Transformation of local Ca\textsuperscript{2+} spikes to global Ca\textsuperscript{2+} transients: the combinatorial roles of multiple Ca\textsuperscript{2+} releasing messengers

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In pancreatic acinar cells, low, threshold concentrations of acetylcholine (ACh) or cholecystokinin (CCK) induce repetitive local cytosolic Ca\textsuperscript{2+} spikes in the apical pole, while higher concentrations elicit global signals. We have investigated the process that transforms local Ca\textsuperscript{2+} spikes to global Ca\textsuperscript{2+} transients, focusing on the interactions of multiple intracellular messengers. ACh-elicited local Ca\textsuperscript{2+} spikes were transformed into a global sustained Ca\textsuperscript{2+} response by cyclic ADP-ribose (cADPR) or nicotinic acid adenine dinucleotide phosphate (NAADP), whereas inositol 1,4,5-trisphosphate (IP\textsubscript{3}) had a much weaker effect. In contrast, the response elicited by a low CCK concentration was strongly potentiated by IP\textsubscript{3}, whereas cADPR and NAADP had little effect. Experiments with messenger mixtures revealed a local interaction between IP\textsubscript{3} and NAADP and a stronger global potentiating interaction between cADPR and NAADP. NAADP strongly amplified the local Ca\textsuperscript{2+} release evoked by a cADPR/IP\textsubscript{3} mixture eliciting a vigorous global Ca\textsuperscript{2+} response. Different combinations of Ca\textsuperscript{2+} releasing messengers can shape the spatio-temporal patterns of cytosolic Ca\textsuperscript{2+} signals. NAADP and cADPR are emerging as key messengers in the globalization of Ca\textsuperscript{2+} signals.

Keywords: cyclic ADP-ribose/inositol trisphosphate/local and global calcium/NAADP/pancreatic acinar cells

Introduction

Ca\textsuperscript{2+} is one of the most versatile and important intracellular messengers, as it is involved in the control of many different cellular functions (Petersen et al., 1994; Berridge et al., 1998). Hormones and neurotransmitters can generate Ca\textsuperscript{2+} signals, such as local and global cytosolic Ca\textsuperscript{2+} elevations, and these can be transient (spiking) or sustained (Petersen et al., 1994; Thomas et al., 1996; Berridge, 1997; Meldolesi and Pozzan, 1998).

The mechanisms involved in the generation of local Ca\textsuperscript{2+} signals have been extensively investigated (Petersen et al., 1994; Parker et al., 1996; Berridge, 1997; Berridge et al., 2000; Jaggar et al., 2000). Local cytosolic Ca\textsuperscript{2+} signals can be generated by opening Ca\textsuperscript{2+} channels located either in the plasma membrane or in the endoplasmic reticulum (ER) membrane (Petersen et al., 1994; Berridge, 1997). Cells possess multiple Ca\textsuperscript{2+} releasing messengers such as inositol trisphosphate (IP\textsubscript{3}), cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) (Berridge, 1997; Guse, 1999; Petersen and Cancela, 1999; Lee, 2000; Cancela, 2001). If the Ca\textsuperscript{2+} release elicited locally by a particular stimulus is sufficiently small, a highly localized cytosolic Ca\textsuperscript{2+} spike can be generated. This is due to cytoplasmic buffers with low mobility. The mitochondria play a particularly important role with respect both to local Ca\textsuperscript{2+} buffering and local Ca\textsuperscript{2+} signal-dependent ATP generation (Pozzan et al., 1994, 2000; Rizzuto et al., 1998; Tinel et al., 1999; Rizzuto et al., 2000; Park et al., 2001a).

Several mechanisms have been proposed to explain the transformation of a local Ca\textsuperscript{2+} spike into a global Ca\textsuperscript{2+} transient. A higher concentration of a second messenger could be produced by a higher extracellular agonist concentration (Parker et al., 1996; Berridge, 1997; Ito et al., 1999) and a local Ca\textsuperscript{2+} signal could be propagated as a global Ca\textsuperscript{2+} wave via a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release mechanism (CICR) (Parker et al., 1996; Berridge, 1997). In order to create a fully sustained Ca\textsuperscript{2+} signal, activation of Ca\textsuperscript{2+} entry must occur (Berridge, 1997; Parekh and Penner, 1997).

