The endoplasmic reticulum as one continuous Ca^{2+} pool: visualization of rapid Ca^{2+} movements and equilibration

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We investigated whether the endoplasmic reticulum (ER) is a functionally connected Ca^{2+} store or is composed of separate subunits by monitoring movements of Ca^{2+} and small fluorescent probes in the ER lumen of pancreatic acinar cells, using confocal microscopy, local bleaching and uncaging. We observed rapid movements and equilibration of Ca^{2+} and the probes. The bulk of the ER at the base was not connected to the granules in the apical part, but diffusion into small apical ER extensions occurred. The connectivity of the ER Ca^{2+} store was robust, since even supramaximal acetylcholine (ACh) stimulation for 30 min did not result in functional fragmentation. ACh could elicit a uniform decrease in the ER Ca^{2+} concentration throughout the cell, but repetitive cytosolic Ca^{2+} spikes, induced by a low ACh concentration, hardly reduced the ER Ca^{2+} level. We conclude that the ER is a functionally continuous unit, which enables efficient Ca^{2+} liberation. Ca^{2+} released from the apical ER terminals is quickly replenished from the bulk of the rough ER at the base.

Keywords: Ca^{2+} movements/ Ca^{2+} uncaging/endoplasmic reticulum/luminal Ca^{2+} /luminal connectivity

Introduction

The endoplasmic reticulum (ER) is the most important dynamic Ca^{2+} store (Berridge and Irvine, 1989; Irvine, 1990; Berridge, 1993; Pozzan *et al.*, 1994). The ER is a continuous tubular network structure (Villa *et al.*, 1991; Terasaki *et al.*, 1994), but work on astrocytes, arterial myocytes and HeLa cells suggests that it is composed of distinct heterogeneous subunits with different Ca^{2+} transport characteristics (Golovina and Blaustein, 1997; Montero *et al.*, 1997a). On the other hand, there is indirect evidence from pancreatic acinar cells suggesting that the ER functions as a lumenally continuous compartment, which would allow movements of Ca^{2+} from the base to the apex (Mogami *et al.*, 1997). It has also been shown that green fluorescent protein (GFP) fusion proteins can diffuse in the aqueous-phase lumen of the ER (Subramanian and Meyer, 1997; Dayel *et al.*, 1999) and this may suggest that the free Ca^{2+} concentration could equilibrate throughout

the cell. However, buffering by Ca^{2+} -binding proteins could slow the movement of Ca^{2+} significantly and lead to the establishment of luminal Ca^{2+} gradients (Subramanian and Meyer, 1997).

In order to test directly whether the ER functions dynamically as a single continuous Ca^{2+} storing unit or is effectively composed of separate sub-compartments, it is necessary to observe the movement of Ca^{2+} in the lumen of the ER under conditions of artificially imposed gradients and during stimulated Ca^{2+} release and re-uptake. So far such data are lacking and we have no information about the speed of Ca^{2+} movement in the ER lumen.

Because the pancreatic acinar cell is both structurally and functionally polarized, it is a good system in which to investigate the movement of Ca^{2+} between different regions within the ER. The acinar cell has an extensive rough ER network dominating its basolateral part. Electron microscope (EM) pictures show that thin ER cisternae are as densely packed as physically possible in the whole of the basolateral region surrounding the nucleus. In contrast, the apical region is densely packed with secretory (zymogen) granules (ZGs) and has almost no space for ER. Only small ER vesicular structures can occasionally be observed (Kern, 1993; Gorelick and Jamieson, 1994). This presents an interesting problem. The physiologically important cytosolic Ca^{2+} signals, elicited by the neurotransmitter acetylcholine (ACh) or the hormone cholecystokinin, are initiated in the apical pole and are confined to this region most of the time (Kasai *et al.*, 1993; Thorn *et al.*, 1993; Petersen *et al.*, 1994). Even in the case of supramaximal agonist stimulation, when the cytosolic Ca^{2+} signal spreads rapidly all over the cell, the Ca^{2+} concentration rise is larger in the apical than in the basal part of the cell (Ito *et al.*, 1997). If the ER is the major intracellular store from which Ca^{2+} is mobilized upon agonist stimulation, then the small Ca^{2+} -releasing ER elements in the apical pole should be functionally connected to the main part of the ER in the basolateral area. Furthermore, this connection must be efficient. Ca^{2+} should be able to move rapidly from the major part of the store, at the base of the cell, into the termini in the granular pole, where stimulation causes opening of Ca^{2+} release channels in the apical ER extensions (Mogami *et al.*, 1997).

We have used fast local photobleaching and local uncaging of substances trapped in the lumen of the ER (NP-EGTA, Mag-fluo 4 and Mag-fura 2, respectively) and studied the movements of Ca^{2+} and small molecular weight fluorescent indicators inside the ER. We show that the whole of the ER in the pancreatic acinar cell is functionally connected. The concentrations of Ca^{2+} and other small molecules appear uniform throughout the ER and suddenly established gradients, induced by local photobleaching or uncaging, equilibrated within a few

Fig. 1. The full connectivity of the whole basolateral ER Ca^{2+} store complex and the connection with the apical extensions is demonstrated by local photobleaching of Mag-fluo 4 in the lumen of the ER and subsequent monitoring of the movement of unbleached dye. (A) One cell [yellow dotted circle in (a)] was selectively bleached with UV laser; (b and c) show fluorescence images before and after bleaching; (d) measurement of fluorescence intensity changes in the three cells. (B) Repetitive local bleaching [R-1 in (a)] resulted in a homogeneous decrease of fluorescence intensity throughout the whole basolateral part of the cell (b and c, before and after bleaching), indicating that the whole basolaterally located Ca^{2+} store was fully interconnected. By contrast, local apical bleaching [R-2 in (a)] affected only the bleached part of the cell (d), suggesting that the granular Ca^{2+} store was not functionally connected to the basolateral ER. (C) After wide apical bleaching [yellow dotted circle in (a)], we monitored the fluorescence intensity changes in the apical and basal pole: (a) transmitted light picture; (b) fluorescence image taken immediately after apical bleaching; (c) 15 s after the bleaching. The length of the horizontal red bar in A(a), B(a) and C(a) represents 10 μm .

