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Active mitochondria surrounding the pancreatic acinar granule region prevent spreading of inositol trisphosphate-evoked local cytosolic Ca^{2+} signals

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Agonist-evoked cytosolic Ca^{2+} spikes in mouse pancreatic acinar cells are specifically initiated in the apical secretory pole and are mostly confined to this region. The role played by mitochondria in this process has been investigated. Using the mitochondria-specific fluorescent dyes MitoTracker Green and Rhodamine 123, these organelles appeared as a bright belt concentrated mainly around the secretory granule area. We tested the effects of two different types of mitochondrial inhibitor on the cytosolic Ca^{2+} concentration using simultaneous imaging of Ca^{2+}-sensitive fluorescence (Fura 2) and electrophysiology. When carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was applied in the presence of the Ca^{2+}-releasing messenger inositol 1,4,5-trisphosphate (IP_3), the local repetitive Ca^{2+} responses in the granule area were transformed into a global rise in the cellular Ca^{2+} concentration. In the absence of IP_3, CCCP had no effect on the cytosolic Ca^{2+} levels. Antimycin and antimycin + oligomycin had the same effect as CCCP. Active mitochondria, strategically placed around the secretory pole, block Ca^{2+} diffusion from the primary Ca^{2+} release sites in the granule-rich area in the apical pole to the basal part of the cell containing the nucleus. When mitochondrial function is inhibited, this barrier disappears and the Ca^{2+} signals spread all over the cytosol.

Keywords: antimycin/Ca^{2+} oscillations/Ca^{2+} store/CCCP/mitochondria

Introduction

Pancreatic acinar cells are structurally and functionally polarized. The zymogen (secretory) granules (ZGs) are concentrated at the apical pole, whereas the endoplasmic reticulum (ER), the nucleus and the Golgi cisternae are localized in the basolateral part of the cells (Amsterdam and Jamieson, 1974). The acinar cells secrete both fluid and enzymes into the lumen in response to stimulation with the neurotransmitter acetylcholine (ACh) and the circulating peptide hormone cholecystokinin. Fluid secretion is dependent on the operation of Ca^{2+}-sensitive Cl^- channels clustered in the luminal (apical) membrane (Petersen, 1992), which can be opened by local agonist-elicited Ca^{2+} spikes in the secretory granule region (Thorn *et al.*, 1993). Such spikes can also be generated by intracellular infusion of the Ca^{2+}-releasing messenger inositol (1,4,5)-trisphosphate (IP_3) (Thorn *et al.*, 1993) as well as by the more recently described putative messengers cyclic ADP-ribose (Thorn *et al.*, 1994; Cancela *et al.*, 1998) and nicotinic acid adenine dinucleotide phosphate (Cancela *et al.*, 1999). Enzyme secretion occurs via Ca^{2+}-dependent exocytosis through the luminal plasma membrane (Palade, 1975) and this process can also be activated by a selective rise in the local Ca^{2+} concentration in the secretory pole (Maruyama *et al.*, 1993).

The Ca^{2+} spike pattern evoked by low ACh concentrations can be mimicked by intracellular infusion of IP_3 or one of its non-metabolizable analogues and can most easily be monitored by measurements of the Ca^{2+}-dependent ionic currents in patch–clamp whole-cell recording experiments (Wakui *et al.*, 1989, 1990; Osipchuk *et al.*, 1990; Petersen *et al.*, 1991a,b; Thorn and Petersen, 1992; Thorn *et al.*, 1993). The secretory pole has a particularly high sensitivity to IP_3 (and indeed also to cyclic ADP-ribose) (Kasai *et al.*, 1993; Thorn *et al.*, 1993, 1994), due to a high concentration of Ca^{2+} release channels in the ER terminals invading the ZG area and possibly also in the ZGs themselves (Nathanson *et al.*, 1994; Petersen *et al.*, 1994; Gerasimenko *et al.*, 1996a; Mogami *et al.*, 1997). However, this does not fully explain how Ca^{2+} is confined to the secretory pole during local Ca^{2+} signalling events. Very substantial Ca^{2+} gradients (up to 400 nM/μm; Gerasimenko *et al.*, 1996b) can be observed between the secretory granule and basal regions and it is not known why Ca^{2+} does not spread throughout the cell.

The aim of the work to be reported here was to investigate the possible role of mitochondria in confining Ca^{2+} signals to the ZG region, since mitochondria are capable of buffering intracellular Ca^{2+} (Simpson and Russell, 1996; Babcock *et al.*, 1997; Hoth *et al.*, 1997). The mitochondrial Ca^{2+} uptake mechanism is a high-capacity, low-affinity uniporter driven by the large electrical potential across the inner membrane. The release of mitochondrial Ca^{2+} occurs mainly via an electroneutral exchange of Ca^{2+} for two Na^+ (Carafoli, 1987). Ca^{2+}-induced release of Ca^{2+} from mitochondria can occur via a transitory opening of the permeability transition pore in a low conductance mode (Ichas *et al.*, 1997). Mitochondria contain very little Ca^{2+} in resting cells, but increases in the cytosolic Ca^{2+} concentration lead to increases in the intramitochondrial Ca^{2+} concentration and this has been shown to be important for stimulation of mitochondrial oxidative metabolism (Denton and McCormack, 1990; McCormack *et al.*, 1990; Pozzan *et al.*, 1994; Pralong *et al.*, 1994; Rizzuto *et al.*, 1994; Hajnoczky

