Subcellular Ca\textsuperscript{2+} Dynamics

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The field of subcellular Ca\textsuperscript{2+} homeostasis is evolving rapidly. In parallel with improvements in spatial and temporal resolution of Ca\textsuperscript{2+} imaging techniques, new methods using the natural cell machinery to target Ca\textsuperscript{2+}-sensitive proteins such as aequorin to precise intracellular locations promise superb specificity to measure [Ca\textsuperscript{2+}] in defined subcellular environments.

Cell activation is often triggered by an increase of the cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{c}). [Ca\textsuperscript{2+}]\textsubscript{c} is kept low (~10\textsuperscript{-7} M) at rest by the operation of Ca\textsuperscript{2+}-dependent ATPases, which pump Ca\textsuperscript{2+} from the cytosol either to the extracellular medium or into the intracellular calcium stores (ICS) where [Ca\textsuperscript{2+}] is kept far above [Ca\textsuperscript{2+}]\textsubscript{c} (~10\textsuperscript{-3} M) (Fig. 1). Different types of Ca\textsuperscript{2+} channels are present in the plasma membrane and in the endomembranes surrounding the ICS. At rest such channels are closed, but during cell activation they open, thus allowing Ca\textsuperscript{2+} to flow to the cytosol. Deactivation of Ca\textsuperscript{2+} channels is followed by extrusion of Ca\textsuperscript{2+} from the cytosol and quick return of [Ca\textsuperscript{2+}]\textsubscript{c} toward resting levels. Thus the transition from the resting condition to activity and to rest again is switched by redistribution of Ca\textsuperscript{2+} among the cytosol, the ICS, and the extracellular medium. Most of the cell calcium is stored inside the endoplasmic reticulum (ER) and the secretory granules. Mitochondria are also able to accumulate Ca\textsuperscript{2+}, but only when [Ca\textsuperscript{2+}]\textsubscript{c} increases above 10\textsuperscript{-6} M.

Why do cells need to mobilize Ca\textsuperscript{2+} from ICS if they have an infinite source of Ca\textsuperscript{2+} at high concentration in the extracellular medium? In recent times research on intracellular Ca\textsuperscript{2+} homeostasis has evolved from global (cellular) to local (subcellular) signaling, produced by activation of a few Ca\textsuperscript{2+} channels located near the effector of a given physiological action (4). Control of cell secretion illustrates this spatiotemporal specificity. The affinity of the secretory machinery for Ca\textsuperscript{2+} is low (10\textsuperscript{-5}-10\textsuperscript{-4} M), and, because of physical constraints imposed by diffusion and by intracellular Ca\textsuperscript{2+} buffering, these concentrations can
only be reached in the vicinity of $\text{Ca}^{2+}$ channels (9). ICS allow $\text{Ca}^{2+}$ release at both the required concentration and the precise location inside the cell. Under this view, a detailed knowledge of the spatial structure and dynamics of $\text{Ca}^{2+}$ inside intracellular organelles becomes essential to understand $\text{Ca}^{2+}$ signaling.

**Measurement of \([\text{Ca}^{2+}]\) inside the intracellular $\text{Ca}^{2+}$ stores**

Low-affinity fluorescent dyes, such as mag-fura 2, have been widely used to measure \([\text{Ca}^{2+}]\) inside ICS (6). A fraction of the dye becomes trapped inside intracellular organelles during loading, and the remainder is removed from the cytosol, usually by cell permeabilization or dialysis through a patch pipette before measurements. Prolonged incubation at 37°C also favors leakage of the cytosolic dye. After such maneuvers, the fluorescent signal comes preferentially from the organelles. Moreover, because of its low $\text{Ca}^{2+}$ affinity ($\sim 50 \, \mu\text{M}$), the dye remaining in cytosol or low-$\text{Ca}^{2+}$ organelles contributes little to the dynamic changes in the overall fluorescence, although it will certainly influence calibration and the final $[\text{Ca}^{2+}]$ estimates. Confocal microscopy has allowed adequate monitoring of nuclear $[\text{Ca}^{2+}]$ (11), but mitochondria and ER cisternae are more difficult to resolve.

