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SPATIAL ASPECTS OF Ca^{2+} SIGNALLING IN PANCREATIC ACINAR CELLS

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Summary

Secretory cells do not only respond to an agonist with a simple rise in $[Ca^{2+}]_i$. It is now clear that complex patterns of $[Ca^{2+}]_i$ elevation in terms of space and time are observed in many cell types and that these patterns may be a cellular mechanism for the regulation of different responses. Ca^{2+} signalling in exocrine cells of the pancreas promotes the secretion of digestive enzymes and fluid. It has been shown that at high concentrations of agonist (acetylcholine or cholecystokinin) the $[Ca^{2+}]_i$ response is initiated in the secretory pole of the cell before spreading across the whole cell. This site of initiation of the $[Ca^{2+}]_i$ elevation is in the region where exocytotic release of enzymes occurs and is also the site of a Ca^{2+}-dependent chloride channel thought to be crucially important for fluid secretion. Lower concentrations of agonist elicit $[Ca^{2+}]_i$ oscillations with complex repetitive patterns characteristic of each agonist. At physiological agonist concentrations, we have recently described repetitive short-lasting Ca^{2+} spikes that are spatially restricted to the secretory pole of the cell. In addition to these spikes, cholecystokinin also promotes slow transient Ca^{2+} rises that result in a global rise in Ca^{2+}. The inositol trisphosphate (InsP_3) receptor plays a crucial role in all of these various agonist responses, most of which can be reproduced by the infusion of InsP_3 into the cell. The high InsP_3-sensitivity of the secretory pole is postulated to be due to a localization of high-affinity InsP_3 receptors. We speculate that in response to cholecystokinin the short-lasting spikes elicit exocytosis from a small ‘available pool’ of vesicles and that the broader oscillations induce both exocytosis and cell changes that involve movement of vesicles into this ‘available pool’.

Introduction

Exocrine glands (such as pancreas or lacrimal) share a common morphology, with a primary fluid secretion from distally located acinar cells modified by proximally located duct cells. The Ca^{2+} signal observed in the acinar cells regulates the secretion of fluid and enzymes (Petersen, 1992). Fluid secretion is dependent on the opening of a Ca^{2+}-dependent chloride conductance thought to be located in the luminal plasma membrane (Petersen and Gallacher, 1988). Enzyme secretion is *via* the Ca^{2+}-dependent exocytosis of secretory zymogen granules on the luminal membrane, releasing the granule contents into the duct. The two main agonists in pancreatic acinar cells that promote an increase in $[Ca^{2+}]_i$ are acetylcholine (ACh) and cholecystokinin (CCK). ACh is released at nerve

Key words: Ca^{2+} oscillations, exocytosis, InsP_3, acinar cell, pancreas.

terminals and CCK acts as a circulating hormone (Petersen, 1992). ACh and CCK, at high concentrations, couple to a different set of G-proteins (Schnefel *et al.* 1988), stimulate phospholipase C (PLC) and both produce increases in InsP_3 (Matozaki *et al.* 1990; Rowley *et al.* 1990). Evidence obtained from microelectrode studies and from the injection of fluorescent markers, such as Lucifer Yellow (Petersen and Findlay, 1987), has shown that individual pancreatic acinar cells are grouped together and are joined by tight junctions into larger multicellular units. Within these units, some cells are thought not to possess secretagogue receptors but, nevertheless, Ca^{2+} increases are observed in these cells, possibly due to diffusion of Ca^{2+} or InsP_3 from neighbouring acinar cells (Dissing *et al.* 1993). These larger units of cells are likely to be of importance in the physiological control of secretion.

The $[Ca^{2+}]_i$ responses of acinar cells to agonists can be categorized into three distinct types. First, at supramaximal agonist concentrations a sustained elevation in $[Ca^{2+}]_i$ is observed. In the majority of reports, this $[Ca^{2+}]_i$ rise is initiated in the secretory pole of the cell and then spreads as a wave towards the basal pole (Kasai and Augustine, 1990). Second, at lower agonist concentrations, oscillations in $[Ca^{2+}]_i$ are recorded. ACh and CCK elicit patterns of oscillations that are different and characteristic for each agonist (Petersen *et al.* 1991*a*). Third, at lower and physiological agonist concentrations (Forster and Dockray, 1992), the combination of whole-cell patch-clamp and digital Ca^{2+} imaging has revealed local repetitive elevations of $[Ca^{2+}]_i$ that occur only in the secretory pole of the cell and do not spread to the basal pole (Thorn *et al.* 1993*a*).

Ca^{2+} oscillations have been proposed to provide a digitally encoded transduction of the stimulus strength (Berridge, 1993). It is still not known whether oscillations are a physiologically important mechanism in acinar cells. It can be said that concentrations of CCK sufficient to promote *in vivo* secretion always elicit oscillations *in vitro*. However, there are a number of possible problems that arise as a result of our normal experimental protocols. For example, intercellular communication is lost in the single-cell preparations normally used. This could be an important difference between experiments that show oscillations and the situation *in vivo*. Single-cell studies will continue to provide insights into Ca^{2+} signalling while questions of physiological relevance will have to be addressed by different experimental approaches.

Sustained Ca^{2+} response

A sustained secretory response in acinar cells is ultimately dependent on extracellular $[Ca^{2+}]$ (Douglas and Poisner, 1963). However, the initial response to high agonist concentrations is known to be the result of the rapid liberation of Ca^{2+} into the cytoplasm from Ca^{2+} stores within the cell (Nielsen and Petersen, 1972). Recordings of Ca^{2+}-dependent currents have suggested that the $[Ca^{2+}]_i$ rise is not homogeneous (Osipchuck *et al.* 1990) but ,with the advent of digital Ca^{2+} imaging, this has been most elegantly demonstrated by Kasai and Augustine (1990).