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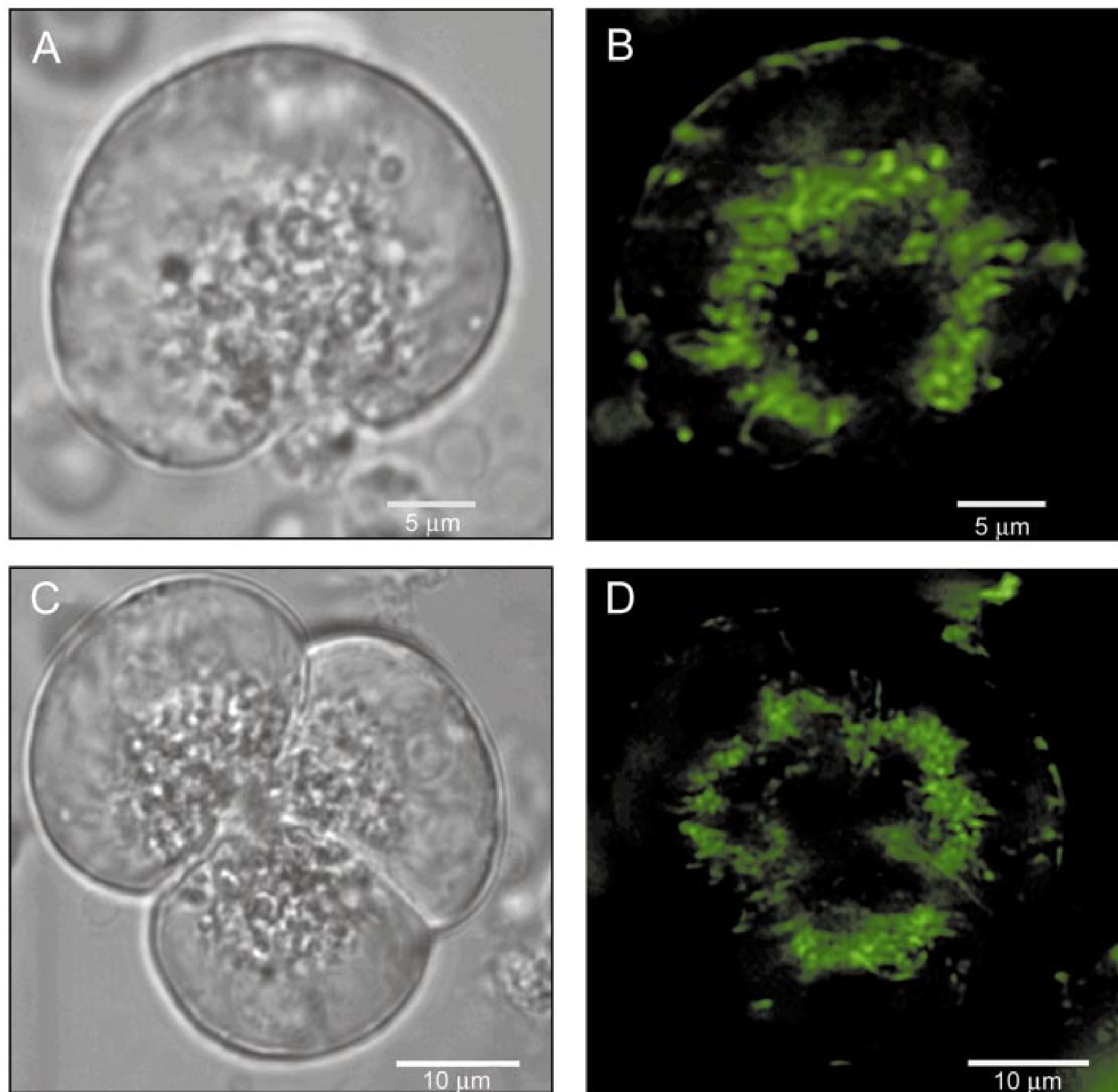


Fig. 1. Active mitochondria surround the secretory granule area**.** Transmitted light and fluorescence images of freshly isolated acinar cells illustrating the intracellular distribution of mitochondria. The left part shows the transmitted light pictures of a single cell (**A**) and a cell triplet (**C**), illustrating the morphological polarity of the isolated cells. The zymogen granules concentrated at the secretory poles can be distinguished easily from the basolateral parts of the cells. The right part of the figure (**B** and **D**) shows the fluorescent images of the same cells stained with the mitochondrion-specific dye MitoTracker Green. Mitochondria appear as a bright belt surrounding the secretory pole, but some distinct spots in the vicinity of the plasma membrane are also seen. All images were obtained using a confocal microscope.

et al., 1995; Robb-Gaspers *et al.*, 1998). Mitochondrial Ca^{2+} uptake can influence the dynamics of stimulant-evoked cytosolic Ca^{2+} signals (Jouaville *et al.*, 1995; Hoth *et al.*, 1997) and Rizzuto *et al.* (1998) have recently demonstrated close contacts between the ER and the mitochondria, which can significantly enhance mitochondrial Ca^{2+} responses to IP_3 stimulation, by allowing mitochondria to sense local domains of high Ca^{2+} concentration generated at the mouth of IP_3 receptors (Rizzuto *et al.*, 1993).

Although the polarized pancreatic acinar cell has been the subject of extensive Ca^{2+} signalling investigations (Petersen *et al.*, 1994), the role of mitochondria in Ca^{2+} signalling has been neglected. The aim of the present work was to localize the functionally active mitochondria in the living acinar cells and to investigate the possibility that functioning mitochondria play a role in confining the IP_3-evoked Ca^{2+} release in the apical pole to this granule-rich region.

Using mitochondria-specific fluorescent dyes and confocal microscopy, we show that active mitochondria in acinar cells are concentrated mainly in a belt surrounding the apical ZG-containing region. We also show that two different ways of inhibiting mitochondrial function (using the protonophore CCCP or inhibition of electron transport at complex III with antimycin) change the pattern of IP_3-evoked local Ca^{2+} spiking in the granule area to a global rise in the cytosolic Ca^{2+} level. In the absence of IP_3 stimulation, mitochondrial inhibition does not evoke any change in the cytosolic Ca^{2+} concentration. We propose that active mitochondria situated on the border of the granule area build a buffer barrier, which prevents IP_3-evoked Ca^{2+} release in the granule-rich apical pole from spreading into the basolateral region.

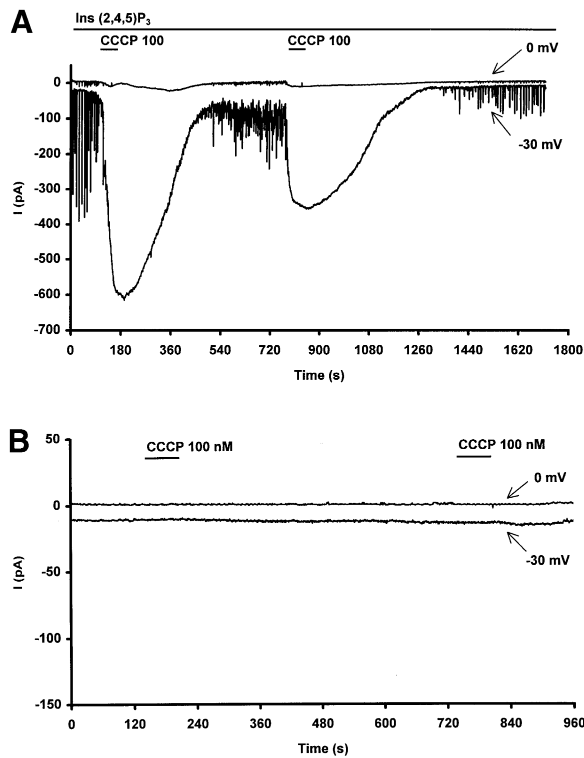


Fig. 2. The mitochondrial inhibitor CCCP evokes an increase in the Ca^{2+}-dependent current, but only when IP_3 is present in the intracellular solution. Whole cell recording of the membrane current at 0 or –30 mV holding potential. (**A**) Application of 100 nM CCCP during 2,4,5-IP_3-evoked current spikes leads to a major increase in the membrane current. The effects of CCCP are reversible and repeatable. (**B**) CCCP does not change the membrane current in the absence of IP_3 (resting state).

