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Termination of cytosolic Ca^{2+} signals: Ca^{2+} reuptake into intracellular stores is regulated by the free Ca^{2+} concentration in the store lumen

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The mechanism by which agonist-evoked cytosolic Ca^{2+} signals are terminated has been investigated. We measured the Ca^{2+} concentration inside the endoplasmic reticulum store of pancreatic acinar cells and monitored the cytoplasmic Ca^{2+} concentration by whole-cell patch-clamp recording of the Ca^{2+}-sensitive currents. When the cytosolic Ca^{2+} concentration was clamped at the resting level by a high concentration of a selective Ca^{2+} buffer, acetylcholine evoked the usual depletion of intracellular Ca^{2+} stores, but without increasing the Ca^{2+}-sensitive currents. Removal of acetylcholine allowed thapsigargin-sensitive Ca^{2+} reuptake into the stores, and this process stopped when the stores had been loaded to the pre-stimulation level. The apparent rate of Ca^{2+} reuptake decreased steeply with an increase in the Ca^{2+} concentration in the store lumen and it is this negative feedback on the Ca^{2+} pump that controls the Ca^{2+} store content. In the absence of a cytoplasmic Ca^{2+} clamp, acetylcholine removal resulted in a rapid return of the elevated cytoplasmic Ca^{2+} concentration to the pre-stimulation resting level, which was attained long before the endoplasmic reticulum Ca^{2+} store had been completely refilled. We conclude that control of Ca^{2+} reuptake by the Ca^{2+} concentration inside the intracellular store allows precise Ca^{2+} signal termination without interfering with store refilling.

Keywords: calcium pump/cytoplasm/endoplasmic reticulum/signal termination

Introduction

Cytosolic Ca^{2+} signals are generated in many different cell types by liberation of Ca^{2+} stored in the endoplasmic reticulum (ER) through special Ca^{2+} release channels known as inositol trisphosphate (IP_3) and ryanodine receptors (Berridge, 1993; Clapham, 1995). The mechanisms by which Ca^{2+} release is controlled have been studied in considerable detail (for reviews, see Berridge, 1993, 1997; Petersen *et al.*, 1994; Bootman and Berridge, 1995; Clapham, 1995), but much less information is available about the equally important control of the mechanism for precisely terminating cytosolic Ca^{2+} signals. An important element in the termination of short-lasting Ca^{2+} spikes, is the negative feedback effect of Ca^{2+} on the IP_3 receptor closing the Ca^{2+} release channel (Bezprozvanny *et al.*, 1991). Ca^{2+} lost from the ER store must be taken up again, and it has been shown that even for the very shortlasting IP_3-evoked local Ca^{2+} spikes in the secretory granule area of pancreatic acinar cells, the duration of individual spikes is prolonged significantly by a specific inhibitor of the ER Ca^{2+} pump (Petersen *et al.*, 1993). With regard to sustained cytosolic Ca^{2+} elevations evoked by sustained high agonist levels, the precise mechanism underlying signal termination is far from clear, but must involve ER Ca^{2+} reuptake (Camello *et al.*, 1996).

Active Ca^{2+} uptake into intracellular stores was discovered by studying the nature of muscle relaxing factor (Kumagai *et al.*, 1955; Ebashi and Lipmann, 1962). The active principle is a Ca^{2+}, Mg^{2+}-activated ATPase in the sarco-endoplasmic reticulum (SR/ER) membrane (Ebashi and Lipmann, 1962), and these enzymes are now generally referred to as SERCA pumps (sarco-endoplasmic reticulum Ca^{2+}-ATPases) (Pozzan *et al.*, 1994). The general principle of operation is simple: when the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) has been elevated due to release from intracellular stores, the SERCA pumps will be activated (by the rise in $[Ca^{2+}]_i$), and as soon as the SR/ER Ca^{2+} release channels have been closed, net Ca^{2+} reuptake will occur. When $[Ca^{2+}]_i$ has been reduced to the resting (pre-stimulation) level, the activation of the SERCA pumps ceases.

There could be an additional control mechanism operating on the luminal side of the SR/ER membrane. Studies of Ca^{2+} uptake into isolated SR or ER vesicles indicate that the SERCA pump-mediated rise in the Ca^{2+} concentration in the intravesicular lumen leads to an inhibition of the SERCA pump in both muscle (Inesi and DeMeis, 1989) and non-muscle cells, where a highly supralinear feedback inhibition of Ca^{2+} uptake by the Ca^{2+} load of ER vesicles has been described (Fauvre *et al.*, 1996).

In intact cells, it is clear that in the period of Ca^{2+} reuptake into stores following an agonist-induced release phase, $[Ca^{2+}]_i$ will be falling, whereas the Ca^{2+} concentration in the lumen of the ER store ($[Ca^{2+}]_{Lu}$) must be rising. It is therefore not easy to determine whether the control from the inside or the outside is the most important or whether both are necessary. In the present study, we tested as directly as possible the hypothesis that Ca^{2+} reuptake into depleted Ca^{2+} stores may be regulated by changes in $[Ca^{2+}]_{Lu}$. We measured $[Ca^{2+}]_{Lu}$ directly with a Ca^{2+}-sensitive fluorescent probe in the store lumen and were able, with the help of the whole-cell recording configuration of the patch-clamp technique, to clamp the cytosolic Ca^{2+} concentration at the resting level by a high buffer concentration. The effectiveness of the cytosolic Ca^{2+} clamping was confirmed directly by measurement of the Ca^{2+}-sensitive ion currents. We show that under such experimental conditions, in which acetylcholine