Digital Ca^{2+} imaging of the agonist-induced $[Ca^{2+}]_i$ response

Kasai and Augustine (1990) determined the secretory pole of the pancreatic acinar cell

as the locus for the initiation of the Ca^{2+} response to supramaximal stimulation, which then spread as a propagated wave across the cell. The secretory pole in acinar cells is easily identified as the region containing the dark (under phase contrast) zymogen granules. These findings have been reproduced by Toescu *et al.* (1992*a*) and Elliot *et al.* (1992) and extended to include a similar pattern for the Ca^{2+} signal evoked by agonists in acinar cells derived from the mouse lacrimal gland. In contrast, Dissing and collaborators have consistently found no evidence for a localized initiation of the Ca^{2+} signal (Dissing *et al.* 1990) and no satisfactory explanation has been found to reconcile the different findings. Kasai and Augustine (1990) proposed, from their data, a region of high InsP_3 sensitivity located in the secretory pole of the cell to explain the site of initiation of the Ca^{2+} signal. The spreading of the Ca^{2+} wave to the basal pole is thought to be by a propagated mechanism of Ca^{2+}-induced Ca^{2+} release (CICR). Ca^{2+} waves in general have been proposed to be due to an autocatalytic process such as CICR (Lechleiter and Clapham, 1992). In this way, Ca^{2+} release from one site acts on a neighbouring Ca^{2+}-sensitive site to promote further Ca^{2+} release and leads to the propagation of a spreading Ca^{2+} wave. In the pancreatic acinar cells, wave velocity has been studied using confocal microscopy (Nathanson *et al.* 1992) and was found to increase with increasing agonist concentrations, but was slowed by caffeine and by a high concentration of ryanodine. These data were interpreted in terms of the presence of ryanodine receptors in the basal pole of the cell that propagate the Ca^{2+} wave. However, it has recently been shown in our laboratory that caffeine can inhibit the production of InsP_3 (Toescu *et al.* 1992*b*) and it may, in fact, act directly to inhibit the InsP_3 receptor (Parker and Ivorra, 1991). Therefore, the effects observed by Nathanson *et al.* (1992) do not provide unequivocal evidence of wave spreading on a ryanodine receptor.

Oscillatory Ca^{2+} response

Ca^{2+} oscillations in electrically nonexcitable cells were first demonstrated in hepatocytes (Woods *et al.* 1986). In acinar cells, the bulk of our knowledge of Ca^{2+} oscillations has been provided through the whole-cell patch-clamp technique (Petersen, 1992). Early patch-clamp studies demonstrated Ca^{2+}-dependent currents (Maruyama and Petersen, 1982*a*; Marty *et al.* 1984) in the plasma membrane of acinar cells. Monitoring of these Ca^{2+}-dependent conductance pathways has been used to gave an indirect measure of the Ca^{2+} signal.

Ca^{2+} -dependent currents

There are three types of Ca^{2+}-dependent currents in the plasma membrane of most acinar cells, namely Cl^-, K^+ and nonselective cation channels (Petersen, 1992). Exceptionally, the rodent pancreas does not possess a Ca^{2+}-dependent K^+ channel, although there is recent evidence for a voltage-dependent K^+ current (Thorn, 1993). Single-channel studies of the nonselective cation channel (equally permeable to Na^+ and K^+) demonstrated activation by cholecystokinin *via* an intracellular elevation of Ca^{2+} (Maruyama and Petersen, 1982*a*,*b*). The Cl^- channel has been directly demonstrated in the lacrimal acinar cell (Marty *et al.* 1984) and there is considerable evidence from

whole-cell recording studies of Cl^- current activation during agonist stimulation (Wakui *et al.* 1989). In the pancreas, the Cl^- current has been demonstrated to be more sensitive to $[Ca^{2+}]_i$ than the nonselective cation current (Randriamampita *et al.* 1988). This, and evidence that $[ATP]_i$ blocked the nonselective cation channel (Suzuki and Petersen, 1988), led to the idea that it was not activated by physiological agonist concentrations. Recently, this conclusion has been shown to be incorrect. We now know that both the Cl^- and the nonselective cation currents are activated by physiological concentrations of agonist (Thorn and Petersen, 1992, 1993*a*).

The validity of using Ca^{2+}-dependent current activation as a measurement of changes in $[Ca^{2+}]_i$ has been demonstrated by dual recording of currents and $[Ca^{2+}]_i$ using whole-cell patch-clamp and photometric Ca^{2+} determination (Osipchuk *et al.* 1990). In these experiments, higher agonist concentrations elicited oscillations with simultaneous rises in $[Ca^{2+}]_i$ and increases in Ca^{2+}-dependent conductance.

Patterns of oscillations

The agonist-evoked oscillatory responses have a characteristic temporal pattern dependent on the agonist used and the concentration of agonist (Petersen *et al.* 1991*a*; Yule *et al.* 1991). With just suprathreshold CCK concentrations, short-lasting spikes of Ca^{2+}-dependent current (approximately 2s duration) are observed that are interspersed with broad transient current activation (approximately 30s duration) (Fig. 1A). At just suprathreshold concentrations of ACh, trains of short-lasting spikes of Ca^{2+}-dependent current are observed, and at higher ACh concentrations the Ca^{2+} signal develops into sinusoidal oscillations (Fig. 1B). At higher CCK concentrations, the slow transients increase in frequency and the short spikes decrease in frequency (Petersen *et al.* 1991*a*). These oscillatory patterns probably reflect the activation of different sets of G-proteins associated with stimulation by ACh or CCK (Schnefel *et al.* 1988).