seconds even over distances of up to $\sim 10 \mu\text{m}$. The whole of the ER, and in particular the bulk of the rough ER in the basal part, provides a homogeneous environment with regard to the luminal Ca^{2+} concentration. This may be crucial for many biological functions such as protein processing and regulation of the sensitivity of the Ca^{2+} release mechanism. We also show that the rapid equilibration of Ca^{2+} throughout the lumen of the ER enables low and physiologically relevant agonist concentrations to evoke repetitive cytosolic Ca^{2+} spikes with hardly any reduction in the ER Ca^{2+} level.

Results

Local photobleaching of Mag-fluo 4 in the lumen of the ER: the lumen of the ER in the basolateral region is continuous

The normal polarity of the acinar cells was well preserved in single cells or clusters after isolation with collagenase treatment [Figure 1A(a), B(a) and C(a)]. In order to visualize the intracellular Ca^{2+} stores, we loaded a low-affinity Ca^{2+} -sensitive dye, Mag-fluo 4 ($K_d = 22 \mu\text{M}$), into

the Ca^{2+} stores and obtained high-resolution images with a confocal microscope. As shown in Figure 1A and B, there was fluorescence from all parts of the cell except the nuclei, indicating a low level of fluorescence in the cytosol (Mogami *et al.*, 1998). This is due to the optical properties of Mag-fluo 4, which are such that at the resting cytosolic free Ca^{2+} concentration there is little fluorescence. In agreement with this, washout of the cytosolic dye component only caused a small decrease in the fluorescence intensity (see below).

To test the connectivity of the lumen of the ER from different parts of the cell, we employed the local bleaching function of the confocal microscope, using a strong UV laser with near full power (351 and 360 nm, 80 mW). (It was unexpected, but helpful, that Mag-fluo 4 could be bleached by UV light. This bleaching did not affect the Ca^{2+} content or Ca^{2+} transport characteristics of the ER store—see below.) Figure 1A shows an example of the bleaching function employed. We bleached only one cell out of a triplet [cell A circled in Figure 1A(a)]. Thereafter, the fluorescence intensity of this cell decreased dramatically, but the other two cells were not affected [Figure 1A(b–d)]. Next, we bleached small areas within

single cells of the doublet as shown in Figure 1B(a–d). At first, we repetitively bleached a small basolateral area (R-1). As a result, the fluorescence intensity of the whole basolateral region decreased homogeneously, whereas the apical granular pole remained almost unaffected and finally disclosed its demarcation [Figure 1B(c)] in agreement with the structure seen in the transmitted light picture [Figure 1B(a)]. In the other cell, we tried deliberately to bleach a small area (R-2) localized within the apical region containing the ZGs. Only the bleached area itself was affected strongly (although a slight decrease in the fluorescence intensity in the basolateral part is just visible), indicating that the granules are not lumenally connected with the ER in the basolateral part of the cell.

Penetration of ER elements into the apical pole of the cell

In order to investigate whether there are ER elements in the granular region, which might be connected to the bulk of the ER in the basolateral region, a wide area containing the apical pole was bleached and the fluorescence intensity changes were monitored. In the experiment shown in Figure 1C(a–c), the whole of the apical region was double bleached. The image shown in Figure 1C(b) was captured immediately after the bleaching and the one shown in Figure 1C(c), 15 s later. Following the bleaching, the fluorescence intensity in the apical pole increased, whereas that of the basolateral part decreased, suggesting that small ER elements in the apical pole can be refilled from the major portion of the ER in the basolateral region. The same result was obtained in all the 11 cells studied and also in a further six cells in which the patch-clamp whole-cell configuration was used to wash out the cytosolic dye component. Figure 1C shows that the fluorescence intensity increase in the apical pole following the bleaching is not homogeneous. However, the spatial resolution of our method is insufficient to measure the fluorescence intensity increase specifically in the tiny ER elements in the apical pole.

Rapid movement of Mag-fluo 4 in the ER lumen

To resolve the speed of dye movement within the ER, we used the fast mode of the confocal microscope, bleached a small circular area and followed the time course of the recovery of fluorescence in that area as well as the decline in fluorescence in an area some distance away. Figure 2 illustrates experiments showing how quickly the dye moved within the ER. In single cells, we bleached a small circular area in the basolateral part [red and yellow circles in Figure 2A(a and c) and B(a and b)] and measured the fluorescence intensities of both the bleached area (red circle) and a remote area across the nucleus [black circle in Figure 2A(a) and B(a)], simultaneously. After each local bleaching event there was a sharp drop in the fluorescence intensity in the bleached field and thereafter an exponential recovery [red curve in Figure 2A(d) and B(c)]. In the remote area across the nucleus, the bleaching event resulted in a decline in fluorescence intensity (black curve). The two curves converged quickly, after each bleaching event, indicating rapid equilibration between the two sites. This could be repeated several times in the same experiment [Figure 2A(d)] and did not depend on the specific positions of the regions in the basolateral area

chosen for analysis. The recovery of fluorescence intensity at the bleached site was well fitted with an exponential function, where the half recovery time was 2.3 s [Figure 2B(c)]. In the 13 cells investigated using this protocol, the average half recovery time was 1.9 ± 0.1 s (SE). After apical bleaching [Figure 2A(b)], we could detect a small decrease of the fluorescence intensity in the basolateral area [Figure 2A(c)].

