Two different but converging messenger pathways to intracellular Ca^{2+} release: the roles of nicotinic acid adenine dinucleotide phosphate, cyclic ADP-ribose and inositol trisphosphate

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Hormones and neurotransmitters mobilize Ca^{2+} from the endoplasmic reticulum via inositol trisphosphate (IP_3) receptors, but how a single target cell encodes different extracellular signals to generate specific cyto-solic Ca^{2+} responses is unknown. In pancreatic acinar cells, acetylcholine evokes local Ca^{2+} spiking in the apical granular pole, whereas cholecystokinin elicits a mixture of local and global cytosolic Ca^{2+} signals. We show that IP_3 , cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (NAADP) evoke cytosolic Ca^{2+} spiking by activating common oscillator units composed of IP_3 and ryanodine receptors. Acetylcholine activation of these common oscillator units is triggered via IP_3 receptors, whereas cholecystokinin responses are triggered via a different but converging pathway with NAADP and cyclic ADP-ribose receptors. Cholecystokinin potentiates the response to acetylcholine, making it global rather than local, an effect mediated specifically by cyclic ADP-ribose receptors. In the apical pole there is a common early activation site for Ca^{2+} release, indicating that the three types of Ca^{2+} release channels are clustered together and that the appropriate receptors are selected at the earliest step of signal generation.

Keywords: cyclic ADP-ribose/inositol trisphosphate/NAADP/oscillator units/pancreatic acinar cells

Introduction

Crabtree (1999), reviewing generic Ca^{2+} signals and specific outcomes, has pointed out that, 'A surprising and yet vexing outcome of the rapid progress made in understanding signal transduction is the observation that while activation of specific receptors leads to highly specific biologic responses, these receptors seem to use ubiquitous signalling intermediates'. One of the ubiquitous signalling intermediates is Ca^{2+} (Pozzan *et al.*, 1994; Berridge, 1997; Parekh and Penner, 1997). It is generally accepted that hormone- or neurotransmitter-elicited intracellular Ca^{2+} release is mediated via inositol 1,4,5-trisphosphate (IP_3) generation and activation of IP_3 receptors in the endoplasmic reticulum (ER) (Berridge, 1997). However, in pancreatic acinar cells, acetylcholine (ACh) and cholecystokinin (CCK) can induce specific cytosolic Ca^{2+} signatures. ACh elicits repetitive short-

lasting cytosolic Ca^{2+} spikes in the apical granular pole. CCK evokes the same type of repetitive short-lasting local Ca^{2+} spikes, but at certain intervals a short spike triggers a much longer lasting global Ca^{2+} transient. The frequency of such global transients increases with increasing CCK concentrations, within the physiological range (1–10 pM) (Osipchuk *et al.*, 1990; Petersen, C. *et al.*, 1991; Thorn *et al.*, 1993; Petersen, O. *et al.*, 1994). These agonist-selective Ca^{2+} signatures cannot be explained simply by the IP_3 pathway. Different patterns of IP_3 generation (Hirose *et al.*, 1999) or phosphorylation of the IP_3 receptors (LeBeau *et al.*, 1999), or different couplings of Ca^{2+} release to Ca^{2+} entry (Parekh and Penner, 1997) could play a role, but it would also seem reasonable to consider the possibility that other Ca^{2+} -releasing messengers may be of functional importance.

Both cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) release Ca^{2+} from sea-urchin egg microsomes (Galione, 1993; Genazzani and Galione, 1997; Lee, 1997), and in pancreatic acinar cells it has been shown that both cADPR (Thorn *et al.*, 1994) and NAADP (Cancela *et al.*, 1999) can elicit cytosolic Ca^{2+} spiking. Furthermore, both functional cADPR and NAADP receptors are essential for the cytosolic Ca^{2+} spiking responses evoked by CCK (Cancela *et al.*, 1998, 1999). Nevertheless, it would appear that the action of CCK is also dependent on functional IP_3 receptors, since the responses are inhibited by blocking these receptors with heparin (Thorn and Petersen, 1993).

In general, the actions of most hormones and neurotransmitters, which release Ca^{2+} from internal stores, are blocked by heparin used as an IP_3 receptor antagonist, but the possible involvement of cADPR and NAADP receptors has not been tested (Petersen and Cancela, 1999). It is therefore not known whether the more complex mechanism of action that has emerged recently for the action of CCK on pancreatic acinar cells (Cancela *et al.*, 1998, 1999) is generally valid or whether different neurotransmitters and hormones could have different ways of mobilizing Ca^{2+} from internal stores. The pancreatic acinar cell represents an excellent model system to investigate this problem, as two different extracellular agonists, the hormone CCK and the neurotransmitter ACh, both act to release Ca^{2+} from intracellular stores (Petersen *et al.*, 1994).