Spatially restricted spikes in the secretory pole

Experiments employing patch-clamp and simultaneous single-cell photon counting (Osipchuck *et al.* 1990) have been used to identify the response to agonists simultaneously recorded from the Ca^{2+}-dependent currents and $[Ca^{2+}]_i$. As mentioned previously, at higher agonist concentrations, $[Ca^{2+}]_i$ increases and Ca^{2+}-dependent current activation occurred simultaneously. However, simultaneous activation of currents and rises in $[Ca^{2+}]_i$ did not always occur. The trains of short-lasting spikes of Ca^{2+}-dependent current observed in response to low ACh concentrations did not induce any rise in the average cellular Ca^{2+} signal measured using photon counting. This was interpreted as an indication that the short-lasting current spikes were due either to a local rise in $[Ca^{2+}]_i$, restricted to one region of the cell, or possibly to a rise in $[Ca^{2+}]_i$ in the subplasmalemmal region (Petersen *et al.* 1991*c*). Such discrete $[Ca^{2+}]_i$ changes would not be picked up in the average $[Ca^{2+}]_i$ signal. Clearly, to obtain data to test the hypothesis of a localized Ca^{2+} signal we require spatial information of the $[Ca^{2+}]_i$ response and so we have turned to combining patch-clamp with digital Ca^{2+} imaging techniques.

There are a number of important advantages in using whole-cell patch-clamp techniques for Ca^{2+} measurement when compared with the usual methods of dye-loading

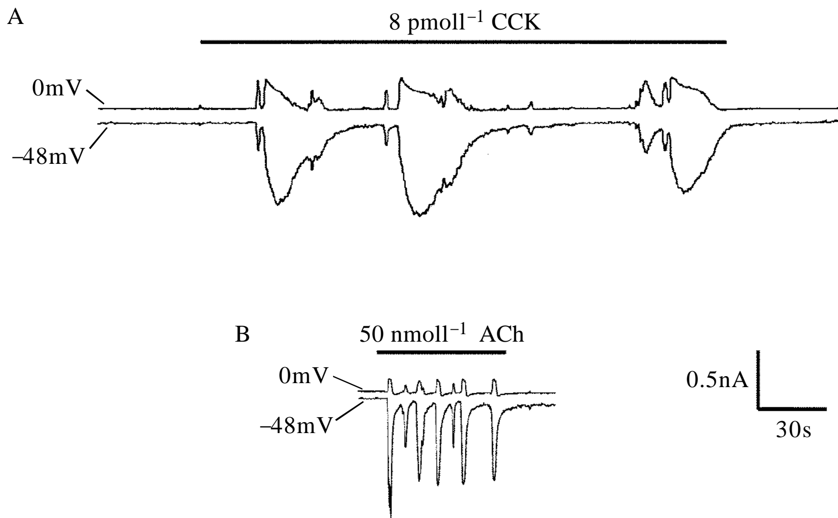

Fig. 1. Cholecystokinin (CCK) and acetylcholine (ACh) evoke different patterns of Ca^{2+}-dependent currents in pancreatic acinar cells. (A) The whole-cell current response to the application of 8pmol l^{-1} CCK. Characteristic long-lasting oscillations (approx. 30s duration) are preceded by short-lasting current spikes. (B) A train of short-lasting spikes typical of a response to a low concentration of ACh. Both of these records were obtained by voltage-clamping at a potential of 0mV (the reversal potential for the cation current) and stepping to −48mV (the reversal potential for the Cl^- current). This method gives a measure of the Cl^- current at 0mV (upper line in each trace) and the nonselective cation current at −48mV (lower line). (Figure adapted from Thorn and Petersen, 1992.)

with acetoxymethyl esters. First, the patch pipette can be used to fill the cell with Fura-2 and thus to avoid the problems of compartmentalization associated with loading the cells with the acetoxymethyl esters (Connor, 1993). Second, it also enables us to control the intracellular buffering directly because we know the concentration of Fura-2 in the patch pipette. Ca^{2+} buffering has a significant influence on the temporal aspects of Ca^{2+} signalling (Neher and Augustine, 1992). In our experiments, it was important to keep the Fura-2 buffering as low as possible (200–400 μmol l^{-1}) consistent with obtaining good images at near video rates of capture.

We focused our interest on the localization of the $[Ca^{2+}]_i$ increase that gives rise to the short-lasting agonist-evoked Ca^{2+}-dependent current spikes (Thorn *et al.* 1993*a*). Our experiments in the pancreas demonstrated repetitive $[Ca^{2+}]_i$ spikes restricted to the secretory pole region of the cell. In the case of low concentrations of CCK (5–30pmol l^{-1}), the restricted $[Ca^{2+}]_i$ spikes often preceded a $[Ca^{2+}]_i$ wave that spread across the whole of the cell (see Fig. 2, Thorn *et al.* 1993*a*). Low concentrations of ACh, which produce trains of short-lasting current spikes (Fig. 1B), induced transient elevations in $[Ca^{2+}]_i$ only in the secretory pole and coincident with each current spike (Thorn *et al.* 1993*a*).