Short photobleaching does not disturb the Ca²⁺ dynamics

Since Mag-fluo 4 is a low-affinity Ca²⁺-sensitive dye and would be expected to have little fluorescence at the low Ca²⁺ concentration in the cytosol under resting conditions, we assumed that there would only be a minor interference by the dye present in the cytosol. In order to test this, the cytosolic dye component was washed out of the cytosol into a patch pipette (whole-cell configuration). During a 10 min period of dye washout, the fluorescence intensity was changed by <10%. As shown in Figure 2C, the bleaching experiments gave the same results under this condition as without the dye washout. After local basal bleaching [Figure 2C(a and b)], the recovery of fluorescence intensity in the bleached area could be fitted by a single exponential [Figure 2C(e)] and the average half recovery time was 1.8 ± 0.2 s ($n = 4$). Local apical bleaching [Figure 2C(c)] caused a major decrease in the fluorescence intensity in the apical part of the cell, which was only partially reversible, and also a smaller fluorescence intensity decrease in the basal part [Figure 2C(d and f)]. In these experiments it was also possible to test whether the photobleaching was associated with any change in the cytosolic Ca²⁺ concentration, by monitoring the Ca²⁺-dependent whole-cell current (Petersen, 1992). In none of these experiments ($n = 4$) did we observe any activation of the Ca²⁺-sensitive current [Figure 2C(e and f)]. However, after the bleaching, ACh could, in the same cell, generate the usual Ca²⁺-sensitive current and release Ca²⁺ from the ER [Figure 2C(g)]. This suggests that short photobleaching does not disturb the Ca²⁺ dynamics.

Photobleaching of Mag-fura 2: confirmation of ER luminal connectivity

As a next step, we used Mag-fura 2 ($K_d = 52$ μ M), which has been used to measure the Ca²⁺ concentration in the ER from different cell types (Hofer and Machen, 1993; Hofer and Schulz, 1996), including pancreatic acinar cells (Mogami *et al.*, 1998; Park *et al.*, 1999). In our experiments we used an excitation wavelength of 351 nm, which is close to the isobestic point of the dye. It was our purpose to test the luminal connectivity within the ER without any possible interference from unwanted Ca²⁺ concentration changes in the cytosol or the ER. To wash out the dye from the cytosol, we used a patch pipette (whole-cell recording configuration) containing 10 mM BAPTA and 2 mM Ca²⁺, so that the cytosolic Ca²⁺ concentration was clamped at the normal resting level. As soon as the patch of plasma membrane covered by the pipette had been ruptured, the whole-cell fluorescence intensity decreased and 10 min later was reduced to ~10% of the level observed before entering the whole-cell configuration (Mogami *et al.*, 1998). Local bleaching experiments of the type shown in Figure 2 were then carried out, with similar results. The

Fig. 2. Time course of Mag-fluo 4 movement in the ER lumen following local bleaching. **(A)** (a) Transmitted light picture of a single cell with the areas of interest identified by the red and black circles. Length of red bar corresponds to 10 μm . (b and c) Fluorescence intensity images before and after wide apical bleach in region marked by large yellow dotted circle in (b). Local basal bleaching [small yellow dotted circle in (c)] was applied repeatedly and the fluorescence intensities measured both in the bleached area [red circle in (a), red curve in (d)] as well as in an unbleached basal area $\sim 10 \mu\text{m}$ away [black circle in (a), black curve in (d)]. **(B)** (a) Transmitted light picture of another cell. Local basal bleaching [red and yellow dotted circles in (a) and (b), respectively] was applied and the fluorescence intensity changes in the marked sites [colour coding corresponds to coloured circles in (a)] are shown in (c). **(C)** In this experiment Mag-fluo 4 was washed out of the cytosol through a patch pipette (a) (whole-cell configuration). Ten minutes after establishing whole-cell configuration, the areas represented by the dotted yellow circles (b and c) were bleached and the fluorescence intensity changes monitored in the areas represented by the marked circles in (a). (a) Transmitted light picture; (b and c) before and after basal bleaching, respectively; (c and d) before and after apical bleaching. The time course of the changes in fluorescence intensity [colour coding according to the marked sites in (a)] and the Ca^{2+} -dependent whole-cell current during the local basal (b) and apical (c) bleaching are shown in (e) and (f), respectively. After the bleaching experiments, ACh elicited an increase in the Ca^{2+} -dependent current (black trace) and released Ca^{2+} from the ER (blue trace) (g).

recovery of fluorescence intensity in a small bleached area was fast, with an average recovery half time of $0.8 \pm 0.1 \text{ s}$ ($n = 10$). The fluorescence intensity in an unbleached basal area, $\sim 10 \mu\text{m}$ away from the bleached area, decreased after each bleaching event and was equilibrated with the bleached area within $\sim 5 \text{ s}$.

Direct measurement of Ca^{2+} movement in the lumen of the ER: Ca^{2+} uncaging in the ER lumen

We loaded the caged Ca^{2+} compound, NP-EGTA, into the lumen of the ER together with Mag-fluo 4. Because the Ca^{2+} concentration in the ER is high, we used a very high dose of NP-EGTA ($20.5 \mu\text{M}$) and a long incubation time (30 min). Thereafter, to wash out the dye and the NP-EGTA in the cytosol, we established the patch-clamp whole-cell configuration. We used a pipette solution containing 10 mM BAPTA and 2 mM Ca^{2+} to clamp the cytosolic Ca^{2+} concentration at the normal resting level and thereby prevented any Ca^{2+} -induced Ca^{2+} release as well as any cytosolic Ca^{2+} change following uncaging of Ca^{2+} inside the ER.

