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Calcium signalling: a historical account, recent developments and future perspectives

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Abstract. Ca^{2+} is a uniquely important messenger that penetrates into cells through gated channels to transmit signals to a large number of enzymes. The evolutionary choice of Ca^{2+} was dictated by its unusual chemical properties, which permit its reversible complexation by specific proteins in the presence of much larger amounts of other potentially competing cations. The decoding of the Ca^{2+} signal consists in two conformational changes of the complexing proteins, of which calmodulin is the most important. The first occurs when Ca^{2+} is bound, the second (a collapse of the elongated protein) when interaction with the targeted enzymes occurs. Soluble proteins such as calmodulin contribute to the buffering of cell Ca^{2+}, but membrane intrinsic transporting proteins are more important. Ca^{2+} is transported across the plasma membrane (channel, a pump, a Na^+/Ca^{2+} exchanger) and across the membrane of the organelles. The endoplasmic reticulum is the most dynamic store: it accumulates Ca^{2+} by a pump, and releases it via channels gated by either inositol 1,4,5-trisphosphate (IP_3) and cyclic adenosine diphosphate ribose (cADPr). The mitochondrion is more sluggish, but it is closed-connected with the reticulum, and senses microdomains of high Ca^{2+} close to IP_3 or cADPr release channels. The regulation of Ca^{2+} in the nucleus, where important Ca^{2+}-sensitive processes reside, is a debated issue. Finally, if the control of cellular Ca^{2+} homeostasis somehow fails (excess penetration), mitochondria 'buy time' by precipitating inside Ca^{2+} and phosphate. If injury persists, Ca^{2+}-death eventually ensues.

Key words. Calcium; second messengers; signalling.

Introduction: origin and development of the concept of Ca^{2+} signalling

The birth of the idea of Ca^{2+} as a carrier of signals is traditionally traced back to the observation made by S. Ringer well over 100 years ago [1] that isolated hearts could only be made to contract if Ca^{2+} was present in the perfusion medium. It is significant that the first experiments showing a signalling role of Ca^{2+} were performed on muscles. For a long time muscle was the favorite, if not the only, playground for Ca^{2+}-centered research, so much so that Ca^{2+} came to be considered a specific factor for muscle contraction. The demonstration that the effect of Ca^{2+} was intracellular and cation-specific, which only came after a long interval of time, also emerged from muscle work performed in 1947 by Heilbrunn and Wiercinski [2]. They elicited the contraction of frog muscles by injecting Ca^{2+} into them, whereas no contraction followed the injection of Na^+, K^+ or Mg^{2+}. While these results unequivocally linked intracellular Ca^{2+} to muscle contraction, the extension of the concept of Ca^{2+} signalling to processes other than muscle contraction was slow in coming. In 1973 Miledi [3] induced neurotrasmitter release by injecting Ca^{2+} into the presynaptic terminal of squid axons, and over the next few years responses were elicited by others by injecting Ca^{2+} into mastocytes [4], salivary gland cells [5] or oocytes [6]. Cellular regulation by Ca^{2+} naturally demands its control in the intracellular environment, then, in parallel with these studies, reports began to appear in the 1960s that linked the control of the Ca^{2+} signals to the reversible complexation of the cation by specific proteins, and gradually made clear that cells possessed soluble systems (i.e. proteins) that buffered Ca^{2+} and also—and espe-

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cially—membrane-linked protein systems that rapidly and efficiently shifted its concentration in given cell domains by transporting it across membrane-boundaries. The membrane-intrinsic Ca^{2+}-transporting systems—plasma membrane, mitochondria, endoplasmic reticulum, nucleus—will be critically described in later sections. A succinct discussion of the soluble (or non-membrane intrinsic) Ca^{2+}-binding proteins, which are crucial to the control of the signalling function of Ca^{2+}, since they process its information, will precede the description. The discussion will help understand the evolutionary rationale of the choice behind Ca^{2+} as a universal cell messenger.

Ca^{2+}-binding proteins

In principle, at least four cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) would have been abundantly available in the environment when the necessity arose of regulating cell activity. It would be reasonable to assume this to have coincided with the emergence of multicellular organisation, which brought with it the division of work among cells and thus the necessity for transmission of messages: this was at variance with monocellular organisms, which essentially only competed for nutrients. Since the essence of regulation is reversibility, the element chosen as a carrier of information had to have properties permitting the rapid and reversible change of its concentration in the environment of the (cell) targets of the message.

