The endoplasmic reticulum: one continuous or several separate Ca\(^{2+}\) stores?

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The Ca\(^{2+}\) store and sink in the endoplasmic reticulum (ER) is important for Ca\(^{2+}\) signal integration and for conveyance of information in spatial and temporal domains. Textbooks regard the ER as one continuous network, but biochemical and biophysical studies revealed apparently discrete ER Ca\(^{2+}\) stores. Recent direct studies of ER luminal Ca\(^{2+}\) movements show that this organelle system is one continuous Ca\(^{2+}\) store, which can function as a Ca\(^{2+}\) tunnel. The concept of a fully connected ER network is entirely compatible with evidence indicating that the distribution of Ca\(^{2+}\)-release channels in the ER membrane is discontinuous with clustering in certain localizations.

The ER is crucial for Ca\(^{2+}\) signalling. The ER can act as a sink for Ca\(^{2+}\) that enters the cell through channels in the plasma membrane, but can also be a source for Ca\(^{2+}\) release into the cytosol in response to intracellular messengers, such as inositol 1,4,5-trisphosphate [Ins(1,4,5)P\(_3\)] (Refs 1, 2); cyclic ADP-ribose or nicotinic acid adenine dinucleotide phosphate, generated following activation by neurotransmitters. Because Ca\(^{2+}\) can interact with many different molecular targets, signal specificity requires subcellular localization of the cytosolic Ca\(^{2+}\) signal\(^{4,5}\) and/or special oscillation and spiking patterns\(^{5,6}\). The ER is essential in this respect, because clustering and mixing of various Ca\(^{2+}\)-release channel types in specific regions can focus Ca\(^{2+}\) signals in space and time\(^{7-9}\). Failure of these processes can lead to serious pathological conditions\(^{6}\). Changes in ER Ca\(^{2+}\) storage and release might also be important in neuronal ageing\(^{10}\).

Here we discuss whether the ER consists of one continuous vesiculo-tubular system or of separate isolated compartments. This issue is separate from, but relevant to, the discussion concerning ER heterogeneity. The question about the continuity and discontinuity of the ER lumen has consequences for cytosolic Ca\(^{2+}\) signal generation and is related to the discussion about the quantal Ca\(^{2+}\) release phenomenon: a sustained elevation of the cytosolic Ins(1,4,5)P\(_3\) concentration to a submaximal level causes only a transient and partial liberation of Ca\(^{2+}\) from the ER and further increments in the Ins(1,4,5)P\(_3\) concentration elicit additional pulses of Ca\(^{2+}\) release\(^{11-13}\). This could be explained by subdivision of the ER into separate compartments,
Each with different Ca\(^{2+}\) transport characteristics\(^{13,12}\), but is probably caused by phasic or adaptive release from a homogeneously sensitive store\(^{2,14,13}\). The ER is not uniform with respect to channel and pump distribution\(^{1,16,18}\). There can be different subtypes of ER Ca\(^{2+}\) pumps [sarcoplasmic endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCAs)] in different parts of the ER (Ref. 19) and Ca\(^{2+}\)-release channels can be specifically localized in certain parts of the cell\(^{4,5,18}\). Such heterogeneity neither shows nor does it by itself indicate that the ER is fragmented into isolated compartments. Here we focus specifically on the question concerning the continuity or discontinuity of the ER lumen. In the evolution of this subject there were distinct phases. First, there were morphological studies; these were followed by imaging experiments in which differences in the Ca\(^{2+}\) concentration between different parts of the ER, and different reactions to various chemicals in different ER regions, were characterized. More recently, the question of continuity or discontinuity has been directly addressed by monitoring movements of Ca\(^{2+}\) within the ER lumen.