The pancreatic acinar cell represents an excellent system in which to investigate the mechanism of Ca\textsuperscript{2+} signal globalization. The pattern of receptor-activated cytosolic Ca\textsuperscript{2+} oscillations depends on the receptor type, the agonist concentration and the intracellular buffering of Ca\textsuperscript{2+} (Petersen et al., 1991a). The two physiologically most important agonists, acetylcholine (ACh) and cholecystokinin (CCK), can generate both local and global Ca\textsuperscript{2+} signals. At high concentrations, both agonists elicit global Ca\textsuperscript{2+} responses, but at low, just suprathreshold concentrations, ACh evokes local repetitive Ca\textsuperscript{2+} spikes, whereas CCK, in addition to such local signals also occasionally induces global and relatively long-lasting Ca\textsuperscript{2+} transients (Petersen et al., 1991a; Thorn et al., 1993).

We have previously investigated the mechanism underlying the generation of local Ca\textsuperscript{2+} spikes in the apical (secretory) pole of the cell. The local cytosolic Ca\textsuperscript{2+} spikes are due to Ca\textsuperscript{2+} release from common oscillator units composed of IP\textsubscript{3} and ryanodine receptors. ACh activation of these common oscillator units is triggered via IP\textsubscript{3} receptors, whereas CCK responses are triggered via a different, but convergent, pathway dependent on NAADP and cADPR receptors (Cancela and Petersen, 1998; Cancela et al., 1998, 1999, 2000; Petersen and Cancela, 1999; Cancela, 2001). However, the mechanisms involved in the generation of global Ca\textsuperscript{2+} transients and global sustained Ca\textsuperscript{2+} elevations remain unclear.
In view of the finding that physiological CCK concentrations evoke very little IP₃ production (Matozaki et al., 1990) and the recent report that ACh stimulation may result in both IP₃ and cADPR generation (Fukushi et al., 2001), we have investigated the functional consequences of the interaction of multiple intracellular messengers in the generation of local Ca²⁺ spikes and global Ca²⁺ transients. We have studied the process that transforms local Ca²⁺ spikes to global Ca²⁺ transients by the patch-clamp whole-cell recording technique combined with confocal Ca²⁺ imaging. The local Ca²⁺ spikes evoked by a low concentration of ACh were transformed into a global sustained Ca²⁺ response by cADPR or NAADP, whereas IP₃ had a much weaker effect. In contrast, the CCK response was strongly potentiated by IP₃, whereas cADPR and NAADP had little effect. In the absence of ACh or CCK stimulation, NAADP alone, like IP₃ and cADPR, evoked cytosolic Ca²⁺ spiking confined to the apical pole of the cell. There were small mutually potentiating effects of cADPR and NAADP, or NAADP and IP₃, whereas a cADPR/IP₃ mixture was only very slightly more effective than either IP₃ or cADPR alone. However, NAADP strongly amplified the local Ca²⁺ release evoked by a cADPR/IP₃ mixture, eliciting a sustained global Ca²⁺ response. Our data demonstrate that different combinations of Ca²⁺ releasing messengers can shape the spatio-temporal pattern of Ca²⁺ signals.

Although all the three Ca²⁺ releasing messengers tested could initiate local Ca²⁺ spikes, globalization of the signals required interactions between them.

**Results**

**Globalization of the ACh response by cADPR and NAADP, but not IP₃**

We have recently reported that CCK potentiates the Ca²⁺-sensitive Cl⁻ current response to low, just suprathreshold concentrations of ACh. This potentiation is dependent on functional cADPR receptors and is blocked by a cADPR antagonist (Cancela et al., 2000). ACh at higher concentrations elicits global Ca²⁺ release, which could be due to increased IP₃ production and/or generation of cADPR (Fukushi et al., 2001). Very high concentrations of IP₃ (>100 μM) (Petersen et al., 1991b) or cADPR (100 μM) (Thorn et al., 1994) can elicit sustained cytosolic Ca²⁺ elevations that are global. Here we investigated whether a low cADPR concentration, which alone would elicit local Ca²⁺ spikes, could transform a local Ca²⁺ signal evoked by ACh into a global Ca²⁺ wave. To do so, we dialysed the cells with an intracellular solution containing 10 μM cADPR and thereafter stimulated with ACh (Figure 1). When cADPR was present in the intracellular solution, ACh evoked long-lasting Ca²⁺-sensitive currents (Figure 1C; n = 8), which were associated with global Ca²⁺ waves.
in which NAADP elicited Ca\textsuperscript{2+} spiking, the subsequent ACh response was markedly potentiated (Figure 1D). Finally, since both the ACh- and CCK-elicited responses depend on functional IP\textsubscript{3} receptors, we decided to investigate whether IP\textsubscript{3} could also potentiate the ACh response. In the four cells tested with 15 μM IP\textsubscript{3} that elicited repetitive short lasting Ca\textsuperscript{2+} spikes, additional stimulation with 25 nM ACh evoked an increase in the spiking frequency and amplitude (Figure 1B). Comparing the typical records shown in Figure 1, it can be seen that the combinations of ACh and cADPR, as well as ACh and NAADP, produced much stronger responses (Figure 1C and D) than the combination of ACh and IP\textsubscript{3} (Figure 1B). This is remarkable, since IP\textsubscript{3} itself was a stronger stimulus than either cADPR or NAADP.