In four out of five cells, we could not detect any significant Ca^{2+} concentration increase after uncaging.

One cell showed tiny changes. This indicates that the amount of Ca^{2+} released by uncaging was small in relation to the already high Ca^{2+} concentration in the resting ER (Mogami *et al.*, 1998). We therefore lowered the Ca^{2+} concentration in the ER by stimulating with $10 \mu\text{M}$ ACh. Figure 3A shows the transmitted light image of the cell used for one of the experiments, with the regions of interest identified by coloured circles, as well as the fluorescence images of the same cell before and after ACh application. The Ca^{2+} concentration inside the ER in the basolateral area decreased markedly after ACh application (Figure 3B), but there was no change in the Ca^{2+} -dependent current (red trace) because of the cytosolic Ca^{2+} clamp. The time course of the Ca^{2+} concentration change inside the ER was monitored, as shown in Figure 3B, to determine the optimal time for uncaging Ca^{2+} . Immediately after the washout of ACh, the Ca^{2+} concentration inside the ER is still low and the Ca^{2+} release channels are closed. At this point we therefore uncaged NP-EGTA in the marked site (dotted circle in Figure 3A) and measured the Ca^{2+} concentration changes around the nucleus in the marked coloured circles (Figure 3A). As seen in Figure 3C, uncaging of Ca^{2+}

Fig. 3. Rapid movement of Ca²⁺ in the ER lumen following local uncaging of caged Ca²⁺. Direct measurement of Ca²⁺ movement in the lumen of the ER. After 30 min loading with Mag-fluo 4-AM and NP-EGTA-AM, the dye and NP-EGTA were washed out of the cytosol into a patch pipette (whole-cell configuration). The cytosolic Ca²⁺ was clamped at the resting level by using a patch pipette containing 10 mM BAPTA and 2 mM Ca²⁺. Thereafter, 10 μ M ACh was applied to lower the Ca²⁺ concentration inside the ER. (A) Transmitted light image of the cell with the patch pipette. The various regions of interest are colour coded. The fluorescence intensity images just before and after ACh application are also shown. NP-EGTA was uncaged in the lumen of the ER in the area represented by the dotted circle. (B) Time course of the ACh-evoked reduction in Ca²⁺ concentration inside the ER (black dots) and also the complete absence of any change in the Ca²⁺-dependent Cl⁻ current (red trace). (C) Time course of the Ca²⁺ concentration changes in the ER, following local uncaging, in the various regions colour coded in (A). It is also seen that the Ca²⁺-dependent current did not change after the intraluminal uncaging.

elicited an instantaneous increase in the Ca²⁺ concentration in the targeted area (black curve). Ca²⁺ concentration changes, with decreasing amplitude and decreasing rate of rise with increasing distance from the site of uncaging, were observed in the other basolateral areas (red, green, dark blue and light blue curves). Thereafter the Ca²⁺ concentration declined in all areas, converging and attaining the same level within ~7 s. Similar results were obtained in all six experiments carried out on separate cells. NP-EGTA has a high affinity for Ca²⁺ ($K_d < 100$ nM). In mouse pancreatic acinar cells, stimulation with 10 μ M ACh has previously been shown to decrease the Ca²⁺ concentration in the lumen of the ER (measured with low-affinity ratiometric indicators) to ~40 μ M (Mogami *et al.*, 1998). At such free Ca²⁺ levels in the stores, NP-EGTA is saturated with Ca²⁺ and consequently cannot rebind Ca²⁺ following photolysis. No change in Ca²⁺ concentration was detected in the nucleus and the Ca²⁺-dependent current remained stable, due to the cytosolic Ca²⁺ clamp. This result (Figure 3C) indicates fast movement of Ca²⁺ through the lumen of the ER. Ca²⁺ concentration equilibration seems to occur, even over distances of ~10 μ m, within a few seconds.

ACh-evoked release of Ca²⁺ from the ER in Mag-fluo 4-loaded cells

Figure 4 shows the ER Ca²⁺ release and refilling processes triggered by a supramaximal dose of ACh in an intact Mag-fluo 4-loaded cell. The structure of the cell, with the granule-containing pole to the right, is shown in the transmitted light picture [Figure 4A(a)]. Figure 4A(b)

shows the colour-coded Ca²⁺-sensitive fluorescence image before the application of ACh, and in Figure 4A(c) the image taken after the maximal release of Ca²⁺ from the ER is shown. Subtraction [Figure 4A(d)] revealed that the main intracellular Ca²⁺ release site was the basolateral region, where the rough ER is densely packed (Kern, 1993; Gorelick and Jamieson, 1994). Nevertheless, the apical pole appears heterogeneously, probably due to penetration of thin ER elements into the apical pole (Figure 1).

As shown in Figure 4B and C, Ca²⁺ release from the ER was slow. It took several minutes to empty the whole ER. Soon after ACh removal, Ca²⁺ refilling began. In the basolateral ER-rich region, all areas essentially shared the same time course of Ca²⁺ release and refilling (Figure 4B and C) due to the relatively rapid equilibration of Ca²⁺ within the lumen of the ER compared with the slow Ca²⁺ release. During the Ca²⁺ release period, focal bleaching in basal areas showed that dye equilibrium was achieved quickly, with results very similar to those shown in Figure 2 ($n = 3$). In the apical region, ACh initially hardly evoked any change in the Ca²⁺ concentration, but later there was a relatively small decrease (Figure 4B), which was spatially non-uniform (Figure 4C). This may be due to the high fluorescence intensity of the granules and the relatively small fraction of the ER extensions in the apical pole.

