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Transformation of local Ca^{2+} spikes to global Ca^{2+} transients: the combinatorial roles of multiple Ca^{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^{2+} spikes in the apical pole, while higher concentrations elicit global signals. We have investigated the process that transforms local Ca^{2+} spikes to global Ca^{2+} transients, focusing on the interactions of multiple intracellular messengers. ACh-elicited local Ca^{2+} spikes were transformed into a global sustained Ca^{2+} response by cyclic ADP-ribose (cADPR) or nicotinic acid adenine dinucleotide phosphate (NAADP), whereas inositol 1,4,5-trisphosphate (IP_3) had a much weaker effect. In contrast, the response elicited by a low CCK concentration was strongly potentiated by IP_3, whereas cADPR and NAADP had little effect. Experiments with messenger mixtures revealed a local interaction between IP_3 and NAADP and a stronger global potentiating interaction between cADPR and NAADP. NAADP strongly amplified the local Ca^{2+} release evoked by a cADPR/IP_3 mixture eliciting a vigorous global Ca^{2+} response. Different combinations of Ca^{2+} releasing messengers can shape the spatio-temporal patterns of cytosolic Ca^{2+} signals. NAADP and cADPR are emerging as key messengers in the globalization of Ca^{2+} signals.

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

Introduction

Ca^{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^{2+} signals, such as local and global cytosolic Ca^{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^{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^{2+} signals can be generated by opening Ca^{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^{2+} releasing messengers such as inositol trisphosphate (IP_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^{2+} release elicited locally by a particular stimulus is sufficiently small, a highly localized cytosolic Ca^{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^{2+} buffering and local Ca^{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^{2+} spike into a global Ca^{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^{2+} signal could be propagated as a global Ca^{2+} wave via a Ca^{2+}-induced Ca^{2+} release mechanism (CICR) (Parker *et al*., 1996; Berridge, 1997). In order to create a fully sustained Ca^{2+} signal, activation of Ca^{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^{2+} signal globalization. The pattern of receptor-activated cytosolic Ca^{2+} oscillations depends on the receptor type, the agonist concentration and the intracellular buffering of Ca^{2+} (Petersen *et al*., 1991a). The two physiologically most important agonists, acetylcholine (ACh) and cholecystokinin (CCK), can generate both local and global Ca^{2+} signals. At high concentrations, both agonists elicit global Ca^{2+} responses, but at low, just suprathreshold concentrations, ACh evokes local repetitive Ca^{2+} spikes, whereas CCK, in addition to such local signals also occasionally induces global and relatively long-lasting Ca^{2+} transients (Petersen *et al*., 1991a; Thorn *et al*., 1993).

We have previously investigated the mechanism underlying the generation of local Ca^{2+} spikes in the apical (secretory) pole of the cell. The local cytosolic Ca^{2+} spikes are due to Ca^{2+} release from common oscillator units composed of IP_3 and ryanodine receptors. ACh activation of these common oscillator units is triggered via IP_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^{2+} transients and global sustained Ca^{2+} elevations remain unclear.