**Globalization of the CCK response by IP\textsubscript{3}, but not cADPR or NAADP**

Since receptors for IP\textsubscript{3}, cADPR and NAADP have all been shown to be involved in the response to CCK (Cancela et al., 2000), we investigated whether addition of one of these messengers could alter the normal Ca\textsuperscript{2+} signal pattern elicited by a physiological CCK concentration. The cells were internally perfused with either control solution or intracellular solutions containing IP\textsubscript{3}, cADPR or NAADP, and then stimulated by 2.5 or 5 pM CCK (Figure 2). As previously documented, CCK itself elicited a mixture of short-lasting repetitive spikes and much more long-lasting transients (Figure 2A; n = 11; Petersen et al., 1991a). When CCK (2.5–5 pM) was added to the external solution, a very large and sustained response was observed (Figure 2B; n = 7). Clearly, IP\textsubscript{3} had markedly potentiated the CCK response. In the presence of IP\textsubscript{3}, the CCK effect was not, as usual, immediately reversible, indicating that the potentiation by IP\textsubscript{3} was so strong that even during the period when CCK was being washed out, a substantial effect remained. During internal stimulation with cADPR (10 μM), low frequency spiking was observed (Figure 2C). When CCK (2.5 or 5 pM) was added to the external solution a fairly normal, non-potentiated, response was seen (Figure 2C; n = 9). Internal stimulation with NAADP (50 nM) also elicited Ca\textsuperscript{2+} spiking with a low frequency (Figure 2D). In this situation, CCK added on top of the internal stimulation again elicited a rather normal, non-potentiated response (Figure 2D; n = 7). These results indicate that NAADP and cADPR, in contrast to IP\textsubscript{3}, are unable to potentiate the response to CCK, although the receptors for all these messengers are involved in the generation of the normal CCK responses (Cancela et al., 2000).

Although low (and most likely physiologically relevant) concentrations of IP\textsubscript{3}, cADPR and NAADP each evoke local, short-lasting Ca\textsuperscript{2+} signals, the data shown in Figures 1 and 2 demonstrate that they can all be involved in global Ca\textsuperscript{2+} signal production. To understand how this can be achieved, we further characterized the functional consequences of multiple combinations of low messenger concentrations.

**NAADP evokes Ca\textsuperscript{2+} spiking localized in the secretory pole**

NAADP is the most potent Ca\textsuperscript{2+} releasing messenger found so far (Chini et al., 1995; Lee and Aarhus, 1995;
Genazzani and Galione, 1997; Cancela et al., 1999, 2000; Lee, 2000). In pancreatic acinar cells NAADP acts as a trigger, apparently eliciting a very small primary Ca\(^{2+}\) release, subsequently recruiting neighbouring IP\(_3\) and ryanodine receptors, which gives rise to the cytosolic Ca\(^{2+}\) elevation; this is observable with currently available methodology (Cancela et al., 1999, 2000). However, the spatial localization of the Ca\(^{2+}\) spikes evoked by NAADP is unknown.

Figure 3 shows the result from an experiment in which the effect of NAADP (50 nM) in the internal pipette solution was assessed both by patch-clamp recording of the Ca\(^{2+}\)-sensitive Cl\(^-\) current and by simultaneous confocal imaging of the Ca\(^{2+}\)-sensitive Fluo 4 fluorescence. As previously documented (Cancela et al., 1999, 2000), NAADP elicited repetitive short-lasting Ca\(^{2+}\) spikes. As shown in Figure 3, the cytosolic Ca\(^{2+}\) rise at the height of the spike was confined to the apical, granule-containing part of the cell (\(n = 7\)). The NAADP-elicited Ca\(^{2+}\) spikes are thus localized in exactly the same part of the cell as those evoked by IP\(_3\) (Thorn et al., 1993) and cADPR (Thorn et al., 1994).