We show here that whereas the CCK-induced Ca^{2+} signalling response is completely dependent on NAADP, cADPR and IP_3 receptors, the ACh-elicited Ca^{2+} spiking cannot be inhibited by blocking NAADP and cADPR receptors, but does depend on functional IP_3 receptors. We also show that both ACh- and CCK-elicited Ca^{2+} signalling are completely dependent on both IP_3 and ryanodine receptors, since all responses can be blocked both by ryanodine and caffeine. We demonstrate that CCK

markedly potentiates ACh-elicited responses, by changing the repetitive local Ca^{2+} spikes to much longer lasting global transients, and show that this novel phenomenon is specifically dependent on functional cADPR receptors. Finally, using a fast confocal acquisition protocol, we have identified a common early activation site for ACh- and CCK-induced Ca^{2+} release in the apical pole. We conclude that in a single target cell, separate neurotransmitter/hormone-evoked cytosolic Ca^{2+} signals are encoded by different combinations of intracellular Ca^{2+} release channels, which serve as separate routes to stimulate common Ca^{2+} oscillator units made up of IP_3 and ryanodine receptors.

Results

The importance of cADPR receptors

The pyridine nucleotide metabolite cADPR releases Ca^{2+} from sea-urchin egg microsomes (Lee, 1997) by acting on ryanodine receptors to stimulate Ca^{2+} -induced Ca^{2+} release (Galione *et al.*, 1991; Meszaros *et al.*, 1993). cADPR can elicit cytosolic Ca^{2+} spiking in pancreatic acinar cells (Thorn *et al.*, 1994) and cADPR receptors are involved in the response to a physiological CCK stimulus, since the cADPR antagonist 8- NH_2 -cADPR (Walseth and Lee, 1993; Cancela and Petersen, 1998) blocks Ca^{2+} spiking induced by this hormone (Cancela *et al.*, 1998). It is not known whether cADPR receptors are generally involved in agonist-elicited Ca^{2+} signalling or only play a role in certain pathways. We have therefore compared the effect of intracellular 8- NH_2 -cADPR on the responses elicited by ACh and CCK, using the patch-clamp whole-cell recording configuration (Hamill *et al.*, 1981). With this technique, putative Ca^{2+} -releasing messengers and antagonists placed in the pipette solution have access to the cytosol, and the Ca^{2+} -dependent current, which has previously been shown to be a sensitive measure of local cytosolic Ca^{2+} concentration changes in the apical pole of pancreatic acinar cells, can be monitored (Petersen, 1992; Thorn *et al.*, 1993, 1994, 1996; Petersen *et al.*, 1994; Tinel *et al.*, 1999).

In all experiments, we used low levels of CCK and ACh, just above threshold concentrations. The CCK concentrations used (2 and 5 pM) are physiological (Walsh, 1994). There are two types of CCK receptor: A and B. The type A receptor mediates the Ca^{2+} signal, which activates the enzyme secretion, and has a much higher affinity for CCK than gastrin. The type B receptor is also referred to as the gastrin receptor, as it has equal affinities for CCK and gastrin (Jensen, 1994). The cytosolic Ca^{2+} signals elicited by CCK in pancreatic acinar cells are clearly due to activation of type A receptors, since: (i) the gastrin concentrations required to elicit Ca^{2+} signals (and the resulting membrane potential and conductance changes) are several orders of magnitude higher than the CCK concentrations needed to induce such effects (Petersen, 1980; Jensen, 1994); and (ii) only the CCK type A, and not the type B receptor is expressed in normal mouse pancreatic acinar cells (Saillan-Barreau *et al.*, 1998).

Figure 1A shows the repetitive spikes of Ca^{2+} -sensitive current evoked by a very low (just above threshold) concentration of ACh in freshly isolated mouse pancreatic acinar cells (Petersen, C. *et al.*, 1991; Petersen, O. *et al.*, 1994). When the cADPR antagonist 8- NH_2 -cADPR was

Fig. 1. Blockade of cADPR receptors by 8- NH_2 -cADPR abolishes Ca^{2+} spiking elicited by CCK, but not by ACh. Repetitive spikes of Ca^{2+} -sensitive current were evoked by a low, just above threshold, concentration of ACh (A). When the cADPR antagonist 8- NH_2 -cADPR was included in the intracellular pipette solution, the ACh-evoked response was not blocked (B). In a series of experiments in which the effects of ACh and CCK were tested in the same cells, CCK was initially applied and a response was observed. Shortly thereafter, ACh was applied and a much larger response than normal was obtained (C). In the presence of 8- NH_2 -cADPR in the intracellular pipette solution, CCK failed to evoke any response, but the subsequent ACh application evoked a normal, non-potentiated, response (D).