**Morphological studies**

Early morphological work on the neuronal ER showed that it was a continuous membrane system\(^1\). The Physiological Control Research Group, The MRC Secretory Laboratory, University of Liverpool, Liverpool, UK

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**Fig. 1.** Photobleaching experiments reveal that the whole of the endoplasmic reticulum (ER) is functionally interconnected. (a) Experiment on pancreatic acinar cell doublet. (i) Transmitted light picture shows the two connected cells with the secretory (zymogen) granules in the middle. (For a schematic view of the structure of pancreatic acinar cells see Fig. 3a). Region (R-1) in the basal part of cell A and region (R-2) in the granular area of cell B were selected for local bleaching. The length of the red bar represents 10 \(\mu\)m. (ii) Confocal image of Mag-fluo 4 fluorescence before bleaching. The cells had been exposed to Mag-fluo 4AM (membrane permeant form of low-affinity Ca\(^{2+}\)-sensitive fluorescent dye). The probe was therefore present both in the cytosol and the intracellular stores, but the optical properties of the dye are such that there is no fluorescence from compartments with a relatively low Ca\(^{2+}\) concentration, such as the cytosol\(^{21}\). In cell A it is seen that there is little fluorescence from a region in the basal part, which corresponds to the site of the nucleus. The nucleoplasm is connected to the cytosol via the nuclear pore complex, which are permeable to Ca\(^{2+}\). Because the cell is densely packed with ER, the best place to measure the cytosolic Ca\(^{2+}\) concentration is the nucleus (see also Fig. 3a). Region (R-1) in the basal part of cell A is bleached, the main decrease in the fluorescence intensity occurs in the granular region. There is only a minor decrease in the basolateral region. This point is explored in more detail in (b). (b) Double bleaching of the Mag-fluo 4 dye in the whole of the granular region is followed by diffusion of some non-bleached dye into parts of the granular area. (i) Transmitted light picture of single pancreatic acinar cell. Yellow dotted circle indicates area of bleaching. The length of the red bar represents a distance of 10 \(\mu\)m. (ii) Fluorescence image taken immediately after double bleaching. (iii) Fluorescence image (at same gain) taken 15 s later. The granular area has become slightly lighter and the basolateral area slightly darker reflecting movement of non-bleached dye from the basal to the apical granule-containing part and movement of bleached dye in the opposite direction. Although the apical area is dominated by granules, there are also tiny ER elements\(^{15}\) and the data presented here indicate a functional connection between these structures and the bulk of the ER in the basal region (see also schematic diagram in Fig. 3a). (c) Time course of Mag-fluo 4 movement in the ER of a pancreatic acinar cell. (i) Transmitted light picture of the cell under investigation. The area occupied by granules is clearly seen. The red and black circles show the two regions of interest. The length of the red bar represents 10 \(\mu\)m. (ii) Confocal image of Mag-fluo 4 fluorescence. The dark area in the upper part of the cell represents the nucleus. The dotted yellow circle, corresponding to the red circle in (i), is the site selected for local bleaching. (iii) Traces of fluorescence intensity from the black and red regions identified in (i). The effects of two local bleaching events are shown. After each event there is a sharp decrease in the fluorescence intensity in the local area of bleaching followed by a slower recovery as non-bleached dye from neighbouring regions diffuse into the bleached area. In the area represented by the black circle in (a), more than 10 \(\mu\)m away from the bleaching site, there is a slow decrease in the fluorescence intensity as non-bleached dye leaves the region to diffuse into the bleached area. After each bleaching event the red and black traces converge. The time constant of re-equilibration was about 2 s. Adapted from Ref. 42.

Suggesting that the ER also works as a single continuous Ca\(^{2+}\) store. Indeed, functional studies in hepatocytes indicated that this was the case\(^{26}\). To work as a dynamic Ca\(^{2+}\) store, the ER needs a luminal low-affinity Ca\(^{2+}\)-binding protein in addition to Ca\(^{2+}\) pumps and Ca\(^{2+}\)-release channels. In Purkinje neurones, the distributions of the major low-affinity Ca\(^{2+}\)-binding protein, calsequstrin, the SERCA and the ER-specific protein Bsp were mapped. The proteins were widely, but unequally, distributed in the cell body, the axon and the dendritic tree\(^{21}\). Calsequstrin was located in the ER lumen and was...
Evidence for separate ER Ca\(^{2+}\) stores: different Ca\(^{2+}\) concentrations in sub-compartments

In cultured astrocytes and arterial myocytes, it has been shown that the ER is heterogeneous, they did not address the issue of tubulo-vesicular continuity. Reese and his collaborators\(^\text{25}\) tackled this question directly in a masterful study of the spreading of a fluorescent lipophilic dye that diffuses within the ER membranes, showing that the ER in cerebellar Purkinje neurons is constituted as a continuous network.

