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Cytoplasmic Ca^{2+} oscillations evoked by receptor stimulation, G-protein activation, internal application of inositol trisphosphate or Ca^{2+}: simultaneous microfluorimetry and Ca^{2+} dependent Cl^- current recording in single pancreatic acinar cells

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The effects of acetylcholine (ACh), cholecystokinin (CCK), internally applied GTP-γ-S, inositol trisphosphate [Ins (1,4,5) P_3] or Ca^{2+} on the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) were assessed by simultaneous microfluorimetry (fura-2) and measurement of the Ca^{2+}-dependent Cl^- current (patch-clamp whole-cell recording) in single internally perfused mouse pancreatic acinar cells. ACh (0.1−0.2 μM) evoked an oscillating increase in $[Ca^{2+}]_i$ measured in the cell as a whole (microfluorimetry) which was synchronous with oscillations in the Ca^{2+}-dependent Cl^- current reporting $[Ca^{2+}]_i$ close to the cell membrane. In the same cells a lower ACh concentration (0.05 μM) evoked shorter repetitive Cl^- current pulses that were not accompanied by similar spikes in the microfluorimetric recording. When cells did not respond to 0.1 μM ACh, caffeine (1 mM) added on top of the sustained ACh stimulus resulted in $[Ca^{2+}]_i$ oscillations seen synchronously in both types of recording. CCK (10 nM) also evoked $[Ca^{2+}]_i$ oscillations, but with much longer intervals between slightly broader Ca^{2+} pulses. Internal perfusion with 100 μM GTP-γ-S evoked $[Ca^{2+}]_i$ oscillations with a similar pattern. Ins (1,4,5) P_3 (10 μM) evoked repetitive shortlasting spikes in $[Ca^{2+}]_i$ that were only seen in the Cl^- current traces, except in one small cell where these spikes were also observed synchronously in the microfluorimetric recording. Caffeine (1 mM) broadened these Ca^{2+} pulses. $[Ca^{2+}]_i$ was also directly changed, bypassing the normal signalling process, by infusion of a low or high Ca^{2+} solution into the pipette. The relatively smooth rise and fall in $[Ca^{2+}]_i$ seen in the microfluorimetric recordings in response to a 3 min Ca^{2+} infusion were accompanied by a similar rise and fall in the Cl^- current. Before the peak and in the following falling phase repetitive short-lasting Cl^- current spikes occurred. We conclude that receptor stimulation generates cytoplasmic Ca^{2+} spikes by Ins (1,4,5) P_3-evoked Ca^{2+} release causing pulses of Ca^{2+}-induced Ca^{2+} release primarily from pools close to the plasma membrane.

Key words: ACh/Ca^{2+}-induced Ca^{2+} release/CCK/G-protein/Ins (1,4,5) P_3/single-cell $[Ca^{2+}]_i$

Introduction

Activation of many different receptors for neurotransmitters or hormones evokes an increase in the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$). There are several mechanisms for receptor activated Ca^{2+} mobilization. In the electrically excitable neurons and endocrine cells, for example, the main process is Ca^{2+} entry from the extracellular space through voltage gated Ca^{2+}-selective channels (Hosey and Lazdunski, 1988). The opening of the voltage gated Ca^{2+} channels is mediated by membrane depolarization due, for example, to agonist evoked closure of K^+-selective channels as recently demonstrated for the action of vasopressin on insulin secreting cells (Martin *et al.*, 1989). In electrically non-excitable cells the Ca^{2+} signals are mediated by inositol (1,4,5) trisphosphate [Ins (1,4,5) P_3] evoking Ca^{2+} release from the endoplasmic reticulum (Streb *et al.*, 1983; Berridge and Irvine, 1984) and this is followed by Ca^{2+} influx from the extracellular fluid through voltage insensitive pathways controlled by both Ins (1,4,5) P_3 and inositol (1,3,4,5) tetrakisphosphate [Ins (1,3,4,5) P_4] (Morris *et al.*, 1987; Berridge and Irvine, 1989; but see also Penner *et al.*, 1988). Ins (1,3,4,5) P_4 is not an independent acute controller of Ca^{2+} movement, but modulates the Ins (1,4,5) P_3 evoked Ca^{2+} mobilization by an unknown mechanism which is only slowly reversible (Changya *et al.*, 1989).

In many different cell types, hormones and neurotransmitters applied in submaximal concentrations evoke repetitive pulses of internal Ca^{2+} release (Berridge and Galione, 1988). It was recently shown that internal perfusion of single pancreatic acinar cells with Ins (1,4,5) P_3 or its non-metabolizable phosphorothioate derivative evoked repetitive pulses of internal Ca^{2+} release as did stimulation of the muscarinic receptors with acetylcholine (ACh) (Wakui *et al.*, 1989). It therefore now seems unlikely that the Ca^{2+} oscillations are due to pulsatile Ins (1,4,5) P_3 formation (Wakui *et al.*, 1989; Berridge and Irvine, 1989). The currently most attractive hypothesis explaining stimulant evoked Ca^{2+} oscillations is based on the existence of at least two separate intracellular non-mitochondrial Ca^{2+} pools, one sensitive and the other insensitive to Ins (1,4,5) P_3 (Thevenod *et al.*, 1989). It has been proposed that an initial release of Ca^{2+} from the Ins (1,4,5) P_3-sensitive pool could act as a primer for further Ca^{2+} release from Ins (1,4,5) P_3-insensitive pools producing a spike that could spread like a wave throughout a cell (Berridge, 1988; Berridge and Irvine, 1989), but although there is evidence for Ca^{2+} induced Ca^{2+} release in the electrically excitable muscle, nerve and chromaffin cells (Endo *et al.*, 1970; Fabiato and Fabiato, 1975; Endo, 1977; Lipscombe *et al.*, 1988; Burgoyne *et al.*, 1989; Kostyuk *et al.*, 1989) the position is unclear in other tissues. In electrically non-

excitable cells, such as the pancreatic acinar cells, there has so far not been evidence for Ca^{2+}-induced Ca^{2+} release.

