# Mini-Review

# The Heterogeneity of ER $Ca^{2+}$ Stores Has a Key Role in Nonmuscle Cell Signaling and Function

**Jacopo Meldolesi* and Tullio Pozzan‡**

*Department of Pharmacology, B. Ceccarelli Center of Neurobiology, University of Milano, CNR Center of Molecular and Cellular Pharmacology and Department of Neurosciences, DIBIT, Istituto Scientifico San Raffaele, 20132 Milano, Italy; ‡Department of Biomedical Sciences, CNR Center of Biomembranes, University of Padova, 35121 Padova, Italy

THE heterogeneity of the endoplasmic reticulum (ER) is well established in cell biology. To a large extent, however, the differences among ER domains (such as the nuclear envelope and the rough- and smooth-surfaced cisternae) are believed to reflect their different involvement in the synthesis, transport, and local degradation of proteins. In contrast, another function of the ER, i.e., its capacity to accumulate $Ca^{2+}$ and to release it quickly in response to specific signals (see reference 32), is often regarded as a property of the whole network. Yet in striated muscles, the heterogeneity of $Ca^{2+}$ handling in the sarcoplasmic reticulum (SR, a specialized version of the ER) was recognized over 20 yr ago. 10 yr later, based on pioneering subcellular fractionation and immunocytochemical results, a group of laboratories first proposed that structures specialized for $Ca^{2+}$ handling, the calciosomes, may also exist in nonmuscle cells (43). Shortly thereafter, a similar concept was put forth to explain the unusual (quantal) kinetics of $Ca^{2+}$ release from microsomes and permeabilized cells when exposed to increasing concentrations of inositol 1,4,5-trisphosphate ($IP_3$; reference 29). Since then, evidence of $Ca^{2+}$ specializations in the ER has grown continuously, yet little effort has been made to generalize the concept and to identify mechanisms not only in biochemical and molecular but also in cell biological terms.

Here, we reconsider critically the developments in the field, focusing primarily on nonmuscle cells. We will first address the nonrandom distribution of the various ER molecular components that sustain the $Ca^{2+}$ homeostatic machinery. We will then discuss the correlations between protein distribution and the complex spatio–temporal features of the $Ca^{2+}$ signaling pathway, ending with a short discussion of the underlying molecular mechanisms. In contrast, we will not deal with other organelles, such as secretory granules and lysosomes, which may also work as dynamic $Ca^{2+}$ storage compartments. Although repeatedly proposed, these possibilities remain open to question, so that any conclusion would be premature.

Address all correspondence to Jacopo Meldolesi, DIBIT, Istituto Scientifico San Raffaele, Via Olgettina 58, 20132 Milano, Italy. Tel.: 39 2 2643 2770. Fax: 39 2 2643 4813. E-mail meldolesi.jacopo@hsr.it

## *Molecular Components*

Heterogeneity of $Ca^{2+}$ storage and exchange within ER subcompartments depends on the nonrandom distribution of the macromolecules governing uptake, release, and binding of the cation.

***Channels.*** There are at least two types of channels through which $Ca^{2+}$ flows from the lumen of the ER to the cytosol: $IP_3$ and ryanodine receptors ($IP_3$Rs and RyRs), both composed of four subunits of high molecular mass (300–500 kD). Additional channels, known only in part, will not be discussed here.

Expression of $IP_3$Rs (three types, each including one or two alternatively spliced variants) is highly variable among cells. The quantity varies from 20,000 to 30 fmol/mg cell protein (Purkinje neurons and epididimus). Moreover, most cells express various types of $IP_3$R subunits assembled into heterotetramers (27). Recent experiments in intact cells have revealed that this multiplicity is reflected in variations in $IP_3$ affinity and in modulation of the release responses by cytosolic $Ca^{2+}$ ($[Ca^{2+}]_c$). In particular, the type II $IP_3$R was shown to be the most sensitive to $IP_3$ and also the most susceptible to modulation. In contrast, type III appears almost insensitive to $[Ca^{2+}]_c$. When composed by heterotetramers, the $IP_3$Rs tend to adopt the responses of the most sensitive/modulatable of their subunits (25).