**IP\(_3\) and cADPR evoke localized Ca\(^{2+}\) spiking without interaction**

Both IP\(_3\) and cADPR elicit local Ca\(^{2+}\) spikes in the secretory pole at low concentrations (<15 μM), whereas higher concentrations of IP\(_3\) or cADPR (>100 μM) elicit global Ca\(^{2+}\) rises (Petersen et al., 1991b, Petersen et al., 1994; Thorn et al., 1994). We investigated the effects of mixing low concentrations of IP\(_3\) and cADPR. IP\(_3\) (10–15 μM) or cADPR (10 μM) evoked repetitive local Ca\(^{2+}\) spikes in the granular part of the cell (Figure 4A and B; \(n = 4\) and 5, respectively). We then perfused the cells internally with a mixture of 10 μM cADPR and 15 μM IP\(_3\), which, in all 10 cells investigated, evoked typical repetitive short-lasting Ca\(^{2+}\)-sensitive currents corresponding to local Ca\(^{2+}\) elevations (Figure 4C). This indicates that there is no major cross-talk between these two messengers.

**NAADP has a modest locally potentiating effect on the local Ca\(^{2+}\) spiking evoked by IP\(_3\)**

The next sets of experiments were designed to test whether a mixture of low concentrations of IP\(_3\) and NAADP could generate larger responses than IP\(_3\) or NAADP alone. Figure 5A shows a typical response to stimulation with IP\(_3\) (15 μM), consisting of repetitive short-lasting spikes (\(n = 9\)) that were associated with Ca\(^{2+}\) elevations confined to the apical granular pole of the cell (\(n = 4\)). In five out of six cells, a mixture of NAADP (50 nM) and IP\(_3\) (15 μM) generated relatively large, repetitive, short-lasting Ca\(^{2+}\)-sensitive currents (Figure 5B), corresponding to local Ca\(^{2+}\) elevations (Figure 5C), whereas in the sixth cell, relatively large spikes on top of a small sustained elevation were observed. The main effect of adding NAADP on top of IP\(_3\) was to increase the amplitude of the local Ca\(^{2+}\) spikes.

**NAADP globalizes cADPR-elicited local Ca\(^{2+}\) spiking**

Both cADPR and NAADP receptors are involved in the Ca\(^{2+}\) response evoked by the hormone CCK (Caneca and Petersen, 1998; Cancela et al., 1999, 2000). The most striking feature of the response to a physiological CCK concentration (2–10 pM) is the mixture of short-lasting local Ca\(^{2+}\) spikes and long-lasting global Ca\(^{2+}\) transients (Petersen et al., 1991a). However, NAADP (Figure 3) and cADPR (Thorn et al., 1994) evoked mainly localized Ca\(^{2+}\) spiking in the granular part of the cell without triggering a long-lasting global Ca\(^{2+}\) wave. During internal perfusion with 10 μM cADPR, all the cells tested displayed short-lasting Ca\(^{2+}\)-sensitive currents (\(n = 14\)). To investigate how CCK could generate global Ca\(^{2+}\) waves using these particular messengers, we performed experiments with both NAADP and cADPR in the pipette solution. Since the same enzyme, ADP-ribosyl cyclase, forms both cADPR and NAADP, this experiment might mimic a relevant physiological situation (Lee, 2000). Figure 6A shows the result of an experiment in which a mixture of NAADP and cADPR elicits repetitive, relatively long-lasting Ca\(^{2+}\)-sensitive currents (\(n = 15\)). Figure 6B shows the result of an experiment simultaneously recording the Ca\(^{2+}\)-sensitive current and the cytosolic Ca\(^{2+}\) concentration in a single pancreatic acinar cell. Initially, the mixture of NAADP and cADPR elicits repetitive local Ca\(^{2+}\) spiking, but gradually there is an evolution to a pattern of relatively long-lasting global Ca\(^{2+}\) waves (\(n = 6\)). This suggests that although both NAADP and cADPR can act separately to initiate local Ca\(^{2+}\) elevations in the secretory pole of the cell, they can also act together to generate the mixed pattern of local Ca\(^{2+}\) spikes and global Ca\(^{2+}\) waves seen in a typical response to a physiological level of CCK.