Results

The intracellular distribution of mitochondria

The intracellular distribution of mitochondria in the pancreatic acinar cells was examined using confocal microscopy. The transmitted light images of freshly isolated single cells (Figure 1A) and of a cell triplet (Figure 1C) show the characteristic polarization of the cells. The ZGs are concentrated in the apical secretory pole. This relatively dark area is surrounded by the brighter basolateral cytoplasm. Figure 1B and D illustrates the intracellular localization of mitochondria in the same cells. Mitochondria were stained using a cell-permeant mitochondrion-selective fluorescent dye MitoTracker Green, which contains a mildly thiol-reactive chloromethyl moiety and is concentrated by active mitochondria (Haugland, 1996). The images show that the distribution of mitochondria in pancreatic acinar cells is not homogeneous. Mitochondria appear as a bright belt concentrated mainly around the secretory granule area. There is relatively little labelling in the basolateral part of the cells, apart from some distinct spots in the vicinity of the plasma membrane. Fourteen experiments of this type were carried out, all giving similar results. Labelling of mitochondria with Rhodamine 123, a cell-permeable cationic fluorescent dye that is readily sequestered by active mitochondria (Haugland, 1996), gave similar results ($n = 5$). However, MitoTracker Green was more photostable and produced a brighter signal at a lower concentration.

The effects of the mitochondrial uncoupler CCCP on Ca^{2+}-dependent current spikes

It has previously been shown that intracellular application of IP_3, or one of its non-metabolizable analogues, evokes Ca^{2+}-dependent current spikes (Wakui *et al.*, 1989) and that these repetitive short-lasting current spikes are associated with local increases in the Ca^{2+} concentration in the secretory pole of the cell (Thorn *et al.*, 1993). Patch–clamp current recording is a sensitive indicator of local Ca^{2+} concentration changes near the cell membrane (Osipchuk *et al.*, 1990; Thorn *et al.*, 1993) and we therefore used the electrophysiological method as our principal means to monitor cytosolic Ca^{2+} signalling events.

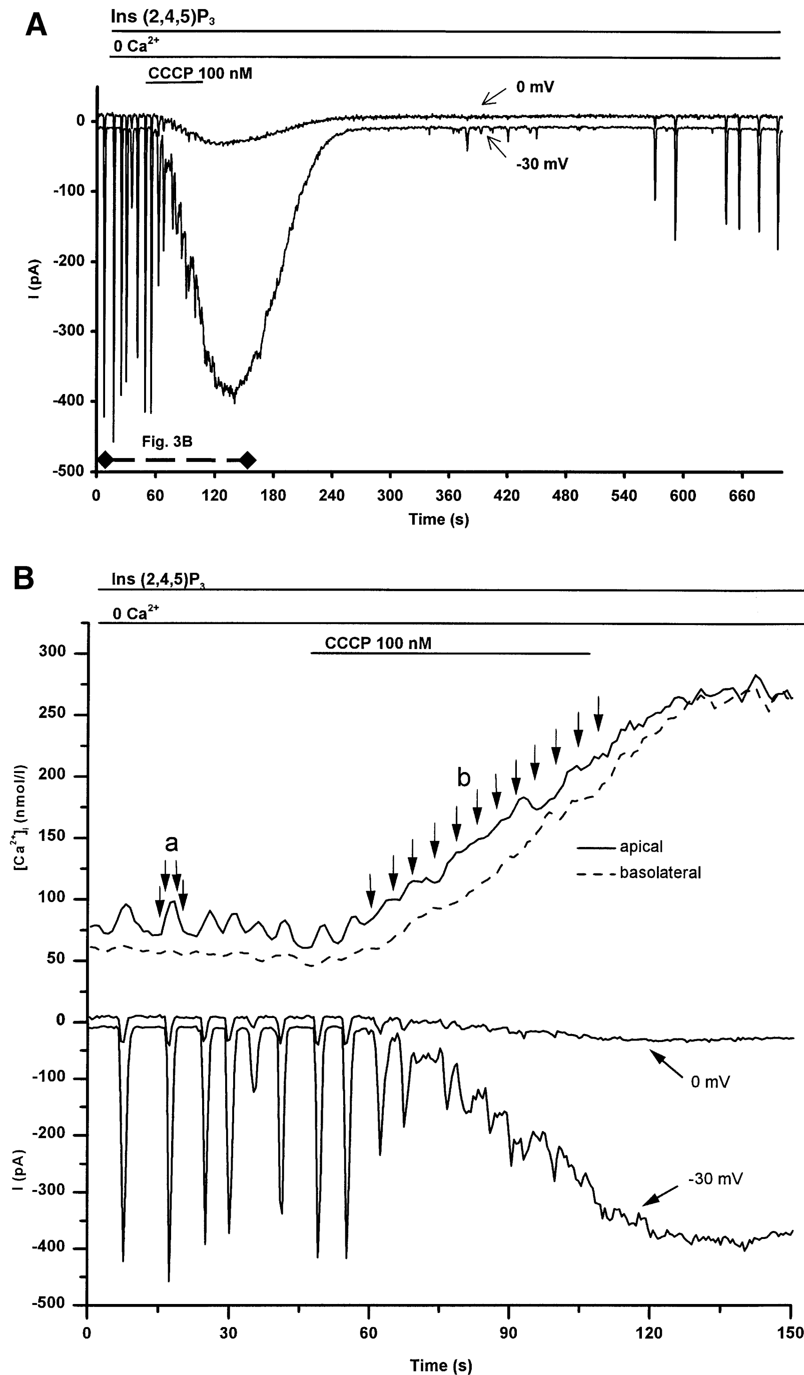
We have tested the hypothesis that mitochondria build a buffer barrier preventing local IP_3-evoked Ca^{2+} release in the apical granule-rich region from spreading into the bulk of the cytosol. The ability of mitochondria to sequester Ca^{2+} depends on the negative mitochondrial membrane potential, serving as the driving force for Ca^{2+} influx. Mitochondrial Ca^{2+} uptake can be inhibited by collapsing the membrane potential with protonophores such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Reyes and Benos, 1984). We tested this in control experiments, using the dual-emission potential-sensitive probe JC-1 (isolated cells were incubated with 10 μg/ml of JC-1 from Molecular Probes for 15 min at 37°C). We established that 100 nM CCCP evoked a rapid and marked depolarization of the inner mitochondrial membrane with the maximal effect being observed within 50 s. Using ratio measurements (Haugland, 1996), we observed that CCCP could evoke a 3-fold change. We then went on to test the effect of CCCP on the Ca^{2+}-dependent current. Figure 2A shows changes in the local Ca^{2+} concentration near the plasma membrane, monitored by patch–clamp whole-cell recording of the Ca^{2+}-dependent currents. The spikes of Ca^{2+}-dependent Cl^- current in the first 60 s of the experiment were evoked by inositol 2,4,5-trisphosphate (2,4,5-IP_3), a non-metabolizable analogue of IP_3, which was present in the patch pipette in a concentration of 10 μM. The pipette solution contained 2 mM ATP (and no ADP) to protect the cell from energy depletion. Extracellular application of 100 nM CCCP led to a sustained increase of the Ca^{2+}-dependent membrane current and inhibition of spiking ($n = 8$). Upon wash-out of CCCP, the cytosolic Ca^{2+} concentration, as monitored by the Ca^{2+}-dependent current, decreased to the normal resting level and later spiking resumed. The effect of CCCP could be observed repeatedly in the same experiment (Figure 2A). CCCP only evoked changes in the Ca^{2+}-dependent current if the protonophore was applied during IP_3-evoked Ca^{2+} spiking. Figure 2B shows the result from an experiment in which 2,4,5-IP_3 was excluded from the pipette solution, so that no Ca^{2+}-dependent spikes occurred. Repeated CCCP applications failed to evoke any rise in the cytosolic Ca^{2+} concentration. Four experiments of this type were carried out, all giving negative results.