(ACh) failed to evoke any rise in $[Ca^{2+}]_i$, even very close to the plasma membrane, Ca^{2+} stores are effectively depleted and upon ACh removal vigorous Ca^{2+} reuptake occurs that is totally dependent on a thapsigargin-sensitive Ca^{2+} pump. In the Ca^{2+} reuptake period, where $[Ca^{2+}]_i$ continues to be clamped, the rate of Ca^{2+} reuptake falls as $[Ca^{2+}]_{Lu}$ rises and stops when the store is full. We have studied the relationship between the rate of active Ca^{2+} reuptake and $[Ca^{2+}]_{Lu}$. Whereas the Ca^{2+} leak is almost independent of $[Ca^{2+}]_{Lu}$ at relatively high levels of $[Ca^{2+}]_{Lu}$, the Ca^{2+} reuptake decreases sharply with an increase of $[Ca^{2+}]_{Lu}$ in the same range. It is therefore the steep dependence of the Ca^{2+} reuptake rate on $[Ca^{2+}]_{Lu}$ that determines the ER calcium content. In experiments in which $[Ca^{2+}]_i$ was not clamped, we show that after ACh removal $[Ca^{2+}]_i$ returns quickly to the pre-stimulation level which is attained long before $[Ca^{2+}]_{Lu}$ has reached its steady-state level. The control of Ca^{2+} reuptake by $[Ca^{2+}]_{Lu}$ explains how a cytosolic Ca^{2+} signal can be terminated abruptly without preventing complete refilling of intracellular Ca^{2+} stores.

Results

ACh-evoked Ca^{2+} loss from intracellular stores and Ca^{2+} reuptake

Hofer and collaborators (Hofer and Machen, 1993; Hofer *et al.*, 1995; Hofer and Schulz, 1996) described a technique to measure $[Ca^{2+}]_{Lu}$ in permeabilized cells by loading the stores with the Ca^{2+}-sensitive fluorescent probe Mag-fura 2. In intact cells, there is a problem with this technique since the probe would be present not only inside the stores, but also in the cytosol. Agonist stimulation would inevitably give rise to a complex signal due to the rise in $[Ca^{2+}]_i$ and the fall in $[Ca^{2+}]_{Lu}$. We therefore used the whole-cell recording configuration of the patch-clamp technique (Hamill *et al.*, 1981) to wash out the fluorescent probe molecules from the cytosol, after the loading of the cell, into the large volume of the patch pipette (Figure 1). In this way, the probe molecules were effectively only remaining inside the intracellular stores, and stimulation with a high concentration of ACh evoked a marked increase in the Ca^{2+}-sensitive fluorescence intensity at the excitation wavelength 380 nm, signalling a marked reduction in $[Ca^{2+}]_{Lu}$ (Figure 1). The patch-clamp whole-cell recording configuration was useful not only for washing out the Ca^{2+}-sensitive probe from the cytosol, but also for allowing us to monitor changes in $[Ca^{2+}]_i$, by recording the current through the Ca^{2+}-sensitive ion channels (Osipchuk *et al.*, 1990; Petersen, 1992).

There are two Ca^{2+}-activated currents in pancreatic acinar cells carried by the Ca^{2+}-sensitive non-selective cation channels (Maruyama and Petersen, 1982) and the Ca^{2+}-sensitive Cl^- channels (Wakui *et al.*, 1989). In our experiments, the recorded current at a membrane potential of –30 mV (Wakui *et al.*, 1989; Osipchuk *et al.*, 1990) will be a mixture of Cl^- and monovalent cation currents (Thorn and Petersen, 1992). The time course of this combined current is well correlated with the time course of changes in $[Ca^{2+}]_i$ under a variety of conditions including stimulation with ACh (Osipchuk *et al.*, 1990). Patch–clamp current recording is actually a more sensitive indicator of $[Ca^{2+}]_i$ changes near the cell membrane than