By combined and simultaneous recordings of changes in $[Ca^{2+}]_i$ near the plasma membrane [assessed by measurement of Ca^{2+} dependent Cl^- current in the patch-clamp whole-cell recording configuration (Hamill *et al.*, 1981; Wakui *et al.*, 1989)] as well as in the cytoplasma as a whole [assessed by measuring fluorescence of fura-2 by photon counting over the surface area of a single cell (Grynkiewicz *et al.*, 1985; Yule and Gallacher, 1988)] we have now further characterized the stimulant evoked Ca^{2+} oscillations in pancreatic acinar cells. We have stimulated the surface membrane receptors for ACh and CCK, or directly activated G-proteins through internal application of the non-hydrolysable GTP analogue GTP-γ-S. We have also infused Ins (1,4,5) P_3, inositol (1,4,5) trisphosphorothioate (Ins (1,4,5) PS_3) or Ca^{2+} into the cells. All these procedures evoke pulsatile Ca^{2+} mobilization, but the pattern of Ca^{2+} spike generation varies considerably. The Ca^{2+} spikes observed during both Ins (1,4,5) P_3 and Ca^{2+} infusion are short and more easily detected near the plasma membrane (Ca^{2+} dependent Cl^- current) than in the cell at large (microfluorimetry). The ACh evoked Ca^{2+} pulses can in some cases be equally brief, but are mostly broader. The Ca^{2+} spikes induced by CCK or GTP-γ-S are generally of somewhat longer duration. Caffeine, a drug long known to enhance Ca^{2+} induced Ca^{2+} release (Endo, 1977), potentiates the action of stimulants so that, for example, brief Ca^{2+} pulses evoked by Ins (1,4,5) P_3 become longer and larger and are seen also in the microfluorimetric traces. Our data indicate that Ca^{2+} induced Ca^{2+} release plays an important role in the generation of pulsatile internal Ca^{2+} release, but also suggest that surface receptor activation may influence the pattern of Ca^{2+} pulse generation by factors additional to Ins (1,4,5) P_3 formation.

Results

The effects of ACh

It is well known that ACh interacting with muscarinic receptors on pancreatic acinar cells evokes an increase in $[Ca^{2+}]_i$ (Schulz, 1980; Muallem, 1989). In single mouse pancreatic acinar cells, stimulation with submaximal ACh concentrations evokes regular oscillations in $[Ca^{2+}]_i$ from the resting level of ~100 nM to concentrations of 300–800 nM (Yule and Gallacher, 1988). In intracellular microelectrode studies on mouse pancreatic acini it has been shown that ACh evokes depolarization by increasing the membrane conductance (Petersen, 1973; Nishiyama and Petersen, 1974). This ACh controlled conductance pathway is mainly permeable to Cl^- (Petersen and Philpott, 1980). The ACh evoked depolarization and conductance increase can be mimicked by intracellular Ca^{2+} application (Iwatsuki and Petersen, 1977) and prevented by intracellular injection of the Ca^{2+} chelator EGTA (Laugier and Petersen, 1980). Later studies employing the patch-clamp whole-cell recording configuration have confirmed that ACh evokes opening of Cl^- conductance pathways in mouse and rat pancreatic acinar cell membranes and that this effect is mediated by an increase in $[Ca^{2+}]_i$ (Randriamampita *et al.*, 1988; Wakui *et al.*, 1989). In addition to Ca^{2+} dependent Cl^- channels the mouse and rat pancreatic acinar cells also possess Ca^{2+} dependent non-selective cation channels that

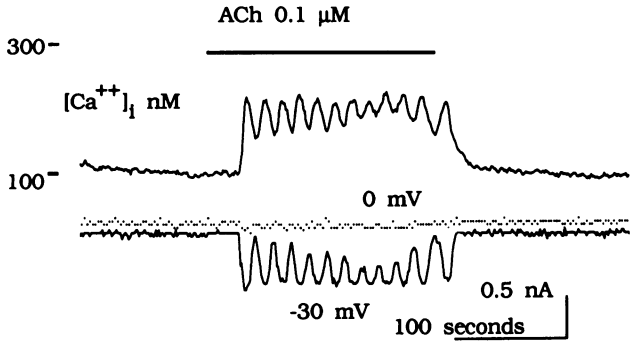

Fig. 1. Single cell microfluorimetric (fura-2) measurement of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ of an enzymatically isolated mouse pancreatic acinar cell (**top trace**) combined with simultaneous whole-cell patch-clamp measurement of Ca^{2+} induced Cl^- current at −30 mV membrane potential (**bottom trace**). Traces show synchronous oscillations in intracellular $[Ca^{2+}]$ and in Ca^{2+} induced Cl^- current in response to 0.1 μM ACh.

can be activated by high ACh concentrations (Maruyama and Petersen, 1982a,b). These pores are, however, closed by intracellular ATP (Suzuki and Petersen, 1988; Petersen and Gallacher, 1988) and since high ATP concentrations are always present in the intracellular perfusion solutions used in our experiments there is no current flow through the non-selective cation channels. Unlike acinar cells from most other exocrine glands, mouse pancreatic acini do not have Ca^{2+} activated K^+ selective channels (Petersen and Maruyama, 1984).