The effects of CCCP on the spatial distribution of cytosolic Ca^{2+}

To determine the origin of the Ca^{2+} increase evoked by CCCP, we combined whole-cell current recording with

simultaneous measurement of Ca^{2+}-sensitive Fura 2 fluorescence. In order to prevent any difficulties in interpretation that could arise from dealing with a mixture of primary intracellular Ca^{2+} release and secondary Ca^{2+} entry from the extracellular solution, these experiments were carried out using Ca^{2+}-free external solutions. It has previously been shown that the local cytosolic Ca^{2+} spikes are independent of extracellular Ca^{2+} for a considerable period (Wakui *et al.*, 1989; Osipchuk *et al.*, 1990). In the first series of six experiments, we recorded simultaneously the Ca^{2+}-dependent current and the average cytosolic Ca^{2+} concentration based on Fura 2 microfluorimetry (Osipchuk *et al.*, 1990). In this type of experiment, as previously shown (Osipchuk *et al.*, 1990), the short-lasting and local Ca^{2+} spikes hardly make any impact on the overall cytosolic Ca^{2+} level and can only be picked up by the electrophysiological method. During the IP_3-evoked spiking there was no measurable increases in the bulk cytosolic Ca^{2+} concentration. Application of CCCP evoked not only a marked increase in the Ca^{2+}-dependent ion current, but also a clear rise in the bulk cytosolic Ca^{2+} level (from 121.3 ± 9.7 nM to 283.2 ± 38.7 nM, $n = 6$). After removal of CCCP the decline in the Ca^{2+}-dependent ion current was matched by a decrease in the cytosolic Ca^{2+} concentration back to the resting level seen before CCCP application ($n = 6$). Since the experiments were performed in nominally Ca^{2+}-free external solutions, the CCCP-evoked increase of $[Ca^{2+}]_i$ was due to release from an intracellular compartment and not due to Ca^{2+} entry.

In order to monitor the spatial distribution of Ca^{2+}, we



carried out experiments with combined electrophysiology and imaging of Ca^{2+}-sensitive fluorescence (Figure 3). Two regions of interest (the granule-rich apical area and the basal clear area) were identified. The IP_3-evoked spikes of Ca^{2+}-dependent current (Figure 3A) were, as previously shown (Thorn *et al.*, 1993), associated with cytosolic Ca^{2+} spikes in the apical pole, but not in the baso-lateral area (Figure 3B). The images shown in Figure 3C(a)