Since the expected ACh-elicited decrease in the Ca²⁺ concentration inside the ER elements in the apical pole could not be resolved (Figure 4B), most likely due to the high fluorescence of the granules, we bleached the apical pole of the cell. The remaining fluorescence would be

Fig. 4. Time course of ACh-evoked Ca²⁺ release and the subsequent refilling of the ER. In a Mag-fluo 4-loaded cell, 10 μ M ACh was applied. (A) (a) Transmitted light picture; (b and c) fluorescence intensity images before and after ACh; (d) subtracted image (b-c). (B) Time course of ACh-induced fluorescence intensity changes in the two basal as well as the apical regions marked in [A(a)]. (C) Time course of Ca²⁺ release following ACh stimulation and subsequent refilling after cessation of stimulation.

expected to come from thin ER elements in the apical pole refilled via the connections to the main part of the basolateral ER. In this situation we could more easily observe changes in the apical pole after 10 μ M ACh application ($n = 5$), but it was difficult to detect possible differences between the Ca²⁺ release rates from various parts of the ER.

The most useful experiments were those in which we dialysed the cell with a high Ca²⁺ buffer solution (10 mM BAPTA and 2 mM Ca²⁺) using the patch-clamp whole-cell configuration. In this condition, the ACh-elicited Ca²⁺ release was much faster (Figure 5), most likely due to the removal of the negative Ca²⁺ feedback on the opening of the inositol trisphosphate (IP₃) receptors (Montero *et al.*, 1997b; Mogami *et al.*, 1998). The transmitted light picture shows the structure of the cell investigated [Figure 5A(a)]. After making the whole-cell configuration, we bleached the apical pole. The fluorescence images taken before establishing the whole-cell configuration [Figure 5A(b)], 10 min after having established this configuration [Figure 5A(c)] and after wide apical bleaching [Figure 5A(d)] are shown. The rather homogeneous Ca²⁺ concentration decrease throughout the basal region evoked by 10 μ M ACh and the relatively homogeneous Ca²⁺ concentration increase following cessation of ACh stimulation are shown in Figure 5A(e). In this condition, we

detected clearly Ca²⁺ concentration decreases both in the basal and apical poles. As seen in Figure 5B(a), there was no difference in time course between the apical pole and the basolateral part of the cell (similar results in six trials). Even in the fast scan mode (89 ms), we could not detect any time lag between the two regions [Figure 5B(b)]. This suggests that the movement of Ca²⁺ inside the lumen of the ER is fast relative to the rate of Ca²⁺ release, so that the whole of the ER is homogeneous with respect to the luminal Ca²⁺ concentration at all times.

Luminal connectivity after supramaximal stimulation with ACh: local uncaging after application of ACh or ionomycin

We tested whether the ER connectivity is maintained under extreme conditions. Figure 6A shows the result from a cell loaded with Mag-fluo 4. After application of a supramaximal ACh dose (10 μ M), the main decrease in the Ca²⁺ concentration inside stores was detected in the basolateral region [Figure 6A(b,c and d)]. Thereafter, we bleached a small area in the basal pole [Figure 6A(a, red circle)] and measured the fluorescence intensity simultaneously in the bleached as well as a remote area (black circle). As shown in Figure 6A(e), the two areas (>5 μ m apart) were connected lumenally. The bleaching was carried out in eight different cells, 3–30 min after start of

Fig. 5. Ca²⁺ release from the ER after ACh stimulation follows the same time course in the basal and apical regions. Mag-fluo 4-loaded cell with subsequent washout of cytosolic dye component through patch pipette in whole-cell configuration. Cytosolic Ca²⁺ concentration clamped at the normal resting level by BAPTA/Ca²⁺ mixture. (A) (a) Transmitted light picture; (b) fluorescence intensity image before cytosolic dye washout; (c) 10 min after establishment of whole-cell configuration. Thereafter wide apical bleaching of apical pole within area represented by dotted yellow circle. (d) After apical bleaching to remove fluorescence from dye in the granules. (e) Line scan image taken from the line shown in (d) illustrating the time course of the Ca²⁺ concentration changes in the ER at the base of the cell. (B) (a) Ca²⁺-dependent fluorescence intensity changes in response to ACh measured in the three areas represented by the colour-coded circles in [A(a)]. (b) Comparison of Ca²⁺ release time in the apical and basal parts of the cell with a fast scan mode (89 ms sampling).

continuous ACh (10 μ M) application. In all cells we found efficient luminal connectivity with an average half recovery time of 1.0 ± 0.1 s ($n = 8$), indicating that the functional connectivity of the ER elements is robust.

Next we tried to increase artificially the cytosolic Ca²⁺ concentration with ionomycin in the presence of 10 mM Ca²⁺ in the extracellular solution. After treatment with 15 μ M ionomycin, local bleaching experiments were carried out and we observed that the ER was connected normally. However, after ~12 min of continuous exposure to ionomycin, the ER became fragmented. Figure 6B shows the result. In this case local bleaching resulted in a permanent reduction in the fluorescence intensity in the bleached area and the local bleaching had no effect on the fluorescence intensity in other parts of the cell. We conclude that the functional ER connection, in normal pancreatic acinar cells, is maintained over the whole range of physiological conditions, whereas prolonged extreme cytosolic Ca²⁺ loading can fragment the ER.