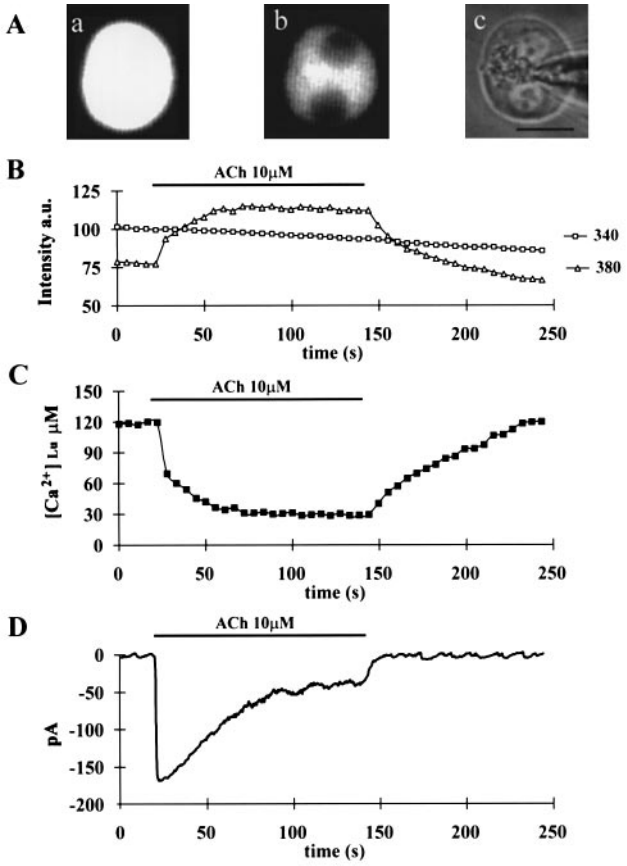

Fig. 1. ACh evokes a sustained reduction in the directly measured Ca^{2+} concentration in the lumen of the intracellular store and a sustained rise in the cytoplasmic Ca^{2+} concentration monitored by recording the Ca^{2+}-sensitive ion current across the cell membrane. ACh removal causes an abrupt termination of the cytoplasmic Ca^{2+} signal and a much slower refilling of the intracellular Ca^{2+} store. (**A**) (a) and (b) are images of Mag-fura 2 fluorescence intensities (excitation 340 nm, emission 510 nm). (a) was obtained just after the 10 min period of incubation with 2.5 μM Mag-fura 2-AM, before the establishment of the patch-clamp whole-cell recording configuration, whereas (b) was obtained 15 min after the start of cytoplasmic probe wash-out into the patch pipette (whole-cell configuration). Images (a) and (b) were taken using the same gain, and show that after establishing the whole-cell patch–clamp configuration there is a significant reduction (>90%) in the overall concentration of the fluorescent probe which is particularly marked in the two nuclei of this particular cell. (c) is the transmitted light picture of the cell with the patch pipette. The two nuclei are clearly seen. The bar represents 10 μm. (**B**) The time course of the changes in fluorescence intensities at the excitation wavelengths 340 and 380 nm. (**C**) The Ca^{2+} concentration in the intracellular store(s) ($[Ca^{2+}]_{Lu}$) calculated from the 340/380 ratio. ACh evokes a marked drop in $[Ca^{2+}]_{Lu}$ which is sustained as long as the agonist is present, but after ACh removal $[Ca^{2+}]_{Lu}$ rises slowly. (**D**) The electrical recording of the whole-cell current at a membrane potential of –30 mV. ACh is seen to evoke a sharp increase in the (Ca^{2+}-sensitive) inward current. Removal of ACh results in a quick return to the pre-stimulation level which is attained at a point in time when the refilling of the intracellular Ca^{2+} store (C) is far from complete.

fluorescence measurements, as shown by Osipchuk *et al.* (1990) who could demonstrate Ca^{2+}-dependent current spikes in response to low doses of ACh which were not associated with any detectable changes in the bulk $[Ca^{2+}]_i$. With digital imaging, it could be shown that the short-lasting Ca^{2+}-dependent current spikes evoked by low ACh concentrations were due to local cytosolic Ca^{2+} rises in the secretory pole (Thorn *et al.*, 1993). With regard to the

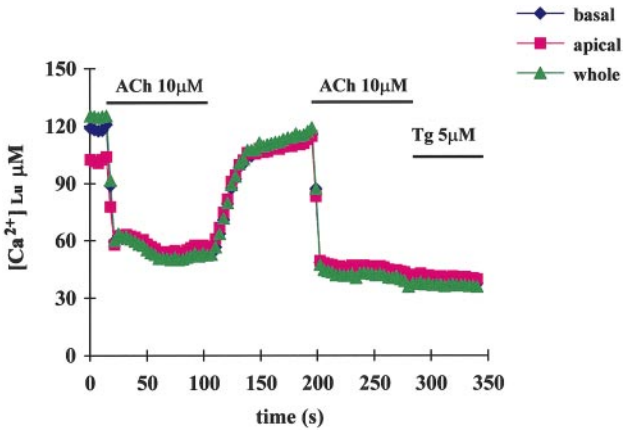

Fig. 2. Ca^{2+} reuptake into intracellular stores following ACh-evoked Ca^{2+} release is acutely blocked by the specific ER Ca^{2+} ATPase inhibitor thapsigargin (Tg). Three regions of interest were selected: the whole of the cell (green trace), the basal part (blue trace; mostly covered by the green trace) and the apical part (red trace). The results were similar in the different regions. ACh evokes a sharp decrease in $[Ca^{2+}]_{Lu}$ and, after ACh removal, there is initially a sharp increase and later a slower rise in $[Ca^{2+}]_{Lu}$. This increase in $[Ca^{2+}]_{Lu}$ following ACh removal is blocked by thapsigargin.

recordings presented here, it is important to emphasize that current measurements, also on a slow minute time scale, are extremely well correlated with $[Ca^{2+}]_i$, even at low levels of $[Ca^{2+}]_i$ elevation. Thus, in experiments in which a slow intracellular Ca^{2+} infusion was followed by a slow infusion of the Ca^{2+} chelator EGTA, it could be shown that in the $[Ca^{2+}]_i$ range 100–300 nM the current is slightly more sensitive to small changes in $[Ca^{2+}]_i$ than the fura-2 fluorescence ratio. The time courses of the $[Ca^{2+}]_i$ changes measured simultaneously with the two methods were virtually identical (see Figure 8 in Osipchuk *et al.*, 1990).

As seen in Figure 1, representing an experiment where a low Ca^{2+} buffer concentration (0.25 mM EGTA) had been included in the pipette solution, ACh evoked a marked rise in the whole-cell inward current in the period where $[Ca^{2+}]_{Lu}$ fell, demonstrating the normally expected rise in $[Ca^{2+}]_i$. $[Ca^{2+}]_{Lu}$ remained low during the period of ACh stimulation but, after ACh removal, $[Ca^{2+}]_{Lu}$ rose slowly and a sharp reduction in the Ca^{2+}-sensitive current was apparent, demonstrating the abrupt termination of the cytosolic Ca^{2+} signal. In seven experiments with a Ca^{2+} buffer (EGTA) concentration of 1 mM in the pipette, ACh still evoked a clear increase in the Ca^{2+}-sensitive current and induced a marked drop in $[Ca^{2+}]_{Lu}$ from 152 $\pm$ 34 μM (SE) to 44 $\pm$ 7 μM. Removal of ACh resulted in a restoration of the pre-stimulation Ca^{2+} level in the store lumen within a 1–2 min period. The maximal apparent rate of Ca^{2+} reuptake was 198 $\pm$ 27 μM/min (n = 7).

Ca^{2+} reuptake is due to a thapsigargin-sensitive Ca^{2+} pump

In order to investigate the nature of the Ca^{2+} reuptake process, the effect of thapsigargin, the specific inhibitor of SERCA pumps (Thastrup *et al.*, 1990), was tested. As seen in Figure 2, thapsigargin abolished Ca^{2+} reuptake in all regions of the acinar cells, indicating that we were, in these experiments, mainly studying Ca^{2+} release and reuptake into ER stores. Three experiments of this type giving similar results were carried out. The slightly lower $[Ca^{2+}]_{Lu}$ in the apical region may reflect a contribution from Ca^{2+} in secretory granules. In a previous study of isolated granules, an average $[Ca^{2+}]$ of ~50 μM was found in these organelles (Gerasimenko *et al.*, 1996). We chose to base all quantification on results obtained from the basal (non-nuclear) region, as this part of the cell is densely packed with ER (Kern, 1993; Gorelick and Jamieson, 1994). The similarity of the ACh-evoked $[Ca^{2+}]_{Lu}$ change in the different regions of the cell shown in Figure 2 does not contradict the previously clearly documented polarization of the agonist-evoked cytosolic Ca^{2+} signals (Kasai *et al.*, 1993; Thorn *et al.*, 1993; Mogami *et al.*, 1997). This phenomenon is seen most clearly when low agonist concentrations are used. When a supramaximal ACh concentration is used, as in the experiments reported here, Ca^{2+} is released all over the cell, but the $[Ca^{2+}]_i$ rise occurs first in the apical region and then spreads across the cell. Under normal conditions, it takes 1–2 s for the Ca^{2+} wave to move from the apical region to the base of the cell (Kasai and Augustine, 1990; Toescu *et al.*, 1992a). The new experiments described here were not designed to study this phenomenon and, because we wanted to study the slower uptake processes and therefore needed long recordings, our experiments did not have sufficient time resolution to reveal differences in the time course of Ca^{2+} release in different regions.