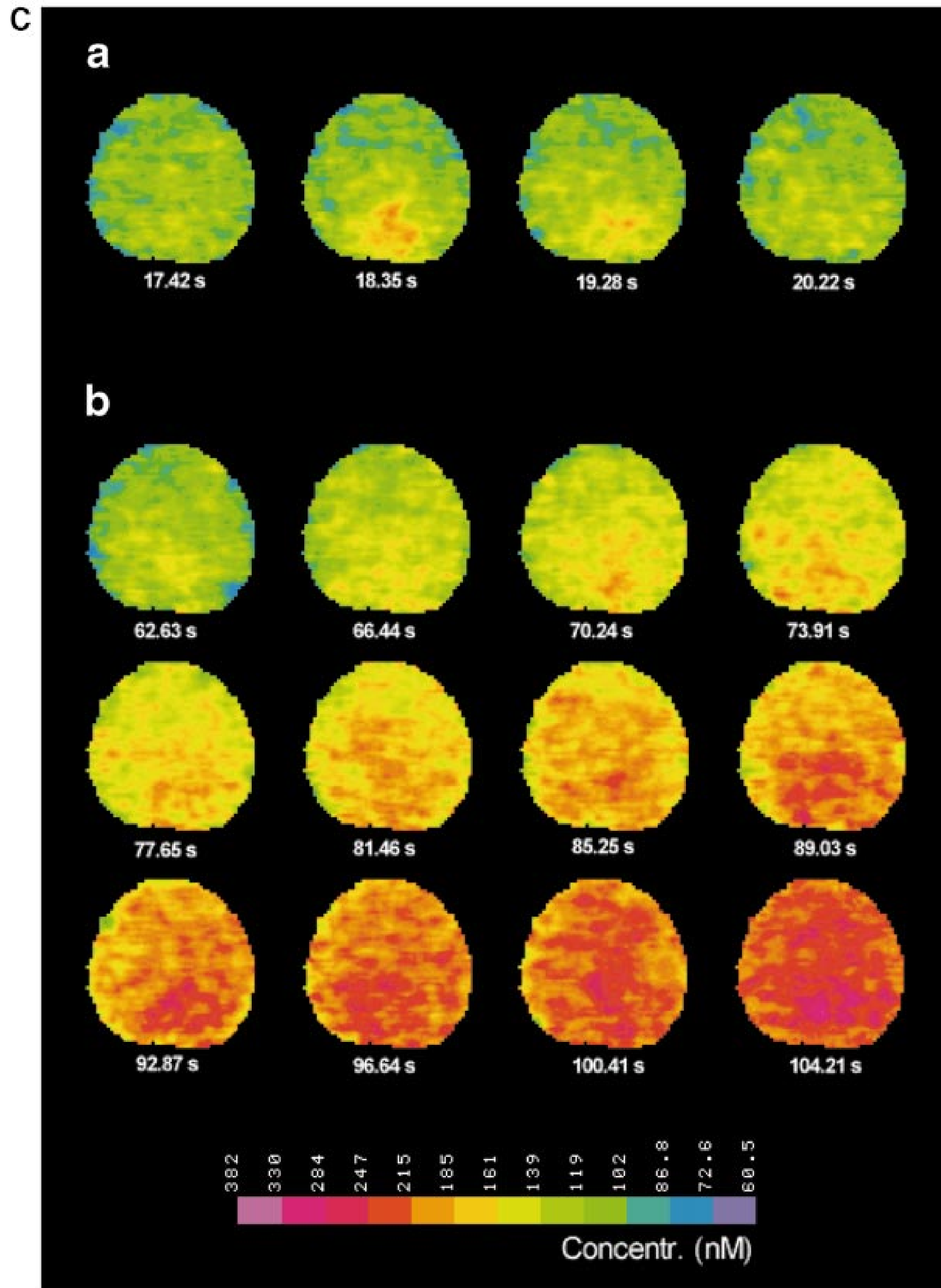


Fig. 3. The mitochondrial inhibitor CCCP transforms the IP_3-evoked pattern of local Ca^{2+} spiking in the apical granule-rich region into a global rise in the cytosolic Ca^{2+} concentration. (**A**) Patch–clamp whole-cell recording of Ca^{2+}-sensitive ion current. 2,4,5-IP_3 (10 μM) was present in the internal pipette solution. The external solution was nominally Ca^{2+} free. It is seen that IP_3 evokes repetitive spikes of Ca^{2+}-sensitive current and that application of CCCP induces a reversible rise in the Ca^{2+}-sensitive current. In this experiment imaging of Ca^{2+}-sensitive Fura 2 fluorescence in the cytosol was also carried out. The interrupted bar labelled Figure 3B indicates the period from which simultaneous fluorimetric and electrical recordings are shown in (B). (**B**) Simultaneous recordings of the cytosolic Ca^{2+} concentrations in two regions of interest (apical and basolateral regions) and the Ca^{2+}-sensitive ion current. It is seen that each spike of Ca^{2+}-dependent current is associated with a small rise in the cytosolic Ca^{2+} concentration in the apical, but not the basolateral region. Application of CCCP causes a slow rise in the cytosolic Ca^{2+} concentration in both regions with a time course similar to the increase in the Ca^{2+}-sensitive ion current. The arrows labelled 'a' and 'b' represent the times at which images showing the cytosolic Ca^{2+} distribution were taken. (**C**) Pseudocolour images showing the cytosolic distribution of Ca^{2+} during a single IP_3-evoked Ca^{2+} spike (a) and the pattern of the Ca^{2+} rise in response to CCCP application (b). The colour scale at the bottom of the panel shows the calibration. The apical secretory granule-containing area is at the bottom of each image. In (a) it is seen that Ca^{2+} only rises in the granule area and that the spike duration is <3 s. In (b) it is seen that the initial rise in Ca^{2+} occurs in the granule-containing area and then spreads out towards the base.

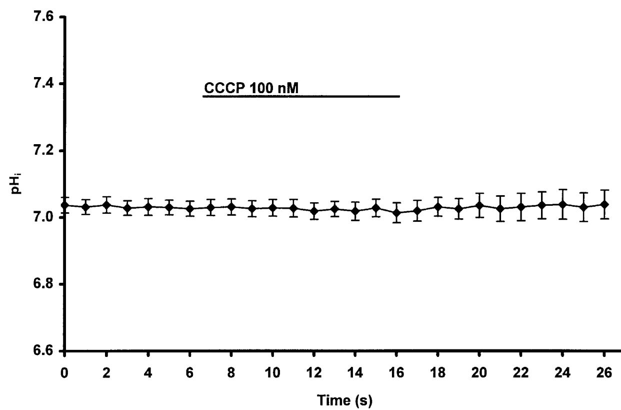


Fig. 4. The mitochondrial inhibitor CCCP does not change the cytosolic pH. Intracellular pH measurement using the fluorescent dye BCECF. Application of 100 nM CCCP for 10 min has no effect on cell pH.