Figure 1 shows an example of simultaneous voltage-clamp current recording and microfluorimetry in a single internally perfused mouse pancreatic acinar cell. The membrane potential was clamped at −30 mV and depolarizing voltage jumps of 100 ms duration to a membrane potential of 0 mV were repetitively applied throughout the experiment. Because of the compression of the current traces the records seem to show the current recorded at −30 and 0 mV simultaneously. The Cl^- concentration was the same inside the cell as on the outside and Cl^- is therefore in equilibrium at 0 mV, whereas there is a large electrical force favouring inward Cl^- current (outward flux) at −30 mV. As seen in Figure 1, ACh (0.1 μM) evoked an oscillatory increase in $[Ca^{2+}]_i$ reflected synchronously in both the microfluorimetry and the Ca^{2+} dependent Cl^- current. The effect of ACh stimulation was completely reversible upon removal of the muscarinic agonist. Similar responses were observed in 15 separate cells from seven different batches of collagenase-isolated acinar cells.

Some cells responded to an ACh concentration as low as 0.05 μM (Figure 2). In seven such cases very brief pulses of Ca^{2+} dependent Cl^- current could be seen, but similar brief Ca^{2+} spikes were absent in the microfluorimetric traces although a tiny elevation in $[Ca^{2+}]_i$ was noticeable. Application of 0.2 μM ACh in the same cell evoked longer pulses of Ca^{2+} dependent Cl^- current and now similar synchronously occurring Ca^{2+} pulses were also detected by the microfluorimetry (Figure 2). Similar results were observed in six other cells.

Some cells did not respond to 0.1 μM ACh. In the recordings shown in Figure 3 a steady resting level of $[Ca^{2+}]_i$ is still observed >5 min after start of stimulation. Addition of 1 mM caffeine on top of the sustained ACh

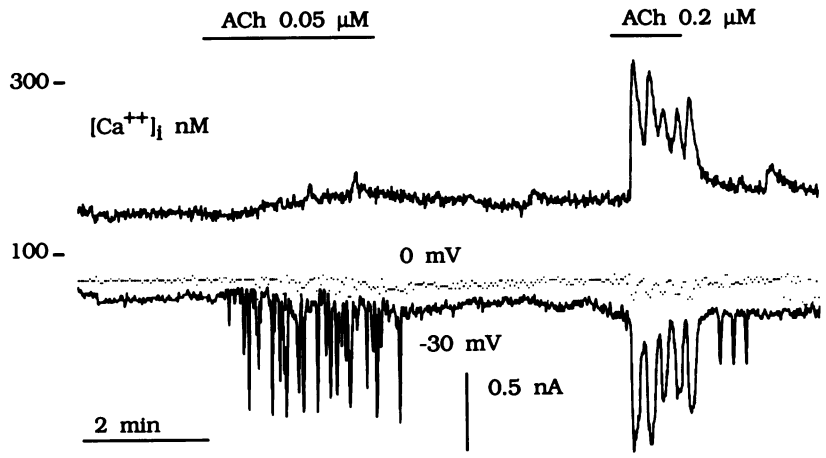

Fig. 2. A low ACh concentration (0.05 μM) induced brief repetitive Ca^{2+} dependent Cl^- current pulses accompanied by tiny elevation of $[Ca^{2+}]_i$ in the cell at large. When the cell is thereafter challenged with 0.2 μM ACh longer-lasting $[Ca^{2+}]$ pulses, recorded simultaneously by both patch-clamp and single cell microfluorimetry are seen.

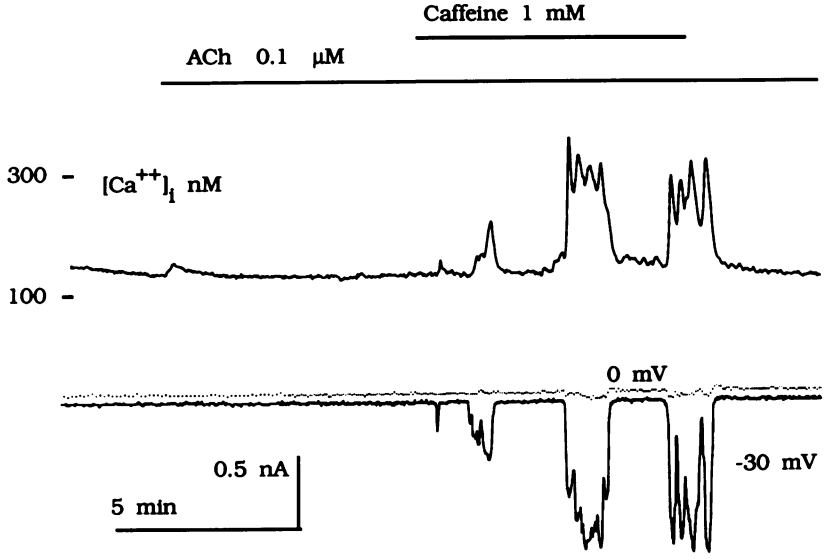

Fig. 3. Lack of response to ACh (0.1 μM) alone. Synchronous oscillations in $[Ca^{2+}]_i$ measured by microfluorimetry and by Ca^{2+} induced Cl^- current evoked by addition of caffeine on top of sustained ACh stimulus.

stimulus did, however, evoke synchronous oscillations in both the current and the microfluorimetric traces. These oscillations were interrupted by silent phases. Caffeine-evoked Ca^{2+} oscillations in the presence of a subthreshold ACh stimulus were observed in all the five separate cells investigated.