Short-lasting repetitive cytosolic Ca²⁺ spikes are only associated with small changes in the Ca²⁺ concentration inside the ER

Physiological agonist concentrations elicit repetitive cytosolic Ca²⁺ spikes rather than a sustained increase in the Ca²⁺ level (Berridge and Irvine, 1989; Berridge, 1993; Petersen *et al.*, 1994). We therefore investigated whether a low ACh concentration, evoking repetitive cytosolic Ca²⁺

spikes, could cause detectable decreases in the Ca²⁺ concentration inside the connected ER store. Figure 7A shows the result of a patch-clamp whole-cell recording experiment in which 50 nM ACh elicited repetitive cytosolic Ca²⁺ spikes, monitored as Ca²⁺-dependent current spikes (Petersen, 1992; Thorn *et al.*, 1993). It is seen that none of these relatively short-lasting cytosolic Ca²⁺ spikes were associated with any detectable change in the ER Ca²⁺ concentration in the basal or the apical regions.

Similar results were obtained in 16 cells investigated with Mag-fluo 4, four cells in Mag-indo 1-loaded cells and six cells in Mag-fura 2-loaded cells. We therefore increased the ACh concentration and generated more substantial cytosolic Ca²⁺ oscillations. In this condition, we were just able to detect, in three out of six cells, a small drop in the ER Ca²⁺ concentration in association with each cytosolic Ca²⁺ spike. Figure 7B shows the result from one of these cells, where the ER Ca²⁺ concentration decrease associated with a Ca²⁺ spike was almost fully restored before the next event. We also tested the effect of ACh on cells exposed to a Ca²⁺-free external solution. In this condition low doses of ACh (25–100 nM) did not generate any Ca²⁺ oscillations ($n = 6$), but 200 nM ACh elicited repetitive spikes. Figure 7C shows one of the results. There was no measurable reduction in the ER Ca²⁺ concentration during each cytosolic Ca²⁺ spike, but after ~2 min of repetitive spiking a gradual reduction in the ER Ca²⁺ concentration was observed. Thereafter, the spike ampli-

Fig. 6. Supramaximal ACh stimulation fails to fragment the ER, but prolonged exposure to ionomycin in the presence of 10 mM extracellular Ca^{2+} abolishes luminal diffusion of Mag-fluo 4. Local bleach experiments were carried out after application of 10 μM ACh (A) and 15 μM ionomycin (B). (A) (a) Transmitted light image of the cell investigated; (b and c) fluorescence images before and after ACh application; (d) ER Ca^{2+} release after 10 μM ACh; (e) a small area in the basal part [red circle in (a)] was bleached and the fluorescence intensity measured at the bleached site (red curve) and a remote site [black circle in (a) and black curve]. Half recovery time is 0.9 s. (B) Local bleaching experiment after 15 min treatment with 15 μM ionomycin in 10 mM Ca^{2+} . We observed luminal connectivity until 10 min after 10 μM ionomycin treatment; however, after ~12 min, the ER appeared to be fragmented. After bleaching two local areas [marked as black and red circles in (a)], there was no recovery of fluorescence intensity (arrows). (a) Transmitted light image of the cells investigated; (b) fluorescence intensity image before bleaching; (c) fluorescence intensity image after bleaching of two local areas (arrows); (d) fluorescence intensities measured at the sites marked by the correspondingly coloured sites in (a).

tude gradually decreased and finally the spiking stopped. Supramaximal ACh stimulation (10 μM) could nevertheless release further Ca^{2+} from the ER (Figure 7C).

The apical pole is dominated by non-ER elements and as we were unable to record specifically from the tiny ER elements in this region, we cannot exclude that there could be very local changes in the apical ER Ca^{2+} concentration during the short-lasting cytosolic spikes, which we could not resolve. Nevertheless it is clear that the amount of Ca^{2+} that has to be released from the ER in order to evoke a

short-lasting cytosolic Ca^{2+} spike is insufficient to make a major impact on the Ca^{2+} concentration in this large continuous store.

Discussion

Our data provide direct evidence for rapid movement within the ER lumen of Ca^{2+} and small molecules. By localized photobleaching of Ca^{2+} -sensitive fluorescent probes and by monitoring at high resolution the subsequent diffusion into the bleached area and away from non-bleached areas, we have characterized quantitatively the rapid movements within the ER lumen. All the basolaterally located ER elements are interconnected efficiently (Figures 1 and 2). Movements of the fluorescent Ca^{2+} -sensitive probes occur rapidly with equilibration over distances of 10–15 μm within a few seconds (Figure 2). Most importantly, we have demonstrated that local uncaging of caged Ca^{2+} , creating suddenly a major Ca^{2+} gradient inside the ER, results in rapid diffusion of Ca^{2+} within the lumen of the ER, followed by equilibration over distances of ~10 μm within a few seconds (Figure 3).

By bleaching the whole of the apical granule-containing area and following the subsequent movements of non-bleached Ca^{2+} -sensitive dye, we have shown that there is non-homogeneous refilling of the apical pole with dye from the basolateral area (Figure 1). Furthermore, ACh stimulation resulted in a reduction of the intra-store Ca^{2+} concentration in the apical pole with exactly the same time course as the reduction in the basal ER Ca^{2+} level (Figure 5). This agrees with the direct demonstration of rapid Ca^{2+} movement through the ER lumen (Figure 3) and illustrates the functional connection between the bulk of the ER Ca^{2+} reservoir at the base and the apical ER terminals.