Evidence for a fully connected ER network: movements of fluorescent substances in the ER lumen

The most direct way of testing the continuity or discontinuity of the ER lumen is to monitor the movements of various probes in this compartment. Subramanian and Meyer\(^\text{37}\) used a luminal GFP (green fluorescent protein) fusion protein and showed, in a tumour mast cell line and in fibroblasts, that such large molecules could diffuse within the ER lumen across the whole cell. Similar results were obtained by Dayel\(^\text{38}\) and collectively, these data indicate that the ER lumen is one continuous space.

It is advantageous to study ER Ca\(^{2+}\) transport characteristics in cells with a well-defined morphology, such as the polarized pancreatic acinar cell. In these cells the ER is densely packed and dominates the intracellular space outside the nucleus, except in the apical granular region in which the secretory (zymogen) granules are prominent\(^\text{39,40}\). In addition, the mitochondrial distribution is much more restricted compared with the ER distribution\(^\text{41}\). As shown in Fig. 1, studies of the movements of small fluorescent probes trapped in the ER lumen indicate that the whole of the ER is luminally connected, allowing widespread diffusion\(^\text{42}\). The ER cannot easily be fragmented, even prolonged supra-maximal stimulation with ACh failed to prevent widespread diffusion of the

Adjacent released Ca\(^{2+}\) asynchronously\(^\text{26}\). These studies imply that ryanodine receptors and SERCAs reside in separate portions of the ER, but how Ca\(^{2+}\) could be accumulated by the ryanodine-sensitive stores was unexplained. In addition, a study of intact HeLa cells indicated that sub-compartments of the ER with low and high luminal Ca\(^{2+}\) concentrations coexist\(^\text{27}\).

Studies with Ca\(^{2+}\)-sensitive fluorescent probes, trapped in the lumen of intracellular Ca\(^{2+}\) stores\(^\text{26}\), allow imaging with high spatial and temporal resolution. However, it is noteworthy that the probes might not only be present in the ER, but also in other Ca\(^{2+}\)-storing organelles. Such organelles, capable of accumulating and releasing Ca\(^{2+}\), include the mitochondria\(^\text{28,29}\) and endosomes\(^\text{30}\). The use of SERCA inhibitors has been important for many studies dealing with ER heterogeneities. Of the available SERCA blockers, thapsigargin is the safest choice\(^\text{11}\). This compound, in contrast to several other SERCA pump inhibitors, has no effect on passive Ca\(^{2+}\) permeability across the ER membranes\(^\text{36}\).

Enriched within small vacuoles that were also equipped with SERCAs. Some, but not all, of these vacuoles (calciosomes\(^\text{22,23}\)) contained Ins(1,4,5)P\(_3\) receptors\(^\text{14}\).

Although the biochemical and structural studies indicated that the ER is heterogeneous, they did not address the issue of tubulo-vesicular continuity. Reese and his collaborators\(^\text{25}\) tackled this question directly in a masterful study of the spreading of a fluorescent lipophilic dye that diffuses in continuous ER membranes, showing that the ER in cerebellar Purkinje neurons is constituted as a continuous network.

Evidence for separate ER Ca\(^{2+}\) stores: different Ca\(^{2+}\) concentrations in sub-compartments

In cultured astrocytes and arterial myocytes, it has been reported that the SERCA inhibitor, cyclopiazonic acid, and the Ca\(^{2+}\) releasing agent, caffeine, could liberate Ca\(^{2+}\) from different, spatially separate ER regions. Stimulation with glutamate caused the Ca\(^{2+}\) concentration to decrease in certain areas of the ER, but to increase in other parts. During Ca\(^{2+}\) oscillations, ER regions that appear to be