We also used thapsigargin in another protocol. In the experiment shown in Figure 3A, ACh induced, as usual, a marked reduction in $[Ca^{2+}]_{Lu}$ and, after ACh removal, Ca^{2+} reuptake occurred. When $[Ca^{2+}]_{Lu}$ had risen to a level close to the pre-stimulation state, thapsigargin was added. This resulted in a gradual reduction of $[Ca^{2+}]_{Lu}$ which became slower and slower as the store was emptied (Figure 3A). Thapsigargin evoked a slow, but complete Ca^{2+} release (to the same extent as ACh), following a Ca^{2+} reaccumulation period of the type shown in Figure 3A, in six experiments.

Ca^{2+} reuptake still occurs when $[Ca^{2+}]_i$ is clamped near the resting level

When the Ca^{2+} buffer EGTA was present in the patch pipette solution in a concentration of 1 mM, it did not prevent ACh, in a maximally activating concentration (10 μM), from evoking an increase in the Ca^{2+}-sensitive ion currents across the plasma membrane. In order to clamp $[Ca^{2+}]_i$ effectively, we therefore replaced the relatively slowly reacting EGTA with the faster Ca^{2+} buffer BAPTA (Tsien, 1980; Roberts, 1993) and also increased the buffer concentration to 10 mM. When the pipette was filled with a nominally Ca^{2+}-free solution containing 10 mM BAPTA, there was no response in two experiments and only a small response to ACh in one of the three experiments carried out and therefore also only a small Ca^{2+} reuptake following ACh removal in that same cell. Clearly, in this series, $[Ca^{2+}]_i$ had been clamped at a level well below the normal resting concentration, probably preventing normal filling of the Ca^{2+} store.

In order to create better conditions for Ca^{2+} release and reuptake, we added 2 mM Ca^{2+} to the 10 mM BAPTA solution in the pipette. When $[Ca^{2+}]_i$ was measured with the help of the fluorescent probe fura-2 in the cytoplasm,

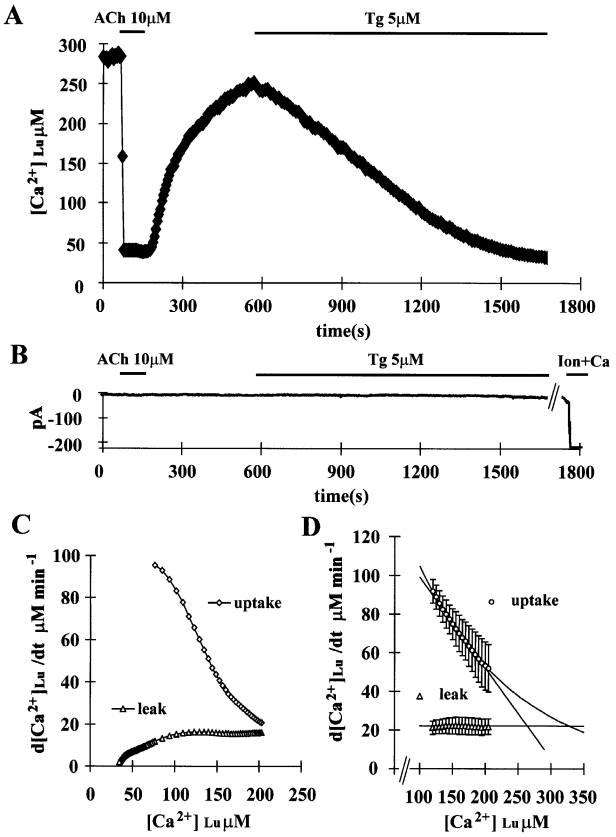

Fig. 3. Ca^{2+} reuptake into intracellular stores following ACh-evoked Ca^{2+} release is not prevented by clamping the cytoplasmic Ca^{2+} concentration at the normal resting level. In this experiment, the patch pipette solution was heavily buffered by a BAPTA–Ca^{2+} mixture, clamping $[Ca^{2+}]_i$ at a concentration of 90 nM. (**A**) The ACh-evoked loss of Ca^{2+} from the ER store and the subsequent rise of $[Ca^{2+}]_{Lu}$ after removal of the agonist. After a period of Ca^{2+} reaccumulation, thapsigargin is added to arrest the ER Ca^{2+} ATPase, and a slow reduction in $[Ca^{2+}]_{Lu}$ follows. (**B**) The electrical recording of the whole-cell (Ca^{2+}-sensitive) current across the plasma membrane at a membrane potential of –30 mV. ACh fails to evoke any current change [despite the marked Ca^{2+} loss from the stores shown in (A)] and thapsigargin also fails to induce any response. To demonstrate the existence of the Ca^{2+}-sensitive current, application of the calcium ionophore ionomycin (10 μM) plus calcium is shown to evoke a sharp inward current in the last part of the trace. (**C**) The relationship between apparent calcium transport rates and $[Ca^{2+}]_{Lu}$ derived from the data shown in (A). (**D**) The mean values (±SE) from four experiments in which the $[Ca^{2+}]_{Lu}$ range 100–200 μM was explored. The apparent uptake rate was leak-corrected. In this $[Ca^{2+}]_{Lu}$ range, the apparent leak rate is independent of $[Ca^{2+}]_{Lu}$ whereas the apparent uptake rate is steeply dependent on $[Ca^{2+}]_{Lu}$.