The effect of caffeine alone, in the absence of ACh stimulation, was also tested. In three experiments caffeine (1 mM) had no effect on $[Ca^{2+}]_i$ as assessed by simultaneous microfluorimetry and measurement of Ca^{2+} dependent Cl^- current. In a further series of experiments, only involving microfluorimetric measurements, caffeine (0.1–30 mM) again had no effect when added prior to stimulation with ACh or cholecystokinin (n = 10). Finally a third series of experiments in which $[Ca^{2+}]_i$ was monitored solely by measurement of the Ca^{2+} dependent Cl^- current once more showed that caffeine (0.2–20 mM) had no effect when added in the absence of other stimuli (n = 6).

In cells that did respond to 0.1 μM ACh, caffeine (1 mM) evoked a transient inhibition (Figure 4). Interestingly, removal of caffeine, still during continued ACh stimulation, again caused transient inhibition of the ACh response. Caffeine effects similar to those shown in Figure 4 were observed in 12 other cells.

The effects of CCK

It has been known for a long time that the peptide hormone CCK (formerly called pancreozymin) is an important stimulant of enzyme secretion from pancreatic acinar cells (Harper and Raper, 1943). CCK, like ACh, evokes depolarization due to an increased membrane conductance (Petersen, 1973), but whereas atropine abolishes the ACh response it has no effect on the action of CCK (Matthews and Petersen, 1973). Extensive intracellular microelectrode studies on mouse pancreatic acini show that activations of the distinct ACh and CCK receptors evoke opening of the same conductance pathways (Petersen, 1973; Nishiyama and Petersen, 1974; Petersen and Ueda, 1975). It is also well known that CCK and its active analogues can evoke an

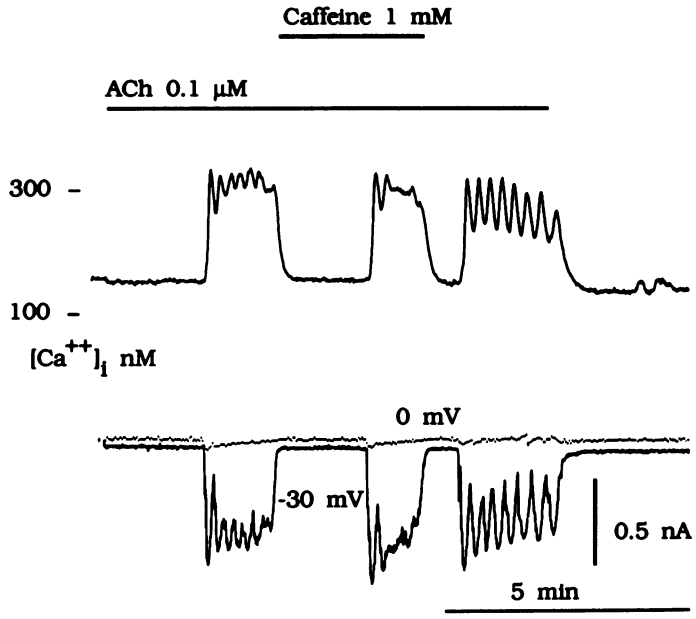

Fig. 4. ACh-evoked oscillating response is transiently blocked by 1 mM caffeine, but note that removal of caffeine during continued ACh stimulation also causes transient inhibition.

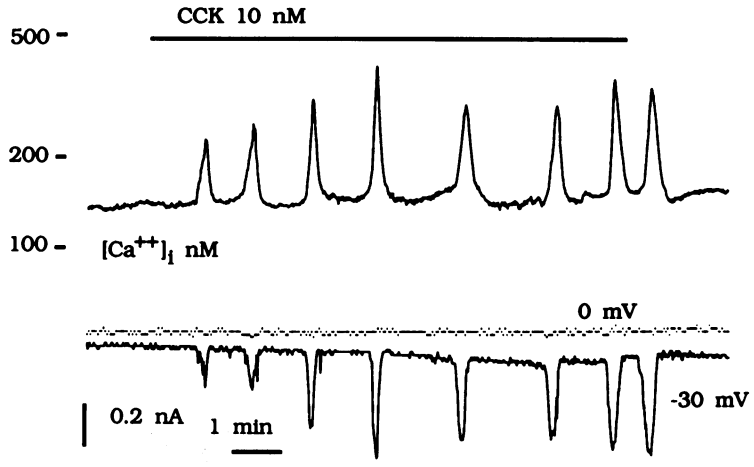

Fig. 5. Regular synchronous pulses of $[Ca^{2+}]_i$ and Ca^{2+} induced Cl^- current evoked by CCK.

increase in $[Ca^{2+}]_i$ in pancreatic acinar cells (Schulz, 1980; Muallem, 1989). Oscillations in $[Ca^{2+}]_i$ in response to the CCK analogue caerulein were recently demonstrated in isolated rat pancreatic acini (Pralong *et al.*, 1988).

Figure 5 shows the effect of 10 nM CCK on $[Ca^{2+}]_i$ assessed by fura-2 microfluorimetry and Ca^{2+} dependent Cl^- current recording. Qualitatively the effect of CCK is similar to that of ACh, but the Ca^{2+} pulses occur with long intervals between them and are relatively broad. Such effects were seen in all the five separate cells investigated.

The effect of GTP-γ-S

ACh and CCK receptors are functionally coupled by different G-proteins to phospholipase C in pancreatic acinar cells (Schnefel *et al.*, 1988). Potentiation of ACh evoked Ca^{2+} dependent current responses by internal application of the non-hydrolysable GTP analogue GTP-γ-S has previously been demonstrated in both lacrimal (Evans and Marty, 1986) and pancreatic (Wakui *et al.*, 1989) acinar cells.