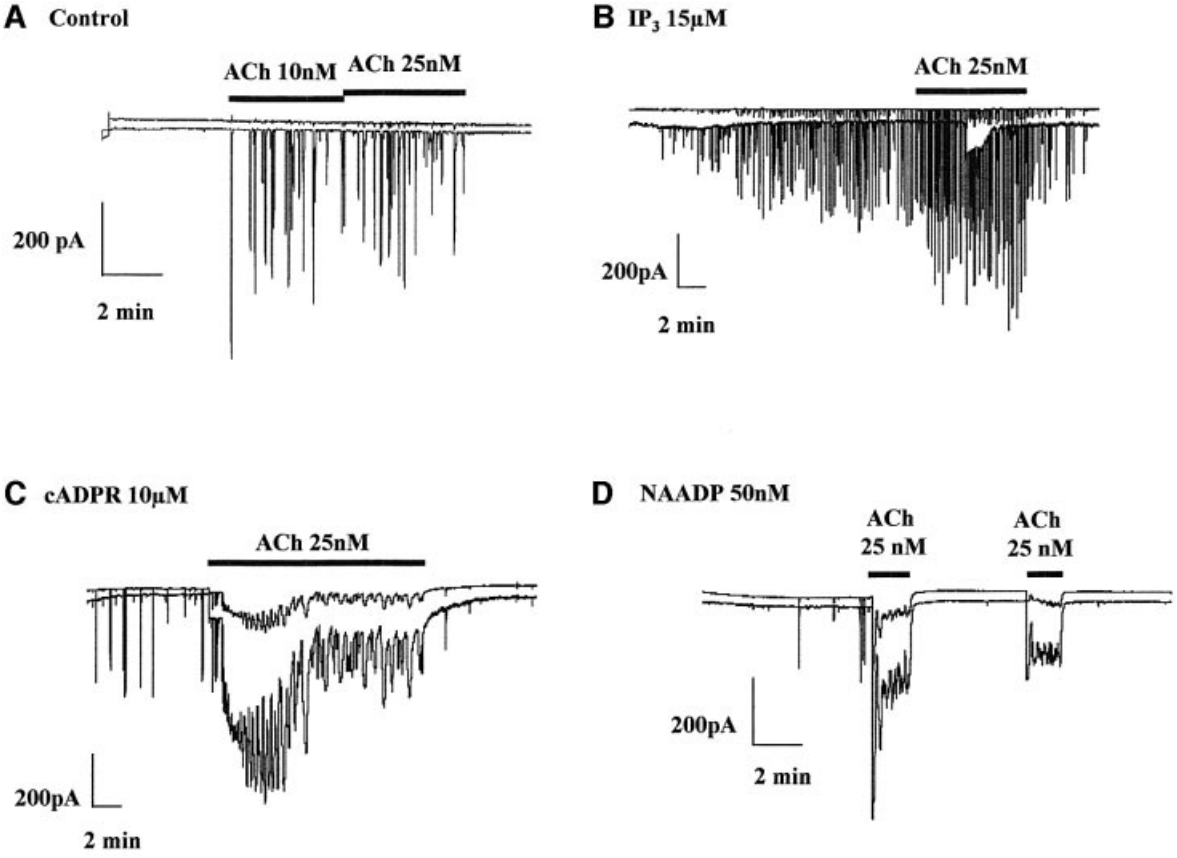

Fig. 1. Globalization of the local ACh response by cADPR and NAADP, but not IP_3. (**A**) Repetitive spikes of Ca^{2+}-sensitive current were evoked by low, just suprathreshold concentrations of ACh. When IP_3 at 15 μM was included in the intracellular pipette solution, the ACh-evoked response was normal (**B**), but when the intracellular pipette solution contained either cADPR (10 μM) (**C**) or NAADP (50 nM) (**D**) ACh elicited larger sustained responses.

In view of the finding that physiological CCK concentrations evoke very little IP_3 production (Matozaki *et al*., 1990) and the recent report that ACh stimulation may result in both IP_3 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 Ca^{2+} spikes and global Ca^{2+} transients. We have studied the process that transforms local Ca^{2+} spikes to global Ca^{2+} transients by the patch–clamp whole-cell recording technique combined with confocal Ca^{2+} imaging. The local Ca^{2+} spikes evoked by a low concentration of ACh were transformed into a global sustained Ca^{2+} response by cADPR or NAADP, whereas IP_3 had a much weaker effect. In contrast, the CCK response was strongly potentiated by IP_3, whereas cADPR and NAADP had little effect. In the absence of ACh or CCK stimulation, NAADP alone, like IP_3 and cADPR, evoked cytosolic Ca^{2+} spiking confined to the apical pole of the cell. There were small mutually potentiating effects of cADPR and NAADP, or NAADP and IP_3, whereas a cADPR/IP_3 mixture was only very slightly more effective than either IP_3 or cADPR alone. However, NAADP strongly amplified the local Ca^{2+} release evoked by a cADPR/IP_3 mixture, eliciting a sustained global Ca^{2+} response. Our data demonstrate that different combinations of Ca^{2+} releasing messengers can shape the spatio-temporal pattern of Ca^{2+} signals. Although all the three Ca^{2+} releasing messengers tested could initiate local Ca^{2+} spikes, globalization of the signals required interactions between them.

Results

Globalization of the ACh response by cADPR and NAADP, but not IP_3

We have recently reported that CCK potentiates the Ca^{2+}-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 Ca^{2+} release, which could be due to increased IP_3 production and/or generation of cADPR (Fukushi *et al*., 2001). Very high concentrations of IP_3 (>100 μM) (Petersen *et al*., 1991b) or cADPR (100 μM) (Thorn *et al*., 1994) can elicit sustained cytosolic Ca^{2+} elevations that are global. Here we investigated whether a low cADPR concentration, which alone would elicit local Ca^{2+} spikes, could transform a local Ca^{2+} signal evoked by ACh into a global Ca^{2+} 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 Ca^{2+}-sensitive currents (Figure 1C; $n = 8$), which were associated with global Ca^{2+} waves