a mean value of 96 ± 3.6 nM (n = 5) was obtained, which corresponds to the normal resting level in intact cells. In this condition, ACh (10 μM) evoked a sharp and very marked reduction in $[Ca^{2+}]_{Lu}$ from 286 ± 63 μM (n = 6) to 38 ± 2 μM. After ACh removal, $[Ca^{2+}]_{Lu}$ increased, first steeply and then more slowly as the store was being filled (Figure 3A). In this, as well as the other five experiments of the same type, $[Ca^{2+}]_i$ had been effectively clamped, since the electrophysiological recording showed that ACh failed to induce any measurable change in the Ca^{2+}-sensitive ion current (Figure 3B). At the end of the experiments, the Ca^{2+} ionophore ionomycin evoked a sharp increase in the inward current, demonstrating the normal operation of the Ca^{2+}-sensitive ion channels. Comparing the response to ACh in Figures 1 and 3, it is clear that the time course of the Ca^{2+} release was different in the two experimental situations. In the experiments where the cytoplasmic Ca^{2+} buffer concentration was relatively low and where $[Ca^{2+}]_i$ rose after ACh stimulation (Figures 1 and 2), it is apparent that the Ca^{2+} loss occurred in two phases: an initial fast Ca^{2+} liberation followed by a very much slower outflow. In contrast, when $[Ca^{2+}]_i$ was clamped near the resting level by a high BAPTA concentration (Figure 3), this late and very slow release phase was completely absent. The rise in $[Ca^{2+}]_i$ that normally occurs provides a negative feedback for the Ca^{2+} release channels (Bezprozvanny *et al.*, 1991; Berridge, 1993; Clapham, 1995), and our finding illustrates that removal of this effect by heavily buffering the cytoplasmic compartment prevents the slowing down of the Ca^{2+} release process. A similar conclusion has been reported very recently by Montero *et al.* (1997).

In spite of the data shown in Figure 3B indicating that the $[Ca^{2+}]_i$ clamp is effective (since there is no activation of Ca^{2+}-dependent ion current during the ACh-evoked Ca^{2+} loss from the ER), it might be postulated that there could be small domains of elevated $[Ca^{2+}]_i$ near the inner mouths of the store-operated Ca^{2+} channels in the plasma membrane that could play a functional role in activating the Ca^{2+} pumps in the ER. We therefore tested the effect of removing external Ca^{2+} immediately after discontinuing ACh stimulation. In each of these four experiments, the Ca^{2+} reuptake proceeded as normal (with a time course similar to what is shown in Figure 3), indicating that with the well-buffered pipette solution, the availability of Ca^{2+} for reuptake into the stores did not depend on Ca^{2+} in the external solution. In these experiments, there cannot of course exist local domains of elevated Ca^{2+} concentration near the inner mouths of the Ca^{2+} channels as there was no supply of external Ca^{2+}. The Ca^{2+} for the refilling of stores in these experiments is therefore provided by the Ca^{2+} in the BAPTA/Ca^{2+} mixture in the large volume of the patch pipette.

The rates of Ca^{2+} reuptake and Ca^{2+} leak depend on the Ca^{2+} concentration in the store lumen

Experiments of the type shown in Figure 3, where thapsigargin application after the period of Ca^{2+} reuptake allowed calculation of the apparent passive Ca^{2+} leak, could be used to derive the relationship between the apparent rate of active Ca^{2+} reuptake into the stores and $[Ca^{2+}]_{Lu}$. Figure 3C shows the graph representing this relationship, derived from the experimental record displayed in Figure 3A. The apparent rate of Ca^{2+} reuptake was high when $[Ca^{2+}]_{Lu}$ was low, and was reduced gradually as $[Ca^{2+}]_{Lu}$ increased. The apparent Ca^{2+} leak was almost independent of $[Ca^{2+}]_{Lu}$ above a concentration of 100 μM, whereas in this range the apparent Ca^{2+} reuptake rate still displayed a relatively steep dependency on $[Ca^{2+}]_{Lu}$. In the $[Ca^{2+}]_{Lu}$ range 40–100 μM, the apparent leak rate was strongly dependent on $[Ca^{2+}]_{Lu}$, rising initially sharply and then more slowly with a rise in this parameter. The maximal leak rate, when the Ca^{2+} store was full, was surprisingly high (19 ± 4 μM/min; n = 6), but nevertheless, relatively small compared with the maximal reuptake rate following ACh removal (95 ± 7 μM/min). The apparent rate of

active Ca^{2+} uptake, mediated by the SERCA pumps, can therefore be increased by a factor of 5 from the resting minimum when the ER store is maximally depleted. The leak rate is, as expected, very small compared with the very rapid loss of Ca^{2+} from the store immediately after ACh application (27 ± 8 μM/s). It is an important assumption underlying the calculation of the Ca^{2+} uptake and leak rates that the thapsigargin action is exclusively on the pump and that it has no effect on the leak. Furthermore, it is important that thapsigargin acts quickly. We used a high concentration of thapsigargin to ensure an immediate effect. On the time scale relevant to our experiments, the action of thapsigargin would appear to be virtually instantaneous (Sagara *et al.*, 1992). There is also clear experimental evidence showing that thapsigargin, in contrast to several other SERCA pump inhibitors, has no effect on passive Ca^{2+} permeability across the ER membranes (Missiaen *et al.*, 1992).

We analysed the four experiments in which the $[Ca^{2+}]_{Lu}$ range 100–200 μM had been fully explored. Figure 3D shows the mean values obtained for apparent Ca^{2+} reuptake (leak corrected) and apparent Ca^{2+} leak. Irrespective of whether linear or exponential extrapolation is used, the curves will cross, giving an equilibrium value for $[Ca^{2+}]_{Lu}$. Figure 3D indicates that this equilibrium would be reached between 260 and 330 μM, which is consistent with the resting $[Ca^{2+}]_{Lu}$ values measured in this series of experiments (Figure 3A).