Spatially restricted rises in $[Ca^{2+}]_i$ have been reported in oocytes (DeLisle and Welsh, 1992; Lechleiter and Clapham, 1992) in response to injection of InsP_3 and nonmetabolizable analogues of InsP_3. These usually gave rise to propagated $[Ca^{2+}]_i$

waves that spread away from the site of initiation, the foci of $[Ca^{2+}]_i$ elevations themselves have not been studied. There is also a report, in neurones, of $[Ca^{2+}]_i$ oscillations restricted to the distal ends of dendrites (Inagaki *et al.* 1991). No functional significance has been ascribed to either of these examples of spatially restricted spiking. We propose that the spatially restricted $[Ca^{2+}]_i$ spikes we describe form an important part of the functional secretory processes in pancreatic acinar cells.

Mechanisms of Ca^{2+} oscillations

A key advantage of the whole-cell patch-clamp technique is the access gained to the cell cytoplasm through the ability to perfuse the cell internally. One crucial series of experiments that has advanced our knowledge of oscillatory mechanisms showed that repetitive oscillations could be evoked by a constant level of InsP_3 (Wakui *et al.* 1989). In these experiments, a nonmetabolizable analogue of InsP_3 was infused through the patch pipette to provide a steady concentration of InsP_3 that could not be hydrolysed. These observations provide evidence against models of oscillations that require fluctuating concentrations of InsP_3 (Meyer and Streyer, 1988).

Other important experiments in the pancreas have shown that the infusion of heparin, a competitive antagonist at the InsP_3 receptor, can abolish the Ca^{2+} response to acetylcholine, InsP_3 (Wakui *et al.* 1990) and CCK (Thorn and Petersen, 1993*b*). These experiments illustrate the importance of this receptor in the oscillatory mechanism.

*Effects of Ins*P$_3$ *infusion*

The agonist responses described in the previous section are thought to be due to $[Ca^{2+}]_i$ release through the action of InsP_3. Combined whole-cell patch-clamp and $[Ca^{2+}]_i$ imaging experiments have enabled us to test the sensitivity of different regions of the acinar cell to the infusion of InsP_3 (Thorn *et al.* 1993*a*).

In a series of experiments, Hassoni and Gray (1992) recorded the combined Ca^{2+}-dependent currents and $[Ca^{2+}]_i$ signal in rat parotid acinar cells and showed the typical agonist-induced response to consist of an initial Cl^- current associated with a luminal $[Ca^{2+}]_i$ elevation followed by the activation of a K^+ current. In separate experiments, they

Fig. 2. Cholecystokinin (CCK) evokes a local $[Ca^{2+}]$ rise in the secretory pole of a pancreatic acinar cell coincident with the short-lasting current spikes, and a global $[Ca^{2+}]$ rise associated with the slower oscillations. Whole-cell patch-clamp and digital Ca^{2+} fluorescent imaging were combined, the cells were filled with Fura-2 through the patch pipette and the Ca^{2+}-dependent currents and the Ca^{2+} signal were simultaneously recorded. The upper trace shows a single complex transient, part of the response elicited by CCK ($17 pmol\,l^{-1}$). The middle trace shows the Ca^{2+} signal obtained from two regions defining the secretory pole and the basal pole of the cell (see inset of bright-field image and diagrammatic representation, scale bar, $20\,\mu m$). At the peak of the current spike, the $[Ca^{2+}]$ rise is restricted to the secretory pole of the cell, whereas during the slow current transient the $[Ca^{2+}]$ increase is seen globally across the whole cell. This is also seen in the three-dimensional maps and pseudocolour images (bottom of the figure) taken at the time points indicated (i–iii). (Figure adapted from Thorn *et al.* 1993*a*.)

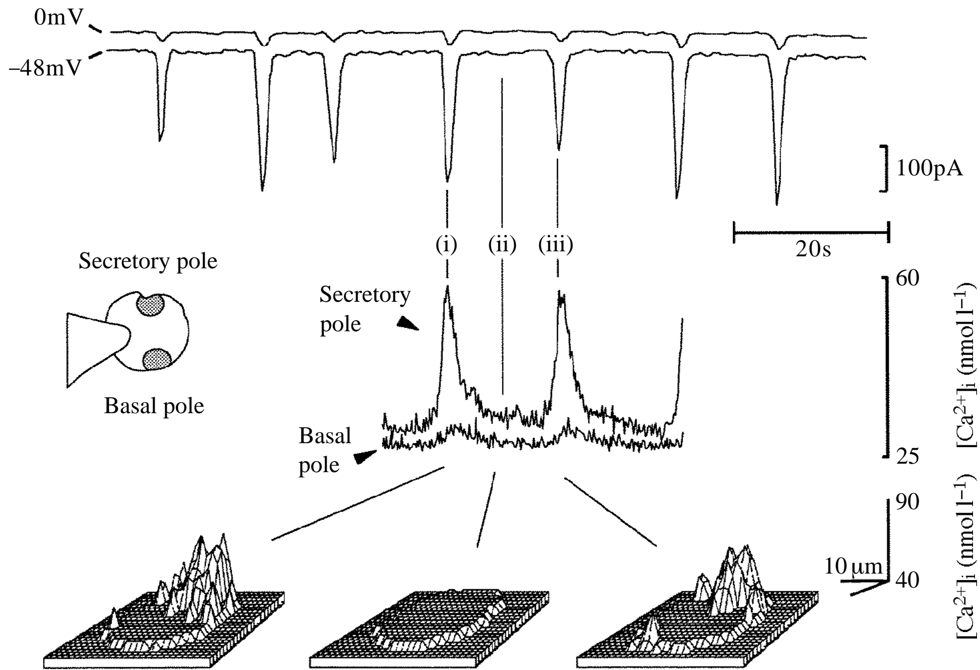

Fig. 3. The nonmetabolizable inositol trisphosphate analogue InsP_3S_3 evokes repetitive Ca^{2+} spikes restricted to the secretory pole of the cell. The reversal potentials for the currents were not separated in these experiments and the current trace, obtained at −48mV, represents both the Ca^{2+}-dependent currents. The Ca^{2+} signal obtained from the secretory and basal poles of the cell shows the restricted $[Ca^{2+}]$ rise associated with each current spike. The three-dimensional Ca^{2+} maps taken at the time points shown (i–iii) confirm the location of the $[Ca^{2+}]$ rise in the secretory pole. (Figure adapted from Thorn *et al.* 1993*a*.)