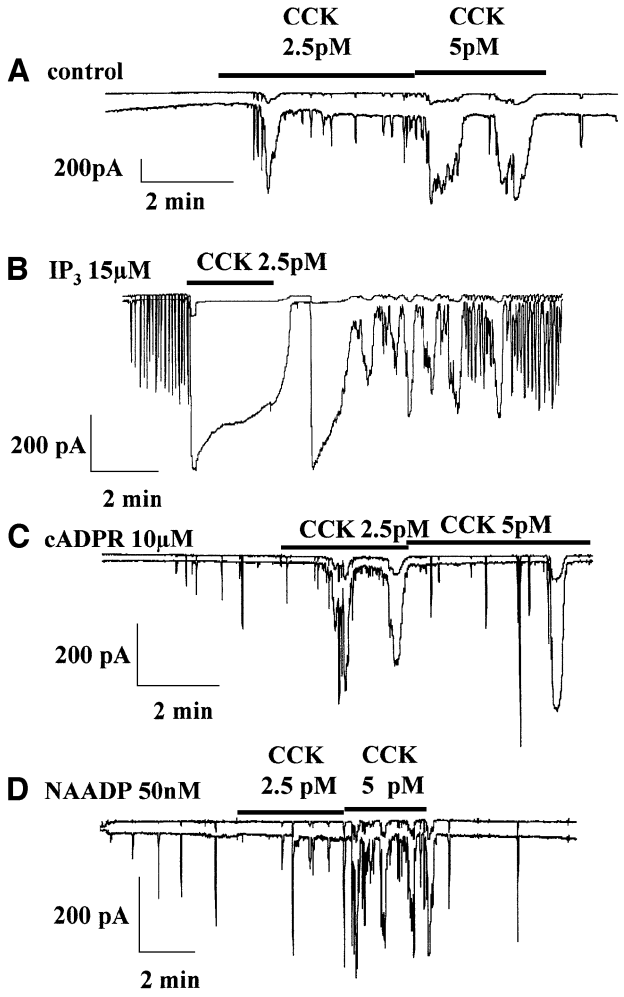

Fig. 2. Globalization of the CCK response by IP_3, but not cADPR or NAADP. (**A**) Repetitive spikes of Ca^{2+}-sensitive current were evoked by low, just suprathreshold concentrations of CCK. (**B**) When IP_3 (15 μM) was included in the intracellular pipette solution, the CCK-evoked response was strongly potentiated (sustained). When the intracellular pipette solution contained either cADPR (10 μM) (**C**) or NAADP (50 nM) (**D**), CCK evoked normal non-potentiated (spiking) responses.

($n = 3$; data not shown). In the absence of cADPR, ACh (as expected, see Petersen *et al*., 1991a; Thorn *et al*., 1993; Cancela *et al*., 2000) elicited short-lasting local Ca^{2+} spikes (Figure 1A; $n = 3$).

In addition to cADPR, the ADP-ribosyl cyclase CD38 may form NAADP, and the NAADP receptors have also been shown to be involved in the CCK response (Cancela *et al*., 2000; Lee, 2000). We therefore investigated whether NAADP could also potentiate the ACh response. In this series of experiments, the cells were perfused internally with 50 nM NAADP. At this concentration, NAADP elicits short-lasting Ca^{2+} spikes (Cancela *et al*., 2000) (Figure 1D). When 25 nM ACh was applied on top of the NAADP stimulus, a sustained Ca^{2+}-sensitive current response was observed, indicative of a global rise in the cytosolic Ca^{2+} concentration (Figure 1D; $n = 12$). In the particular cell giving rise to the trace shown in Figure 1D, the NAADP response was very weak, but the potentiation of the ACh response was remarkably strong. In all 12 cells in which NAADP elicited Ca^{2+} spiking, the subsequent ACh response was markedly potentiated (Figure 1D). Finally, since both the ACh- and CCK-elicited responses depend on functional IP_3 receptors, we decided to investigate whether IP_3 could also potentiate the ACh response. In the four cells tested with 15 μM IP_3 that elicited repetitive short lasting Ca^{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_3 (Figure 1B). This is remarkable, since IP_3 itself was a stronger stimulus than either cADPR or NAADP.

Globalization of the CCK response by IP_3, but not cADPR or NAADP

Since receptors for IP_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^{2+} signal pattern elicited by a physiological CCK concentration. The cells were internally perfused with either control solution or intracellular solutions containing IP_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 on top of an IP_3 (15 μM) stimulus, a very large and sustained response was observed (Figure 2B; $n = 7$). Clearly, IP_3 had markedly potentiated the CCK response. In the presence of IP_3, the CCK effect was not, as usual, immediately reversible, indicating that the potentiation by IP_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^{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_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_3, cADPR and NAADP each evoke local, short-lasting Ca^{2+} signals, the data shown in Figures 1 and 2 demonstrate that they can all be involved in global Ca^{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^{2+} spiking localized in the secretory pole

NAADP is the most potent Ca^{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 (Cancela 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