included in the intracellular pipette solution, the ACh-evoked response was not blocked (Figure 1B). In the control cells, ACh (25 nM) evoked repetitive spiking responses in all nine cells tested. When 8- NH_2 -cADPR was present in the intracellular pipette solution at a concentration of 18 μM [a concentration previously shown to block Ca^{2+} spiking evoked by 5 pM CCK (Cancela *et al.*, 1998) and 10 μM cADPR, but not 10 μM IP_3 (Cancela and Petersen, 1998)], all 10 cells tested also responded to ACh by firing repetitive spikes of Ca^{2+} -dependent current.

We also tested the effects of ACh and CCK in the same cells. In the control experiments, CCK in a physiological concentration (5 pM) evoked a clear response (Figure 1C). When ACh (25 nM) was applied shortly after a period of CCK stimulation, the response obtained was much larger than normal (Figure 1C). The ACh-elicited short-lasting Ca^{2+} spikes (Figure 1A and B) had been converted into a prolonged continuous Ca^{2+} increase (Figure 1C). This CCK-evoked potentiation of the ACh response was observed in five of the seven cells tested using this

protocol. In the presence of 18 μM 8- NH_2 -cADPR in the intracellular pipette solution, 5 pM CCK failed, as expected (Cancela and Petersen, 1998; Cancela *et al.*, 1998), to evoke any response in the five cells tested, but subsequent ACh application evoked a normal, non-potentiated, response in all of the same five cells (Figure 1D). The CCK-evoked potentiation of the ACh response therefore depends on cADPR receptor activation. In a previous study (Petersen *et al.*, 1991), in which the actions of CCK and ACh were studied in the same cells, CCK potentiation of a subsequent ACh response was not observed. Interestingly, in that study (Petersen *et al.*, 1991), the intracellular pipette solution always contained 10 mM glucose, which is now known to abolish cADPR-elicited, but not IP_3 -induced, Ca^{2+} spiking (Cancela *et al.*, 1998). Although potentiation of ACh responses involves cADPR receptors, it is clear that the basic action of ACh, in contrast to the case for CCK, does not depend on functional intracellular cADPR receptors (Figure 1).

The importance of intracellular NAADP receptors

The next step was to investigate the role of NAADP, a novel Ca^{2+} -releasing agent in sea urchin eggs (Chini *et al.*, 1995; Lee and Aarhus, 1995; Genazzani and Galione, 1997; Lee, 1997). In sea urchin eggs, it would appear that the NAADP-sensitive stores are physically separated from those sensitive to IP_3 and cADPR (Genazzani and Galione, 1997; Lee, 1997). In pancreatic acinar cells, NAADP has recently been shown to be involved in CCK-evoked Ca^{2+} signalling (Cancela *et al.*, 1999), but it is not known whether NAADP receptors are generally involved in agonist-evoked Ca^{2+} release, like IP_3 and ryanodine receptors, or whether they are only used in certain specific pathways. NAADP-sensitive signalling exhibits a remarkable self-desensitization mechanism (Aarhus *et al.*, 1996; Genazzani *et al.*, 1996; Cancela *et al.*, 1999), which provides a good tool. When a high desensitizing concentration (100 μM) of NAADP was included in the pipette solution, the response to CCK (2 and 5 pM) was markedly inhibited or abolished (Cancela *et al.*, 1999). However, as seen in Figure 2, the response to ACh was not inhibited by the high intracellular NAADP concentration. In the control series (without NAADP), 11 of the 16 cells tested responded to ACh (25 nM). In the test series of 16 cells, with 100 μM NAADP in the pipette, ACh evoked the usual responses in 11 cells.

We also tested the effects of both CCK and ACh in the same cells with or without NAADP in the pipette solution. As seen in Figure 2A, CCK (2 pM) potentiated the ACh response, while ACh did not potentiate the CCK response ($n = 4$). The CCK-mediated potentiation was not blocked by 100 μM NAADP in the cell interior, even though NAADP had abolished the Ca^{2+} signal normally evoked by this CCK concentration (Figure 2C). CCK (2 pM) evoked responses in four of the five test cells tested, but no responses were recorded in the three test cells (with NAADP) although they all subsequently responded vigorously to ACh (potentiated response). These results indicate that ACh-evoked Ca^{2+} signal generation, in contrast to CCK-induced Ca^{2+} spiking, is independent of NAADP receptors. It is also clear that NAADP receptors, in contrast to cADPR receptors, do not play any role in CCK-evoked potentiation of the ACh response. In pan-

Fig. 2. Blockade of NAADP receptors by a high desensitizing concentration of NAADP abolishes Ca^{2+} spiking in response to CCK, but not to ACh stimulation. A low, physiological, CCK concentration (2 pM) evoked a potentiation of the ACh response (A). The response to ACh was not inhibited by 100 μM NAADP in the intracellular pipette solution (B). The CCK mediated potentiation was not blocked by NAADP, even though NAADP had abolished the Ca^{2+} signal evoked by CCK (C).

creatic acinar cells, the NAADP receptors are therefore specifically of importance for CCK-elicited Ca^{2+} signal generation.