Figure 6 shows the result of an experiment in which GTP-γ-S (100 μM) was present in the pipette solution. Several minutes after establishment of the patch-clamp whole-cell configuration and initiation of internal cell perfusion, pulses of Ca^{2+} release were observed in both the current and microfluorimetric recordings. The pattern of Ca^{2+} pulse generation was closer to that seen with CCK (Figure 5) than with ACh (Figure 1) stimulation since there were long intervals between relatively broad Ca^{2+} spikes. The Ca^{2+} pulse pattern evoked by GTP-γ-S stimulation tended however, to be more irregular than that seen when CCK was used and in between long Ca^{2+} pulses there were also much briefer spikes that in some cases were only picked up in the electrical current recording. In all the four GTP-γ-S experiments carried out a similar pattern was found and in all these cases 1 mM caffeine augmented the GTP-γ-S effect so that the individual pulses partially fused to generate a quasi-sustained oscillating response (Figure 6).

The effects of Ins (1,4,5) P_3 and Ins (1,4,5) PS_3

In a previous study of mouse pancreatic acinar cells it was

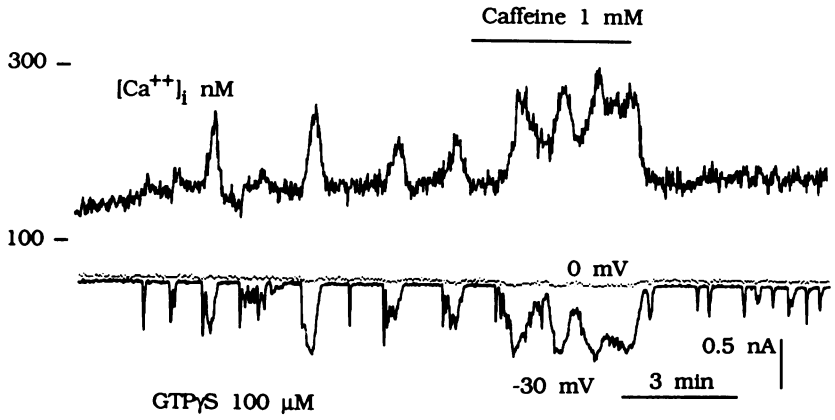

Fig. 6. Intracellularly applied GTP-γ-S induced irregular short and longer lasting pulses of Ca^{2+} dependent Cl^- current in a pancreatic acinar cell. Only the longer pulses could be detected by the microfluorimetric measurement of $[Ca^{2+}]_i$. Caffeine, added extracellularly, enhanced the response to GTP-γ-S. This effect was reversible.

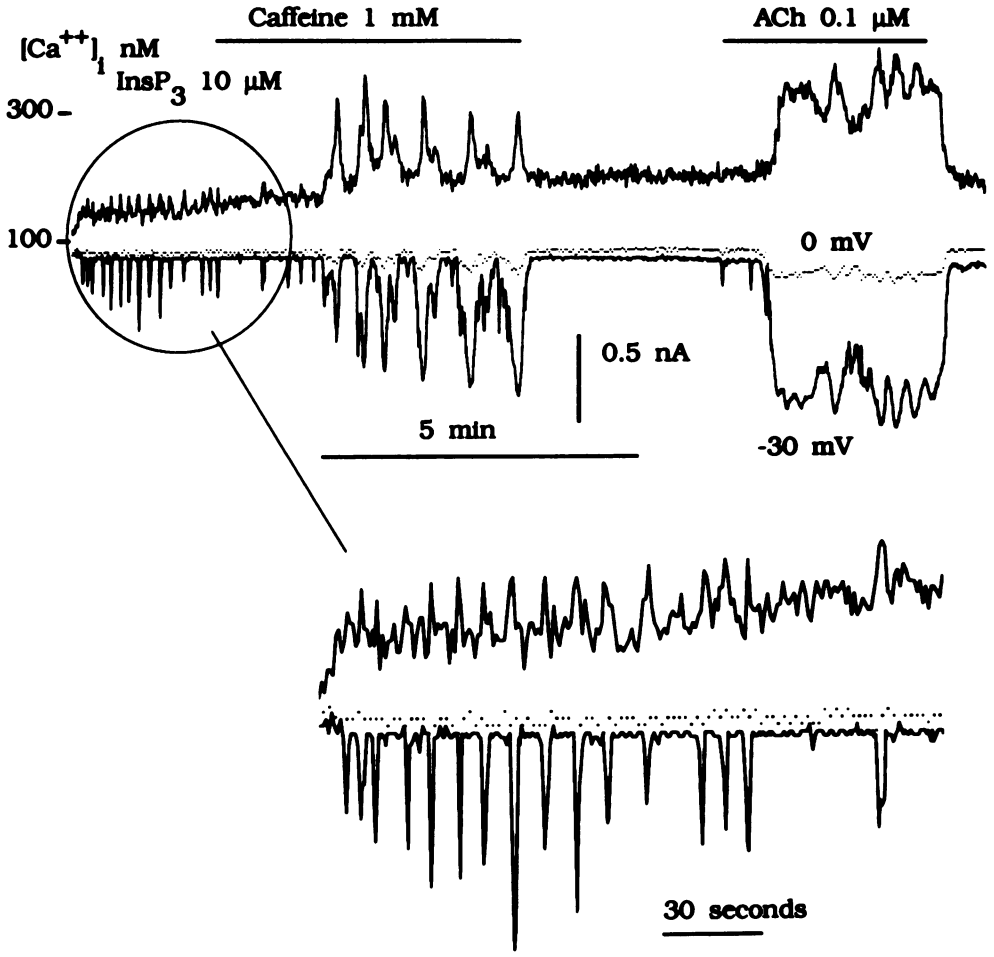

Fig. 7. Ins (1,4,5) P_3, present in the pipette solution, produced short-lasting regular pulses of Ca^{2+} induced Cl^- current with corresponding brief elevations in the microfluorimetric recording. Addition of caffeine or ACh markedly broadened $[Ca^{2+}]$ pulses, making them easily detectable by microfluorimetry.