then infused 'caged' InsP_3 through the patch pipette and recorded the Ca^{2+}-dependent current response to the flash release of InsP_3 in rat parotid cells. Their results demonstrated current activation with a time course similar to that induced by agonist, suggesting a luminal-to-basal Ca^{2+} wave induced by this rapid release of InsP_3.

In our experiments on the pancreatic acinar cells, application of low concentrations of InsP_3 and other analogues elicited trains of current spikes (Wakui *et al.* 1989) and, after waiting for some minutes following breakthrough to whole cell, to allow for equilibration of the InsP_3, we then simultaneously recorded these Ca^{2+}-dependent currents and the $[Ca^{2+}]_i$ response of the cell. An example of the results from an experiment infusing a nonmetabolizable analogue of InsP_3, InsP_3S_3, into a cell is shown in Fig. 3. Trains of Ca^{2+}-dependent current spikes are seen in Fig. 3 (top traces); also shown are mean $[Ca^{2+}]_i$ changes measured from the basal pole of the cell and the secretory pole. The peak of each short-lasting Ca^{2+}-dependent current spike is associated with a rise in $[Ca^{2+}]_i$ in the secretory pole of the cell with a return to basal levels of $[Ca^{2+}]_i$ between each of the spikes. The lower part of Fig. 3 shows three-dimensional plots of $[Ca^{2+}]_i$ obtained at the three time points shown and demonstrates again the spatially restricted nature of each $[Ca^{2+}]_i$ spike. In these experiments, we would expect the InsP_3 concentration to have

equilibriated across the whole of the cell. The observation that only the secretory pole region responds to this level of InsP_3 provides direct evidence that this region of the cell has a higher sensitivity to InsP_3. In further experiments, we have shown that under certain conditions InsP_3 infusion can also reproduce the slow waves that spread across the cell (Thorn *et al.* 1992*a*). The results we have obtained can explain the regional initiation of the $[Ca^{2+}]_i$ signal at high agonist concentrations and the repetitive spatially restricted Ca^{2+} spikes observed at lower agonist concentrations.

Further evidence of the high sensitivity to InsP_3 in the secretory pole was obtained from experiments in which we perfused ACh focally through a patch pipette after the formation of a cell-attached patch on the basal pole of the cell (Thorn *et al.* 1993*a*). Fig. 4 shows an example of one experiment: the elicited Ca^{2+} rise indicates that ACh receptors are located in the basal plasma membrane. The first Ca^{2+} response of the cell after ACh application showed a $[Ca^{2+}]_i$ rise simultaneously in both the secretory pole and the basal pole of the cell (Fig. 4 middle). It would be expected that, at this time, at the very initiation of the Ca^{2+} response, the InsP_3 concentration would have formed a gradient across the cell, high in the region immediately beneath the patch pipette and low in the secretory pole. The similarity in the time course and amplitude of the Ca^{2+} response in the two poles of the cell therefore provides further evidence that the Ca^{2+} store in the secretory pole has a higher sensitivity to InsP_3.

What is the basis of the high sensitivity of the secretory pole region to InsP_3? We have addressed this issue by studying the CCK-induced response, which consists of both the short-lasting local spikes and the broad transients. Infusion of a high concentration of the competitive InsP_3 receptor antagonist heparin (>250mgml^{-1}) (Ghosh *et al.* 1988) totally abolished the response (Thorn and Petersen, 1993*b*). Lower concentrations of heparin (<250 μg ml^{-1}) abolished only the local spikes, leaving the broad transients essentially unaffected. There are a number of problems associated with this use of heparin (Berridge, 1993; Thorn *et al.* 1993*b*), but one interpretation is that heparin is binding specifically to an InsP_3 receptor in the secretory pole that displays a higher heparin affinity. The affinity of the InsP_3 receptor for heparin has been correlated with its affinity for InsP_3 (Khan *et al.* 1992). A clustering of high-affinity InsP_3 binding sites in the secretory pole is consistent with the idea that a single cell may possess InsP_3 receptors of different affinity (Sharp *et al.* 1992).

In conclusion, Ca^{2+} imaging studies have provided evidence for initiation of the $[Ca^{2+}]_i$ signal in the secretory pole of the cell. Our studies using combined whole-cell patch-clamp and Ca^{2+} imaging have revealed local cytosolic spikes confined to the secretory pole region. InsP_3 infusion through the patch pipette also elicits $[Ca^{2+}]_i$ spikes restricted to the secretory pole and provides evidence for a region of high sensitivity to InsP_3 which may be explained by the regional localization of high-affinity InsP_3 receptors.