The importance of ryanodine receptors

There is evidence for the existence of intimate functional relationships between ryanodine and IP_3 receptors (Boittin *et al.*, 1998). In pancreatic acinar cells, ryanodine has been shown to inhibit responses elicited by ACh, CCK or cADPR, but in a previous study, ryanodine (10 μM) failed to inhibit IP_3 -induced Ca^{2+} spiking, although ruthenium red (50 μM) markedly reduced the spiking frequency (Thorn *et al.*, 1994). We have tested the effects of ryanodine on the Ca^{2+} spiking responses elicited by NAADP and reinvestigated the actions of ryanodine on the Ca^{2+} oscillations elicited by ACh, IP_3 , CCK and cADPR. In general, ryanodine has complex effects on the ryanodine receptors, since low concentrations tend to open the channels, whereas higher concentrations are more likely to evoke closure. Ryanodine also binds more easily to open channels than to closed ones; this causes the use-dependence phenomenon (Sutko *et al.*, 1997). Initially using ryanodine concentrations of 10–50 μM , we observed enhanced spiking in the presence of 10 μM ryanodine, whereas at 50 μM , ryanodine evoked inhibition of Ca^{2+} spiking; we therefore focused our attention on this concentration in later studies [25 nM ACh, $n = 7$; 10 μM IP_3 (the non-metabolizable analogue inositol 2,4,5-trisphosphate was used), $n = 3$; 5 pM CCK, $n = 6$; 10 μM cADPR, $n = 5$; 50 nM NAADP, $n = 3$]. Figure 3 illustrates the strongly inhibitory or blocking effects of ryanodine on Ca^{2+} spiking. It would appear that ryanodine receptor involvement is required for all Ca^{2+} spiking responses in pancreatic acinar cells.

Fig. 3. Blockade of ryanodine receptors by ryanodine inhibits all Ca^{2+} spiking responses. Effects of ryanodine (Rya) (applied externally) on Ca^{2+} spiking in response to ACh (A), inositol 2,4,5-trisphosphate [Ins(2,4,5) P_3] (B), CCK (C), cADPR (D) and NAADP (E).

The importance of IP_3 receptors

Recent investigations have indicated that not only CCK, but also cADPR and NAADP, could recruit IP_3 receptors through a Ca^{2+} -induced Ca^{2+} release process (Cancela *et al.*, 1999; Petersen and Cancela, 1999). However, the conclusion that the IP_3 receptor is involved was based on the inhibitory effect of heparin. Unfortunately, heparin is not only an IP_3 antagonist, but also has other effects. For example, it can interact with GTP-binding proteins and therefore uncouple the plasma membrane receptors (Petersen and Cancela, 1999). Caffeine, although best known as an activator of ryanodine receptors, also inhibits the opening of IP_3 receptors (Wakui *et al.*, 1990; Parker and Ivorra, 1991; Brown *et al.*, 1992; Ehrlich *et al.*, 1994; Petersen and Cancela, 1999). This effect is clearly not mediated by an increase in the intracellular cyclic AMP concentration (Wakui *et al.*, 1990; Brown *et al.*, 1992), but is likely to be a direct effect on the IP_3 receptor or a closely associated protein, since it has been observed in single-channel current studies of isolated IP_3 receptors from cerebellum (Ehrlich *et al.*, 1994). Furthermore, caffeine has the advantage of being extremely membrane permeant. It can therefore be applied externally and its effects are rapidly reversible (Wakui *et al.*, 1990; Petersen and Cancela, 1999). It has previously been shown that high caffeine concentrations (10–20 mM) inhibit Ca^{2+} spiking evoked by ACh, IP_3 and various CCK analogues, as well

Fig. 4. Blockade of IP_3 receptors by caffeine inhibits Ca^{2+} spiking responses. Effect of caffeine on Ca^{2+} -dependent current spikes evoked by CCK, cADPR and NAADP. Caffeine (20 mM) was applied externally. CCK (5 pM)-evoked Ca^{2+} spikes were reversibly inhibited by caffeine (A). cADPR and NAADP were present in the internal patch pipette solution at 10 μM or 50 nM, respectively, and application of caffeine reversibly blocked the Ca^{2+} spikes evoked by both cADPR (B) and NAADP (C).