shown that internal perfusion with Ins (1,4,5) P_3 and Ins (1,4,5) PS_3 evoked repetitive pulses of Ca^{2+} dependent Cl^- current (Wakui *et al.*, 1989). In the majority of the experiments described by Wakui *et al.* (1989) the Ca^{2+} dependent Cl^- current pulses obtained during internal stimulation with Ins (1,4,5) P_3 or Ins (1,4,5) PS_3 were shorter than those seen in the presence of ACh stimulation. This finding was confirmed in the present study. In four experiments Ins (1,4,5) P_3 (10 μM) evoked repetitive and short-lasting pulses of Ca^{2+} dependent Cl^- current that were not accompanied by similar narrow spikes in the microfluorimetric traces. Addition of caffeine (1 mM) in all cases made the pulses broader with a larger amplitude and they were thereafter also observed in the microfluorimetric recordings. Typically the potentiating effect of caffeine was preceded by an inhibitory phase (Figure 7). Very similar

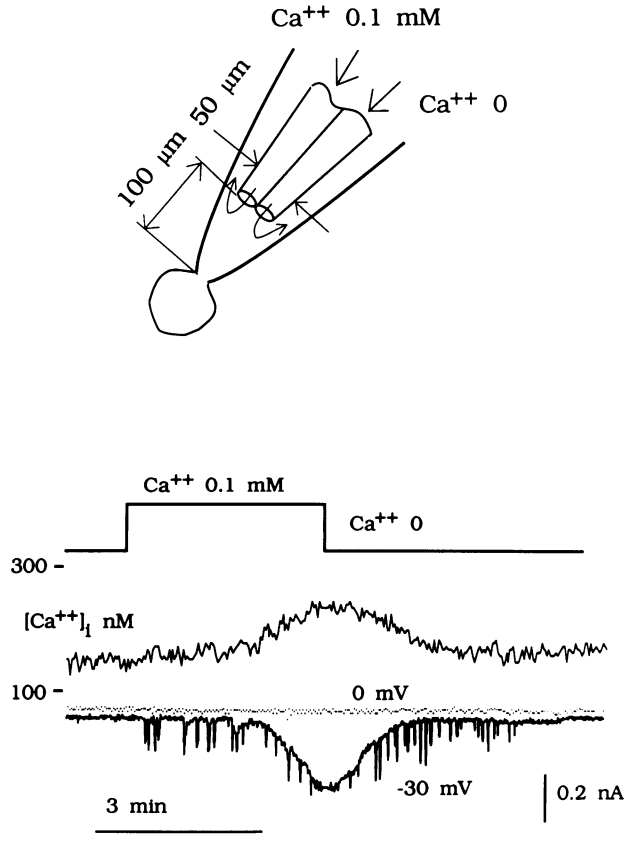

Fig. 8. Two plastic tubes with opening diameter of 20 μm were used to exchange solution at the tip of the patch pipette. Application of 200 mm Hg pressure to the tube containing 0.1 mM Ca^{2+} resulted in a slow rise of $[Ca^{2+}]_i$ and Ca^{2+} induced Cl^- current and in repetitive short-lasting spikes of Ca^{2+} dependent Cl^- current. Application of pressure to the tube filled with Ca^{2+} free solutions reversed the effect.

results were obtained in 21 separate cells stimulated by internal perfusion of Ins (1,4,5) PS_3 (20−50 μM). In one relatively small acinar cell where exactly the same Cl^- current pulse pattern evoked by 10 μM Ins (1,4,5) P_3 was observed, it was just possible to see the Ins (1,4,5) P_3-evoked shortlasting Ca^{2+} spikes also in the microfluorimetric recording (Figure 7). Caffeine (1 mM) enhanced both the magnitude and the duration of the Ca^{2+} pulses as did ACh (0.1 μM) (Figure 7). The short-lasting repetitive Ca^{2+} pulses generated by Ins (1,4,5) P_3 (Figure 7) seem very similar to those evoked by stimulation with 0.05 μM ACh (Figure 2).

The effects of intrapipette Ca^{2+} infusion

Figure 8 shows that Ca^{2+} infusion into the tip of the recording micropipette causes a rise in $[Ca^{2+}]_i$ which is fully reversible after discontinuation of the internal Ca^{2+} application. While the slow rise and fall in $[Ca^{2+}]_i$ evoked by the Ca^{2+} injection is reflected in both the microfluorimetric and current traces there are additional repetitive short-lasting spikes of Ca^{2+} dependent Cl^- current in both the ascending and descending phases, but absent at the peak of $[Ca^{2+}]_i$. These spikes, which resemble those evoked by Ins (1,4,5) P_3 and the very low ACh concentration, were not discernible in the microfluorimetric recording (Figure 8). Three successful Ca^{2+} infusions of the type shown in Figure 8 were carried out in two separate cells with similar results.