Ca^{2+} influx

Ca^{2+} influx is not directly important in the mechanism of Ca^{2+} oscillations, which will persist for some time in the absence of extracellular Ca^{2+}. We do know that, during each oscillation, a component of the Ca^{2+} released into the cytoplasm is actively extruded from

the cells (Tepikin *et al.* 1992). The Ca^{2+} lost from the cell through extrusion eventually leads to the abolition of the oscillations during incubating in Ca^{2+}-free extracellular media (Yule *et al.* 1991; Petersen *et al.* 1991*a*). In the presence of a normal extracellular

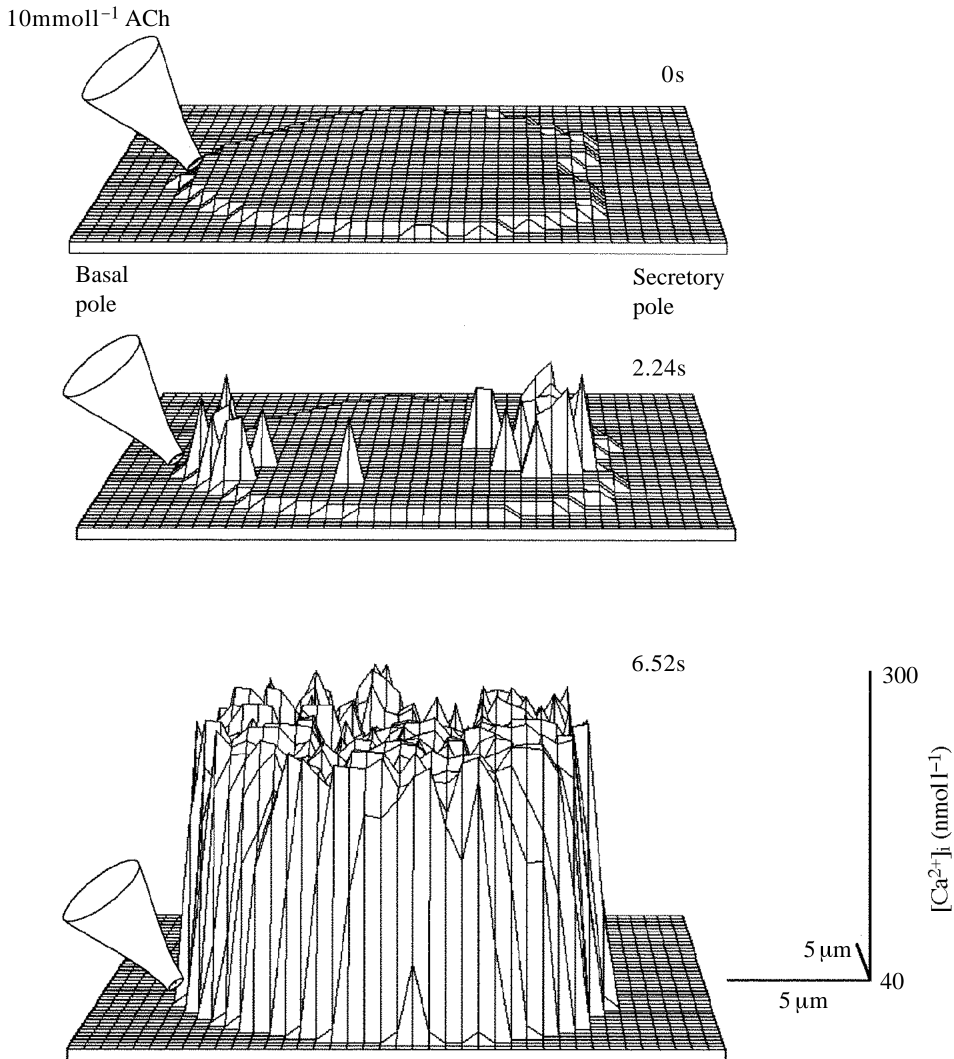

Fig. 4. Focal application of acetylcholine (ACh) to the basal pole of the cell promotes a simultaneous rise in $[Ca^{2+}]$ in both the secretory pole and in the region immediately below the patch pipette. A cell-attached patch was formed on the basal pole of a cell filled with Fura-2 (preloading with Fura-2 acetoxymethyl ester). The patch pipette was filled with ACh ($10 mmol\,l^{-1}$) and a retaining voltage was applied to prevent receptor activation. Before ACh application (time 0) the cell was quiescent. Reversal of the holding potential allowed ACh access to the plasma membrane receptors. The first Ca^{2+} response was recorded 2.24s after ACh application and was seen as a simultaneous rise in the secretory pole and in a region beneath the patch pipette. After a few seconds, a global $[Ca^{2+}]$ rise was observed. (Figure adapted from Thorn *et al.* 1993*a*.)

Ca^{2+} concentration, therefore, the Ca^{2+} extruded must be balanced by an influx of Ca^{2+}. In the pancreatic acinar cells, the Ca^{2+} influx pathway is not known but in the lacrimal acinar cells evidence has accumulated for a role for inositol tetrakisphosphate (InsP_4) in the activation of Ca^{2+} entry. It was originally shown that InsP_4 was able to facilitate Ca^{2+} entry in the presence of InsP_3 (Morris *et al.* 1987). Subsequent work demonstrated that a very high concentration of InsP_3 alone, infused into lacrimal cells, could evoke a Ca^{2+} influx (Bird *et al.* 1991). However, even at this very high concentration of InsP_3, there is still a demonstrable further potentiation of Ca^{2+} influx in response to the addition of InsP_4 (Smith, 1992). Much work remains to be done to clarify the nature and regulation of Ca^{2+} influx into exocrine acinar cells.