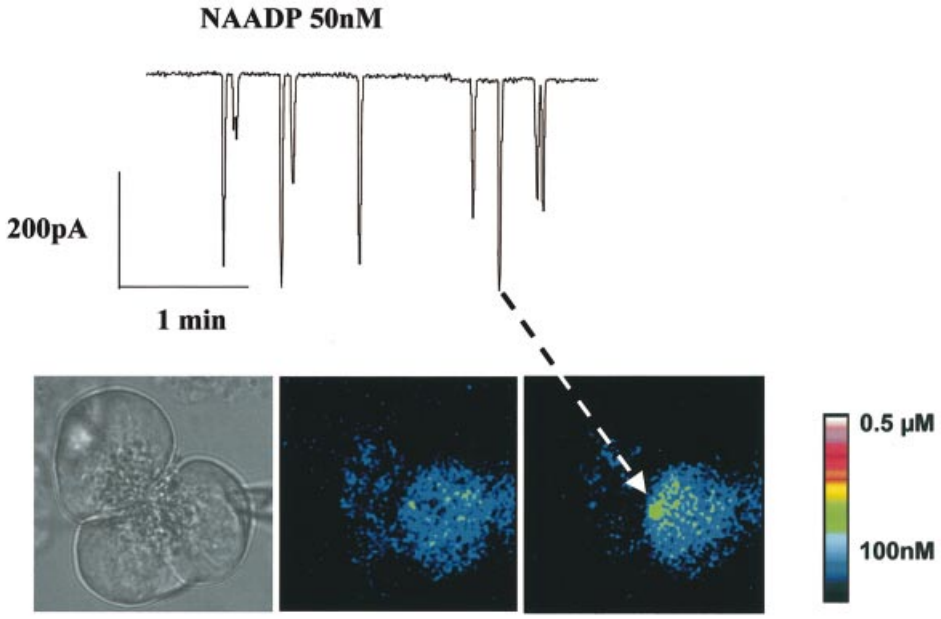

Fig. 3. NAADP evokes Ca^{2+} spiking in the secretory pole. Confocal fluorescence microscopy reveals that NAADP, at 50 nM in the intracellular pipette solution, evokes repetitive Ca^{2+} spikes localized in the apical pole. In the picture panel below the current trace is shown (left) the transmitted 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.

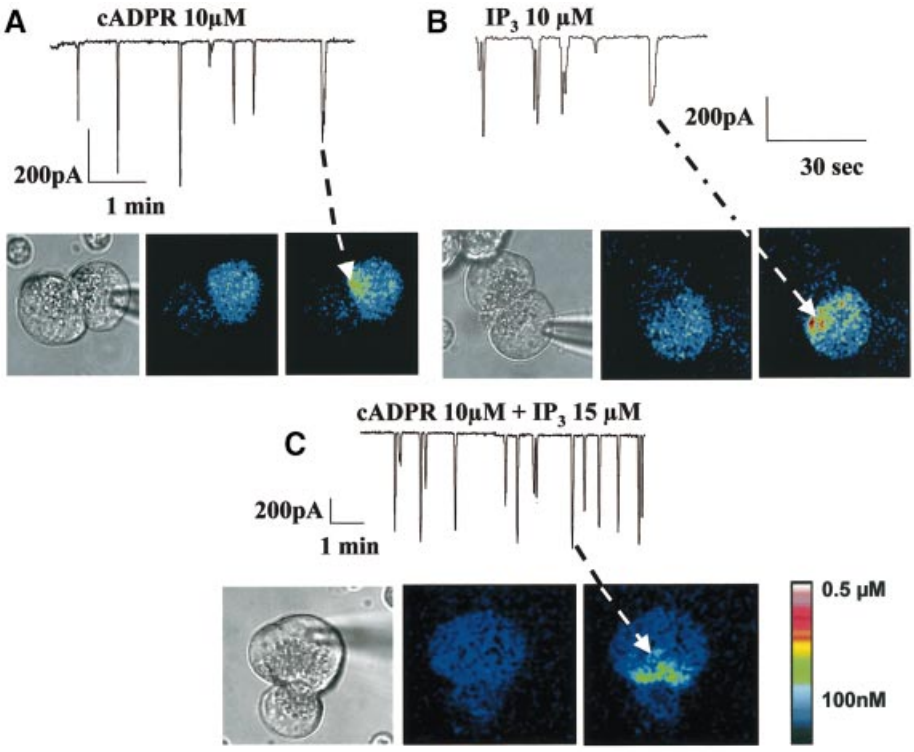

Fig. 4. A mixture of IP_3 and cADPR only evokes local Ca^{2+} release. (**A**) Confocal fluorescence microscopy reveals that cADPR, at 10 μM in the intracellular pipette solution, evokes repetitive Ca^{2+} spikes localized in the apical pole. The repetitive Ca^{2+} spikes evoked by IP_3 (10 μM) are localized 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

