels permeable to $Ca^{2+}$, through which a more significant amount of $Ca^{2+}$ can flow into the cell [81,82]. At relatively low physical pressures this might not in itself be sufficient to cause toxic $[Ca^{2+}]_i$ elevations. However, in the presence of a low just subthreshold CCK concentration, a relatively modest $Ca^{2+}$ influx generated by a small physical pressure could lead to dramatic and dangerously toxic $Ca^{2+}$ signals. Toxic $Ca^{2+}$ signals are always global and involve activation not only of the relatively few $IP_3$Rs in the basal part of the acinar cells, but also of the ryanodine receptors in that area (Fig. 7). $Ca^{2+}$ release through the basal ryanodine receptors may be of particular significance for the intra-acinar zymogen activation that is an important feature of AP [83].

The pronounced structural [1] and functional [5] polarity of the acinar cells is essential for safeguarding the pancreas. The apical granular pole contains the complete machinery for activation of both enzyme and fluid secretion, whereas the basal part provides the logistics, re-supplying the cell with $Ca^{2+}$ lost from the stores during signaling as well as importing amino acids and glucose needed for the energy production that sustains the energy-consuming secretion and signaling processes [5]. An important factor in the development of AP is the loss of signaling polarity and an increasing sustained and global $[Ca^{2+}]_i$ elevation [5]. The principal instigators of AP, bile acids or alcohol combined with fat, elicit large global $[Ca^{2+}]_i$ elevations, causing mitochondrial $Ca^{2+}$ overload and marked reduction in ATP formation [5,84]. $Ca^{2+}$

overloading of the mitochondria leads to opening of the large permeability transition pore and, therefore, depolarization of the inner mitochondrial membrane, preventing ATP formation [84]. Pharmacological blockade of the permeability transition pore could become an effective therapy against AP [85].

The loss of ATP is a crucial step in the development of AP. As already mentioned, mitochondrial $Ca^{2+}$ overload is an important factor, but reduced sugar metabolism - due to inhibition of the initial molecular step in the disposal of intracellular glucose, namely 6-phosphorylation [5,86] - also plays a role. There is strong evidence indicating that the excessive intracellular $Ca^{2+}$ release elicited by, for example, the combination of ethanol and fatty acids, leading to fatty acid ethyl ester formation, depends on functional $IP_3Rs$ [87]. The mechanism by which the fatty acid ethyl esters activate the $IP_3Rs$ is still unknown. The finding highlighted in this review, that a high intracellular glucose concentration - which would occur when the glucokinase is blocked [5,86] - markedly enhances the sensitivity of $IP_3Rs$ to $IP_3$ (Fig. 3) may provide one possible explanation.

The reduced ATP production that is a cardinal feature of AP creates a vicious circle. Lack of ATP prevents active $Ca^{2+}$ pump function, inhibiting both $Ca^{2+}$ extrusion from the cell as well as reuptake of released $Ca^{2+}$ into the ER. Inhibition of $Ca^{2+}$ extrusion changes the balance between $Ca^{2+}$ inflow and outflow across the plasma membrane, inevitably leading to an increased resting $[Ca^{2+}]_i$, which, as discussed above, by itself promotes $Ca^{2+}$ signal globalization, worsening an already dangerous situation.

Although both physiological and pathological $Ca^{2+}$ signals are primarily generated by release of $Ca^{2+}$ stored inside the acinar cells, this causes obligatory secondary opening of $Ca^{2+}$ entry channels (CRAC – $Ca^{2+}$ Release Activated $Ca^{2+}$ – channels) [88]. It is opening of these pores that ultimately is responsible for the cellular and then mitochondrial $Ca^{2+}$ overload that kills the pancreatic acinar cells in AP. In the absence of ATP, the acinar cells can only die by necrosis, because apoptosis requires ATP [5]. The $Ca^{2+}$ overload generated by excessive activation of CRAC channels can be specifically reduced by certain small molecule inhibitors [5,88].

Exocytosis, in general, requires intracellular $Ca^{2+}$ and Mg-ATP [89] and this is also the case for pancreatic secretion [5]. In AP, the balance between these two key elements is distorted with a too high $[Ca^{2+}]_i$ and a too low Mg-ATP concentration [5]. Any rational therapy must take this into account and aim to restore the natural balance. It has been demonstrated directly that it is possible to reduce excessively high levels of $[Ca^{2+}]_i$ by targeting CRAC channels with a small molecule specific inhibitor, thereby markedly reducing intracellular trypsin activation and necrosis [88]. This approach has subsequently been shown to have beneficial effects in treating experimental AP [90]. Trials have been in progress for some time to determine if this works in the clinic [91] and very recently the result of a phase2b clinical trial has been published [92]. The trial demonstrated a statistically significant reduction in the median time it took hyper-inflamed patients to tolerate solid food and also showed that the CRAC channel inhibitor prevented severe breathing problems, reduced the incidence of pancreatic dead tissue, and led to better overall outcomes compared to placebo [92].

The reduction of ATP production in AP, which is partly due to inhibition of the glucose-6 phosphorylation step [86], can be overcome by supplying galactose, which is taken up into the acinar cells via the same transporters as glucose, but can bypass the defective 6-phosphorylation step via the so-called Lelois pathway [5,86]. In animal experiments *in vivo*, it has been shown that galactose can restore depleted ATP levels caused by experimental AP [86]. Combining CRAC channel inhibition - thereby preventing cellular $Ca^{2+}$ overload - with galactose - thereby restoring metabolism and ATP production - may turn out to be a particularly advantageous therapy for severe AP [93].