as the responses induced by low caffeine concentrations, which are most likely initiated by activation of ryanodine receptors (Wakui *et al.*, 1990; Thorn *et al.*, 1994; Petersen and Cancela, 1999). This inhibitory effect of a high caffeine concentration is not due to store depletion as the effect is rapidly reversible even in the complete absence of external Ca^{2+} (Wakui *et al.*, 1990). We have now tested the effects of caffeine on the actions of normal CCK, cADPR and NAADP (Figure 4).

When caffeine was applied externally to the bath solution, during continuous CCK application, the Ca^{2+} -dependent current spiking was abolished; this inhibitory effect was fully reversible ($n = 4$). Caffeine reversibly inhibited the cADPR-evoked Ca^{2+} spikes ($n = 7$) and reversibly inhibited the Ca^{2+} spikes evoked by 50 nM NAADP ($n = 12$). Caffeine and heparin are not very selective drugs, but to our knowledge the only action shared by these two very different compounds is the antagonism of IP_3 receptors. Our new caffeine data, taken in conjunction with the previously published heparin data (Wakui *et al.*, 1990; Cancela *et al.*, 1999; Petersen and Cancela, 1999), therefore provide fresh evidence for the involvement of IP_3 receptors in the CCK, cADPR and NAADP responses.

Localization of early activation sites

We investigated whether the Ca^{2+} signalling responses to ACh and CCK share the same Ca^{2+} release sites. High (supramaximal) concentrations of ACh or CCK elicit global cytosolic Ca^{2+} increases and these always start in the apical area and then spread as waves towards the basal part of the cells (Kasai and Augustine, 1990; Mogami *et al.*, 1997; Ito *et al.*, 1999). We have now looked at the initiation of the Ca^{2+} signals at the low concentrations of ACh and CCK (just above threshold) employed in our electropharmacological experiments (Figures 1–4). Figure 5A shows a band-scan experiment, where the

Fig. 5. Confocal fluorescence microscopy reveals similar positions of early activation sites for Ca^{2+} release by ACh and CCK. Using band scan (A, 8 bands/s; bar corresponds to 10 μm) or line scan (B, horizontal 10 μm , vertical 200 ms), ACh and CCK released Ca^{2+} from similar early activation sites in the same cells. At the top of each panel are shown, on the right, a transmitted light picture of the cell investigated (the bar corresponds to 2 μm) and, on the left, the fluorescence image showing the position of the nucleus (nuclei). Calibration of the colour coding of the cytosolic Ca^{2+} concentration for both panels is shown on the right.

effects of ACh and CCK have been compared in the same cell. In both cases the signal is initiated in a part of the secretory pole ($\sim 4 \times 4 \mu\text{m}$) close to the apical luminal membrane and spreads out in the secretory region in much the same way ($n = 4$). The band-scan experiments do not allow us to follow the evolution of the Ca^{2+} signals continuously. We therefore also carried out line-scan

experiments. We applied ACh or CCK twice in the same cell and observed that repeated application of the same agonist led to similar initial signal evolutions (ACh, $n = 5$; CCK, $n = 5$). We then compared the effects of ACh and CCK in the same cells. Figure 5B shows the result of such an experiment where the scan line goes through one of the nuclei and the apical region. An area with a width of 2 μm ,

Fig. 6. Schematic model showing that CCK and ACh initiate Ca^{2+} spiking from units of ryanodine and IP_3 receptors (InsP_3R) via separate intracellular pathways. The peptide hormone CCK initiates Ca^{2+} release through a specific combination of intracellular Ca^{2+} release channels with separate functions, where the NAADP receptor (NAADPR) is the trigger, the cADPR receptor via the ryanodine receptor (RyR) is an amplifier and the combination of IP_3 receptors and RyRs is the oscillator. The neurotransmitter ACh also acts through a combination of intracellular Ca^{2+} channels, where the InsP_3R is the trigger and the RyR- IP_3 complex is the amplifier/oscillator. The RyR is probably recruited by the action of Ca^{2+} released from the InsP_3R , but another unknown messenger cannot be excluded. CaM, calmodulin.

in which early activation sites for Ca^{2+} release can be seen, has been highlighted. As for other cells (Parker and Yao, 1991; Yao *et al.*, 1995; Parker *et al.*, 1996; Berridge, 1997; Marchant *et al.*, 1999), the evolution of a Ca^{2+} signal starts with a low frequency of small and short-lasting discrete events in one (or a few) area(s). The earliest events in the pancreatic acinar cells last for ~20–50 ms, have amplitudes of ~100–150 nM and spread out less than ~2 μm . The frequency and amplitude of these events then gradually increase and events begin to occur also in other neighbouring areas, so that there is Ca^{2+} release in virtually all parts of the granular region (Figure 5B), in general agreement with descriptions in other systems (Parker *et al.*, 1996; Berridge, 1997). As seen in Figure 5, the initial parts of the ACh- and CCK-elicited Ca^{2+} signal generation were very similar ($n = 16$), suggesting the existence of converging early release sites stimulated by different routes.