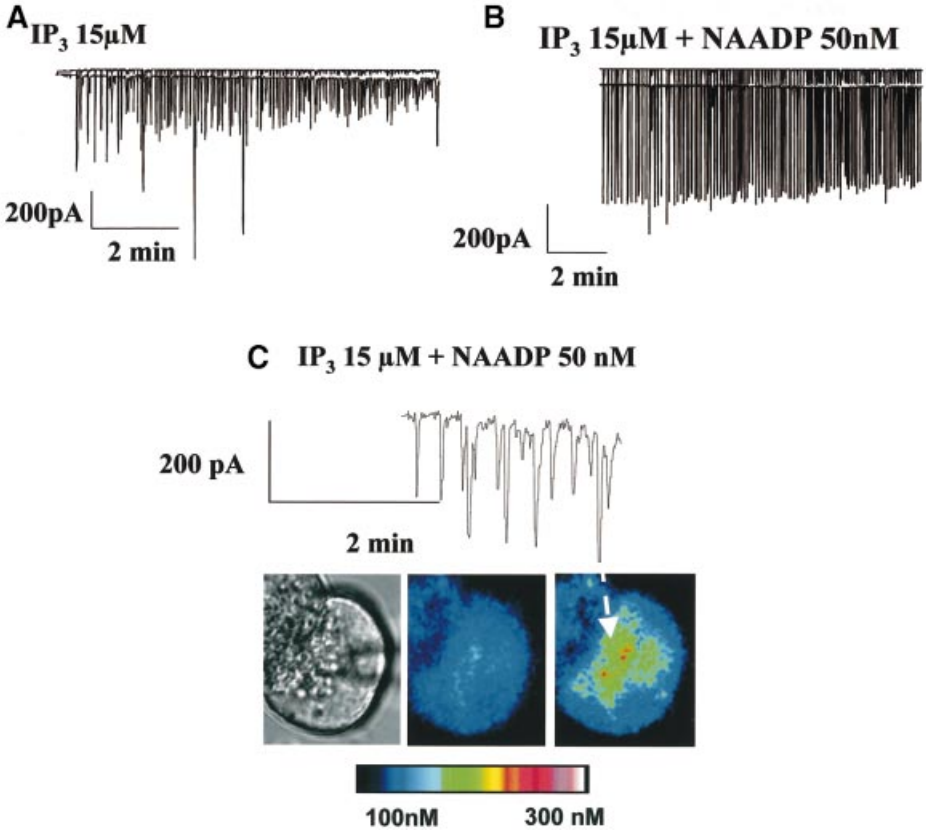

Fig. 5. A mixture of NAADP and IP_3 evokes locally amplified Ca^{2+} release. (**A**) IP_3 (15 μM in the intracellular pipette solution) evokes repetitive spikes of Ca^{2+}-sensitive current. The underlying Ca^{2+} spikes are localized in the apical pole (see Figure 4B). (**B**) A mixture of IP_3 (15 μM) and NAADP (50 nM) evokes somewhat amplified repetitive spikes of Ca^{2+}-sensitive current. (**C**) The Ca^{2+} spikes elicited by the mixture of IP_3 and NAADP are localized in the apical pole. Calibration of the colour coding of the cytosolic Ca^{2+} concentration is shown at the bottom.

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

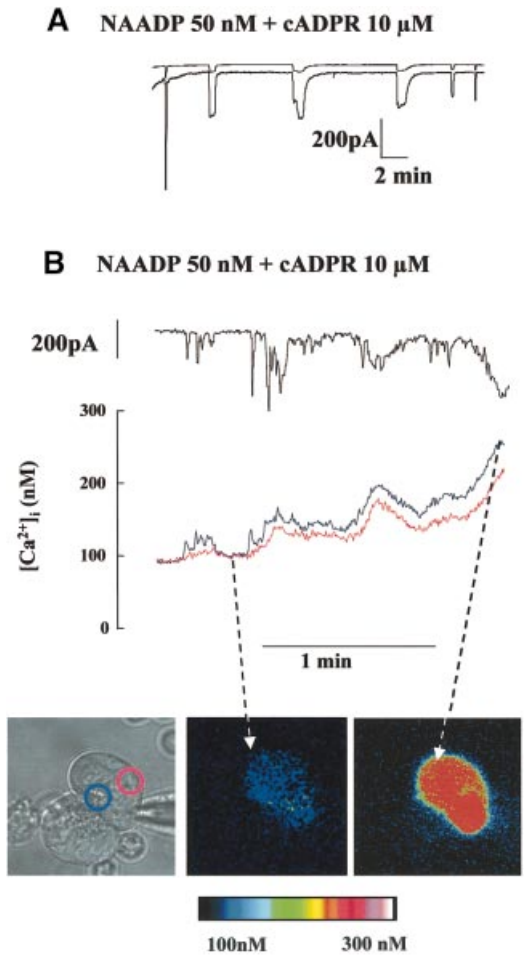

Fig. 6. NAADP transforms the short-lasting Ca^{2+} spikes evoked by cADPR into long-lasting Ca^{2+} spikes. (**A**) A mixture of NAADP (50 nM) and cADPR (10 μM) in the intracellular pipette solution evokes repetitive, long-lasting spikes of Ca^{2+}-sensitive current with relatively long intervals between the spikes. (**B**) The result of an experiment simultaneously recording the Ca^{2+}-sensitive Cl^- current and the cytosolic Ca^{2+} concentration measured by microfluorimetry in a single pancreatic acinar cell. The short-lasting spikes are local and the long-lasting spikes are global. Calibration of the colour coding of the cytosolic Ca^{2+} concentration is shown at the bottom.