**NAADP globalizes localized Ca\(^{2+}\) spiking evoked by a mixture of cADPR and IP\(_3\)**

The CCK response depends on functional NAADP and cADPR receptors, but there is also, as for ACh, a requirement for operational IP\(_3\) receptors. Since ACh can stimulate the ADP-ribosyl cyclase CD38 (Fukushi et al., 2001), both CCK and ACh may generate cADPR, NAADP and IP\(_3\), although the precise levels are likely to depend very much on the agonist and its concentration. However, the functional consequences of the concerted action of the three messengers have never been explored. The next sets of experiments were therefore designed to determine whether NAADP triggers a globalization of the local Ca\(^{2+}\) spikes evoked by a mixture of cADPR and IP\(_3\). Figure 7A and B shows that NAADP strongly potentiates the Ca\(^{2+}\) release evoked by the cADPR/IP\(_3\) mixture. Infusion of cells with the triple mixture NAADP (50 nM), cADPR (10 μM) and IP\(_3\) (15 μM) induced a sustained Ca\(^{2+}\) sensitive current, often with superimposed spikes, in six out of seven cells investigated. The sustained Ca\(^{2+}\) sensitive current corresponds to a sustained global Ca\(^{2+}\) elevation (\(n = 4\)) (Figure 7B). Comparing the responses to the mixture of NAADP and cADPR (Figure 6) with those to the triple mixture NAADP + cADPR + IP\(_3\) (Figure 7), it is apparent that whereas NAADP + cADPR typically elicited repetitive pulses of local and global Ca\(^{2+}\) transients, the triple mixture evoked a sustained global Ca\(^{2+}\) rise.

In order to assess the possible functional consequence of adding IP\(_3\) to a mixture of NAADP and cADPR, we measured the maximal amplitude of the Ca\(^{2+}\)-activated Cl\(^-\) current in the two sets of experiments. It has recently been demonstrated that all Ca\(^{2+}\)-sensitive Cl\(^-\) channels in pancreatic acinar cells are located in the apical (secretory) part of the plasma membrane (Park et al., 2001b). The
Fig. 3. NAADP evokes Ca\(^{2+}\) spiking in the secretory pole. Confocal fluorescence microscopy reveals that NAADP, at 50 nM in the intracellular pipette solution, evokes repetitive Ca\(^{2+}\) spikes localized in the apical pole. In the picture panel below the current trace is shown (left) the transmitted light picture of the cell investigated (tip of attached patch pipette is seen), (middle) the fluorescence image between spikes, and finally (right) the fluorescence image demonstrating the position of one of the Ca\(^{2+}\) spikes evoked by NAADP. By comparison with the transmitted light image, it can be seen that the spike occurs in the apical granular pole of the lower right cell. Calibration of the colour coding of the cytosolic Ca\(^{2+}\) concentration is shown at the right-hand side.

Fig. 4. A mixture of IP\(_3\) and cADPR only evokes local Ca\(^{2+}\) release. (A) Confocal fluorescence microscopy reveals that cADPR, at 10 μM in the intracellular pipette solution, evokes repetitive Ca\(^{2+}\) spikes localized in the apical pole. The repetitive Ca\(^{2+}\) spikes evoked by IP\(_3\) (10 μM) are localized in the apical pole (B) and the repetitive short-lasting Ca\(^{2+}\) spikes evoked by a mixture of cADPR (10 μM) and IP\(_3\) (15 μM) (C) are also localized in the apical pole (no mutual potentiation).

Ca\(^{2+}\)-dependent Cl\(^{-}\) current is responsible for driving acinar fluid secretion, which together with the exocytotic secretion of digestive enzymes (Nemoto et al., 2001) represents the major functional consequence of the
agonist-elicited cytosolic Ca$^{2+}$ elevation. The mean peak amplitude of the Ca$^{2+}$-dependent current response to NAADP + cADPR was (±SE) 435 ± 118 pA (n = 8), whereas the corresponding value for the triple mixture was 1283 ± 227 pA (n = 9). Thus, addition of IP$_3$ to the mixture of NAADP + cADPR markedly enhanced the Cl$^{-}$ current across the apical membrane, most likely due to an enhanced amplitude of the Ca$^{2+}$ concentration rise very close to the inner mouths of the Cl$^{-}$ channels.