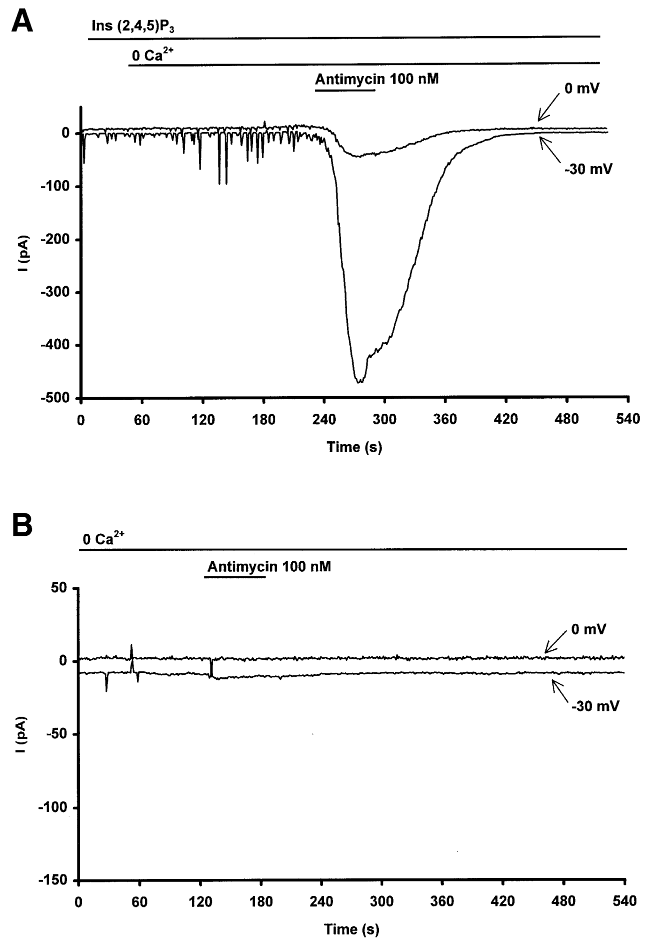


Fig. 5. The mitochondrial inhibitor antimycin evokes a marked rise in the cytosolic Ca^{2+} concentration, but only in the presence of the Ca^{2+}-releasing messenger IP_3. (**A**) Antimycin (100 nM) applied during IP_3-evoked Ca^{2+} spiking in Ca^{2+}-free external solution evokes an increase in the Ca^{2+}-dependent current. (**B**) Application of antimycin in the absence of IP_3 stimulation (resting state) fails to evoke any changes in the Ca^{2+}-dependent current.

demonstrate a single IP_3-evoked local Ca^{2+} spike in the granule-rich region. Application of CCCP (100 nM) evoked a marked rise in the cytosolic Ca^{2+} concentration in both the apical and the basolateral areas (Figure 3B). The images shown in Figure 3C(b) illustrate the effect of CCCP, in the presence of IP_3, on the subcellular Ca^{2+} distribution. The rise in the cytosolic Ca^{2+} concentration clearly starts in the apical pole, but then spreads out towards the base. After ~30 s the cytosolic Ca^{2+} concentration is uniformly elevated throughout the cell. Four separate experiments of the type shown in Figure 3 were carried out, all giving similar results.

CCCP does not change intracellular pH

CCCP is a protonophore and the changes in $[Ca^{2+}]_i$ observed during its application might be influenced by changes in the intracellular pH. To assess this possibility we used the pH-sensitive fluorescent dye (2′,7′-bis-2-carboxyethyl)-5-(and-6)carboxyfluorescein (BCECF) to measure cell pH during CCCP application. The resting intracellular pH of pancreatic acinar cells was 7.04 ± 0.02 (n = 10) and did not change significantly during 10 min of exposure to 100 nM CCCP (Figure 4).

Antimycin evokes a prolonged cytosolic Ca^{2+} rise

The mitochondrial membrane potential is built up in functional organelles by the electron transport associated with oxidative phosphorylation. Antimycin is a well known and well characterized inhibitor of this transport (Singer, 1979). In control experiments with the dual-emission potential-sensitive probe JC-1, we established that 100 nM antimycin evoked a clear depolarization of the inner mitochondrial membrane, although the amplitude of the signals was smaller than in response to CCCP stimulation. Using ratio measurements (Haugland, 1996) antimycin could at most evoke a doubling of the ratio, whereas CCCP could elicit a 3-fold increase. We then went on to test the effect of antimycin on the Ca^{2+}-dependent current. Application of 100 nM antimycin, during IP_3-evoked Ca^{2+} spiking, evoked a substantial increase in the cytosolic Ca^{2+} concentration, as monitored by the Ca^{2+}-dependent membrane current (n = 6; Figure 5A). The major antimycin-evoked increase in the cytosolic Ca^{2+} level was, as in the case of CCCP, associated with inhibition of Ca^{2+} spike generation. As also seen in the CCCP experiments, Ca^{2+} spiking did not resume immediately after the bulk cytosolic Ca^{2+} concentration had been restored to the normal resting level following removal of the mitochondrial inhibitor. Within the time frame of our measurements in the antimycin experiments, we did not observe resumption of Ca^{2+} spiking after inhibitor removal. Antimycin application in the absence of IP_3 stimulation had no effect on the Ca^{2+}-dependent currents (n = 4; Figure 5B).

Despite the virtually limitless supply of 2 mM ATP to the cell interior in our patch–clamp whole-cell recording studies, it could be postulated that a local depletion of ATP might occur in certain critical regions. In the presence of antimycin it is possible that ATP hydrolysis by the ATPase could take place, which might result in local ATP depletion and perhaps help sustain in part a proton electrochemical gradient. We therefore also carried out a series of experiments in which the combined effect of 100 nM antimycin and 500 nM oligomycin (inhibitor of ATP synthase) was investigated. The protocol for these experiments was the same as in Figure 5A. In three separate experiments the effect of antimycin + oligomycin was similar to the effect of antimycin alone. The spikes