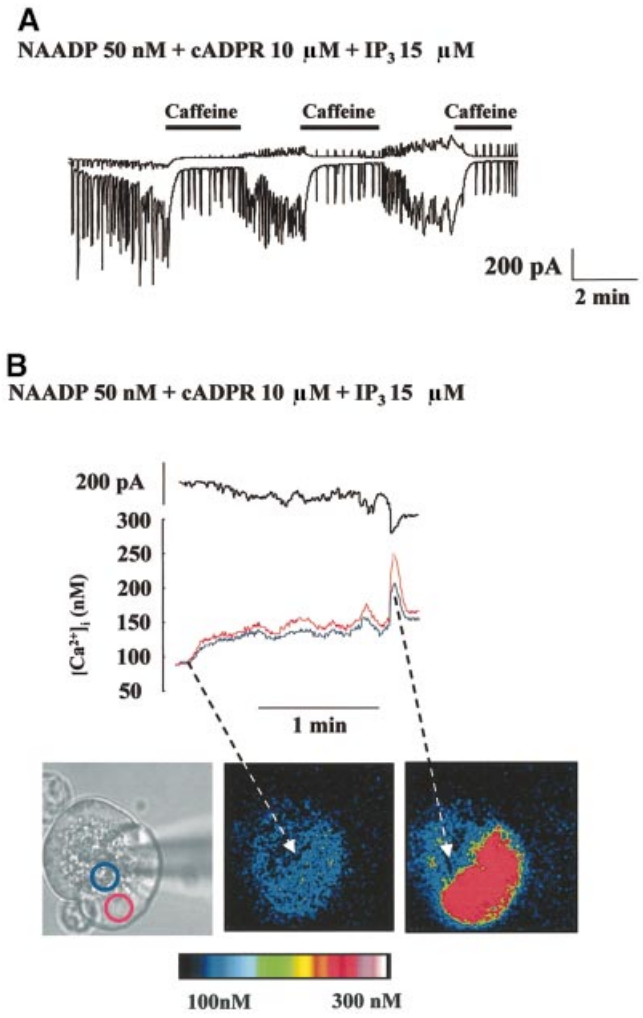

Fig. 7. NAADP transforms the local Ca^{2+} spikes evoked by a cADPR + IP_3 mixture into a sustained global Ca^{2+} elevation. (**A**) A mixture of short-lasting spikes and sustained activation of Ca^{2+}-sensitive current evoked by a triple mixture of NAADP (50 nM), cADPR (10 μM) and IP_3 (15 μM). External application of 20 mM caffeine, used as an IP_3 antagonist, abolished the sustained activation of the Ca^{2+}-sensitive current, indicating that functional IP_3 receptors are required to generate the sustained Ca^{2+} signal. (**B**) The result of an experiment simultaneously recording the Ca^{2+}-sensitive current and the cytosolic Ca^{2+} concentration measured by microfluorimetry in a single pancreatic acinar cell. Confocal fluorescence microscopy reveals that the triple mixture of NAADP (50 nM), cADPR (10 μM) and IP_3 (15 μM) in the intracellular pipette solution evokes a sustained global Ca^{2+} elevation. Calibration of the colour coding of the cytosolic Ca^{2+} concentration is shown at the bottom.

between these messengers. The strongest Ca^{2+} signals were obtained by a triple mixture containing IP_3, cADPR and NAADP.

Agonist-specific Ca^{2+} signal patterns and messenger interactions

In pancreatic acinar cells, ACh and CCK induce specific Ca^{2+} signal signatures (Petersen *et al*., 1991a, Petersen *et al*., 1994). Low and physiological concentrations of both agonists elicit repetitive, short-lasting Ca^{2+} spikes confined to the apical granular pole (Thorn *et al*., 1993). These local Ca^{2+} 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 Ca^{2+}-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 Ca^{2+} 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 pM) there are very infrequent, long, global transients, and the response essentially consists of repetitive local Ca^{2+} 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

principally triggered by IP_3, since it can be markedly potentiated by cADPR and NAADP, but not by IP_3, whereas the response to a physiological CCK concentration must be triggered by cADPR and NAADP, since it can be dramatically potentiated by IP_3, but not by cADPR or NAADP. Clearly only complementary messengers can effectively potentiate a response. One important conclusion 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 Ca^{2+} 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 potentiation by CCK of the ACh response (Cancela *et al*., 2000).