Discussion

Our experiments demonstrate both the unity and diversity in the agonist-induced cytosolic Ca^{2+} signalling systems operating in a classic cell biological model, namely the normal pancreatic acinar cell (Figure 6). The common feature of the ACh and the CCK response is the short-lasting Ca^{2+} spike in the apical pole (Petersen *et al.*, 1991; Thorn *et al.*, 1993). Our new results show that these local Ca^{2+} spikes evoked by ACh or CCK stimulation always depend on concerted activity of IP_3 and ryanodine receptors (Figures 3 and 4). Although IP_3 , cADPR and NAADP are each capable of eliciting cytosolic Ca^{2+} spikes (Petersen and Cancela, 1999), our data show that the Ca^{2+} -releasing actions of directly injected IP_3 , cADPR or NAADP all depend on concerted activation of IP_3 and ryanodine receptors. Closely clustered IP_3 and ryanodine

receptors therefore function as common oscillator units (Figure 6).

Our data demonstrate that the route to the common oscillator units is quite different for ACh and CCK stimulation. Although functional cADPR and NAADP receptors clearly are not required in order for ACh to evoke repetitive cytosolic Ca^{2+} spikes, these receptors are obligatory for the Ca^{2+} spiking responses elicited by CCK (Figures 1 and 2). In sea urchin eggs, NAADP-induced Ca^{2+} release does not behave as a Ca^{2+} -induced Ca^{2+} release system, suggesting that the only mechanism for activation of these channels would be by NAADP itself (Chini and Dousa, 1996). Furthermore, in pancreatic acinar cells, NAADP-induced Ca^{2+} spiking is blocked by a cADPR antagonist, whereas the cADPR-evoked response is not blocked by a high desensitizing concentration of NAADP (Cancela *et al.*, 1999). For the responses initiated by CCK, the NAADP receptors are therefore probably the triggers and recruit cADPR/ryanodine and finally IP_3 receptors (Figure 6). Since it is clear that neither cADPR nor NAADP receptors are involved in the pathway initiated by ACh stimulation, it would be simplest to propose that IP_3 receptors are activated initially and therefore are the triggers in this pathway (Figure 6).

In pancreatic acinar cells, the stimulant-evoked, local short-lasting Ca^{2+} spikes in the apical pole can continue for a long time in the complete absence of external Ca^{2+} (Wakui *et al.*, 1989, 1990). This can most easily be explained by effective Ca^{2+} tunnelling through a highly interconnected ER store, providing a large mobilizable intracellular Ca^{2+} reservoir (Villa *et al.*, 1991; Terasaki *et al.*, 1994; Mogami *et al.*, 1997). However, there are systems in which Ca^{2+} stores may not be so well coupled (Gennazani and Galione, 1997; Golovina and Blaustein, 1997; Lee, 1997) and such differences may account for observations showing that Ca^{2+} spiking from ryanodine receptors can be independent of IP_3 (Malgaroli *et al.*, 1990).

or, as for pancreatic acinar cells, depend on concerted action of the IP_3 and ryanodine receptors (Figure 6). Separate dynamic Ca^{2+} stores may also exist (Villa *et al.*, 1991; Gennazani and Galione, 1997; Lee, 1997). We cannot, therefore, exclude the possibility that the high NAADP concentration, which blocks the CCK, but not the ACh effect in our experiments (Figure 2), empties a small Ca^{2+} pool specifically linked to the NAADP receptor. This particular interpretation of our result would not, however, change the conclusion that the CCK response, in contrast to the ACh response, depends specifically on intracellular functional NAADP receptors (Figure 6).