Conclusions

Ca^{2+} oscillations in acinar cells have provided key information contributing to our understanding of the mechanisms of Ca^{2+} handling in cells. Recent work has shown that regional differences in the Ca^{2+} signal exist within the acinar cells and has identified the secretory pole as an area of high sensitivity to InsP_3. The local spikes that we observe in this region in response to low agonist concentrations may provide a cellular mechanism for restricting the spread of the Ca^{2+} signal and therefore limiting the cell response to the elevation in [Ca^{2+}]. The crucial role of InsP_3 receptors in the agonist-induced response is clear, but the mechanisms of the oscillations and the possible role of other types of Ca^{2+}-releasing receptors remain unknown. We do not know whether oscillations are present *in vivo* and it could be that, at room temperature (used in the majority of studies), the normal processes of Ca^{2+} regulation are slowed so as to reveal oscillatory responses (Gray, 1988). It can be said that the activation of the Ca^{2+}-dependent currents would occur physiologically and that these currents are crucial to all models of fluid secretion.

Models of fluid secretion

Kasai and Augustine (1990) proposed a model that depends on fluctuating membrane potential to provide a unidirectional flow of Cl^- through the cell. In this model, the cell at rest is more hyperpolarized than the Cl^- equilibrium potential. Agonist-induced oscillations evoke an initial opening of Cl^- channels in the luminal plasma membrane, promoting Cl^- efflux into the lumen. These channels then close, and nonselective channels and Cl^- channels open in the basal plasma membrane. The nonselective channel depolarizes the cell to a value more positive than the Cl^- equilibrium potential and Cl^- flows into the cell through the Cl^- channel on the basal membrane. The whole process is repeated at each oscillation. This model for fluid secretion is restricted to pancreatic acinar cells, which do not possess a Ca^{2+}-dependent K^+ channel. In other acinar cell types, this latter channel is thought to hold the cells hyperpolarized and to provide the outward driving force for Cl^- exit to the lumen (Petersen and Gallacher, 1988). Against the Kasai and Augustine model, secretion still occurs during sustained elevations of [Ca^{2+}] and does not therefore require the Ca^{2+} oscillations that are necessary in the 'push–pull' hypothesis. In models of secretion, the Cl^- channels play a role in Cl^- efflux, but active ion transport must be crucially important to provide the electrochemical

gradients for fluid secretion to take place. Further work is necessary to build up a good model of fluid secretion for the rodent pancreas.

Local Ca^{2+} spikes

The high sensitivity to InsP_3 of the Ca^{2+} stores in the secretory pole region would be maintained *in vivo* and the repetitive local restricted Ca^{2+} spikes that we observe as a consequence of this sensitivity have a number of important implications. Phasic exocytotic secretion has been observed during Ca^{2+} oscillations in acinar cells (Maruyama, 1988) and also in gonadotrophs (Tse *et al.* 1993), with each global $[Ca^{2+}]$ rise being correlated with a burst of exocytosis. It is also known that exocytosis in acinar cells can be elicited when Ca^{2+} concentration is elevated in the secretory pole (Maruyama *et al.* 1993). Therefore, it would seem probable that the restricted Ca^{2+} spikes would elicit exocytotic secretion of digestive enzymes. The effective localization of the spikes in the secretory pole would limit the cellular consequence of Ca^{2+} stimulation and prevent activation of Ca^{2+}-dependent processes, some of which are known to be damaging, in other parts of the cell. There are also energetic advantages for the cell in utilizing a local Ca^{2+} signal. The extrusion of Ca^{2+} after each global oscillation is a process that is energetically expensive (Tepikin *et al.* 1992); such costs would be reduced in the recovery from local spikes.

Relationship of patterns of oscillation to exocytosis

It has been proposed that oscillations may provide a means of digitally encoding an analogue stimulus (Berridge, 1993). It appears that in the pancreas there may be some elements of this, with an increased frequency of oscillations at increasing InsP_3 concentrations (Petersen *et al.* 1991*b*). However, it is also evident from the different patterns of oscillation that the spatial characteristics of the Ca^{2+} response are also very important.

It is known that there are a number of Ca^{2+} -dependent processes involved in exocytosis with different temporal characteristics (Neher and Zucker, 1993; Thomas *et al.* 1993). In response to a step change in $[Ca^{2+}]_i$, three kinetically distinct phases of secretion were observed in rat pituitary cells (Thomas *et al.* 1993). An initial rapid phase of exocytotic release occurred within 40ms, followed by two slower phases, with the bulk of secretion occurring over 400–1000ms and a steady slow secretion for up to 8s. These time courses have been explained in terms of the relative position of the secretory vesicles to the plasma membrane. The initial release involves vesicles closely associated with the plasma membrane, followed by two subsets of vesicles positioned farther away (Thomas *et al.* 1993). In addition to the relative vesicle position, the process of regulated exocytosis is thought to involve changes in the cell cytoskeleton, with disassembly of actin filaments in a Ca^{2+}-dependent process (Cheek and Burgoyne, 1991). This disassembly then allows vesicle access to the plasma membrane. Estimates of the time course of changes in the actin filaments (Cheek and Burgoyne, 1986) indicate that they are slow compared with the rapid exocytotic release described by Thomas *et al.* (1993). Initial cytoskeletal changes were observed after 5s and maximal changes after 30s,