Caffeine (20 mM), used as a permeant IP$_3$ receptor antagonist, was employed to test the need for functional IP$_3$ receptors in the concerted activity of the three messengers. Although best known as an activator of ryanodine receptors, caffeine 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). In the pancreatic acinar cells, caffeine (20 μM) does not release Ca$^{2+}$ via ryanodine receptors and does not deplete intracellular Ca$^{2+}$ stores. With IP$_3$ present in the pipette solution there is no Ca$^{2+}$ spiking when caffeine is present from the beginning of an experiment, but immediately (within seconds) after caffeine removal, Ca$^{2+}$ spiking starts (see Figure 5 in Wakui et al., 1990). 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; Cancela et al., 2000). Extracellular application of 20 mM caffeine dramatically, repeatedly and reversibly reduced the Ca$^{2+}$ release evoked by the triple messenger mixture (Figure 7A; n = 7). This indicates that functional IP$_3$ receptors are required for sustained Ca$^{2+}$ release.

**Discussion**

Our experiments demonstrate that the generation of global cytosolic Ca$^{2+}$ signals depend on interaction between different Ca$^{2+}$ releasing messenger pathways, which can be activated separately or in combination. We analysed the functional consequence of the concerted activity of IP$_3$, cADPR and NAADP. Our new results demonstrate that the spatio-temporal pattern of a cytosolic Ca$^{2+}$ signal can be shaped by different combinations of Ca$^{2+}$ releasing messengers. One important finding from this work is that although every messenger can initiate local Ca$^{2+}$ spikes, globalization of the Ca$^{2+}$ signal requires interaction
between these messengers. The strongest Ca²⁺ signals were obtained by a triple mixture containing IP₃, cADPR and NAADP.

**Agonist-specific Ca²⁺ signal patterns and messenger interactions**

In pancreatic acinar cells, ACh and CCK induce specific Ca²⁺ signal signatures (Petersen et al., 1991a; Petersen et al., 1994). Low and physiological concentrations of both agonists elicit repetitive, short-lasting Ca²⁺ spikes confined to the apical granular pole (Thorn et al., 1993). These local Ca²⁺ spikes are sufficient to elicit exocytosis, as assessed by capacitance measurements (Maruyama et al., 1993; Maruyama and Petersen, 1994), and fluid secretion, as assessed by monitoring the Ca²⁺-dependent Cl⁻ current across the apical membrane (Thorn et al., 1993; Park et al., 2001b). CCK, but not ACh, at physiological concentrations also elicits much longer-lasting global Ca²⁺ transients (Petersen et al., 1991a; Thorn et al., 1993). The frequency with which the global transients occur is concentration dependent. At the lower end of the physiological concentration range (1–10 µM) there are very infrequent, long, global transients, and the response essentially consists of repetitive local Ca²⁺ spikes, whereas at the top end long transients are seen regularly. The physiological importance of these global transients has not been clarified, but one possibility is that they are connected to the CCK-induced pancreatic growth response (Petersen et al., 1994). The results presented in Figures 1 and 2 indicate that the response to a low ACh concentration is
principal triggers by IP₃, since it can be markedly potentiataed by cADPR and NAADP, but not by IP₃, whereas the response to a physiological CCK concentration must be triggered by cADPR and NAADP, since it can be dramatically potentiataed by IP₃, but not by cADPR or NAADP. Clearly only complementary messengers can effecttively potentiatae a response. One important concluison to be drawn from our new data is that increasing the intensity of stimulation with any one agonist is likely to produce all three messengers and consequently to generate substantial Ca²⁺ waves. To do so, ACh and CCK may recruit these messengers in a different sequence. These findings also provide a mechanism to explain the marked potentiataion by CCK of the ACh response (Cancela et al., 2000).

The experiments with messenger mixtures revealed only a relatively minor interaction between IP₃ and cADPR (Figure 4), and a modest interaction between IP₃ and NAADP (Figure 5). There was a somewhat stronger potentiating interaction between cADPR and NAADP, but in order to obtain the type of global and sustained response that can result from combining CCK with IP₃ (Figure 2) or ACh with cADPR or NAADP (Figure 1), it was necessary to use all three messengers together (Figure 7). Using the membrane-permeant IP₃ receptor antagonist caffeine (Petersen and Cancela, 1999), we were able to demonstrate that activation of IP₃ receptors is essential for maintaining a sustained response. Application of caffeine led to a fully reversible transformation from a sustained cytosolic Ca²⁺ elevation to repetitive baseline spiking (Figure 7).