In the case of the four simple ions mentioned above, reversible complexation by ligands having sufficient structural complexity to ensure specificity of interaction (i.e. proteins) would evidently be the ideal mechanism to achieve the goal: the unique fitness of Ca^{2+} as a biological messenger, which becomes clear if one considers its chemistry vis-à-vis that of the three other cations, in fact made its evolutionary choice obligate. This is so because the combination of size and charge allows Ca^{2+} to interact with the coordinating (oxygen) atoms of the binding site in the complexing protein with a large variability in bond length and angle, and thus makes it fit to accept binding cavities of very irregular shapes, such as those one necessarily expects in proteins. This becomes particularly evident if one compares Ca^{2+} with Mg^{2+}: the latter is much smaller and thus tends to attract much more strongly the coordinating (oxygen) atoms of the binding cavity. As a result, Mg^{2+} demands perfectly octahedral cavities, which evidently do not exist in proteins.

Proteins able to bind Ca^{2+} with the affinity and specificity required for the regulation of its concentration in the intracellular environment as a rule belong to the so-called EF-hand family [7]. The family now contains hundreds of members: some are committed to the regulation of only one Ca^{2+}-dependent process (enzyme), e.g. recoverin, troponin C, whereas others are not target-specific. The most important and most thoroughly studied EF-hand protein is undoubtedly calmodulin. The structural principles underlying its Ca^{2+}-binding function (and that of all other proteins of the family) which had been originally extrapolated from the crystal structure of parvalbumin [8] were later confirmed by the solution of its three-dimensional (3D) structure [9]. According to these principles, domains made of two orthogonal α-helices interrupted by a 10–12 amino acid loop coordinate Ca^{2+} to oxygens of carboxylic side chains and (more rarely) to carbonyl and water oxygens. This helix-loop-helix Ca^{2+}-binding motif, which is normally repeated several times in the molecule, has been optimised in the course of evolution, and it is now found in hundreds of proteins, some of them even extracellular. Since these motifs are used to bind Ca^{2+}, EF-hand proteins are evidently used to control its free intracellular concentration. Cells contain up to 10 μM calmodulin and 80 μM troponin C [10], i.e. amounts which can buffer impressive amounts of Ca^{2+}, considering that one molecule of calmodulin (or of troponin C) binds four Ca^{2+} atoms. Important as it may be, however, the Ca^{2+} buffering function of EF-hand proteins is secondary only to their ability to process the Ca^{2+} signal and to transmit its information to targets. The essence of the decoding process [11, 12] is a double conformational change of the protein (calmodulin). The first, which does not alter the general shape of the molecule, exposes hydrophobic domains on its surface upon binding Ca^{2+}, enabling it to interact with targets. The second collapses the elongated structure of the protein around its binding domain in the target enzyme, completing the processing of the Ca^{2+} signal (fig. 1).

Clearly, the decoding of the Ca^{2+} signal by EF-hand proteins is a sophisticated operation which goes beyond the relatively uncomplicated process of reversibly binding Ca^{2+} for mere buffering purposes. A quantitative argument must also be considered: the total cellular amount of EF-hand proteins (and of other types of Ca^{2+}-binding proteins which are not discussed in this contribution, e.g. annexins), however large, is still finite and could in principle become inadequate to buffer large swings in Ca^{2+} concentration generated by physiological stimuli in given cell domains. Clearly, these quantitative restrictions do not apply to membrane-intrinsic Ca^{2+}-binding (and transport) proteins, which complex Ca^{2+} at one membrane side, transport it across and discharge it at the other side, continuously repeating the operation. These proteins thus play the most important role in the buffering of intracellular Ca^{2+}, satisfying both the demands for rapid and high-

affinity regulation, i.e. for the fine tuning of cell Ca^{2+} and for lower affinity regulation. For high-affinity Ca^{2+} regulation, cells depend solely on adenosine triphosphate (ATP)-driven pumps; for low-affinity regulation they have several options: exchangers, channels and (in mitochondria) electrophoretic uniporters (fig. 2).