Heterogeneous distribution of $IP_3$Rs throughout the ER was demonstrated by both subcellular fractionation and immunocytochemistry. In the late 1980s, $IP_3$-binding sites were reported to be enriched in rapidly sedimenting membrane fractions, caused not by plasmalemma fragments but by aggregation of specialized microsomes. In conventional microsomes $IP_3$ binding levels were much lower (see reference 32). More recently, Western blots of microsomal subfractions obtained by velocity and density gradient centrifugation have shown that $IP_3$Rs do partially segregate from the ER $Ca^{2+}$ pumps (sarcoplasmic–endoplasmic reticulum $Ca^{2+}$ ATPases [SERCAs]) and especially from the lumenal $Ca^{2+}$-binding proteins (35, 41). In cells overexpressing $IP_3$Rs (Purkinje neurons) or after cDNA transfection, the receptors appear as cylindrical structures, ~18 nm in diameter, sticking out from the membranes, concentrated (densities up to 500/$\mu m^2$, i.e., over 100-fold greater than the rest of the ER) in stacks of parallel cisternae (39, 44). In other cell types, $IP_3$R was concentrated in vesicles and cisternae distributed either in the juxtaplasmalemma

layer (smooth muscle fibers: references 7, 42; endothelia: reference 7) or in the apical area (submandibular duct cells: reference 48; pancreatic acinar cells: reference 19) of the cytoplasm. In blood neutrophils, a redistribution of $IP_3R$-rich ER vesicles towards the phagosome takes place after exposure to opsonized particles (37). Moreover, clustering of $IP_3Rs$ into discrete puncta, corresponding to enriched ER membrane areas, has been revealed in a variety of cells whenever their $[Ca^{2+}]_c$ was raised (47), documenting the dynamics of $IP_3R$ distribution.

Compared with the $IP_3Rs$, the information about RyRs in nonmuscle cells is still limited. There are three subtypes of RyRs, but they are assembled as homotetramers, not heterotetramers. Type I, believed for a long time to be specific to skeletal muscle, is abundant in the brain, while types II and III are widely but not ubiquitously expressed (1, 10). Direct coupling of RyRs with the plasmalemma L-type $Ca^{2+}$ channels, i.e., the interaction that drives the quick discharge of muscle SR, has been hypothesized in a few neurons showing ER cisternae parallel to the cell surface (8; see also reference 3). Elsewhere, activation of RyRs is probably triggered by rapid increases of the local $[Ca^{2+}]_c$ ($Ca^{2+}$-induced $Ca^{2+}$ release). Activation of type II and possibly also of type III is modulated by a messenger, cyclic ADP ribose.

Knowledge about RyR distribution is less extensive than for $IP_3Rs$, also because the levels of expression are lower and thus difficult to reveal, especially by immunocytochemistry. Except for the neuron superficial cisternae (8), high receptor concentrations, such as in muscle SR, have not been observed. Distribution, however, does not appear to be random, as documented by $[Ca^{2+}]_c$ imaging in many cell types, where discharge by caffeine (the alkaloid that activates RyRs) and $IP_3$ was shown to take place from different cisternae (12). At fine dendrites and dendritic spines of neurons, differential distribution of the two channels has been observed also by immunocytochemistry. In chicken Purkinje neuron spines, $IP_3Rs$ are numerous, whereas RyRs remain below detection (45). In contrast, RyRs are also abundant in the spines of hippocampal neurons (36), playing a considerable role in the shaping of dendritic action potentials (16).

***$Ca^{2+}$ Pumps.*** Accumulation of $Ca^{2+}$ within the stores is due to the SERCAs, the products of at least three genes (23). ER non-SERCA $Ca^{2+}$ pumps have also been reported but not characterized. Among SERCAs, type 1 and 2a are expressed at high levels in skeletal and heart muscle, respectively. SERCA 2b, an alternatively spliced version of 2a, is widespread and often coexists with type 3. These pumps maintain a high free $[Ca^{2+}]$ within the ER/SR lumen, in equilibrium with $Ca^{2+}$ bound to the lumenal proteins (resting values in the $10^{-4}$–$10^{-3}$ [free] and $10^{-2}$ [total] mol/liter, respectively: references 14, 26, 28, 31, 33).

The best information on SERCA distribution is in striated muscle, where the pump is known to occupy the SR surface, except for the junctional face occupied by RyRs. In a few nonmuscle cells, immunocytochemistry has shown a widespread distribution (20). However, subcellular fractionation has revealed dissociations of SERCAs 2a and 3 from both the $IP_3Rs$ and ER lumenal proteins (35, 41). Thus, it is likely that $Ca^{2+}$ uptake is not uniformly distributed but can vary considerably among ER domains.