Following the same strategies used by cells to send their own proteins to specific compartments, it is possible to target $\text{Ca}^{2+}$-sensitive proteins to specific subcellular locations and thereby monitor $[\text{Ca}^{2+}]$ in them. The use of aequorin, a $\text{Ca}^{2+}$-sensitive photoprotein from *Aequorea victoria*, was the first and still the most fruitful approach to this end. Aequorin has been successfully targeted to mitochondria (5, 13), nucleus (5), endoplasmic reticulum (3), and different locations in the cytosol (13). Targeting is achieved by constructing chimeric aequorin cDNAs in which either a minimal targeting sequence or a portion of a resident organellar protein is fused to the amino-terminal end of the photoprotein. For example, portions of the cytochrome oxidase, the glucocorticoid receptor, and the immunoglobulin heavy chain were used for mitochondrial matrix, nucleus, and ER targeting, respectively. Immunofluorescence or electron microscopy demonstrates in each case that the fusion protein is sent with high specificity to the desired subcellular location. Measurements of high $[\text{Ca}^{2+}]$ within the ER have been made possible by a two-step reduction of the $\text{Ca}^{2+}$ affinity of aequorin, combining the effects of mutation in one of the $\text{Ca}^{2+}$-binding sites and reconstitution with a semisynthetic prothetetic group (3). Among the advantages of aequorin, we should mention that luminescence can increase by a factor of up to 10³ in the presence of $\text{Ca}^{2+}$, thus providing a wide dynamic range for $[\text{Ca}^{2+}]$ measurements and high sensitivity to $[\text{Ca}^{2+}]$ changes. Calibration is also relatively simple (3). On the negative side, aequorin is irreversibly consumed during the experiments, thus limiting the measuring time in some cases, and photoluminescence is not well adapted for single-cell imaging. Even though some single-cell studies have been performed with targeted aequorin (6), expensive high-sensitivity detection techniques are required and spatial and temporal resolutions are still poor.

More recently, cameleon, a fusion protein containing two modified green fluorescent proteins (GFP), calmodulin, and the calmodulin-binding peptide M13, has been constructed (7). Binding of $\text{Ca}^{2+}$ to calmodulin makes it wrap around the M13 peptide and increases fluorescence resonance energy transfer between the two GFPs. Calmodulin mutations allow construction of cameleons with different $\text{Ca}^{2+}$ affinities, suitable for $[\text{Ca}^{2+}]$ measurements in different ranges. Cameleons have been successfully targeted to cytosol, nucleus, and ER (7). One of the advantages of cameleons over aequorins is that fluorescence is more suitable than photoluminescence for single-cell imaging. However, although this approach is promising, there is still little experience with cameleons and some of their properties may require further improvement before they can..."
replace aequorins. In particular, the change in fluorescence on Ca²⁺ binding is small, and the low-Ca²⁺ affinity cameleon designed to measure ER [Ca²⁺] (\([\text{Ca}^{2+}]_{\text{ER}}\)) has two dissociation constants, which makes calibration difficult.

Endoplasmic reticulum

ER [and the sarcoplasmic reticulum (SR) in muscle cells] is regarded as the most important ICS, at least in terms of Ca²⁺ signaling, because it contains a large amount of calcium and is able to exchange it quickly with the cytosol. Electron microscopy techniques reveal total calcium concentrations as large as 5–50 mM and the ⁴⁰Ca pools mobilized by inositol 1,4,5-trisphosphate (InsP₃) and/or the ER Ca²⁺ pump [sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA)] inhibitors amount to 0.3–1.5 mmol per liter of cells, corresponding to a total ER calcium concentration of 5–10 mM (6, 12). Most of this calcium is bound, mainly with low affinity (\(K_d 1–4 \text{ mM}\)), to calreticulin (calsequestrin in SR) and other Ca²⁺-binding proteins, some with known roles as chaperones. This large calcium content and high-capacity/low-affinity buffering suggests that the free \([\text{Ca}^{2+}]_{\text{c}}\) in the ER should be in the millimolar range. Reliable measurements in this range require the use of probes with a low affinity for Ca²⁺.