Prior to describing these transporters, important developments that have made possible the precise measurement of free Ca^{2+} in cells, and in their subcompartments, must be briefly discussed. Two techniques which have rapidly become of general use have permitted spectacular advances. In the first, fluorescent indicators (quin-2 molecule was the precursor), whose basic structure is an acetoxymethyl tetraester of a Ca^{2+} chelator, are allowed to diffuse into the cell to be hydrolysed inside by esterases which regenerate the four COOH groups and thus trap the chelator inside [13]. A fluorophore group associated with the carboxylic group confers to the molecule fluorescent properties sensitive to the binding of Ca^{2+} to the carboxylic cage. The first generation of these indicators was later chemically modified [14] to permit their optimal use in physiological studies. Thanks to the greater molar fluorescence intensity of the new generation of indicators, it became possible to utilise smaller amounts of them, thus reducing their cytosolic Ca^{2+} buffering effect: concentrations of the widely used fura2 10 times lower than those originally used for quin-2 are sufficient for accurate measurements. The new generation of Ca^{2+} indicators have lower Ca^{2+} affinity, making them better suited to monitor the Ca^{2+} concentrations in the range of 1–3 μM, which are normally reached in the cytoplasm of stimulated cells. Finally, binding of Ca^{2+} to the indicator not only changes its fluorescence intensity but also induces a spectral shift: i.e. the free and the Ca^{2+}-bound form of the dye have different spectral characteristics. Since the Ca^{2+} concentration can be calculated from the ratio between the fluorescence intensities at the two wavelengths, the measurement becomes independent of the absolute quantity of indicator loaded into the cells. This becomes important in single-cell experiments where the loading of the dye may be inhomogeneous in the various cell regions.

In the second technique, cloning of the coelenterate photoprotein aequorin has allowed its use as a specific probe for the measurement of $[Ca^{2+}]$ in individual organelles. When cells are transfected with the complementary DNA (cDNA) coding for aequorin, the photoprotein is expressed constitutively within the cytosolic compartment [15]. However, the aequorin cDNA may be modified by adding a nucleotide sequence coding for the targeting amino acid sequence of a specific compartment, i.e. mitochondria [16], nucleus [17], endoplasmic

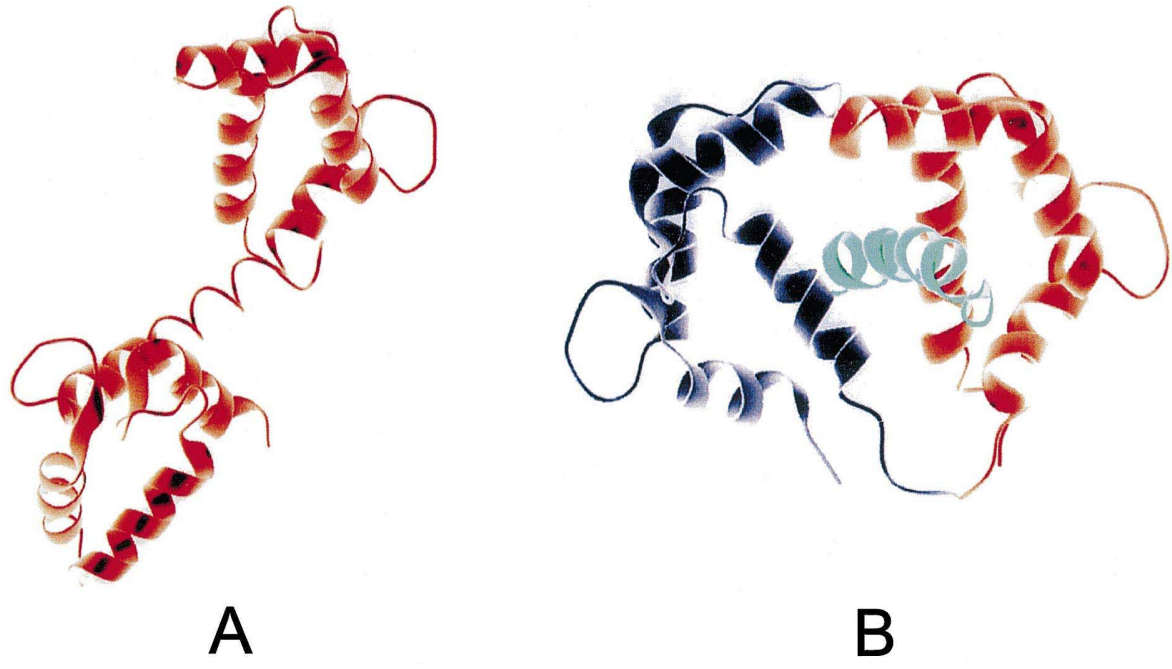


Figure 1. Ribbon representation of the crystal structure of calmodulin. (*A*) Uncomplexed calmodulin, (*B*) calmodulin in complex with the calmodulin binding domain of a calmodulin-dependent protein kinase [11]. As the interaction with the binding peptide (pale blue) occurs, the extended protein molecule collapses around the binding peptide to a hairpin conformation.