Localization of intracellular Ca²⁺ release channels

IP₃, cADPR and NAADP each simply evoke Ca²⁺ release in the apical pole. This has been well documented for IP₃ and cADPR (Thorn et al., 1993, 1994). Our data (Figure 3) indicate that the short-lasting Ca²⁺-dependent currents evoked by NAADP are associated with Ca²⁺ elevations specifically in the apical pole of the cells. This result demonstrates directly that infusion of NAADP into a cell can release Ca²⁺ in one specific region without affecting other parts. This could be due to the exclusive presence of NAADP receptors in the apical pole or to a much higher concentration of NAADP receptors in this part of the cell than in the basal region. It could also be explained by a more diffuse presence of NAADP receptors throughout the cell, since the NAADP response is completely dependent on functional IP₃ and ryanodine receptors (Cancela et al., 2000). It is known that ryanodine receptors are present throughout the acinar cell (Leite et al., 1999; Fitzsimmons et al., 2000; Straub et al., 2000), whereas IP₃ receptors are preferentially localized in the apical pole (Nathanson et al., 1994; Lee et al., 1997). Most likely, cADPR elicits local Ca²⁺ spikes in the apical pole (Thorn et al., 1994) because of the concentration of IP₃ receptors in this region, since the cADPR responses are completely dependent on functional IP₃ receptors (Cancela et al., 2000). It is therefore entirely possible, and indeed likely, that the general primary localization of Ca²⁺ signals to the apical pole, irrespective of which Ca²⁺ releasing messenger is used, is principally due to the dominant presence of IP₃ receptors in this part of the cell. Since all Ca²⁺ signal initiation depends on interaction between at least ryanodine and IP₃ receptors, it must occur at sites where both these Ca²⁺ release channels co-exist, and the only such region is the apical pole.

The general concept concerning the organization of the major intracellular Ca²⁺ store, the ER, is that it forms a continuous sheet enclosing a single internal space. The continuous lumen allows Ca²⁺ and other small molecules to diffuse rapidly over relatively long distances (Terasaki et al., 1994; Mogami et al., 1997; Subramanian and Meyer, 1997; Park et al., 2000; Petersen et al., 2001). In the pancreatic acinar cells, the primary localization of cytosolic Ca²⁺ signal generation in the apical granular pole (Kasai et al., 1993; Thorn et al., 1993) is due to clustering of IP₃ receptors in the most apical parts of the ER extensions into the granular area (Lee et al., 1997). Opening of these channels can mobilize Ca²⁺ from the whole of the ER, and particularly from the major part of this store in the basolateral part of the cell due to the ability of Ca²⁺ to diffuse within the lumen of the ER, from the base of the cell to its apex (Mogami et al., 1997; Park et al., 2000; Petersen et al., 2001).

Globalization of Ca²⁺ signals

There would appear to be two separate aspects of the globalization process. Unlike the luminal continuous ER, the cytosol is effectively compartmentalized with respect to Ca²⁺ diffusion by a major mitochondrial Ca²⁺ buffer placed on the border between the apical granular pole and the rest of the cell (Tinel et al., 1999; Park et al., 2001a). This barrier undoubtedly plays a major role in mostly confining Ca²⁺ signals generated in the apical region to this part of the cell (Tinel et al., 1999; Park et al., 2001a). In order for a Ca²⁺ signal to become global, this barrier has to be overwhelmed by a substantial amount of Ca²⁺ released in the granular region. However, it is also known that when a Ca²⁺ wave progresses through the cell from the apical to the basal pole, there is a regenerative process, almost certainly due to Ca²⁺-induced Ca²⁺ release (Kasai and Augustine, 1990; Toescu et al., 1992, 1994). This means that under these circumstances Ca²⁺ release channels also open in the basal pole. The mechanism by which silent Ca²⁺ release channels in the basal pole become activated during globalization of Ca²⁺ signalling is not fully understood, but our data indicate that a combination of IP₃, cADPR and NAADP plays an important role in this process (Figure 8). A global Ca²⁺ wave is generated by the concerted activity of elementary Ca²⁺ release units, which act as ‘building blocks’ (Parker et al., 1996; Marchant et al., 1999; Berridge et al., 2000). The distance between these elementary units may vary between cell types, but the Ca²⁺ wave propagates by recruiting, in a salutary manner, neighbouring Ca²⁺ release sites (Parker et al., 1996; Berridge, 1997; Boitton et al., 1998; Cannell and Soeller, 1999; Koizumi et al., 1999; Marchant et al., 1999; Berridge et al., 2000). The Ca²⁺ release units are recruited by several mechanisms, including Ca²⁺ diffusion, Ca²⁺-induced Ca²⁺ release and increase of IP₃ production. All these mechanisms increase the frequency of elementary Ca²⁺ release events, which, once the threshold is reached, will trigger a Ca²⁺ wave. However, if poorly sensitive Ca²⁺ release units surround the most sensitive Ca²⁺ release units, then the Ca²⁺ signal remains localized and the Ca²⁺ wave is aborted (Parker et al., 1996; Marchant et al., 1999;
Berridge et al., 2000). This is exactly what happens in our experiments with the infusion of a low concentration of either IP$_3$, cADPR or NAADP, each of which is able to generate short-lasting Ca$^{2+}$ spikes in the apical pole of the cell without triggering a Ca$^{2+}$ wave. In this situation, the basolateral part of the cell contains poorly sensitive Ca$^{2+}$ release units that cannot trigger a wave (Figure 8). A Ca$^{2+}$ wave across the cell is generated by a combination of potentiated Ca$^{2+}$ release in the apical pole, helping to overcome the mitochondrial barrier, and sensitization of Ca$^{2+}$ release channels in the basolateral by coincident activation of ryanodine, IP$_3$ and NAADP receptors by their respective messengers (Figure 8).