Ca²⁺ redistribution through the ER membrane on stimulation by inositol 1,4,5-trisphosphate (InsP₃)-producing agonists. A: stimulation by histamine or acetylcholine (ACh) results in the opening of the InsP₃ receptor (InsP₃R) channels and Ca²⁺ release from the ER. By the end of the stimulation sarcoplasmic and endoplasmic reticulum Ca²⁺-ATPase (SERCA) pumps Ca²⁺ inside ER again. Modulation of InsP₃R by \([\text{Ca}^{2+}]_{\text{c}}\) and \([\text{Ca}^{2+}]_{\text{ER}}\) is also indicated. B: simultaneous measurements of \([\text{Ca}^{2+}]_{\text{ER}}\) (with targeted aequorin) and \([\text{Ca}^{2+}]_{\text{c}}\) (with fura 2). HeLa cells with emptied Ca²⁺ stores were allowed to refill by addition of external Ca²⁺ and then stimulated with histamine. Original experiments by the authors. C: simultaneous measurements of \([\text{Ca}^{2+}]_{\text{ER}}\) (using mag-fura 2) and Ca²⁺-dependent Cl⁻ current (\(I_{\text{Cl}}\)) used here as an index of \([\text{Ca}^{2+}]_{\text{c}}\) in pancreatic acinar cells stimulated by ACh. Redrawn with permission from Ref. 8. The trace for \(I_{\text{Cl}}\) has been inverted. D: measurement of \([\text{Ca}^{2+}]_{\text{ER}}\) in a single HeLa cell transfected with yellow cameleon-4-er stimulated with histamine. Reprinted by permission from Ref. 7. Note in A and B the opposite changes of \([\text{Ca}^{2+}]_{\text{ER}}\) and \([\text{Ca}^{2+}]_{\text{c}}\) and the relatively rapid kinetics (faster for \([\text{Ca}^{2+}]_{\text{c}}\).

“ER . . . contains a large amount of calcium . . . .”
using wild-type aequorin and/or native coelenterazine had reported \([\text{Ca}^{2+}]_\text{ER}\) values that were grossly underestimated because of fast aequorin consumption and heterogeneity of \([\text{Ca}^{2+}]_\text{ER}\) within the ER (6). Fluorescent dyes usually report resting \([\text{Ca}^{2+}]_\text{ER}\) values around 100–300 \(\mu\)M (Ref. 6; Fig. 2C). Although lower, these values are in the same range as those obtained with ER-targeted aequorin. Moreover, difficulties in calibration and the presence of some dye in other organelles with low \([\text{Ca}^{2+}]_\text{c}\) such as mitochondria may lead to underestimation of \([\text{Ca}^{2+}]_\text{ER}\) (6). ER-targeted cameleons reported resting \([\text{Ca}^{2+}]_\text{ER}\) values of 60–400 \(\mu\)M in HeLa cells (Ref. 7; Fig. 2D).