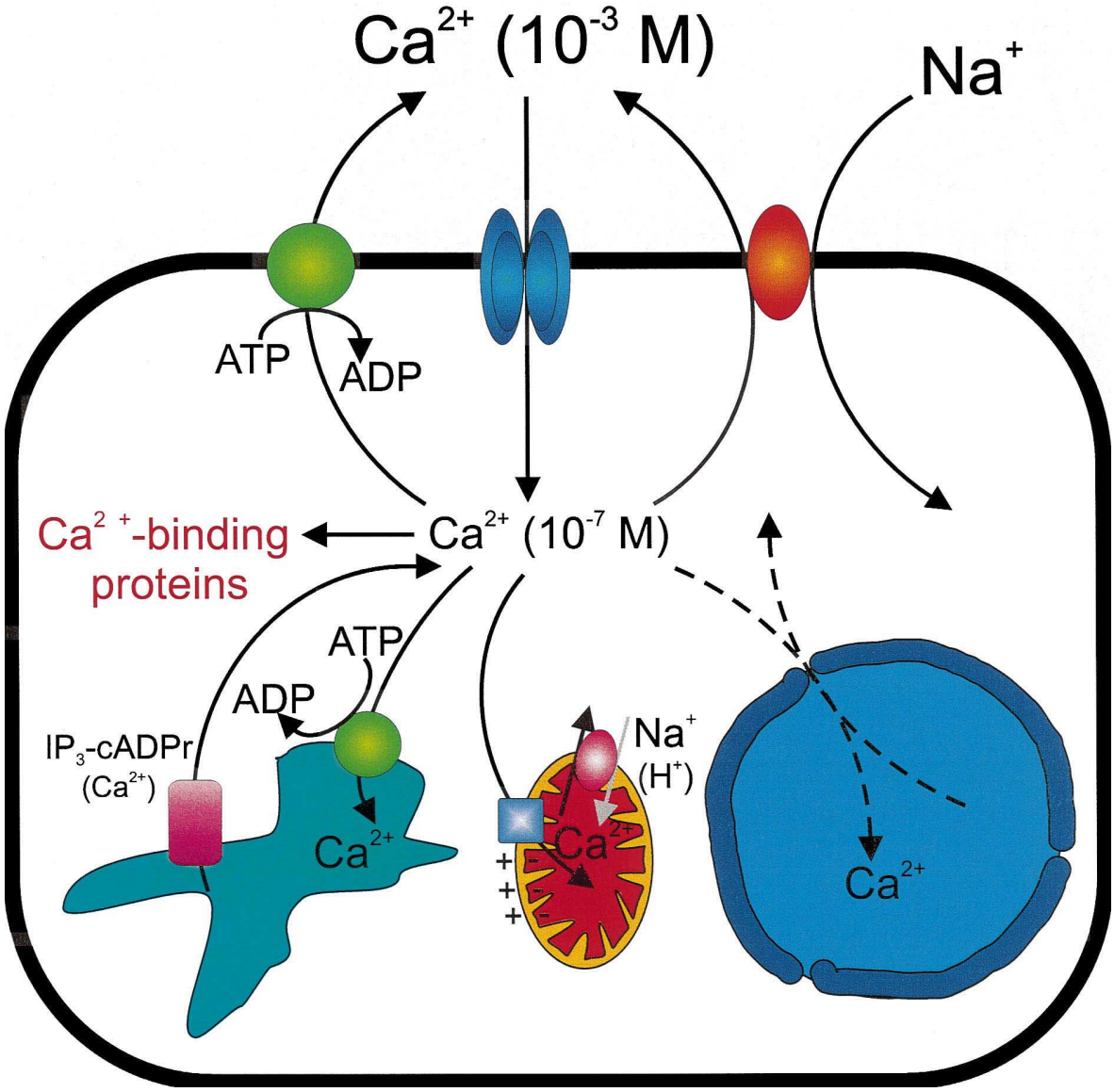


Figure 2. A schematic representation of the structures involved in cellular Ca^{2+} homeostasis. The model shows the Ca^{2+}-transporting systems and also indicates the Ca^{2+}-buffering proteins. The former are Ca^{2+}-ATPases (plasma membrane and sarco/endoplasmic reticulum), plasma membrane Ca^{2+} channels, Na^{+}/Ca^{2+} exchangers (plasma membrane and mitochondria), the electrophoretic mitochondrial uptake uniporter, the IP_3-sensitive and the cyclic ADP-ribose (cADPr)-sensitive (ryanodine receptor) Ca^{2+} release channels and the nuclear pores.