Discussion

The results presented in this study indicate that cytoplasmic Ca^{2+} spikes in pancreatic acinar cells are primarily generated by Ca^{2+} release from stores close to the cell membrane and reveal a role for Ca^{2+} induced Ca^{2+} release. The most important evidence is the demonstration that Ca^{2+} infusion into single cells can generate repetitive Ca^{2+} spikes near the cell membrane (Figure 8) similar in nature to those evoked by Ins (1,4,5) P_3 infusion (Figure 7) or low doses of externally applied ACh (Figure 2). The simplest explanation for these findings is that ACh via Ins (1,4,5) P_3 formation evokes a steady release of Ca^{2+} that induces Ca^{2+} release from pools close to the cell membrane. The Ca^{2+} release from these superficially located pools would inactivate and this turn-off could, as demonstrated in muscle cells (Simon *et al.*, 1989), be slowed down by caffeine in this way explaining the marked prolongation and enhancement of Ins (1,4,5) P_3 evoked Ca^{2+} spikes by this drug (Figure 7). It could be argued that intracellular Ca^{2+} infusion evokes Ca^{2+} spikes similar to those evoked by Ins (1,4,5) P_3 infusion not because of Ca^{2+} induced Ca^{2+} release, but by generation of Ins (1,4,5) P_3 due to Ca^{2+} activation of phospholipase C. We do not believe this is likely to be the true explanation since Takemura *et al.* (1989) working with parotid acinar cells, have shown that marked elevation of $[Ca^{2+}]_i$ evoked by the tumour promoter thapsigargin is not associated with breakdown of phosphatidylinositol (4,5) bisphosphate (PIP_2) or formation of Ins (1,4,5) P_3. Takemura *et al.* (1989) also showed that thapsigargin did not inhibit PIP_2 breakdown or Ins (1,4,5) P_3 formation evoked by muscarinic receptor activation. In pancreatic acinar cells Matozaki and Williams (1989) have shown that the Ca^{2+} ionophore A23187 does not increase Ins (1,4,5) P_3 levels. These results indicate that an elevation of $[Ca^{2+}]_i$ in the absence of receptor occupation does not activate phospholipase C sufficiently to generate measurable quantities of Ins (1,4,5) P_3.

It would appear that the cytoplasmic Ca^{2+} spikes under many circumstances primarily occur close to the cell membrane (Figures 2 and 7). This is in agreement with a recent study on parotid acinar cells employing conventional intracellular microelectrode recording combined with microfluorimetry using fura-2. Foskett *et al.* (1989) showed that high concentrations of carbachol evoked a rapid membrane hyperpolarization, due to activation of Ca^{2+} dependent K^+ channels (Petersen and Maruyama, 1984), significantly preceding the rise in $[Ca^{2+}]_i$ as measured by microfluorimetry and they concluded that the agonist sensitive Ca^{2+} pool is located at or near the cell membrane.

Our conclusion that the Ca^{2+} induced Ca^{2+} release primarily occurs close to the cell membrane may at first seem to contradict the recent findings of Burgoyne *et al.* (1989) on adrenal chromaffin cells in which caffeine (10 mM) alone evoked a rise in $[Ca^{2+}]_i$ throughout the cytoplasm of the cells. What we have shown is that direct Ca^{2+} induced Ca^{2+} release is most easily observed close to the cell membrane (Figure 8) as is Ca^{2+} release evoked by low doses of ACh (Figure 2) and Ins (1,4,5) P_3 (Figure 7). Caffeine allows the Ca^{2+} signal to invade the whole of the cell (Figure 7) and this finding is therefore in agreement with the results of Burgoyne *et al.* (1989) suggesting that caffeine sensitive Ca^{2+} stores are not only present at the cell membrane. Our result do indicate, however, that the Ca^{2+}

stores close to the cell membrane are the most Ca^{2+} sensitive.

Functionally distinct G-proteins can selectively couple different receptors to PIP_2 hydrolysis in the same cell (Ashkenazi *et al.*, 1989). In pancreatic acinar cells Schnefel *et al.* (1988) have shown that CCK and muscarinic ACh receptors are functionally coupled to phospholipase C by two different GTP-binding proteins. Our finding that GTP-γ-S evokes a pattern of Ca^{2+} release more similar to that induced by CCK than ACh (Figures 1, 5 and 6) fits well with the observation of Schnefel *et al.* (1988) that pretreatment of permeabilized acinar cells with activated cholera toxin inhibits both CCK- and GTP-γ-S-, but not carbachol-induced Ins (1,4,5) P_3 production. Although it is well documented that CCK-receptor activation evokes PIP_2 breakdown and Ins (1,4,5) P_3 formation in pancreatic acinar cells (Streb *et al.*, 1985) it is now evident that in this system (Matozaki and Williams, 1989) as well as in others (Martin and Michaelis, 1989) there is also significant phosphatidyl choline hydrolysis. Since the CCK concentrations we had to use in our dialysed cells in order to observe Ca^{2+} responses (10 nM) are surprisingly high (Gardner and Jensen, 1987) it cannot be excluded that messengers other than Ins (1,4,5) P_3 may be involved in mediating the CCK evoked Ca^{2+} responses. If other messengers are involved this might explain the very different pattern of Ca^{2+} pulses obtained after CCK stimulation as compared to that induced by ACh. On the other hand, it is entirely possible that an Ins (1,4,5) P_3-evoked Ca^{2+} release generating repetitive bursts of Ca^{2+} induced Ca^{2+} release could be differentially modulated by hormone sensitive influences on Ca^{2+} uptake into the various internal Ca^{2+} compartments, occurring via different mechanisms (Schulz *et al.*, 1989; Thevenod *et al.*, 1989), as well as Ca^{2+} extrusion through the cell membrane (Muallem, 1989). The spatio-temporal control of $[Ca^{2+}]_i$ is very complex in the acinar cells as it is in many other cell types (Lipscombe *et al.*, 1988; O'Sullivan *et al.*, 1989) and although the present study indicates that the receptor-mediated cytoplasmic Ca^{2+} spikes are primarily generated via Ca^{2+} induced Ca^{2+} release from a pool localized close to the cell membrane, many finer points particularly concerning the mechanisms by which different agonists create different Ca^{2+} 'signatures' remain obscure.