In pancreatic acinar cells, ACh and CCK elicit specific Ca^{2+} signals (Osipchuk *et al.*, 1990; Petersen, C. *et al.*, 1991; Petersen, O. *et al.*, 1994). CCK not only stimulates secretion of digestive enzymes, but also promotes pancreatic growth, whereas ACh mainly stimulates secretion (Petersen *et al.*, 1994). It is possible that the growth-promoting action of CCK is due to global Ca^{2+} transients, although there is no specific evidence for this. It is well established that global increases in cytosolic Ca^{2+} , which can display complex patterns, such as waves and oscillations, result from the co-ordinated activity of elementary Ca^{2+} release units (Lechleiter *et al.*, 1991; Parker *et al.*, 1996; Thomas *et al.*, 1996; Berridge, 1997). Elementary Ca^{2+} release units are of two types: (i) ' Ca^{2+} sparks', involving only ryanodine receptors in cardiac muscle cells (Cheng *et al.*, 1993; Cannell and Soeller, 1999); and (ii) ' Ca^{2+} puffs' in, for example, *Xenopus* oocytes and PC12 cells (Yao *et al.*, 1995; Parker *et al.*, 1996; Reber and Schindelholtz, 1996), involving only IP_3 receptors. In portal vein smooth muscle cells, it has been shown that elementary Ca^{2+} release sites can also be composed of a mixture of IP_3 and ryanodine receptors (Boittin *et al.*, 1998). Our imaging data from pancreatic acinar cells (Figure 5) indicate that the Ca^{2+} spikes originate from the same part of the apical region and are basically similar, irrespective of agonist type. The earliest Ca^{2+} release events last for 20–50 ms and have an amplitude of 100–150 nM with a spread of 2 μm . Like all channel events, the small Ca^{2+} releases occur stochastically, but the probability of observing such events increases throughout the period following the start of agonist application (Parker *et al.*, 1996).

It would appear that early Ca^{2+} release sites contain IP_3 , ryanodine, cADPR and NAADP receptors, and represent the basic units of Ca^{2+} signal generation in pancreatic acinar cells. We have, therefore, in one unique normal cell, two different intracellular Ca^{2+} mobilization pathways, which converge on the same oscillator units consisting of IP_3 and ryanodine receptors. The agonist can therefore specifically pick up the appropriate receptors giving rise to a specific Ca^{2+} signal. In view of the limited range of Ca^{2+} diffusion (Baker, 1978; Allbritton *et al.*, 1992; Kasai and Petersen, 1994), an interesting consequence of this particular organization of the intracellular receptors is the possibility of cross-talk between agonists, as observed in our study. In this particular case, CCK markedly potentiates the ACh response and this potentiation phenomenon requires cADPR receptors (Figure 1). Previous studies of pancreatic acinar cells show that whereas short-lasting (a few seconds duration) Ca^{2+} spikes are local, longer lasting transients inevitably spread throughout the whole cytosol (Osipchuk *et al.*, 1990;

Thorn *et al.*, 1993; Tinel *et al.*, 1999). The cADPR-mediated potentiation of the ACh response, elicited by CCK (Figure 1), must therefore involve a major spatial extension of the cytosolic Ca^{2+} increase from the apical pole to the whole of the cell.

On the basis of our data, a general model for Ca^{2+} spike generation in the apical pole can be produced. To generate a Ca^{2+} spike by ACh or CCK, concerted activity of IP_3 and ryanodine receptors is needed. ACh stimulates the production of IP_3 , which in turn releases Ca^{2+} through IP_3 receptors and thereby also recruits ryanodine receptors, via Ca^{2+} -induced Ca^{2+} release, although the involvement of another unknown messenger cannot be excluded. Physiological concentrations of CCK (5–10 pM) may not generate IP_3 (Matozaki *et al.*, 1990), but may instead activate NAADP and cADPR receptors by messenger formation or by sensitization of these Ca^{2+} release channels via other unknown mechanisms. The enzyme ADPR cyclase, which is responsible for NAADP (and cADPR) formation, has been found in pancreatic acinar cells (Cancela *et al.*, 1999). In these cells, it is not known whether CCK stimulation results in an increase in intracellular NAADP or cADPR concentration, but in longitudinal intestinal smooth muscle cells, it has been shown that the CCK receptor is coupled to ADPR cyclase (Kuemmerle and Makhlof, 1995). Ca^{2+} release from channels regulated by NAADP and cADPR (ryanodine receptors) in the vicinity of the IP_3 receptors could sensitize the latter and lead to the major Ca^{2+} release generating the spikes.