**NAADP as a key messenger in Ca$^{2+}$ signal globalization**

From our present work, NAADP is emerging as a key messenger in the globalization of Ca$^{2+}$ signals. NAADP itself has a modest effect, since it only releases Ca$^{2+}$ in the apical part of the cell; however, its unique ability to interact with ryanodine and IP$_3$ receptor activity allows a substantial increase in the medium excitability to further activation by either cADPR or IP$_3$. Recent work in other cell types has shown that not only pancreatic acinar cells possess several Ca$^{2+}$ releasing messengers (Churchill and Galione, 2000, 2001; Cancela, 2001; Lee, 2001), suggesting that our model may be more generally valid. In systems such as ascidian oocytes, starfish oocytes, T lymphocytes and sea urchin eggs, NAADP but also cADPR and IP$_3$ release Ca$^{2+}$ from the internal stores in the same target cell (Albreux et al., 1998; Guse et al., 1999; Berg et al., 2000; Churchill and Galione, 2000, 2001; Santella et al., 2000). These cells may have one continuous Ca$^{2+}$ store or separate multiple Ca$^{2+}$ stores located in different regions (Malgaroli et al., 1990; Golovina and Blaustein, 1997; Lee, 1997, 2001; Hofer et al., 1998; Churchill and Galione, 2000, 2001; Patel et al., 2001).

Our work with IP$_3$, cADPR and NAADP has demonstrated the functional consequences of the actions of
different Ca\(^{2+}\) releasing messengers in one target cell (Figure 8). They can be recruited individually or in combination to give an important diversity of Ca\(^{2+}\) signals (Figure 8). The new types of messenger interaction unravelled in our work may represent the building blocks for the more complex associations seen during stimulation with agonists. This is important, because cells in their native environment are constantly surrounded by multiple stimuli and must respond in an appropriate manner.

Materials and methods

Isolation of pancreatic acinar cells

Isolated single and double mouse pancreatic acinar cells were prepared and loaded with Fluo 4 at 60 \(\mu\)M in the pipette solution as described previously (Park et al., 2001a,b).

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 CF- and non-selective cation channels (Thorn and Petersen, 1992). The CF-current is by far the most important quantitatively (Park et al., 2001b). Using our solutions, the reversal potential of both the CF- and non-selective cation currents were at 0 mV (Petersen et al., 1991a). Small deviations in \(E_{CF}\) 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; Tiné 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 were 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 and ACh was performed by means of a gravity perfusion system.

Confocal imaging

Fluorescence measurements and calcium concentration calibration on Fluo 4-loaded cells (Takahashi et al., 1999; Park et al., 2001a,b) were done using a Zeiss LSM510 confocal system. The \(K_0\) for Fluo 4–Ca\(^{2+}\) at room temperature was assumed to be 400 nM ( Molecular Probes). An objective (60\(\times\)) with NA 1.4 was used in all experiments. For fast scanning experiments, five frames per second final scanning speed was used. Fluo 4 was excited using a 488 nm laser light. Emitted light was collected using a BP505-550 filter. Image analysis was performed using the Zeiss confocal 510 image software as well as software developed by us. Images were divided by the first image. A linear colour scale was used in all cases.

Chemicals

NAADP, cADPR, 2,4,5-IP\(_3\), caffeine, CCK and ACh were purchased from Sigma. Fluo 4 and NAADP were from Molecular Probes.

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