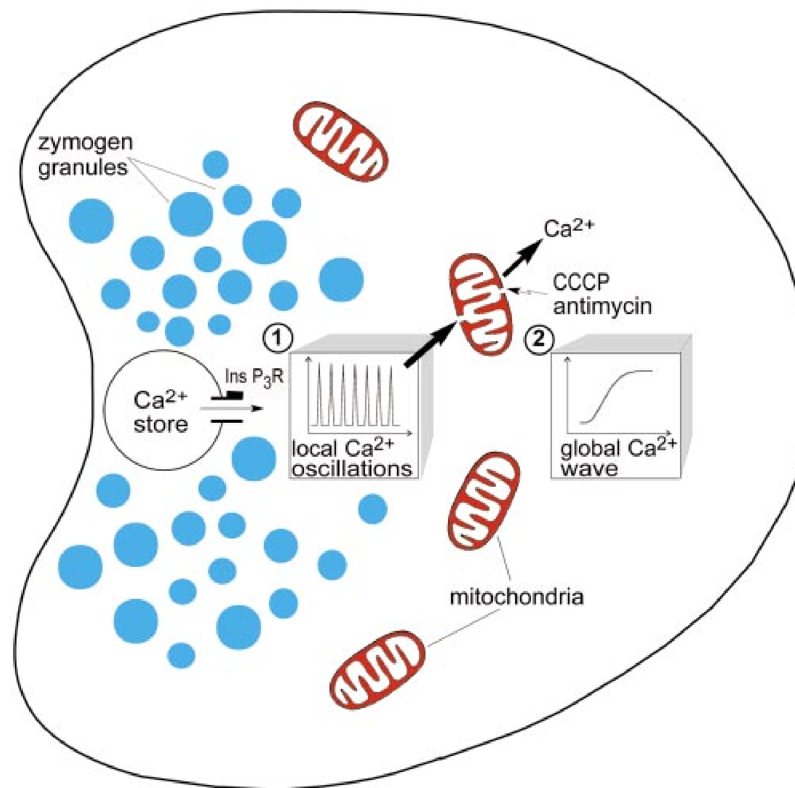


Fig. 6. Schematic diagram showing in simplified form the mitochondrial Ca^{2+} barrier hypothesis. At low levels of agonist stimulation, or by direct infusion intracellularly of IP_3, local cytosolic Ca^{2+} spikes (oscillations) confined to the granular region are produced. The figure indicates that part of the Ca^{2+} released in the granule region, which potentially could diffuse into the basal part of the cell, is normally prevented from doing so by uptake into the mitochondrial belt surrounding the granule-rich part. When mitochondrial function is inhibited, by CCCP or antimycin, this mitochondrial Ca^{2+} uptake is severely reduced or abolished and Ca^{2+} does move into the basal area and spreads throughout the cytosol creating a global Ca^{2+} wave.

were abolished and there was a substantial increase in the steady inward current, indicating an overall generalized increase in the cytosolic Ca^{2+} concentration.

Discussion

The new data presented here clarify the mechanism underlying cytosolic Ca^{2+} signal polarization in epithelial cells. Our results explain how IP_3-evoked cytosolic Ca^{2+} signals, generated in the apical granule-rich region of the highly polarized pancreatic acinar cells, remain confined to this part of the cell. We have found that active mitochondria are mainly localized as a belt surrounding the granule-rich apical pole (Figure 1). When mitochondrial function is inhibited, the pattern of IP_3-evoked local Ca^{2+} spiking in the apical pole is transformed into a gradually rising global elevation of the cytosolic Ca^{2+} concentration (Figure 3). This indicates that when the mitochondrial barrier is functionally inactive, the cell is no longer able to confine messenger-mediated Ca^{2+} signalling to the strategically important granule-containing region.

During IP_3-evoked local Ca^{2+} spiking, Ca^{2+} is released from ER terminals in the granule-containing region and again taken up into the ER by thapsigargin-sensitive Ca^{2+} pumps (Petersen *et al.*, 1993). Our new results could be explained most simply by assuming that during each local spike there must also be Ca^{2+} uptake into the adjacent mitochondria. Since IP_3-evoked local Ca^{2+} spiking can continue for a long time in the complete absence of external Ca^{2+} (Figure 3) (Wakui *et al.*, 1989), there should be a dynamic equilibrium between release and uptake of Ca^{2+} from both the ER and the mitochondria.

The intracellular Ca^{2+} release evoked by the protonophore CCCP is most likely coming from the mitochondria. It is generally recognized that these structures contain little Ca^{2+} in the resting cell, but take up Ca^{2+} whenever there is an increase in the cytosolic Ca^{2+} concentration (Pozzan *et al.*, 1994). This is consistent with our data showing the absence of any CCCP-evoked cytosolic Ca^{2+} rise, when the cells were not stimulated by IP_3. In contrast, the other main intracellular Ca^{2+} stores, the ER and the secretory granules, would be full under resting conditions (Gerasimenko *et al.*, 1996a; Mogami *et al.*, 1998). Blocking the ER Ca^{2+}-pump by the selective inhibitor thapsigargin evokes a major Ca^{2+} release into the cytosol in the resting condition, but such a cytosolic Ca^{2+} rise, unlike the one caused by CCCP in the presence of IP_3 (Figure 3), starts in the baso-lateral rather than the apical part of the cell (Toescu *et al.*, 1992; Gerasimenko *et al.*, 1996a). Our conclusion that CCCP evokes Ca^{2+} release from the mitochondria is further strengthened by the results showing that another mitochondrial inhibitor, antimycin, acting by a completely different mechanism (blocking electron transport through the respiratory chain between cytochromes *b* and *c*1), also releases Ca^{2+} into the cytosol and also does so selectively in the presence of IP_3 (Figure 5).

Although our working hypothesis (Figure 6) seems to

be the simplest way of accounting for the new data, we cannot exclude completely an alternative explanation. One could postulate that the effect of uncouplers, or respiratory chain inhibitors, depends on ATP depletion, and thus an effect on Ca^{2+} re-uptake into the ER. Landolfi *et al.* (1998) have shown that in intact cells an FCCP-induced reduction of the ATP/ADP ratio has an effect that is similar to inhibitors of the ER Ca^{2+} pumps. However, in our experiments, the effect of CCCP is most likely not secondary to ATP depletion, since ATP (2 mM) was continuously supplied to the cell interior via the vast reservoir of the patch pipette. In order to explain a possible CCCP-elicited local ATP depletion around the ER terminals in the apical pole, one would need to postulate a permeability barrier to the small water-soluble ATP molecule, which clearly does not exist for the small water-soluble IP_3 molecule, since IP_3 from the pipette readily reaches the apical pole to induce the local Ca^{2+} spikes. Furthermore, Landolfi *et al.* (1998) have shown that in permeabilized cells, the addition of 0.5 mM ATP to the solution surrounding the cells is sufficient for normal function of the ER Ca^{2+} pumps. In our experiments, the interior of the patch pipette, containing 2 mM ATP and no ADP, is in direct continuity with the cell interior and there is no reason why ATP should not be present throughout the cytosol in this concentration. However, our conclusion that active mitochondria prevent spreading into the basolateral area of Ca^{2+} released locally in the granule region, would still be valid even in the unlikely event that an ATP-selective barrier, preventing access of ATP (dialysed into the cell via the patch pipette) to the critical apical region where Ca^{2+} release from and re-uptake into the ER occur, actually existed. In that case the role of the strategically placed mitochondria surrounding the granular region would be more indirect than envisaged in our model (Figure 6), namely to supply ATP locally to allow the ER Ca^{2+} pumps to operate optimally so that they could effectively recapture released Ca^{2+}.