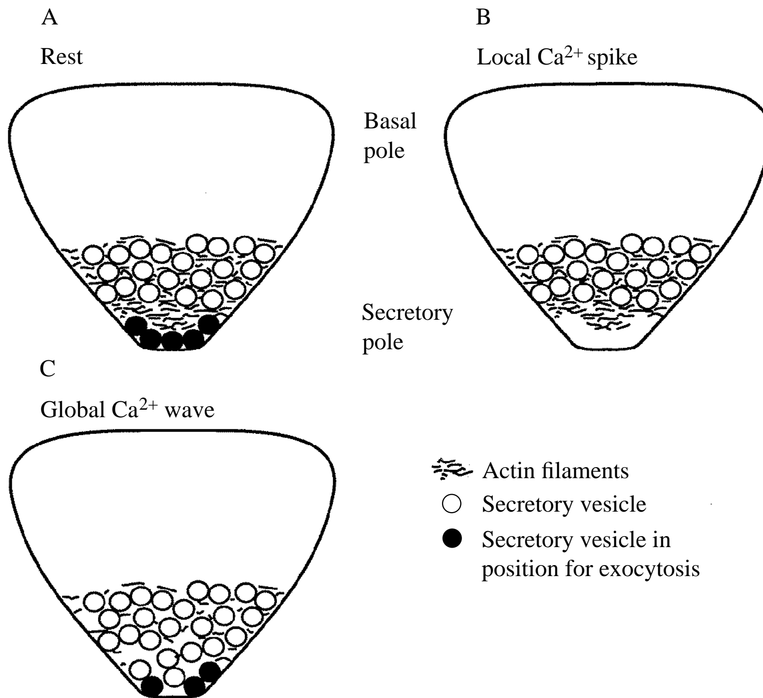

Fig. 5. Diagram of the proposed events that take place during stimulation with cholecystokinin (CCK). At rest (A), a small population of secretory vesicles lies close to the plasma membrane (represented in black), while the bulk of the vesicles (represented in white) are held in an actin filament network away from the luminal plasma membrane. During a local Ca^{2+} spike (B), a small burst of exocytosis of the vesicles immediately adjacent to the plasma membrane occurs. The spike, however, is too spatially restricted and too short to promote significant changes in the actin filament structure. However, during the longer, global Ca^{2+} wave (C), actin filament disassembly can occur and this, along with other possible Ca^{2+}-dependent changes, such as release of proteins binding the vesicles to the actin, leads to movement of some vesicles into a position ready for further exocytosis.

which shows some overlap with the slowest time course observed in the study of Thomas *et al.* (1993).

Actin filaments have been shown in the pancreatic acinar cells to be concentrated in the secretory pole (Drenckhahn and Mannherz, 1983) and cytoskeletal changes have been observed in parotid acinar cells during active secretion (Perrin *et al.* 1992). Comparison of the patterns of the Ca^{2+} oscillations evoked by CCK in the pancreas with the observed time courses of secretion allows some speculation as to the functioning of the Ca^{2+} signal. The short-lasting spikes in the pancreas were, on average, 2s in duration, which is close to the middle time course that elicits maximal exocytotic secretion described by Thomas *et al.* (1993). The broad transient oscillations that we observe have a width of approximately 30s. It is possible that these broad transient $[Ca^{2+}]$ rises are involved in priming the exocytotic process through a mechanism of cytoskeletal change and movement of vesicles closer to the plasma membrane (Fig. 5). Such a mechanism would be able to regulate secretion over long periods. If secretion took place by a simple $[Ca^{2+}]$

rise, the rate of secretion would be limited by the kinetics of the exocytotic events. However, in mechanisms of periodic exocytosis, the rate of secretion would be determined precisely by the frequency of oscillations.

Intracellular Ca^{2+}-releasing receptors

The evidence from our laboratory (Thorn *et al.* 1993*a*) indicates that infusion of InsP_3 can simulate the agonist response in acinar cells. Unfortunately, heparin and decavanadate, both used as antagonists at the InsP_3 receptor, have rather nonspecific actions and better tools are required to push our knowledge further. It has already been shown that monoclonal antibodies can be used against the InsP_3 receptor to block propagated waves in oocytes (Miyazaki *et al.* 1992). In the future, antibodies may be raised against specific subtypes of the InsP_3 receptor (Ross *et al.* 1992) to test the hypotheses we have put forward about receptor distribution.

In addition to the InsP_3 receptor, there is evidence in acinar cells for ryanodine effects (Foskett and Wong, 1991; Nathanson *et al.* 1992) and Ca^{2+} effects (Wakui *et al.* 1990) and there is also evidence from single-channel studies of reconstituted internal membranes (Schmid *et al.* 1990) that indicate a diversity of other Ca^{2+}-releasing receptors. The role of these latter receptors remains unknown and may involve 'sensitizing agents', such as cyclic ADP ribose, in their activation (Galione, 1993). One example of Ca^{2+} mobilization from another unknown Ca^{2+} pool comes from experiments on a CCK analogue, JMV-180, which induces a Ca^{2+} release in permeabilized cells that is apparently independent of caffeine- or InsP_3-sensitive stores (Saluja *et al.* 1992). In our own studies, we have shown that JMV-180 elicits oscillations of mixed pattern like those induced by CCK and that these can be blocked by heparin (Thorn and Petersen, 1993*c*). Once again, this highlights the role of the InsP_3 receptor in oscillations. However, it does not address the issue of a separate Ca^{2+} pool that could release Ca^{2+} and subsequently sensitize the InsP_3 receptors (Bezprozvanny *et al.* 1991). Sensitization of InsP_3 receptors by sulphydryl reagents has recently gained some significance (Missien *et al.* 1991; Thorn *et al.* 1992; Bootman *et al.* 1992), and we have proposed that a similar sensitizing action may be involved in the agonist-induced response (Thorn *et al.* 1993*a*).

In summary, recent experiments have revealed a complex temporal and spatial pattern of Ca^{2+} signalling. Evidence is accumulating that intracellular variation in sensitivity to InsP_3 can play a role in the production of different Ca^{2+} signals. This complexity would allow the cell a repertoire of different physiological responses. The challenge for future investigation will be to determine the links between the Ca^{2+} signal and the physiological processes.

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