reticulum [18] or by fusing it with the cDNA coding for a resident protein, i.e. calsequestrin in the sarcoplasmic reticulum [19], the SNAP-25 of the plasma membrane [20] and the sialyl transferase in the Golgi apparatus [21]. This enables aequorin to be expressed in the desired cell compartment, reporting the Ca^{2+} concentration in it. Unlike the natural protein, recombinant aequorin does not contain the prosthetic group coelenterazine necessary for the chemiluminescent reaction. Coelenterazine is highly hydrophobic, and it is sufficient to add it to intact cells to reconstitute the active photoprotein. Moreover, chemically modified coeleterazines, which reduce the Ca^{2+} affinity of aequorin, can be employed for measurements in compartments with high Ca^{2+} concentration, e.g. the lumen of the endoplasmic reticulum or the subplasma membrane district.

Ca^{2+} transport across membranes

The plasma membrane

The plasma membrane possesses channels which control the downhill diffusion of Ca^{2+} into cells, and two systems that extrude it: a high-affinity, low-capacity pump, and a lower-affinity, large-capacity Na^+/Ca^{2+} exchanger. The plasma membrane is evidently responsible for the long-range maintenance of the 10,000-fold gradient of Ca^{2+} normally measured between the extracellular space and the cytoplasm. However, as a general rule, cells exchange across the plasma membrane only a minor portion of the total Ca^{2+} they use for their activities, using instead for most of their signalling needs Ca^{2+} stored in the intracellular deposits (organelles). However, the minor amounts of Ca^{2+} penetrating through the plasma membrane are essential, since they trigger cascades of events which are vital to cell activity, including the liberation of massive amounts of Ca^{2+} from the intracellular stores of some cell types (see below). Another point on the traffic of Ca^{2+} across the plasma membrane deserves to be mentioned: the very large gradient of Ca^{2+} across the plasma membrane ensures its unlimited availability, and is dynamically beneficial, since in its presence even minor changes in the Ca^{2+} permeability of the plasma membrane would ensure significant swings in the intracellular Ca^{2+} concentration. On the other hand, the large gradient also creates a potential situation of danger: if the Ca^{2+} permeability barrier of the plasma membrane were somehow to fail, which is a frequent event in pathology, the cell would be inundated by Ca^{2+}, and its reversible messenger function would come to an end.

Many types of Ca^{2+} channels operate in the plasma membrane: although some are gated, i.e. activated, by ligands (e.g. the channels activated by glutamate in numerous postsynaptic membranes or even, in a process that is still very poorly understood, by the emptying of the cytoplasmic Ca^{2+} stores (the so-called capacitative Ca^{2+} entry [22]), the most important and the best-known Ca^{2+} channels are voltage-gated. They are typical of excitable tissues, e.g. heart, and are subdivided into two subclasses: the T (tiny) channels, which are activated and inactivated at low membrane potentials, and the channels that are activated and inactivated at high membrane potentials. In turn, the latter are subdivided into four classes: the B (brain), the L (long-lasting), the N (neither nor B nor L) and the P (Purkinje cell) channels (see [23] for a recent review). The best known are the L-type channels, which have a conductance of 15–25 pS (3×10^6 Ca^{2+} ions per second, [24]) and begin to open when the depolarisation of the plasma membrane increases the resting transmembrane potential from about -70 to about -40 mV (maximum current at about 0 mV). The L-channels are important pharmacologically because they are blocked by several classes of widely used Ca^{2+} antagonists [25], the most popular being the dihydropyridines. Like all other voltage-gated Ca^{2+} channels, several of which have now been cloned [23], the L-channels are composed of five subunits: $\alpha 1$, $\alpha 2$, β, γ, δ, $\alpha 2$ and δ being linked by disulphide bonds. The $\alpha 1$ subunit, which contains the Ca^{2+} channel proper, is intrinsic to the membrane, like the γ and δ subunits, whereas the $\alpha 2$ and β subunits are extrinsic and located at opposite sides of the membrane (fig. 3, see [26], for a review). The critical unit in channel function is $\alpha 1$: it follows the architectural motif of other voltage-gated ion channels, i.e. four repeats of six-transmembrane domains, the fourth of each containing several positively charged residues, and thus presumably acting as a voltage sensor. The channel is assumed to be formed by the loop connecting transmembrane domains 5 and 6 of each repeat, which would fold within the membrane to provide the path for Ca^{2+} across the protein. The role of the other subunits is not clear, but the β subunit is phosphorylated by protein kinase A, mediating the increased probability of channel opening, e.g. following β-adrenergic stimulation of (heart) cells.