The ER luminal connectivity is robust (Figure 6). Previously, experiments on RBL cells have indicated that an increase in the cytosolic Ca^{2+} concentration can reversibly fragment the ER tubules and thereby prevent luminal diffusion of a GFP fusion protein (Subramanian and Meyer, 1997). However, our experimental results indicate that even sustained stimulation of the acinar cells for up to 30 min with a supramaximal ACh concentration (10 μM) does not cause any reduction in the ability of Mag-fluo 4 to diffuse rapidly through the ER lumen. Only when the cytosol was flooded with Ca^{2+} in an unphysiological manner (ionomycin and high Ca^{2+} for >10 min) could we detect signs of functional ER fragmentation (Figure 6). During physiologically relevant Ca^{2+} signalling events there was no sign of ER fragmentation and the ER remained an efficiently connected ER store.

The major Ca^{2+} store that can be mobilized by agonist stimulation is the rough ER (Kern, 1993; Gorelick and Jamieson, 1994) in the basolateral region (Figures 4 and 5). The connection with apical ER extensions allows efficient cytosolic Ca^{2+} signalling in the strategically important apical region, without depleting any ER element of Ca^{2+} . This may be important, since a high Ca^{2+} concentration inside the ER is necessary for the retention of proteins (Wileman *et al.*, 1991). The nuclear envelope contains a mobilizable Ca^{2+} store (Gerasimenko *et al.*, 1995; Stehno-Bittel *et al.*, 1995) and is lumenally connected to the ER (Petersen *et al.*, 1998). Ca^{2+} depletion

Fig. 7. Repetitive short-lasting cytosolic Ca²⁺ spikes evoked by a low ACh concentration are associated with no or very small changes in the Ca²⁺ concentration inside the ER. (A) (a) Transmitted light picture of the cell investigated, with the two regions of interest identified by the coloured circles. The fluorescence intensity images before (b) and after (c) the establishment of the patch-clamp whole-cell recording configuration as well as after the apical bleaching (d) are shown. (e) The Ca²⁺-sensitive fluorescence intensity from Mag-fluo 4 inside the ER before and during stimulation with 50 nM ACh in both the basal and apical regions (upper part) as well as the Ca²⁺-dependent whole-cell current (lower part). (B) 100 nM ACh generated more substantial Ca²⁺-dependent current oscillations close to the maximal activation of the current with 10 μM ACh. In this particular cell, we observed small decreases in the Ca²⁺ concentration of the ER, recorded using the ratiometric dye Mag-fura 2 (351 and 364 nm excitation) and calibrated as described previously (Park *et al.*, 1999). (C) Measurement of the ER Ca²⁺ concentration (Mag-fluo 4) and the Ca²⁺-dependent current oscillations in a cell exposed to an external Ca²⁺-free solution. In Ca²⁺-free solutions, low doses of ACh (25–100 nM) failed to evoke Ca²⁺ oscillations and 200 nM ACh had to be used to generate repetitive cytosolic Ca²⁺ spikes. Individual short-lasting Ca²⁺-dependent current spikes were not associated with any measurable drop in the Ca²⁺ concentration in the ER store, but after ~2 min of repetitive spiking the ER Ca²⁺ concentration began to decrease. As the ER was losing Ca²⁺, the spikes became smaller and finally disappeared. After the Ca²⁺ oscillations had ceased, there was a further decrease in the ER Ca²⁺ concentration during a period of non-oscillatory elevation in the cytosolic Ca²⁺ concentration. Finally, a supramaximal ACh (10 μM) application caused a marked further Ca²⁺ release from the ER.

of the nuclear envelope store is associated with changes in the morphology and permeability of the nuclear pore complexes (Perez-Terzic *et al.*, 1996), but the functional connection of the whole of the ER Ca²⁺ store complex makes it unlikely that the nuclear envelope store could be depleted under physiological conditions.

The short-lasting cytosolic Ca²⁺ spikes, elicited by low (and probably physiological) levels of agonist, are due to release of Ca²⁺ from ER elements in the apical pole (Kasai *et al.*, 1993; Thorn *et al.*, 1993). Since the total ER volume in the apical pole is small (Kern, 1993; Gorelick and Jamieson, 1994), the total amount of Ca²⁺ held in this part of the store must also be small. The functional connection of the whole of the ER is undoubtedly essential for the repetitive Ca²⁺ spiking in the apical pole. As seen in Figure 3, the Ca²⁺ concentration in the ER lumen equilibrates over distances of >5 μm within <7 s after a local perturbation. The highest frequency of ACh-induced

cytosolic Ca²⁺ spiking is about one spike every 10 s (Figure 7C). Hence, Ca²⁺ movements within the ER are rapid enough to ensure that Ca²⁺ availability in the apical ER lumen is preserved at all stages during oscillations. Any tendency towards Ca²⁺ depletion of the small ER terminals would be countered by rapid diffusion from the main bulk of the store in the basal region.

Our data also have relevance for the quantal Ca²⁺ liberation phenomenon: a low IP₃ concentration causes only partial Ca²⁺ release from the ER and further Ca²⁺ liberation requires an increase in the messenger concentration (Muallem *et al.*, 1989; Bootman *et al.*, 1992). Two models have been proposed to account for this phenomenon: (i) all-or-none release from stores exhibiting heterogeneous sensitivities (Muallem *et al.*, 1989; Bootman *et al.*, 1992) and (ii) phasic or adaptive release from homogeneously sensitive stores (Irvine, 1990; Nunn and Taylor, 1992). Our data, demonstrating directly