Direct measurements of \([\text{Ca}^{2+}]_\text{ER}\) have allowed estimations of the rates of leak and filling of the ER (3, 8). Incubation of HeLa cells in \(\text{Ca}^{2+}\)-free medium promotes a quick decrease of \([\text{Ca}^{2+}]_\text{ER}\) with a half-time of 3 min at 37°C or 12 min at 22°C (3). Inhibition of SERCAs accelerates the emptying to half-times of 1–2 min (3). Full refilling of the ER after \(\text{Ca}^{2+}\) readdition requires 2–3 min at 37°C (Fig. 2) and 5–8 min at 22°C in several cell types studied (1, 3, 8). The same steady-state \([\text{Ca}^{2+}]_\text{ER}\) is reached at both temperatures, probably because uptake and leak have a similar thermal dependence (3). Refilling does not require an increase of the bulk \([\text{Ca}^{2+}]_c\) (Fig. 2). Even in 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetracetic acid (BAPTA)-loaded cells, in which there is essentially no change in \([\text{Ca}^{2+}]_c\) on \(\text{Ca}^{2+}\) readdition, the ER refills with \(\text{Ca}^{2+}\) nearly as fast as in the control cells not loaded with BAPTA (3). This may be attributed to local submembrane \([\text{Ca}^{2+}]_c\) increases, not detectable in the bulk \([\text{Ca}^{2+}]_c\), or to the capacity of SERCA to pump \(\text{Ca}^{2+}\) even at resting \([\text{Ca}^{2+}]_c\) provided that \(\text{Ca}^{2+}\) entry through the plasma membrane makes available enough calcium to fill the ER. On the other hand, it is remarkable that the rate of \(\text{Ca}^{2+}\) leak from ER is comparable in magnitude to the maximum rate of uptake through SERCA (3, 8). This indicates that there is a fast and continuous energy-expending futile cycle of \(\text{Ca}^{2+}\) in and out of the ER, even in the resting condition, which may result in the loss of up to 3% of the cell energy production (12). Both the physiological role and the molecular substrate for the leak are unknown. This may perhaps be an unavoidable consequence of the operation of the molecular machinery required for the transport of nascent ER-proteins across the ER membrane, the so-called translocon.

SERCAs take up 2 cytosolic calcium ions for every ATP hydrolyzed (12). For a typical free energy of hydrolysis of ATP of 50–60 kJ/mol, the thermodynamic limit for \(\text{Ca}^{2+}\) accumulation into ER/SR would be of 4–5 orders of magnitude, equivalent to a \([\text{Ca}^{2+}]_c\) of 1–10 mM for a \([\text{Ca}^{2+}]_c\) near 100 nM. However, the efficiency of free energy transformation can never reach 100%, and the large \(\text{Ca}^{2+}\) leak from the ER contributes to decreasing it. It has been shown that SERCAs use the free energy of ATP to accumulate \(\text{Ca}^{2+}\) with an apparent efficiency of only 74% in isolated cardiac microsomes (14). This corresponds to a maximum distribution ratio \([\text{Ca}^{2+}]_\text{ER}/[\text{Ca}^{2+}]_c\) of ~7,000, equivalent to a \([\text{Ca}^{2+}]_\text{ER}\) of 700 mM. This value is close to those reported recently with ER-targeted aequorin, and this suggests that the resting \([\text{Ca}^{2+}]_\text{ER}\) may be regulated just by thermodynamic constraints. If this were the case, it would be strongly affected by the metabolic state, and low-energy conditions, such as those occurring during hypoxia, would lead to a substantial decrease. For example, \(\Delta G_{\text{ATP}}\) within cardiomyocytes drops from 59 to 47 kJ/mol after oxidative phosphorylation blockade, a reduction that would lead to a sevenfold decrease in \([\text{Ca}^{2+}]_\text{ER}\) (14). Alternatively, it has been proposed that \(\text{Ca}^{2+}\) uptake through SERCAs may be negatively modulated by \([\text{Ca}^{2+}]_\text{ER}\) (8), a mechanism that would allow a much finer control of \([\text{Ca}^{2+}]_\text{ER}\). Further work will be needed to unravel the mechanisms that control the steady-state \([\text{Ca}^{2+}]_\text{ER}\).