Fig. 2. Rapid movement of Ca\(^{2+}\) in the endoplasmic reticulum (ER) lumen following local uncaging of caged Ca\(^{2+}\): (a)(i) Transmitted light picture of pancreatic acinar cell under investigation with the colour coded regions of interest. The tip of a patch clamp pipette (whole cell configuration) is seen to the right. The cytosolic Ca\(^{2+}\) concentration was clamped at the resting level with the help of a BAPTA/Ca\(^{2+}\) mixture in the patch pipette solution. The cytosolic component of the caged Ca\(^{2+}\) compound NP-EGTA, which had been loaded into the cell in its membrane permeant form together with Mag-fluo 4, was washed out into the large volume of the patch pipette. (ii) and (iii) Two Mag-fluo 4 fluorescence images are also shown, before (ii) and after (iii) application of a supramaximal ACh concentration (10 µM). Two dark nuclei are seen. (iii) After ACh stimulation, there was a marked decrease in the fluorescence intensity from all extranuclear areas of the cell, except for the part occupied by the granules. This is caused by ACh-elicited release of Ca\(^{2+}\) from the ER. (b) Time course of the Ca\(^{2+}\) concentration changes in the ER, following local uncaging in the area marked by the dotted yellow circle in the lower fluorescence image in (a)(iii), in the various regions colour coded in the transmitted light picture in (a)(i). Uncaging of caged Ca\(^{2+}\) was carried out immediately after removal of ACh, so that the Ca\(^{2+}\)-release channels in the ER membrane were already closed, but before refilling of the store with Ca\(^{2+}\) had occurred. The whole-cell Ca\(^{2+}\)-dependent Cl\(^{-}\) current did not change after the intra-lumenal uncaging, demonstrating the effectiveness of the cytosolic Ca\(^{2+}\) clamp, which is also reflected in the absence of any rise in the nucleoplasma Ca\(^{2+}\) concentration. Adapted from Ref. 42.
fluorescent probe in the ER lumen. However, it is possible to fragment the ER in several cell types, including in pancreatic acinar cells, by prolonged treatment with the Ca\(^{2+}\)-ionophore ionomycin and under these circumstances the fluorescent probes do not diffuse in the ER lumen\(^{37,42}\). Such Ca\(^{2+}\)-induced ER fragmentation can be counteracted by protein kinase C activation\(^{43}\).

These diffusion studies on different cell types indicate that the free Ca\(^{2+}\) concentration in the ER lumen should equilibrate throughout the cell. However, as highlighted by Subramanian and Meyer\(^{37}\), buffering by Ca\(^{2+}\)-binding proteins could significantly slow ER luminal movements of Ca\(^{2+}\), enabling luminal Ca\(^{2+}\) gradients to be established. To what extent this occurs can only be determined by direct measurements of Ca\(^{2+}\) movements in the ER lumen.

The ER is one continuous Ca\(^{2+}\) pool: visualization of rapid Ca\(^{2+}\) movements and equilibration in the ER lumen

Figure 2 shows a direct approach to answering the question about the ability of Ca\(^{2+}\) to move within the ER lumen. In these experiments it was possible to uncage caged Ca\(^{2+}\) in a highly localized region of the ER luminal space. The Ca\(^{2+}\) concentration changes at the site of uncaging, in addition to many neighbouring sites up to a distance of 10 \(\mu\)m away, were monitored. This directly showed rapid Ca\(^{2+}\) movements inside the ER along a chemical gradient. As shown in Fig. 2, there was a sharp rise in the Ca\(^{2+}\) concentration in the ER lumen in the area of uncaging, followed by a decline to the previous level. In the immediate neighbouring area there was also a substantial, but smaller and slower, rise in the Ca\(^{2+}\) concentration, which subsequently declined back towards the resting level. In general, the further removed from the site of Ca\(^{2+}\) uncaging, the smaller and slower the rise in the Ca\(^{2+}\) concentration in the ER lumen. All areas were fully equilibrated, with a uniform Ca\(^{2+}\) concentration, within 10 s of the perturbation. In the experiment shown in Fig. 2, the cytosolic Ca\(^{2+}\) concentration was clamped chemically at the resting level to avoid the problems associated with changes secondary to the primary Ca\(^{2+}\) uncaging event, which could be a consequence of Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Uncaging of Ca\(^{2+}\) in the ER lumen therefore did not have any influence on the
Ca^{2+} concentration in the nucleoplasm, which, because of its connection with the cytosol via the nuclear pore complexes, reflects the cytosolic concentration.