Ca^{2+} signal initiation in the apical secretory granule pole occurs very close to the luminal cell membrane (Kasai *et al.*, 1993; Thorn *et al.*, 1996; Mogami *et al.*, 1997) and is almost certainly due to primary release from ER terminals invading the granule region (Mogami *et al.*, 1997). Ca^{2+} release channels must therefore be clustered in the extreme apical part of these ER terminals, a site that is some distance away from the main localization of the mitochondria at the border between the granule and basal regions (Figure 1). Kasai *et al.* (1993) showed that the whole secretory granule region exhibits Ca^{2+}-induced Ca^{2+} release and Gerasimenko *et al.* (1996a) demonstrated that both IP_3 and cyclic ADP-ribose can evoke Ca^{2+} release from single isolated ZGs. Although this conclusion has been disputed by Yule *et al.* (1997), the idea that Ca^{2+} can also be released from non-ER stores (Petersen, 1996) has recently been reinforced by studies showing agonist-evoked Ca^{2+} release from the Golgi apparatus (Pinton *et al.*, 1998) and from direct demonstrations of IP_3-evoked Ca^{2+} oscillations inside and outside secretory granules in goblet cells (Nguyen *et al.*, 1998). These recent data provide support for our hypothesis that the initial Ca^{2+} release from ER terminals is magnified by further Ca^{2+} release from the granules themselves (Mogami *et al.*, 1997). This would then bring the Ca^{2+} rise in the apical pole right up to the border with the basal region, exactly where the mitochondria are located and explain how they can be loaded with Ca^{2+} during local spiking events. Kasai *et al.* (1993) showed that the rapidly propagating Ca^{2+} wave in the granule area, set up by supramaximal agonist stimulation, suddenly slows when it crosses the border between the granular and basal areas. Our new results show that this is the region of the cell where the mitochondria are mostly concentrated. The high buffer capacity of mitochondria, which is ~400 times higher than in the cytosol (Babcock *et al.*, 1997), prevents the spreading of a Ca^{2+} wave at a low agonist concentration and slows down the speed of the wave at higher agonist concentrations. Ca^{2+} uptake by mitochondria may in this way build a buffer barrier confining Ca^{2+} spikes to the granule area.

Materials and methods

Cell preparation

Single isolated mouse pancreatic acinar cells were prepared using collagenase (Worthington, 200 U/ml, 20 min, 37°C) in the presence of trypsin inhibitor (Sigma, 3 mg/ml) as described previously (Osipchuk *et al.*, 1990).

Solutions

The extracellular (bath) solution contained (mM): NaCl 140, KCl 4.7, $CaCl_2$ 1.0, $MgCl_2$ 1.13, glucose 10 and HEPES–NaOH 10 (pH 7.2). In some experiments, when specifically indicated, $CaCl_2$ was not included (Ca^{2+}-free solution). The pipette solution contained (mM): KCl 140, $MgCl_2$ 1.13, ATP (Na) 2.0 and HEPES–KOH 10 (pH 7.2). Ca^{2+} was buffered with 100 μM EGTA. The cells, placed on a glass coverslip coated with polylysin (0.2%) attached to an open perifusion chamber, were perifused continuously from a gravity-fed system. Bath solution changes at the cell occurred after <7 s in this system. All experiments were performed at room temperature.

Confocal laser scanning microscopy

Acinar cells were loaded with 200 nM MitoTracker Green AM for 15 min or 1 μM Rhodamine 123 (Molecular Probes) for 20 min at room temperature. The fluorescent signals were recorded using a Noran Odyssey confocal microscope (Noran Instruments, USA) with an excitation wavelength of 488 nm and a 515 nm long-pass barrier filter.

Patch–clamp whole-cell current recording

Standard patch–clamp whole-cell current recording was used (Hamill *et al.*, 1981; Osipchuk *et al.*, 1990; Thorn and Petersen, 1992). The electrophysiological recording of Ca^{2+}-sensitive Cl^- and non-selective cation currents is a very sensitive measure of $[Ca^{2+}]_i$ changes (Osipchuk *et al.*, 1990; Thorn *et al.*, 1993, 1996). We recorded the Ca^{2+}-sensitive currents when the membrane potential was clamped at –30 mV. Under our experimental conditions, the equilibrium potentials for both the Ca^{2+}-sensitive cation current and the Cl^- current were close to 0.

Cytosolic Ca^{2+} measurements

For the cytosolic Ca^{2+} measurements the patch pipette solution contained 50 μM Fura 2-free acid (Sigma). EGTA was excluded from this solution. The Ca^{2+} concentration was estimated by means of a ratio fluorescence imaging system (PTI, Wedel, Germany) that was based on an inverted microscope (Zeiss, Oberkochen, Germany) equipped with a Zeiss Fluar ×40 oil-immersion objective. Monochromator settings, chopper frequency and complete data acquisition were controlled by software for a microcomputer system (PTI). The fluorescence emission of the cells was recorded with a ICCD camera (Hamamatsu Photonics, Japan) using a 510 nm wide band filter (Omega Optical, Brattleboro, VT, USA). The images were ratioed (340:380) pixel by pixel and the resultant ratio was proportional to the intracellular free Ca^{2+} concentration. Fura 2-fluorescence was calibrated using cells loaded with the dye and exposed to 5 mM EGTA or 10 mM Ca^{2+} in the presence of 20 μM ionomycin (Sigma) using a dissociation constant for Ca^{2+}-Fura 2 at room temperature of 150 nM.

Intracellular pH measurement

Intracellular pH was monitored by use of the fluorescent dye BCECF (Molecular Probes, Inc., Eugene, OR, USA). Cells were exposed to the probe in its acetoxymethyl ester form at a final concentration of 1 μM for 15 min. Cell fluorescence was excited by use of the 488-nm band of an argon ion laser and the 442-nm band of a helium–cadmium laser on a confocal laser scan unit (MRC-600, Bio-Rad, Hemel Hempstead, UK). This device was coupled to a Nikon Diaphot microscope with a ×20 Nikon lens. Images were acquired every 60 s. Cell pH was determined as the fluorescence ratio from both excitation wavelengths. For calibration at the end of each experiment, cells were exposed to 140 mM KCl and 10 μM nigericin (Sigma) in HEPES-buffered solutions in the pH range 6.4–7.8.

Statistical analysis

Mean values ± SE are presented, with n denoting the number of experiments. Paired t-tests were applied. $P < 0.05$ was considered significant.

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