The experiments with messenger mixtures revealed only a relatively minor interaction between IP_3 and cADPR (Figure 4), and a modest interaction between IP_3 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 IP_3 (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 IP_3 receptor antagonist caffeine (Petersen and Cancela, 1999), we were able to demonstrate that activation of IP_3 receptors is essential for maintaining a sustained response. Application of caffeine led to a fully reversible transformation from a sustained cytosolic Ca^{2+} elevation to repetitive baseline spiking (Figure 7).

Localization of intracellular Ca^{2+} release channels

IP_3, cADPR and NAADP each simply evoke Ca^{2+} release in the apical pole. This has been well documented for IP_3 and cADPR (Thorn *et al*., 1993, 1994). Our data (Figure 3) indicate that the short-lasting Ca^{2+}-dependent currents evoked by NAADP are associated with Ca^{2+} elevations specifically in the apical pole of the cells. This result demonstrates directly that infusion of NAADP into a cell can release Ca^{2+} 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 IP_3 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 IP_3 receptors are preferentially localized in the apical pole (Nathanson *et al*., 1994; Lee *et al*., 1997). Most likely, cADPR elicits local Ca^{2+} spikes in the apical pole (Thorn *et al*., 1994) because of the concentration of IP_3 receptors in this region, since the cADPR responses are completely dependent on functional IP_3 receptors (Cancela *et al*., 2000). It is therefore entirely possible, and indeed likely, that the general primary localization of Ca^{2+} signals to the apical pole, irrespective of which Ca^{2+} releasing messenger is used, is principally due to the dominant presence of IP_3 receptors in this part of the cell. Since all Ca^{2+} signal initiation depends on interaction between at least ryanodine and IP_3 receptors, it must occur at sites where both these Ca^{2+} release channels co-exist, and the only such region is the apical pole.

The general concept concerning the organization of the major intracellular Ca^{2+} store, the ER, is that it forms a continuous sheet enclosing a single internal space. The continuous lumen allows Ca^{2+} 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 Ca^{2+} signal generation in the apical granular pole (Kasai *et al*., 1993; Thorn *et al*., 1993) is due to clustering of IP_3 receptors in the most apical parts of the ER extensions into the granular area (Lee *et al*., 1997). Opening of these channels can mobilize Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} signals

There would appear to be two separate aspects of the globalization process. Unlike the lumenally continuous ER, the cytosol is effectively compartmentalized with respect to Ca^{2+} diffusion by a major mitochondrial Ca^{2+} buffer barrier 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 Ca^{2+} signals generated in the apical region to this part of the cell (Tinel *et al*., 1999; Park *et al*., 2001a). In order for a Ca^{2+} signal to become global, this barrier has to be overwhelmed by a substantial amount of Ca^{2+} released in the granular region. However, it is also known that when a Ca^{2+} wave progresses through the cell from the apical to the basal pole, there is a regenerative process, almost certainly due to Ca^{2+}-induced Ca^{2+} release (Kasai and Augustine, 1990; Toescu *et al*., 1992, 1994). This means that under these circumstances Ca^{2+} release channels also open in the basal pole. The mechanism by which silent Ca^{2+} release channels in the basal pole become activated during globalization of Ca^{2+} signalling is not fully understood, but our data indicate that a combination of IP_3, cADPR and NAADP plays an important role in this process (Figure 8).

A global Ca^{2+} wave is generated by the concerted activity of elementary Ca^{2+} 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 Ca^{2+} wave propagates by recruiting, in a saltatory manner, neighbouring Ca^{2+} release sites (Parker *et al*., 1996; Berridge, 1997; Boittin *et al*., 1998; Cannell and Soeller, 1999; Koizumi *et al*., 1999; Marchant *et al*., 1999; Berridge *et al*., 2000). The Ca^{2+} release units are recruited by several mechanisms, including Ca^{2+} diffusion, Ca^{2+}-induced Ca^{2+} release and increase of IP_3 production. All these mechanisms increase the frequency of elementary Ca^{2+} release events, which, once the threshold is reached, will trigger a Ca^{2+} wave. However, if poorly sensitive Ca^{2+} release units surround the most sensitive Ca^{2+} release units, then the Ca^{2+} signal remains localized and the Ca^{2+} wave is aborted (Parker *et al*., 1996; Marchant *et al*., 1999;