All the $[Ca^{2+}]_{Lu}$ measurements described so far in this report have been based on the use of the Ca^{2+}-sensitive fluorescent probe Mag-fura-2. This probe is, however, also sensitive to Mg^{2+} (although with a much lower affinity than for Ca^{2+}), and we therefore decided to carry out some experiments with the Ca^{2+}-selective probe fura-2FF. These five experiments gave results of the type shown in Figure 3. The $[Ca^{2+}]_i$ was clamped exactly as in the Mag-fura 2 experiments (10 mM BAPTA/2 mM Ca^{2+}). Following ACh-evoked Ca^{2+} store depletion, removal of ACh led to a fast Ca^{2+} reuptake into the ER. The apparent rate of uptake declined sharply as $[Ca^{2+}]_{Lu}$ approached the normal resting (equilibrium) level, whereas the leak was virtually linear at $[Ca^{2+}]_{Lu}$ above ~50 μM. The maximal apparent Ca^{2+} uptake rate was 138 ± 40 μM/min (n = 5), whereas the maximal apparent leak rate was 19 ± 4 μM/min (n = 5).

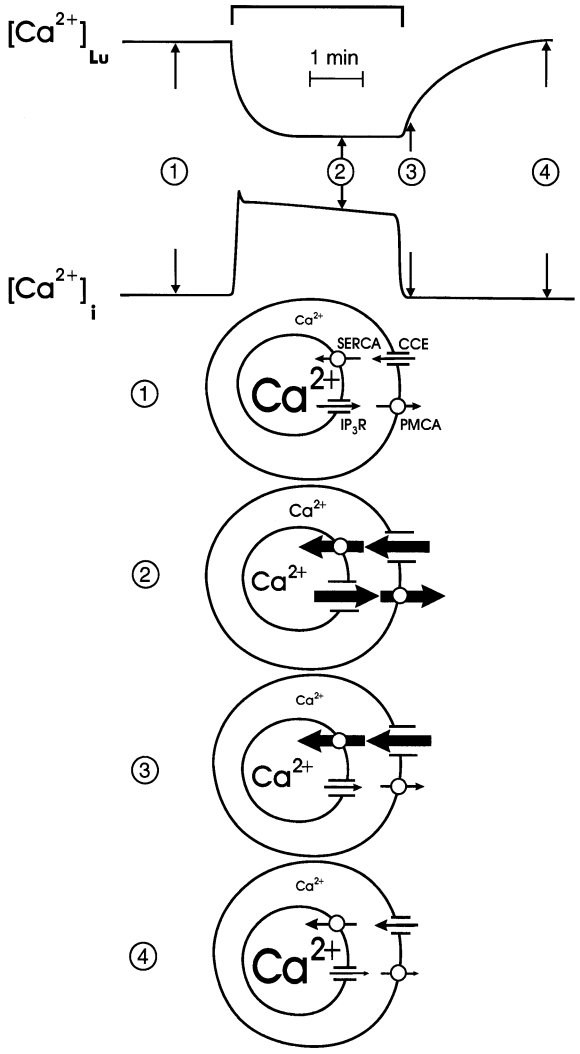

Fig. 4. Abrupt cytosolic Ca^{2+} signal termination does not prevent continued refilling of the intracellular Ca^{2+} store. A simplified diagram explaining the main Ca^{2+} transport events across the plasma membrane (PM) and ER membrane before, during and after an agonist-evoked rise in $[Ca^{2+}]_i$. The two traces illustrate idealized, but nevertheless quite typical, time courses of ACh-evoked changes of $[Ca^{2+}]_i$ and $[Ca^{2+}]_{Lu}$. Below relative Ca^{2+} transport rates through the four main transport pathways are shown in the four different phases. SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase; PMCA, plasma membrane Ca^{2+} ATPase; CCE, capacitative Ca^{2+} entry (store-operated Ca^{2+} entry); IP_3R, inositol trisphosphate receptor.

Discussion

Ca^{2+} reuptake into intracellular stores, after agonist-evoked depletion, is potentially the most powerful mechanism for terminating a cytosolic Ca^{2+} signal (Petersen *et al.*, 1994; Pozzan *et al.*, 1994; Camello *et al.*, 1996). We have now demonstrated that this Ca^{2+} reuptake cannot be prevented by clamping $[Ca^{2+}]_i$ at the resting level. This indicates that there must be control of Ca^{2+} store reuptake from the inside of the store membrane. We have provided direct evidence for the effectiveness of our $[Ca^{2+}]_i$ clamp and excluded the possibility that store reloading depends on local domains of elevated Ca^{2+} near the inner mouths of store-operated Ca^{2+} channels in the plasma membrane. Our findings, that Ca^{2+} reuptake does not depend on an elevated cytosolic Ca^{2+} level (Figure 3) and largely occurs during a period when $[Ca^{2+}]_i$ has already returned to the pre-stimulation resting level (Figure 1), should not be taken to indicate that the ER Ca^{2+} pumps cannot be stimulated by a rise in $[Ca^{2+}]_i$. The initial apparent maximal Ca^{2+} reuptake rate was higher in the experiments where $[Ca^{2+}]_i$ was not clamped (~200 μM/min) than in the experiments where $[Ca^{2+}]_i$ was clamped at the resting level (~100–150 μM/min). Under normal conditions, the elevated $[Ca^{2+}]_i$ that still exists immediately after cessation of agonist exposure most likely provides an additional stimulus for the ER Ca^{2+} pump.