***Lumenal $Ca^{2+}$-binding Proteins and Lumenal $Ca^{2+}$.*** Most ER resident proteins bind $Ca^{2+}$ with low affinity. They are thus able to buffer the $Ca^{2+}$ concentration within the lumen, $[Ca^{2+}]_l$, in the $10^{-4}$–$10^{-3}$ M range and to rapidly release their bound $Ca^{2+}$ when $[Ca^{2+}]_l$ drops, thus sustaining for seconds the efflux through $IP_3R$ and RyR channels. Accumulation at discrete sites could be important for the functioning of the ER $Ca^{2+}$ homeostatic machinery. So far, however, this property has been observed only for calsequestrin (CSQ), the major lumenal protein of striated muscle, which is also expressed by Purkinje neurons (avian) and smooth muscle fibers (42, 44). In nonmuscle cells, the distribution of other $Ca^{2+}$-binding proteins, such as calreticulin (which binds ~50% of the $Ca^{2+}$ store in the ER) and BiP, was found to be widespread by both subcellular fractionation and immunocytochemistry (31, 42). Surprisingly, however, when high-resolution maps were developed by either energy loss spectroscopy or electron probe x-ray microanalysis (applied to quick-frozen cells to preserve the in vivo localization of the cation), the distribution of total calcium within the ER lumen was found to be far from homogeneous, with calcium-rich cisternae in continuity with cisternae remaining below threshold (31; Fig. 1). In addition, long stimulations of neurons induced considerable loading of some cisternae, whereas others remained unaffected, as if they were not participating in the evoked $Ca^{2+}$ fluxes (33). In terms of $Ca^{2+}$ handling, therefore, the functional continuity of ER cisternae looks much less extensive than commonly believed. The apparent conflict with the results of rapid diffusion of ER lumenal proteins implies the existence of calcium segregation mechanisms, which, however, remain largely mysterious.

## *Cell Physiology*

In the past, distribution of $Ca^{2+}$-governing proteins and $[Ca^{2+}]_c$ events taking place within intact cells have often been investigated in parallel and interpreted together. Progressively, however, it has become clear that $[Ca^{2+}]_c$ responses do not result only from the activation of individual receptors but constitute coordinate processes in which localized initiation events are followed by the activation of autoregenerative $Ca^{2+}$ release (see references 2, 15, 32). The properties of these responses depend largely on the molecular properties and distribution of the proteins (especially the channels described above) governing the ER $Ca^{2+}$ homeostasis.

The events that follow channel openings are governed by two general rules: the slow rate of $Ca^{2+}$ diffusion throughout the cytosol and the positive modulation of most (but not all) $IP_3Rs$ and RyRs by $[Ca^{2+}]_c$. The first, caused by the high $Ca^{2+}$ buffering of the cytosol, tends to keep $[Ca^{2+}]_c$ increases in the proximity of the releasing organelles; the second imparts increased opening probability to adjacent channels. The resulting local $[Ca^{2+}]_c$ increases (spikes, puffs, or sparks) can be important not only as the trigger of waves, but also by themselves. In the case of mitochondria, the affinity of $Ca^{2+}$ uptake is low, in the micromolar range, and a high level is attained only in the proximity of active $IP_3Rs$. $Ca^{2+}$ accumulation by juxta-ER mitochondria can thus be rapid, resulting in strong, $Ca^{2+}$-dependent activation of key dehydrogenases and conse-

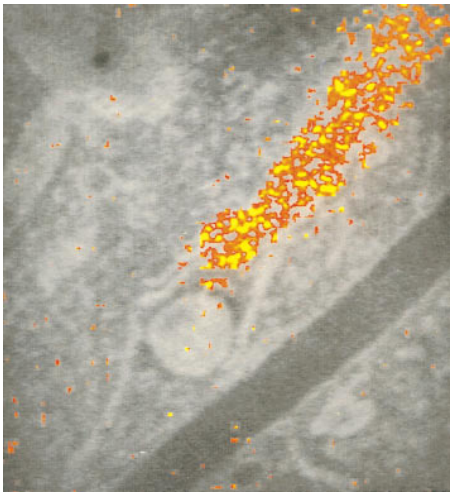

*Figure 1.* Distribution of total calcium in a PC12 pheochromocytoma cell, as revealed by electron energy loss imaging analysis. The calcium signal, expressed in false colors, is superimposed on a conventional image from a thin section of a quick frozen–freeze dried cell, including a mitochondrion and a few parallel running ER cisternae. Notice the calcium-rich ER cisternae in continuity with other cisternae where the signal remains below threshold, as it is the case with the mitochondrion and the plasmalemma. Reproduced with permission from Pezzati et al. (31).