Of the two plasma membrane Ca^{2+}-exporting systems, the Na^+/Ca^{2+} exchanger, which has low Ca^{2+} affinity but high Ca^{2+}-transporting velocity, is particularly active in excitable cells, e.g. heart, which periodically experience the need to rapidly eject large amounts of Ca^{2+} (see [27] for a recent review). The exchanger operates electrogenically, exchanging three Na^+ for one Ca^{2+}, and thus responds both to the gradients of Ca^{2+} and Na^+ across the plasma membrane and to the transmembrane potential. Under some conditions, the exchanger also mediates the influx, rather than the efflux, of Ca^{2+}, a function that is widely assumed to be the basis of the positive ionotropic effect of digitalis, the classical inhibitor of the Na^+/K^+ pump. Since the exchanger has low Ca^{2+} affinity ($K_m > 1$ μM), it must necessarily be exposed to areas of localised high Ca^{2+} concentrations to operate efficiently. In heart, for example, it appears to be specifically concentrated in the T-system portion of the plasma membrane, where it would have immediate access to the high local concentrations of Ca^{2+} produced by the activation of the Ca^{2+} release channels in the terminal cisternae of sarcoplasmic reticulum.

Three basic exchanger gene products are known, NCX1, NCX2 and NCX3. The first two are ubiquitously distributed in tissues; NCX3 is restricted to brain. All three are organised in the membrane, with 11 transmembrane domains and a large cytosolic loop separating transmembrane domains 5 and 6. The loop contains regulatory sites, e.g. a putative calmodulin binding domain and a Ca^{2+} binding site. Its C-terminal

portion contains a region where a complex pattern of alternative splicing occurs, producing a large number of potential isoforms (however, no alternative splicing has so far been reported for NCX2). Surprisingly, the loop can be deleted from the exchanger molecule in heterologous expression experiments without total loss of exchanger activity [28]. A particular variant of the exchanger is active in retinal photoreceptors. Although its membrane topology corresponds to that of the other exchangers, the retinal exchanger bears no particular sequence homology to the latter. In addition, it is mechanistically different, since it exchanges four Na^+ against one Ca^{2+} and one K^+. A serious problem with the exchangers is the difficulty of measuring their activity. Since no enzymatic tests are possible, the measurements necessarily demand the purification and reconstitution of the exchanger proteins, obviously a difficult task. For this reason, hardly any information is presently available on the (probable) functional differences of the various isoforms, alternatively spliced or otherwise. One can predict with confidence that all isoforms will transport Ca^{2+} with low affinity and high capacity, but beyond this the territory is largely essentially unknown.

The other Ca^{2+}-exporting system of the plasma membrane is the Ca^{2+} pump (PMCA), a member of the P-type ion motive ATPase family (see [29] for a recent review). As mentioned, the pump has high Ca^{2+} affinity ($K_m < 0.5$ μM), but only when complexed with calmodulin. In its absence the pump has a Ca^{2+} affinity comparable to that of the Na^+/Ca^{2+} exchanger. Other treatment alternatives to calmodulin, which could be significant in vivo, also confer high Ca^{2+} affinity to the pump, i.e. exposure to acidic phospholipids, several kinase-mediated phosphorylations, C-terminal truncations, e.g. by calpain and oligomerization. The C-terminal calmodulin binding domain interacts with the cytoplasmic portion of the pump containing the active site, and maintains it in an inhibited state until calmodulin removes it, reestablishing full activity of the pump. This inhibition/disinhibition mechanism is reminiscent of that operating in the SERCA pump, where the accessory protein phospholamban maintains the enzyme inhibited until removed from its pump interacting site by two kinase-mediated phosphorylations (see below). The analogy of the two mechanisms has recently been made