Molecular studies have revealed ER heterogeneity in terms of the subcellular distribution of the different components of the machinery responsible for \(\text{Ca}^{2+}\) uptake, binding, and release (12). Whether this is important for the \(\text{Ca}^{2+}\) storage function and whether it may lead to differences in \([\text{Ca}^{2+}]_\text{ER}\) among different subcompartments are questions that remain unanswered. High-resolution total calcium mapping in PC12 cells by electron-loss spectroscopy has revealed large heterogeneities, with strong positive cisternae lying in the proximity of or even in direct continuity with other apparently negative cisternae (6). On the other hand, large molecules, such as ER-targeted GFPs, diffuse rapidly across the luminal storage space defined by the ER and the nuclear envelope membranes in the intact cell (15). This structural continuity is preserved during short cytosolic \(\text{Ca}^{2+}\) pulses, but persistent (>10 min) elevations of \([\text{Ca}^{2+}]_c\) lead to reversible vesiculation and prevent luminal \(\text{Ca}^{2+}\) diffusion. This luminal continuity suggests that no gross heterogeneity in \([\text{Ca}^{2+}]_\text{ER}\) should be expected under resting conditions. Aequorin is a very sensitive tool for detecting \([\text{Ca}^{2+}]_c\) heterogeneities. Its consumption rate is proportional to \([\text{Ca}^{2+}]_c\), and \([\text{Ca}^{2+}]_\text{ER}\) heterogeneities should be evidenced by an abrupt decrease of the relative rate of light output once aequorin inside high-[\(\text{Ca}^{2+}\)] areas has been consumed. The fraction of unconsumed aequorin at this time gives us an estimate of the size of the ER space with low \([\text{Ca}^{2+}]_c\). These kinds of measure-
ments have shown that more than 95% of the ER has a high {[Ca$^{2+}$]}$_{c}$, in the millimolar range (3).

Even if {[Ca$^{2+}$]}$_{c}$ was homogeneous under resting conditions, some degree of heterogeneity could appear after cell stimulation if Ca$^{2+}$ release occurred preferentially at defined subcellular locations. Ca$^{2+}$ release takes place through two families of Ca$^{2+}$ channels, In$P_{3}$ receptors (InsP$_{3}$R), and ryanodine receptors (RyR). Both share homologies in the primary structure, particularly in the transmembrane domain (12), and they are both modulated by {[Ca$^{2+}$]}$_{c}$ and {[Ca$^{2+}$]}$_{ER}$ (4, 6, 12). Modulation by {[Ca$^{2+}$]}$_{c}$ is bell shaped, although in a different range of concentrations for both receptors. Activation by {[Ca$^{2+}$]}$_{c}$ takes place below 1-2 µM for the InsP$_{3}$R or below 100 µM for the RyR, whereas higher concentrations are inhibitory. Activation of RyR by {[Ca$^{2+}$]}$_{c}$ constitutes the classical Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) mechanism that follows Ca$^{2+}$ entry through voltage-dependent Ca$^{2+}$ channels in cardiac muscle cells. Evidence for the presence and the role of CICR in nonmuscle excitable cells is difficult to interpret because of the difficulties in separating the contributions of Ca$^{2+}$ release and Ca$^{2+}$ entry to the {[Ca$^{2+}$]}$_{c}$ signal and the nonspecificity of some pharmacological agents used to activate RyR, such as caffeine. Direct measurements of {[Ca$^{2+}$]}$_{ER}$ during CICR will help answer this question. The concept of CICR is also sometimes used to refer to the activation of In$P_{3}$R by {[Ca$^{2+}$]}$_{c}$. Although different from the classical cardiac CICR, this mechanism may be the substrate for regenerative Ca$^{2+}$ release underlying phenomena such as {[Ca$^{2+}$]}$_{c}$ oscillations and waves (4). On the other hand, inhibition of In$P_{3}$R by local microdomains of high {[Ca$^{2+}$]}$_{c}$ appears to be a very important factor limiting Ca$^{2+}$ release (3) and may also be essential for shaping Ca$^{2+}$ oscillations and waves. The modulatory effect by the luminal {[Ca$^{2+}$]}$_{c}$ is more controversial, although there is much evidence suggesting that high {[Ca$^{2+}$]}$_{ER}$ facilitates activation of Ca$^{2+}$ release through both RyR and In$P_{3}$R. As a corollary, low {[Ca$^{2+}$]}$_{ER}$ would limit Ca$^{2+}$ release, thus avoiding complete Ca$^{2+}$ depletion of the ER, a condition that may induce processes leading to cell damage or death (see below).