quently more efficient ATP synthesis. Mitochondria thus change their role depending on their partnership with the ER, in line with a spatial regulation of cellular energy metabolism (34). The distribution of $Ca^{2+}$ spikes is also important with respect to the nucleus. Only the spikes generated in the proximity of the nucleus have high probability to invade the nucleoplasm, whereas most of those generated at some distance extinguish in the cytoplasm (22), with different effects on gene expression. Transcription of some genes is in fact controlled locally by $Ca^{2+}$ rises, whereas the $Ca^{2+}$ activation of others is indirect, mediated by cytoplasmic enzymes (e.g., the MAP kinases) or factors transferred to the nucleus in response to $[Ca^{2+}]_c$ rises. Thus, $Ca^{2+}$ regulation of gene expression can be defined as a space-dependent process (11). Even in the cytoplasm, differently localized $[Ca^{2+}]_c$ events can have different, even opposite effects. In smooth muscle fibers, the sparks in the deep cytoplasm contribute to the tone, whereas those near the plasmalemma can induce relaxation via activation of $Ca^{2+}$-dependent $K^+$ channels (30).

$Ca^{2+}$ waves expand from the initiating spike to the rest of the cell, inducing $Ca^{2+}$ release also from ER areas of low receptor density. Rhythmic repetition of the process can result in trains of oscillations. In pancreatic and salivary acinar cells initiation of $Ca^{2+}$ release takes place at the apical area, where $IP_3R$-rich vesicles (see before) are intermingled with secretory granules. The $Ca^{2+}$ signal thus generated can trigger two important responses: exocytosis of granules and generation of a wave directed towards the base (18, 40), sustained by the abundant, but $IP_3R$-poor, ER cisternae of that area (19).

News about $[Ca^{2+}]_c$ oscillations, which occur at frequencies around 1/min, are also exciting. Expression of single genes, mediated by the phosphorylation/dephosphorylation with ensuing nuclear-cytoplasmic migration of transcription factors, has been shown to vary specifically according to distinct $Ca^{2+}$ frequency codings (4, 21). Similar mechanisms regulate the action potential–dependent gene expression in cultured neurons (5).

## *Mechanisms*

This section is focused more on muscle because the information about nonmuscle cells is still primitive. Developmental studies of skeletal muscle fibers have revealed that clustering of L type $Ca^{2+}$ channels in the plasmalemma T tubules and of RyRs in the junctional face of SR terminal cisternae occur synchronously, establishing excitation–contraction coupling units (see reference 6). In animals and cells lacking either one of the channels, some clustering of the other still takes place. The master drive of the process, therefore, cannot be one of the channels (intracellular and surface located) but may be another as yet unidentified component (6). Coordinate clustering of SR and plasmalemma channels also occurs in the heart, however, without establishment of physical coupling (38).

Aggregation of CSQ appears to be a molecular property of the lumenal $Ca^{2+}$-binding protein (9, 46). Its localization within the terminal cisternae appears, in contrast, to be caused by the interaction of CSQ aggregates with membrane proteins protruding into the lumen, the triadins, and junctins (13, 17). The latter form a complex with the RyR and thus share its localization (49).

The information available for the SR might be taken as a model to orient the studies of nonmuscle cells. $IP_3Rs$, RyRs, and SERCAs are all known to interact with multiple proteins, at both their cytosolic and lumenal domains. So far, however, interest has been focused mostly on the functional regulation of these macromolecules rather than on their heterogeneous distribution, which therefore remains unexplained.

The hypothesis that $Ca^{2+}$ in nonmuscle cells is stored in distinct organelles (calciosomes), largely independently from the ER (43), has proven to be incorrect. The idea, however, has been seminal for subsequent studies demonstrating the heterogeneity of ER structures in terms of $Ca^{2+}$ homeostasis, a concept now widely accepted, but still incompletely understood. In the future, rapid progress is expected in many fields, including the identification of the mechanisms by which heterogeneity is established and the integration of ER $Ca^{2+}$ stores in cells physiology (see reference 24). From the information already available, we predict that the role of $Ca^{2+}$ within the stores will emerge as more fundamental for the cell than presently believed, paralleling from this stand point the well-known role played by the cation within the cytosol.

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