Our finding has considerable implications for the Ca^{2+} signalling process. Figure 4 summarizes our conclusions. A prolonged cytosolic Ca^{2+} signal generated by a maximal agonist concentration is illustrated. We focus attention on the four main Ca^{2+} transport events across

the plasma and ER membranes in the various phases. In the steady-state resting situation (1), the Ca^{2+} fluxes are at a minimum, $[Ca^{2+}]_i$ is ~100 nM and $[Ca^{2+}]_{Lu}$ is at its maximum (100–300 μM). ACh evokes opening of the Ca^{2+} release channels in the ER, elevating $[Ca^{2+}]_i$. The elevated $[Ca^{2+}]_i$ activates the plasma membrane (PM) Ca^{2+} pumps markedly enhancing the extrusion rate (Tepikin *et al.*, 1992a,b). The fall in $[Ca^{2+}]_{Lu}$ will remove an inhibitory influence on the ER Ca^{2+} reuptake mechanism, but because the Ca^{2+} release channels remain open during agonist application, there is no net Ca^{2+} reuptake (Camello *et al.*, 1996) (2). When the agonist is removed, the ER Ca^{2+} release channels close. Because $[Ca^{2+}]_{Lu}$ is low, Ca^{2+} reuptake is maximal and net uptake can now occur. $[Ca^{2+}]_i$ falls rapidly and has returned to the resting pre-stimulation level at a point in time when $[Ca^{2+}]_{Lu}$ is still far from its resting pre-stimulation level (3). In order to understand this, it is necessary to appreciate that a major proportion of the Ca^{2+} lost from the store is not in the cytosol, but has been removed from the cell by the PM Ca^{2+} pumps. Phase (3) in Figure 4 illustrates the crucial point established by our new results. Because Ca^{2+} reuptake proceeds vigorously at resting $[Ca^{2+}]_i$, the cytosolic Ca^{2+} signal can be terminated very sharply upon agonist removal (Figure 1) (as indeed documented in very many investigations, for example, Yule *et al.*, 1991; Toescu *et al.*, 1992b; Camello *et al.*, 1996) without impairing Ca^{2+} refilling of the store, which continues after $[Ca^{2+}]_i$ has returned to the pre-stimulation level (Figure 1). In this phase (3), Ca^{2+} for store refilling comes from the external solution, due to opening of the capacitative Ca^{2+} entry channel (store-operated Ca^{2+} channel) (Putney, 1990; Berridge, 1997). The quick termination of the cytosolic Ca^{2+} signal after agonist removal is not only essential from the point of view of signalling precision but, because it terminates Ca^{2+} extrusion by the PM Ca^{2+} pumps, it is also energy saving and enhances the rate of net Ca^{2+} entry thereby helping to supply the Ca^{2+} necessary for refilling the store. As $[Ca^{2+}]_{Lu}$ increases, the degree of capacitative Ca^{2+} entry decreases and the rate of Ca^{2+} reuptake slows down until phase (4), which is identical to (1), has been reached.

Figure 4 makes it clear that the at first sight surprising observation, that Ca^{2+} reuptake does not require an elevated $[Ca^{2+}]_i$, is in fact an essential feature of effective Ca^{2+} signal termination. If Ca^{2+} reuptake could only occur at an elevated $[Ca^{2+}]_i$, the cytosolic Ca^{2+} signal could not be fully terminated until the store had been entirely refilled and the ER Ca^{2+} reuptake pump would compete with the PM Ca^{2+} extrusion pump. Our results also point to an important correlation between the Ca^{2+} reuptake into the ER and the capacitative Ca^{2+} entry process. In our model, these two transport events cannot be dissociated. During the refilling phase (phase 3 in Figure 4), the rates of Ca^{2+} reuptake into the ER and the capacitative Ca^{2+} entry must be exactly matched at all levels of $[Ca^{2+}]$ in the ER store.

Our findings may also be relevant to a situation in which part of the Ca^{2+} released from the ER has been taken out of the cytosol by uptake into the mitochondria (Pozzan *et al.*, 1994). Ca^{2+} refilling of the ER store from mitochondria could occur without any $[Ca^{2+}]_i$ elevation and would stop exactly when the ER store was full, due to the steep dependence of Ca^{2+} reuptake on $[Ca^{2+}]_{Lu}$ (Figure 3C and D). If ER Ca^{2+} reuptake were dependent on a $[Ca^{2+}]_i$ elevation, Ca^{2+} accumulated in the mitochondria could not be given back to the ER without generating at least local Ca^{2+} signals, and this process might involve an undesirable activation of PM Ca^{2+} pumps.

Although the ER operates as one luminally continuous store for both Ca^{2+} (Renard-Rooney *et al.*, 1993; Mogami *et al.*, 1997) and macromolecules (Subramanian and Meyer, 1997), there can be differences in the Ca^{2+} transport characteristics in different parts of the cell. In the polarized pancreatic acinar cells, the Ca^{2+} release mechanism operates with the highest sensitivity in the apical granule region, and low agonist concentrations can evoke cytosolic Ca^{2+} signals that are confined entirely to this part of the cell (Kasai *et al.*, 1993; Thorn *et al.*, 1993). It is possible to create conditions under which the Ca^{2+} reuptake into the store, that has been depleted primarily in the apical granule region, occurs exclusively in the basal part of the cell without causing any $[Ca^{2+}]_i$ rise there (Mogami *et al.*, 1997). Our new results explain how this is possible. The process whereby substantial Ca^{2+} reuptake into the ER can take place in the absence of any $[Ca^{2+}]_i$ elevation ensures that Ca^{2+} signals only occur where and when Ca^{2+} release channels open and not during phases of Ca^{2+} store refilling in places where Ca^{2+} signals are not intended. It is helpful in this respect that the crucial Ca^{2+} export from the pancreatic acinar cells during the phase of agonist-induced Ca^{2+} release from internal stores (phase 2 in Figure 4) occurs preferentially in the apical region (Belan *et al.*, 1996, 1997; Lee *et al.*, 1997) close to the primary Ca^{2+} release sites (Kasai and Augustine, 1990; Toescu *et al.*, 1992a; Kasai *et al.*, 1993; Thorn *et al.*, 1993, 1996; Mogami *et al.*, 1997).