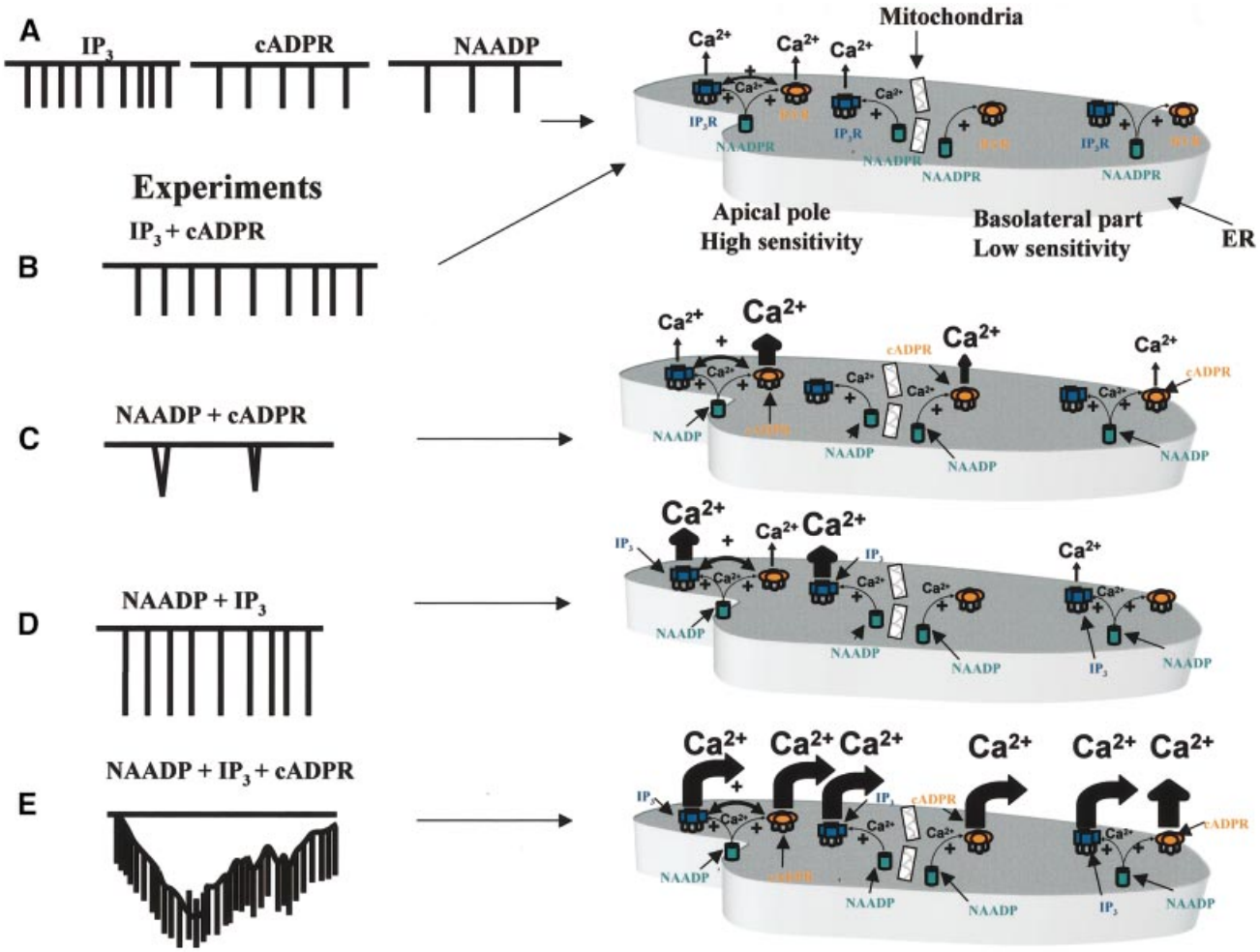

Fig. 8. (**A**) Schematic model showing that every messenger on its own can initiate local Ca^{2+} spikes. (**B**) There is little interaction between IP_3 and cADPR. (**C**) NAADP does potentiate the action of cADPR producing long-lasting global spikes at long intervals. (**D**) In contrast, NAADP only has a locally potentiating effect on the local IP_3-evoked Ca^{2+} spikes. (**E**) When all three messengers act together a large, sustained, global Ca^{2+} elevation is observed. The apical pole is the most sensitive part of the cell. In the models shown to the right, the basolateral part of the cell contains poorly sensitive Ca^{2+} release units that cannot trigger a wave in the presence of either IP_3, cADPR or NAADP alone. To generate a Ca^{2+} wave across the cell, a combination of potentiated Ca^{2+} release in the apical pole, helping to overcome the mitochondrial barrier, and sensitization of Ca^{2+} release channels by coincident activation of ryanodine, IP_3 and NAADP receptors by their respective messengers in the basal pole is necessary.

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 whithout 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 basal pole 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 (Albrieux *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 μM 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 Cl^- and non-selective cation channels (Thorn and Petersen, 1992). The Cl^- current is by far the most important quantitatively (Park *et al*., 2001b). Using our solutions, the reversal potential of both the Cl^- and non-selective cation currents were at 0 mV (Petersen *et al*., 1991a). 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 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 perifusion 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_D for Fluo 4–Ca^{2+} at room temperature was assumed to be 400 nM (Molecular Probes). An objective (60×) 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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