One characteristic of CCK signals is that the Ca^{2+} release becomes global at some point (Thorn *et al.*, 1993). IP_3 receptors of all three subtypes are localized in the apical pole (Nathanson *et al.*, 1994; Lee *et al.*, 1997), in agreement with the finding that the local ACh-evoked response is blocked by caffeine and heparin (Wakui *et al.*, 1990). However, the existence of a low density of IP_3 receptors in the basolateral part of the cell cannot be excluded and is indeed likely (Kasai *et al.*, 1993; Thorn *et al.*, 1993). There are at present no data about NAADP receptors or their localization in pancreatic acinar cells or in any other cell type. cADPR activates ryanodine receptors of subtype 2 (Meszaros *et al.*, 1993), which are known to be present in pancreatic acinar cells (Leite *et al.*, 1999). In contrast to what has been shown for IP_3 receptors, ryanodine receptors seem to be widely distributed in acinar cells (Leite *et al.*, 1999). It would appear, therefore, that there are different receptor ratios in different parts of the cell. The apical pole seems to be richer in IP_3 than in ryanodine receptors, whereas the basolateral part of the cell is richer in ryanodine receptors. This could explain the global response to CCK stimulation, which is normally dependent on cADPR activation of ryanodine receptors.

Active mitochondria play a crucial role in confining IP_3 -elicited Ca^{2+} spikes generated in the apical granular pole to this part of the cell (Tinel *et al.*, 1999). In general, the ability of mitochondria to take up Ca^{2+} released from internal stores would depend on close proximity between the part of the ER store providing the released Ca^{2+} and the mitochondria (Pozzan *et al.*, 1994; Rizzuto *et al.*, 1998; Csordas *et al.*, 1999). Because of the different receptor pathways used by ACh and CCK stimulation, it is possible

that the ability of CCK to produce global responses, which becomes more pronounced at higher agonist concentrations (Petersen *et al.*, 1994), could be due to part of the intracellular Ca^{2+} release channel population being relatively far away from the mitochondrial ring surrounding the granular pole (Tinel *et al.*, 1999).

Finally, the ability of CCK to produce global responses may also rely on the particular distribution of NAADP receptors. Being a trigger, the localization of NAADP receptors may be of crucial importance in order to initiate the Ca^{2+} response at the appropriate time and place. In more general terms, the specific location of the trigger, whether it is IP_3 or NAADP, may be crucial in shaping the Ca^{2+} signals generated by the agonists.

Materials and methods

Isolation of pancreatic acinar cells

Isolated single and double mouse pancreatic acinar cells were prepared and loaded with Fura red AM as described previously (Gerasimenko *et al.*, 1996).

Patch-clamp recordings

Cells were investigated using the whole-cell patch-clamp configuration. From a holding potential of -30 mV, steps were made to 0 mV, the reversal potential of the two Ca^{2+} -dependent currents through Cl^- and non-selective cation channels (Thorn and Petersen, 1992). Using our solutions, the reversal potential of both the Cl^- and non-selective cation currents was at 0 mV (for use as a potential control) (Petersen *et al.*, 1991). Small deviations in E_{Cl} and E_{cation} and in the holding potential sometimes produce small inward or outward currents at 0 mV. At -30 mV we obtained a measure of both the Ca^{2+} -dependent currents, which are an index of the cytosolic Ca^{2+} changes (Thorn *et al.*, 1993; Tinel *et al.*, 1999). The extracellular Na^+ -rich solution contained (in mM): 140 NaCl, 4.7 KCl, 1.13 MgCl_2 , 10 glucose, 1 CaCl_2 and 10 HEPES–NaOH pH 7.2 . CCK octapeptide or ACh was added to the external solution as indicated. The internal solution contained (in mM): 140 KCl, 1.13 MgCl_2 , 0.05 EGTA, 2 ATP and 10 HEPES–KOH pH 7.2 . Extracellular application of CCK, ACh, ryanodine and caffeine was performed by means of a gravity perfusion system.

Confocal imaging

Fluorescence measurements and calcium concentration calibration on Fura red loaded cells (Gerasimenko *et al.*, 1996) were performed using a Noran Odyssey confocal microscope. The K_D for Fura red– Ca^{2+} at room temperature was assumed to be 140 nM (Molecular Probes). An objective ($60\times$) with n.a. 1.4 and slit $25\ \mu\text{m}$ was used in all experiments. For fast scanning experiments, 8 or 16 frames/s final scanning speed was used together with quarter of screen mode and online averaging 4 in slow mode (400 – 800 ns). For line scanning experiments, the slowest speed, 4 ms/line in slow mode, was used. Image processing was performed using TwoD Analysis (Noran Inc.). Images were shade corrected (divided by first image) and inverted (subtracted from saturated image) (Gerasimenko *et al.*, 1996). A linear colour scale was used in all cases.

Chemicals

NAADP, cADPR, CCK, ACh and caffeine were purchased from Sigma. Ryanodine mixture was obtained from Calbiochem. Fura red and $8\text{-NH}_2\text{-cADPR}$ were from Molecular Probes.

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