The Ca^{2+} leak from the ER to the cytosol is substantial. The apparent maximal leak rate is only about five times smaller than the apparent maximal uptake rate. This means that there must be considerable energy expenditure in order to ensure the high $[Ca^{2+}]_{Lu}$ that is necessary in order both to achieve rapid messenger-mediated Ca^{2+} mobilization (Montero *et al.*, 1995) and to prevent accelerated protein degradation (Wileman *et al.*, 1991). It is possible that the translocons (Simon and Blobel, 1991) in the rough ER, which dominates the basolateral regions of the pancreatic acinar cells where the major Ca^{2+} release (leak) following thapsigargin inhibition occurs (Toescu *et al.*, 1992a; Gerasimenko *et al.*, 1996), impose limitations on the degree of impermeability to ions, but we do not know the nature of the leak pathway. Since the Ca^{2+} content of the ER is so important for preserving essential proteins (Wileman *et al.*, 1991), it must be finely regulated. Our study shows that it is the relatively steep dependence of the Ca^{2+} reuptake rate on $[Ca^{2+}]_{Lu}$ that determines the store Ca^{2+} content (Figure 3D). In this context, the high Ca^{2+} leak rate, although slightly uneconomical, is helpful in defining $[Ca^{2+}]_{Lu}$ relatively precisely.

Materials and methods

Cell preparation

Single isolated mouse pancreatic acinar cells were prepared using collagenase (Worthington) digestion as described previously (Osipchuk *et al.*, 1990).

Solutions

The extracellular (bath) solution contained (mM): NaCl 140, KCl 4.7, $MgCl_2$ 1.13, $CaCl_2$ 1, glucose 10 and HEPES-NaOH 10 (pH 7.3). In some experiments, $CaCl_2$ was not included (Ca^{2+}-free solution). Pipette solution I contained (mM): KCl 120, NaCl 20, $MgCl_2$ 1.13, ATP 2 and HEPES-KOH 10 (pH 7.2). $[Ca^{2+}]$ was buffered with EGTA (0.25 or 1 mM). Pipette solution II contained (mM): KCl 110, NaCl 20, $MgCl_2$ 1.13, ATP 2 and HEPES-KOH 10 (pH 7.2). $[Ca^{2+}]$ was clamped using a BAPTA/Ca^{2+} mixture containing 10 mM BAPTA and 2 mM $CaCl_2$. The cells, placed on a glass coverslip attached to an open perifusion chamber, were perifused continuously from a gravity-fed system. Bath solution changes at the cell occurred after <7s in this system. All experiments were performed at room temperature.

Ca^{2+} measurement in intracellular stores

The isolated acinar cells were incubated with 2.5 μM Mag-fura 2 AM and 0.01% pluronic F-127 for 10–15 min at 37°C, then washed twice and used within 4 h. Before the establishment of the patch–clamp whole-cell recording configuration, there was intense fluorescence throughout the cell (Figure 1Aa). It is reasonable to assume that at this stage the fluorescent probe is present both inside organelles and in the cytoplasm. In order to wash out the cytoplasmic probe content, a patch pipette was sealed to the cell membrane and the membrane area covered by the pipette disrupted by suction to establish the whole-cell recording configuration (Hamill *et al.*, 1981). The overall fluorescence intensity was markedly reduced by this procedure and, as seen in Figure 1Ab, 15 min after starting the wash out into the pipette, the two nuclei (in this particular cell) had a very low concentration of the probe.

Fluorescence images were captured using the set-up previously described (Mogami *et al.*, 1997). Alternate excitation wavelengths of 340 and 380 nm from a Xenon light source were used. Three regions of interest were selected: the apical pole, the basal pole including the nucleus and the basal pole excluding the nucleus. The ratio (340/380) of fluorescence intensities in these regions was used to calculate $[Ca^{2+}]$ in the stores. The Mag-fura 2 fluorescence ratio was calibrated using exposure to 10 μM ionomycin and 15 mM Ca^{2+} or 10 mM EGTA, assuming a dissociation constant for Ca^{2+}–Mag-fura 2 at room temperature of 53 μM. Since Mag-fura 2 is also Mg^{2+}-sensitive (although with a much lower affinity than for Ca^{2+}), we attempted to assess to what extent Mg^{2+} had an influence on our measurements by lowering $[Mg^{2+}]$ in the pipette solution to 0.113 mM. In three experiments of this type, the ACh (10 μM)-evoked reduction in $[Ca^{2+}]_{Lu}$ and the subsequent reuptake were very similar to those obtained in the presence of the standard 1.13 mM $MgCl_2$ solution, indicating that the influence of Mg^{2+} on the fluorescence ratio is relatively minor. We also carried out five experiments with the Ca^{2+}-selective probe fura 2-FF. The procedures for loading the cells with the probe (2.5 μM fura 2-FF-AM and 0.01% pluronic F-127, 10–15 min at 37°C) and washing it out from the cytoplasm were similar to what has been described above for Mag-fura 2. The excitation wavelengths were 340 and 380 nm and the fluorescence was recorded at 510 nm. Calibration was carried out as described above for Mag-fura 2, assuming a dissociation constant of 35 μM for Ca-fura 2-FF (Hajnoczky and Thomas, 1997). With regard to the precision of $[Ca^{2+}]_{Lu}$ measurements, the reader is referred to the discussion of this point by Hofer and Schulz (1996).

An initial analysis (Figure 2) indicated that $[Ca^{2+}]_{Lu}$ changes in response to ACh application and removal were very similar in the three selected regions of interest. Since the basal non-nuclear region is totally dominated by ER (Kern, 1993; Gorelick and Jamieson, 1994), this suggests that with our experimental protocol we are essentially measuring $[Ca^{2+}]_{Lu}$ in the ER. The quantitative part of our analysis was based exclusively on measurements from the basal region of the cells excluding the nuclei. It cannot be excluded that the kinetics of Ca^{2+} release and uptake are influenced by the Ca^{2+} buffer component contributed by Mag-fura 2 or fura 2-FF; however, our estimates indicate that the additional buffering due to the presence of these dyes is probably only a small proportion of the overall buffering.

Cytosolic Ca^{2+} measurements

Calibration of $[Ca^{2+}]_i$ was carried out using fura 2 applied through the patch pipette. The R_{min} value was obtained with a pipette solution containing 10 mM BAPTA whereas R_{max} was obtained when the cell was incubated with 15 mM $CaCl_2$ in the presence of 10 μM ionomycin using a K_d for Ca^{2+}–fura 2 of 150 nM.

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 usually 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.

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