Both In$P_{3}$Rs and RyRs coexist in many cell types. Some years ago the general belief was that In$P_{3}$Rs were almost universally distributed whereas RyRs were mostly restricted to muscle cells. However, the presence of RyRs has been detected now in many tissues, including nonexcitable cells (12). The reasons for the presence of multiple Ca$^{2+}$-release mechanisms in the same cell are not clear. It has been proposed that they could be activated by two different signaling pathways leading to the production of two different second messengers, In$P_{3}$ for the In$P_{3}$R and cyclic adenosine diphosphate ribose (cADPR) for the RyR. Although In$P_{3}$R is a well-established physiological activator, the role of cADPR as activator or modulator of RyR in the presence of physiological concentrations of ATP remains controversial. On the other hand, it has been proposed that In$P_{3}$S and cADPR could release Ca$^{2+}$ from separate compartments of the ICS, thus conferring on both messengers a different physiological significance. There is extensive but controversial information regarding the presence of separate (or common) Ca$^{2+}$ pools responsive to In$P_{3}$S or to caffeine or cADPR (taken usually as RyR activators, although this is also controversial). However, details on the precise subcellular distribution of these receptors are only known in a few cases, such as the concentration of RyR in the junctional membrane of the terminal cisternae of skeletal muscle cells, where they contact physically with the dihydropyridine receptors of the transverse tubules (6). Much more ultrastructural and functional information is needed to define the relationships between these Ca$^{2+}$-release channels and their physiological role.

[Ca$^{2+}$]$_{ER}$ is also involved in the activation of the plasma membrane store-operated Ca$^{2+}$ channels (SOC, also called capacitative Ca$^{2+}$ entry and, since the first patch-clamp studies, CRAC, from Ca$^{2+}$ release-activated channels; Ref. 10). SOC open when [Ca$^{2+}$]$_{ER}$ decreases, but the signaling pathway leading from the ER to the plasma membrane is unknown. There have been two main hypotheses in dispute for the last decade. One of them proposes that there may be a physical contact between an integral ER protein (perhaps the In$P_{3}$R) and the SOC, and the other suggests that there may be a soluble messenger produced in the ER on Ca$^{2+}$ depletion that diffuses to the plasma membrane and activates Ca$^{2+}$ entry. The first concrete proposal of a putative messenger, a product of cytochrome P-450, was made in 1991. Since then, many other possible messengers have been proposed (10). At the present moment, we must say that no definitive evidence exists for any particular mechanism of activation of SOC.

Depletion of Ca$^{2+}$ of the ER also induces several actions unrelated to Ca$^{2+}$ homeostasis (6). Protein synthesis is inhibited by phosphorylation of the initiation factor 2a, while the expression of several proteins related to the stress response is induced. Membrane traffic between the ER and Golgi compartments is also affected, and nucleocytoplasmic transport of molecules >500 kDa is blocked. Finally, depletion of Ca$^{2+}$ of the ER has been shown to induce apoptosis independently of the behavior of [Ca$^{2+}$]$_{c}$. The mechanisms responsible for these actions are unknown, but...
these findings suggest that $[\text{Ca}^{2+}]_{\text{ER}}$ is important not only for releasing Ca$^{2+}$ to the cytosol, but also by itself, as a general requirement essential for the integration of many different cellular functions.