The most dangerous aspect of AP is the potential development of systemic organ failure [94]. It is not the intra-acinar trypsin activation - which is an important feature of AP - that is responsible for the systemic inflammatory response that can be fatal [95], but rather the acinar necrosis, principally due to lack of ATP and toxic $[Ca^{2+}]_i$ elevation. This triggers invasion of inflammatory cells, including macrophages [5,9]. Inflammation spreads, also helped by production of the pro-inflammatory and vaso-dilatory peptide bradykinin, split off from bradykininogen by the enzyme kallikrein liberated from necrotic acinar cells [5]. The root cause of AP therefore seems to be acinar necrosis, triggering a major inflammatory response. Protection against the generation of toxic $Ca^{2+}$ signals and loss of ATP therefore remain important therapeutic objectives.

## 12. Future perspective

Although there has been significant progress in our understanding of the mechanism by which CCK elicits the cytosolic $Ca^{2+}$ signals that activate pancreatic acinar secretion, as reviewed in this article, there are still important gaps in our knowledge. The mechanism underlying the intriguing intracellular glucose switch between the cADPR and $IP_3$ pathways (Fig. 3) is currently obscure. We also do not know whether the requirement for operational NAADP receptors can be changed by glucose. If we were able to understand the glucose switch phenomenon, it could provide groundbreaking insights into regulation of messenger pathways, not only in the pancreatic acinar cells, but also more widely. One specific issue is that we lack good data about the free glucose levels inside the acinar cells and how these may change during CCK stimulation. Without such information, there will be little progress in this area.

A more general problem is the lack of reliable information about $[Ca^{2+}]_i$ levels and intracellular messenger concentrations during prolonged CCK stimulation. It is understandable that there has been an experimental focus on the acute effects of CCK, as these can be robustly determined, but CCK action will, under normal physiological conditions, be long lasting, and we know very little about events in this longer timeframe. Specifically, with regard to determinations of intracellular levels of NAADP and cADPR, it would be very advantageous to be able to determine these in individual cells, so that the precise time course could be observed which, in the studies so far carried out on populations of cells, will be obscured due to mixing of individual events with different timings. Progress will depend on the development of new sensitive methods.

The mechanism(s) by which NAADP and cADPR are produced in response to CCK stimulation have still not been fully clarified [55], but there is evidence for CD38 playing an important role [96]. However, there are also indications that there may be involvement of other pathways [96]. In this context, it is intriguing that CCK-elicited $Ca^{2+}$ signaling is not only dependent on CD38 [96], but also on Gq/11 [12]. The potential interrelationships are obscure and require clarification.

Our understanding of the different spatio-temporal $Ca^{2+}$ signaling patterns elicited by CCK and ACh is also far from complete. Given the specific requirement for functional NAADP receptors in the CCK pathway, it was reasonable to assume that the more frequent $Ca^{2+}$ signal globalization evoked by CCK stimulation as compared to what is observed with ACh, was due to the influence of NAADP receptor activation. However, the data discussed in this review with regard to the action of the frog peptide bombesin, which also evokes frequent $Ca^{2+}$ signal globalization,

but does not depend on NAADP, questions this assumption. Clearly more detailed studies of the action of bombesin, and – in particular – of its mammalian counterpart, gastrin releasing peptide, could be illuminating.

With regard to the 'real' physiology of CCK in the human body, there is also a need to further clarify the sites of action of CCK. Although, as reviewed in this article, there is no longer any doubt about direct CCK action on CCK1 receptors on the plasma membrane of human pancreatic acinar cells, it is possible that CCK also acts on CCK receptors on nerve endings surrounding the acinar units. This should be investigated directly by two-photon microscopy studies on lobules of human pancreatic tissue, like those previously done on mouse pancreatic lobules [97].

Whereas we have gained considerable insight into the role of $Ca^{2+}$ in the initiation and development of acute pancreatitis [5,9,84] and now even have the prospect of a rational therapy based on inhibition of $Ca^{2+}$ entry channels of the Orai1 type [5,88,92], we still do not know much about the potential role of $Ca^{2+}$ in the development of pancreatic cancer. There is some evidence linking CCK receptor activation to cancer development [98,99], but there are few studies of $Ca^{2+}$ homeostasis in pancreatic cancer cells. In the pancreatic cancer cell line AR42J, derived from a rat pancreatic tumor, neuronal characteristics never observed in normal pancreatic acinar cells were demonstrated [100], including the presence of voltage-activated $Ca^{2+}$ channels. In this cell line, bombesin elicited cytosolic $Ca^{2+}$ signals due to $IP_3$-mediated intracellular $Ca^{2+}$ release as in normal cells, whereas Substance P caused $Ca^{2+}$ signals by activating the voltage-gated $Ca^{2+}$ channels [100]. These cells did not respond to CCK. Similar detailed studies have not yet been carried out in other pancreatic cancer cells. In pancreatic acinar cells isolated from KPC mice (with 'driver mutations' in *KRAS* and *TP53* genes), the principal defect was a slowing down in the rate of $Ca^{2+}$ extrusion after $Ca^{2+}$ signal generation due to decreased expression of plasma membrane $Ca^{2+}$-activated ATPase ($Ca^{2+}$ pump) [101]. Given that pancreatic cancer cells may have certain neuronal characteristics, one should also investigate the possible presence of $Na^+/Ca^{2+}$ exchangers in the plasma membrane. This exchanger is important for the rapid extrusion of $Ca^{2+}$ from cells that possess voltage-gated $Ca^{2+}$ channels and does not exist in normal pancreatic acinar cells [102] but could potentially play an important role in cancer cells. In general, the role of $Ca^{2+}$ in the development of pancreatic cancer is an open field that should attract a lot of attention in the coming years.

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