Experiments of the type shown in Fig. 2, represent the strongest current evidence for a continuous ER lumen allowing Ca^{2+} diffusion throughout the extensions of the ER (ER Ca^{2+} tunnel function). So far such experiments have only been carried out on normal pancreatic acinar cells and it would be desirable to use this approach in many other preparations, particularly from the nervous system.

In general, the relative ease with which Ca^{2+} moves in the ER lumen and in the cytosol would depend on the relative Ca^{2+}-binding capacities in these two compartments and the relative mobilities of the various Ca^{2+}-binding proteins. In mouse pancreatic acinar cells, the Ca^{2+} binding capacity turned out to be considerably higher in the cytosol (~2000) than in the ER (~20) (Ref. 46). The cytosolic Ca^{2+}-binding capacity in rat cerebellar Purkinje cells was also high (~2000) (Ref. 47), whereas lower values were found in chromaffin cells and in invertebrate neurones. Comprehensive knowledge of the Ca^{2+}-binding capacity in the ER lumen of various cell types is lacking, and we do not have a full picture of the mobilities of the various Ca^{2+}-binding proteins in the cytosol and the ER.

Conclusions

Although different cell types might be organized differently, the textbook view that the ER 'forms a continuous sheet enclosing a single internal space' (Ref. 50) would appear, on the basis of the best currently available evidence, to be remarkably accurate. What advantages would Ca^{2+} signalling have, by constituting the ER as one continuous Ca^{2+} pool that allows rapid Ca^{2+} movements in the lumen? In pancreatic acinar cells, the Ca^{2+} binding capacity inside the ER is lower than in the cytosol (~2000) (Fig. 3a). Should the ER nevertheless become depleted of Ca^{2+}, it is possible, with the help of such a connected system, to refill the whole of this organelle system through store-operated Ca^{2+} channels and ER cisterns close to the plasma membrane. This can be carried out without a general elevation of the cytosolic Ca^{2+} concentration (ER Ca^{2+} tunnel from base to apex) and is important for the rapid termination of cytosolic Ca^{2+} signals after removal of a Ca^{2+}-releasing neurotransmitter. This mechanism, by which the cytosolic Ca^{2+} signal can be terminated without preventing ER Ca^{2+} reuptake, is important because the refilling process is slow.

In neuronal and glial systems there is at present no direct information about the movements of Ca^{2+} in the ER lumen. As indicated in Fig. 3b, we can imagine that it could be useful to refill depleted ER Ca^{2+} stores via the sub-surface cisterns, which might come as close as 20 nm to the plasma membrane. In this way, we would have a mechanism for refilling the entire neuronal ER store with Ca^{2+}, without any need for a general elevation of the cytosolic Ca^{2+} concentration. Separating, in time and space, the generation of cytosolic Ca^{2+} signals from the refilling of internal Ca^{2+} stores might be of considerable importance in neuronal systems.

It is possible that movements of Ca^{2+} inside the ER tubules that connect the spine apparatus to the dendritic ER (Fig. 3c) could be important for the local Ca^{2+} release in response to activation of glutamate receptors, which is required for LTD (Ref. 57). Ca^{2+} that is released from the spine apparatus, in response to local stimulation, could be replenished via connections to the dendritic ER, thus maintaining full local response capability (Fig. 3c).

The ER could also act as a Ca^{2+} sink. Local cytosolic Ca^{2+} signals, as a result of localized Ca^{2+} entry, could be buffered by SERCA-mediated uptake into the ER. A fully connected ER network would clearly enhance the capacity for local Ca^{2+} clearance, by distributing the Ca^{2+} taken up locally throughout the whole of the ER.

In general, it might be proposed that the ER lumen could provide an important pathway for information transfer mediated by Ca^{2+}. If the findings in pancreatic acinar cells, indicating that the Ca^{2+} binding capacity inside the ER is lower than in the cytoplasm, are also true for cells in the nervous system, then Ca^{2+} movement through the ER lumen could be an attractive route for communication, perhaps even between remote areas. As direct studies of the type recently carried out in pancreatic acinar cells become feasible in neuronal and glial systems, we shall no doubt begin to understand in some detail many more important aspects of the connected ER.

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