**Mitochondria**

Studies with aequorin targeted to mitochondria have shown conclusively that it has a resting $[\text{Ca}^{2+}]$ in the same range as the cytosol (5, 13). Therefore, these organelles are not Ca$^{2+}$ stores under resting conditions and they cannot contribute to the release of Ca$^{2+}$ during cell stimulation. However, they can take up Ca$^{2+}$ when $[\text{Ca}^{2+}]_{c}$ increases. The behavior of mitochondria is quite particular in this respect. The membrane potential across the inner mitochondrial membrane ($-180$ mV) provides a strong driving force for Ca$^{2+}$ entry and accumulation in the matrix. Ca$^{2+}$ uptake takes place through a ruthenium red-sensitive uniporter that moves Ca$^{2+}$ according to the electrochemical gradient. This would mean a matrix $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_{M}$) of about 6 orders of magnitude above $[\text{Ca}^{2+}]_{c}$ at thermodynamic equilibrium (around 100 mM). However, the resting $[\text{Ca}^{2+}]_{c}$ is well below the Ca$^{2+}$ affinity of the uniporter, keeping Ca$^{2+}$ influx slow. On the other hand, the operation of two electroneutral exchangers, the 2Na$^{+}$/Ca$^{2+}$ and the 2H$^+$/Ca$^{2+}$ antiporters, prevents any Ca$^{2+}$ accumulation in the matrix in the resting condition (Fig. 3A). When $[\text{Ca}^{2+}]_{c}$ increases, the bulk cytosol may reach an average value around 1 $\mu$M, a value still too low to produce much Ca$^{2+}$ uptake by mitochondria, but a much higher $[\text{Ca}^{2+}]_{c}$ is produced in some local areas near the Ca$^{2+}$ channels of the plasma membrane or of the ER. These hot spots of $[\text{Ca}^{2+}]_{c}$ induce in the mitochondria nearby a rapid Ca$^{2+}$ accumulation leading to $[\text{Ca}^{2+}]_{M}$ much higher than the mean $[\text{Ca}^{2+}]_{c}$ (3,13). Then, as the hot spot of $[\text{Ca}^{2+}]_{c}$ dissipates, mitochondria rapidly extrude Ca$^{2+}$ through the antiporters, so that $[\text{Ca}^{2+}]_{M}$ returns to resting values (Fig. 3B). This mechanism is intimately

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"Therefore, these organelles are not Ca$^{2+}$ stores under resting conditions..."
related to the ultrastructural relationship between mitochondria and the Ca\(^{2+}\) channels, either at the plasma membrane or at the ER. Recent data obtained with GFP targeted to mitochondria suggest that they form a highly interconnected and dynamic network, which, in some areas, is in close contact with the ER (13). This emphasizes the importance of the subcellular architecture and the spatial relationship among different organelles for Ca\(^{2+}\) homeostasis and Ca\(^{2+}\) signaling. The portion of the mitochondrial network closer to ER will be exposed to a higher [Ca\(^{2+}\)]\(_{m}\), thus leading to a greater increase in [Ca\(^{2+}\)]\(_{m}\). In conclusion, regarding [Ca\(^{2+}\)]\(_{c}\) signaling, mitochondria can be considered under physiological conditions as a transient Ca\(^{2+}\) buffer able to delay cytosolic Ca\(^{2+}\) diffusion after Ca\(^{2+}\) release or Ca\(^{2+}\) entry (2, 5, 13).

The effect of [Ca\(^{2+}\)]\(_{m}\) increase on the mitochondrial function is probably even more important. Changes in [Ca\(^{2+}\)]\(_{m}\) seem large enough to activate mitochondrial dehydrogenases, with the ensuing activation of electron transport and oxidative phosphorylation. This mechanism may be designed to increase ATP production to cover the increased cell need after cell stimulation. Finally, if [Ca\(^{2+}\)]\(_{c}\) undergoes a prolonged increase, e.g., under pathological conditions such as ischemia, accumulation of Ca\(^{2+}\) inside mitochondria may occur, thus leading to increased production of oxidative radicals, opening of the mitochondrial permeability transition pores, and cell damage, including apoptosis. This kind of physiopathological mechanism has been proposed to be involved in the phenomena of ischemia-reperfusion and in the development of some degenerative diseases.