Fig. 8. Simplified schematic model illustrating that the whole of the basal ER is functionally connected and also that this compartment is connected to thin extensions deep into the apical granular pole. The bulk of the intracellular Ca^{2+} pool is located in the basal part of the cell. During agonist stimulation Ca^{2+} is released primarily from the thin ER extensions in the apical granular pole, where the Ca^{2+} release channels are located, but the apical ER elements can recruit Ca^{2+} from the basal store. SERCA, sarco-endoplasmic reticulum Ca^{2+} -activated ATPase. SOC, store-operated Ca^{2+} channel.

efficient connectivity of the whole of the ER lumen with regard to Ca^{2+} movements, seem incompatible with the first hypothesis. A recent study in which subcellular Ca^{2+} puffs evoked by IP_3 were imaged in *Xenopus* oocytes, showed that individual sites responded repeatedly to successive increments of the IP_3 concentration. It was shown that the amplitude of a second puff was little diminished even when it arose within a few hundred milliseconds of a preceding event, indicating that the local Ca^{2+} concentration inside the ER was not appreciably reduced (Callamaras and Parker, 2000). This could be explained by Ca^{2+} replenishment of local ER regions, from which release had occurred, by rapid diffusion from other parts of the ER.

With regard to the overall pancreatic acinar cell Ca^{2+} homeostasis, we must differentiate between two entirely different situations. During supramaximal agonist stimulation (Figures 4 and 5), there is substantial Ca^{2+} depletion of the ER. In this situation there is transcellular Ca^{2+} transport (Figure 8). Ca^{2+} is taken up through store-operated Ca^{2+} channels (Parekh and Penner, 1997) in the basal membrane and is pumped into the basal ER by Ca^{2+} ATPases (Mogami *et al.*, 1997, 1998). Ca^{2+} then diffuses through the lumen of the ER from the base to the apical region, where it is released into the cytosol via specific Ca^{2+} channels (IP_3 and ryanodine receptors; Cancela *et al.*, 2000). The route through the lumen of the ER is attractive because of the substantial buffering power of the mitochondria, which impedes Ca^{2+} flow through the cytosol (Tinel *et al.*, 1999). Finally Ca^{2+} is pumped into

the acinar lumen by plasma membrane Ca^{2+} ATPases concentrated in the apical membrane (Belan *et al.*, 1996).

In contrast, during submaximal agonist stimulation there is little ER Ca^{2+} depletion (Figure 7). Ca^{2+} spiking can continue for some time in the complete absence of Ca^{2+} entry from the extracellular solution. Some Ca^{2+} must be pumped out of the cell via the plasma membrane Ca^{2+} ATPases, but Ca^{2+} released during the spikes must also be temporarily stored in the mitochondria, helping to ensure that the Ca^{2+} spikes remain confined to the apical granular area (Tinel *et al.*, 1999). In this signalling mode there can be re-uptake of the small amounts of Ca^{2+} lost from the apical ER terminals after each spike (Figure 7B) via Ca^{2+} ATPases in the ER (Petersen *et al.*, 1993).

Materials and methods

Cell preparation

Fresh mouse pancreatic acinar cells were isolated after collagenase treatment, as described previously (Osipchuk *et al.*, 1990) and used within 4 h. All experiments were carried out at room temperature (22–24°C). Mag-fluo 4, Mag-fura 2 and NP-EGTA were purchased from Molecular Probes and the other chemicals from Sigma Co.

Solutions

The extracellular bathing solution contained (concentration in mM): NaCl 140, KCl 4.7, MgCl_2 1.13, CaCl_2 1, glucose 10 and HEPES 10. pH was adjusted to 7.3 by NaOH. We used two different intracellular pipette solutions. One contained (concentration in mM): KCl 135, MgCl_2 1.13, NaCl 20, HEPES 10, Na_2ATP 2, EGTA 0.1. The pH was 7.2, adjusted by KOH. In the other solution we replaced 0.1 mM EGTA with 10 mM BAPTA + 2 mM Ca^{2+} .

Imaging of Ca^{2+} in intracellular stores

Isolated pancreatic acinar cells were incubated with 4–6 μM Mag-fluo 4-AM and 0.01% pluronic acid for 20–30 min, or Mag-fura 2-AM and 0.01% pluronic acid for 20–25 min at 37°C. NP-EGTA (caged calcium) was loaded into pancreatic acinar cells by incubation in a solution containing 20.5 μM NP-EGTA/AM for 30 min. To remove indicators and caged probe from the cytosol we used the patch-clamp whole-cell configuration (Mogami *et al.*, 1998). The same pipette was used to introduce Ca^{2+} buffer into the cell. The patch pipette contained 0.1 mM EGTA or a 10 mM BAPTA/2 mM Ca^{2+} mixture. Some experiments with Mag-fluo 4 were carried out on intact cells, without unloading the cytosolic Ca^{2+} indicator. For the image analysis, we used Zeiss confocal 510 image software as well as software developed by ourselves.

Photobleaching and uncaging experiments

We used near maximal power of UV laser (lines 351 and 364 nm, 80 mW) to induce fast bleaching of Mag-fluo 4, Mag-fura 2 or uncaging of NP-EGTA, mostly localized to small regions of the cells. Uncaging and photobleaching were combined with confocal imaging. The 488 nm laser line was used for measurements involving Mag-fluo 4 (emission filter: 505–550 or >505). The 351 nm laser line was used to excite and image the Mag-fura 2 distribution (emission filter: 505–550 or >505).

Electrophysiology

Standard patch-clamp whole-cell current recording (Hamill *et al.*, 1981) was used. The electrophysiological recording of Ca^{2+} -dependent current (Petersen, 1992) was made using the EPC-8 amplifier and Pulse software (HEKA). The pipette resistance was 2–3 M Ω . The detailed procedure has been described previously (Wakui *et al.*, 1989; Thorn and Petersen, 1992).

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