Materials and methods

Cell isolation

Fragments of mouse pancreas were digested by pure collagenase (Worthington, 200 units/ml, 20 min, 37°C) in the presence of 1 mM Ca^{2+}. After washing with physiological saline, the cell suspension was gently pipetted to obtain further separation and then again washed with control solution. Only single cells were chosen for experiments.

Media for experiments

The extracellular (bath) solution contained 140 mM NaCl, 4.7 mM KCl, 1.13 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose. pH was adjusted to 7.2. The intracellular (pipette) solution contained 140 mM KCl, 1.13 mM $MgCl_2$, 10 mM HEPES, 5 mM Na_2ATP and 0.2–0.5 mM EGTA. pH was adjusted to 7.2. In many experiments fura-2 (Molecular probes) was present in the pipette solution, in other experiments fura-2 was loaded in from the outside solution by preincubating the cells with fura-2-acetoxymethyl ester (fura-2 AM) (Molecular probes) (see section on Ca^{2+} measurements). Ins (1,4,5) P_3 (gift from Dr R.Irvine, AFRC Cambridge, UK or Sigma), Ins (1,4,5) PS_3 (Cooke *et al.*, 1987) (gift from Dr B.V.L.Potter, University of Leicester, UK) or GTP-γ-S (Boehringer) were present in the pipette solution as and when indicated in the figure legends. ACh (Sigma), CCK (Sigma) and caffeine (Sigma) were present in the extracellular solution in the periods shown and at the concentrations indicated in the figures. All experiments were carried out at room temperature (22–25°C).

Whole-cell current recording

The tight-seal whole-cell recording configuration of the patch-clamp method was used (Hamill *et al.*, 1981; Marty and Neher, 1983; Jauch *et al.*, 1986). Patch-clamp pipettes (type 101PB, Ceebee Glass, Denmark) had final resistances of between 2 and 5 MΩ when filled with the intracellular solution. In a few experiments exchange of solution at the tip of the patch pipette was done by a dual perfusion method employing two fine tubes inserted into the pipette (see Figure 8). One tube was filled with an intracellular solution containing 1 mM $CaCl_2$ ($[Ca^{2+}]$ = 0.1 mM) the other with a Ca^{2+}-free intracellular solution containing 0.5 mM EGTA. The appropriate solution was made to flow into the pipette by pressure application (50–300 mm Hg). The transmembrane currents under voltage clamp were recorded by a LM-EPC-5 (List Electronics, Darmstadt, FRG) patch-clamp amplifier and stored on magnetic tape (Racal 4DS, Southampton, UK). Data was subsequently digitized and transferred to a microcomputer so that they could be displayed together with the microfluorimetric data simultaneously obtained.

Microfluorimetric Ca^{2+} measurement

Ca^{2+} measurements were made simultaneously with the whole-cell current recording in a chamber (volume 0.2 ml) on the stage of a Nikon Diaphot microscope. The cells were continuously superfused in a stream of solution from any one of the eight inlet tubes converging into the chamber containing control and relevant test solutions. A system of taps allowed exchange of solutions in the chamber, within ~20 s. Two different protocols were used in loading the cells with the fluorescent indicator fura-2. In the first protocol fura-2 was present in the intracellular pipette solution at a concentration of 20 μM and diffused into the cell following the establishment of the whole-cell recording configuration (Almers and Neher, 1985). The time constant of fura-2 diffusion into the cell was ~3 min. This protocol was mainly used to investigate extracellularly applied stimuli, as it was possible to delay the stimulation to insure that cells had been adequately loaded with fura-2. In the case of intracellularly applied stimuli, a second protocol was mostly used. Cells were pre-incubated for 15 min at 22°C in the extracellular solution containing 1 μM fura-2 AM so that values for $[Ca^{2+}]_i$ could be obtained immediately after starting whole-cell current recording. Even when the intracellular pipette solution contained no fura-2, loss from the pipette solution was relatively slow (time constant ~20 min) thus allowing microfluorimetric measurement of $[Ca^{2+}]_i$ for a considerable time. In some experiments both methods were combined. The results obtained did not differ with the different fura-2 loading protocols.

$[Ca^{2+}]_i$ was measured in the internally perfused acinar cells by dual-excitation microfluorimetry (Grynkiewicz *et al.*, 1985; Schlegel *et al.*, 1987; Yule and Gallacher, 1988) using a Spex (Glen Spectra) DM 3000 CM system providing alternating excitation wavelengths of 340 and 380 nm at 3 Hz. Fluorescence emitted at 505 nm was monitored by photon counting from single cells isolated optically by means of a diaphragm. $[Ca^{2+}]_i$ was calculated from the ratios of fluorescence intensity at the two excitation wavelengths as previously reported (Schlegel *et al.*, 1987; Yule and Gallacher, 1988), but the limitations inherent in the calibration procedure should be noted (Mollard *et al.*, 1988; Tsien, 1989).

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