**Nucleus, secretory granules, and other subcellular locations**

Nucleoplasmic [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{n}\)) has been proposed to play a role in the regulation of several key nuclear processes, such as gene expression, breakdown of the nuclear envelope, or apoptosis. Resting [Ca\(^{2+}\)]\(_{n}\) is in the same range as the [Ca\(^{2+}\)]\(_{c}\) (100–200 nM), as seen with both nuclear-targeted aequorin (5) or confocal imaging with fluorescent dyes (11). After cell stimulation, [Ca\(^{2+}\)]\(_{n}\) follows the same pattern as [Ca\(^{2+}\)]\(_{c}\), suggesting that Ca\(^{2+}\) rapidly diffuses from the cytosol to the nucleus through the nuclear pores (Fig. 3, A and C). Under some circumstances, however, it has been proposed that [Ca\(^{2+}\)]\(_{n}\) may behave differently from [Ca\(^{2+}\)]\(_{c}\). In particular, it has been proposed that the nuclear envelope may have Ca\(^{2+}\) release channels, either InsP\(_{3}\)R or RyRs, looking toward the nucleoplasm, so that they may directly release Ca\(^{2+}\) into the nucleus (11). This would allow a more precise and independent regulation of [Ca\(^{2+}\)]\(_{n}\), but more information is still needed, on both the structural and the physiological side.

Secretory granules contain large amounts of Ca\(^{2+}\), and it has been suggested that functional InsP\(_{3}\)Rs and RyRs could be present in their membranes (11). Such a positive feedback would provide a mechanism for sharpening the Ca\(^{2+}\) threshold for exocytic secretion, but this attractive hypothesis remains controversial (12). Secretory granules also accumulate large amounts of other divalent cations, notably Zn\(^{2+}\), but their possible mobilization and interactions with the secretory machinery during cell activation have been scarcely investigated.

Apart from intracellular organelles, targeted Ca\(^{2+}\)-sensitive proteins have also been used to study [Ca\(^{2+}\)] in defined subcellular cytosolic environments. The spatial resolution of the imaging techniques does not allow measurement of the [Ca\(^{2+}\)] at precise subcellular regions such as the inner side of the plasma membrane, the outer side of the inner mitochondrial membrane, or the outer side of the membrane of the secretory granules. [Ca\(^{2+}\)] in all these regions has been explored using specifically targeted aequorin, and valuable information has been obtained regarding the presence of microdomains with [Ca\(^{2+}\)] higher than that in the bulk cytosol (13). We may therefore envisage that in the near future additional targeted Ca\(^{2+}\)-sensitive proteins will be sent to specific locations to obtain a map of subcellular [Ca\(^{2+}\)].

**Conclusion**

The dynamics of [Ca\(^{2+}\)] inside intracellular organelles, particularly ER and mitochondria, is becoming increasingly important to the understanding of cellular Ca\(^{2+}\) homeostasis. The ER is now recognized to be the main cellular Ca\(^{2+}\) store, and details on the subcellular structure of the ER, its structural and functional relationship with other organelles, and the precise distribution of the proteins involved in Ca\(^{2+}\) uptake and release are now required to complete the picture of subcellular Ca\(^{2+}\) dynamics. Moreover, recent evidence suggests that [Ca\(^{2+}\)]\(_{ER}\) controls multiple signaling pathways outside the ER, some of them essential for cell survival. Similarly, [Ca\(^{2+}\)]\(_{m}\) is probably a key regulator of mitochondrial function and may lead to cell damage in case of Ca\(^{2+}\) overload. Therefore, the availability of techniques to measure [Ca\(^{2+}\)] inside organelles is revealing several new important roles for Ca\(^{2+}\) as a second messenger and, in the near future, may throw new light on different physiological and pathological processes.
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