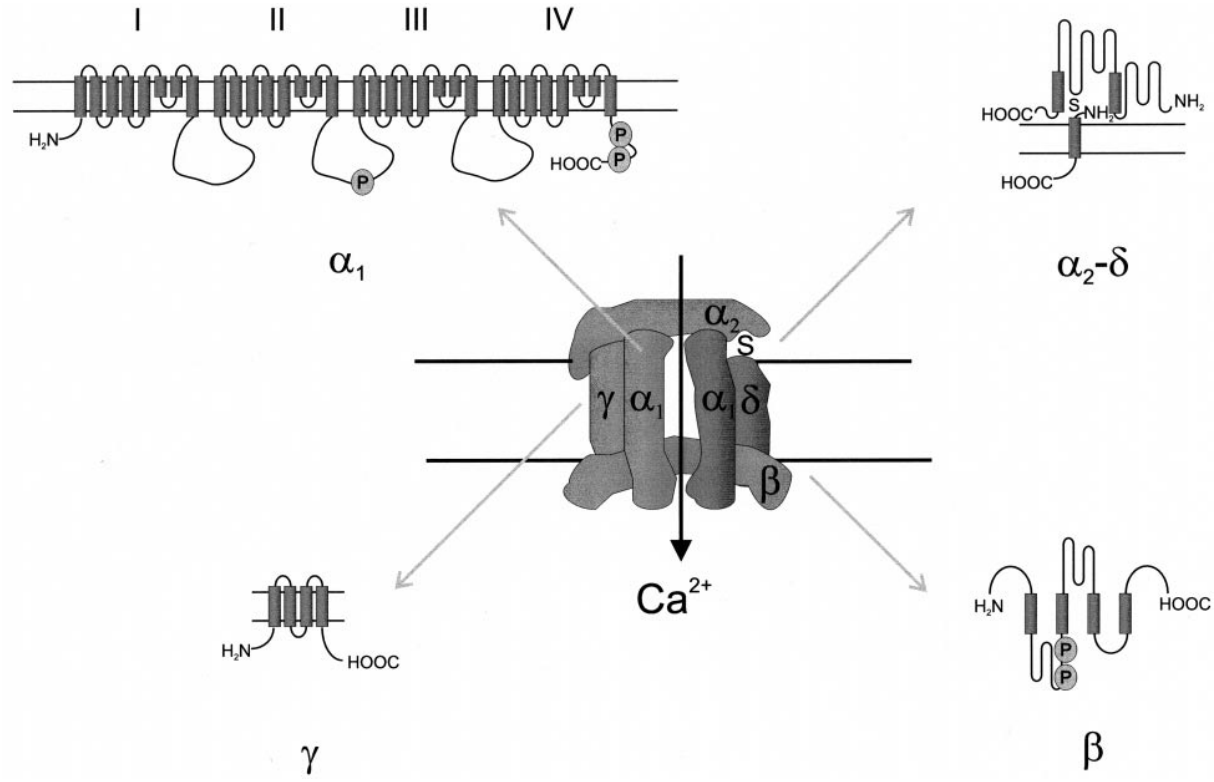


Figure 3. Membrane topology of the subunits of a L-type Ca^{2+} channel (modified from [26]). The channel contains five subunits, $\alpha 1$, $\alpha 2$, δ, β and γ. The $\alpha 1$ subunit is a large transmembrane protein that contains the channel pore, the voltage sensor and the gating machinery. The $\alpha 2$-δ subunit is a heterodimer linked by disulfide bonds (S). The β subunit is entirely intracellular; the γ subunit is intrinsic to the membrane.

even more striking by the finding that a phosphorylation step by protein kinase C also abolishes the ability of the C-terminal portion of the PMCA pump to interact with its cytoplasmic portion containing the active site [30]. The pump is organised in the membrane with 10 transmembrane domains, with most of its mass protruding into the cytoplasm. Whereas calmodulin interacts with the C-terminal protruding unit, the activating acidic phospholipids interact mainly with a predominantly basic portion of the pump located in the unit protruding between transmembrane domains 3 and 4 [31]. The matter of acid phospholipids is probably important physiologically, since the pump is presumably always exposed to them: it has been calculated that under the conditions prevailing in vivo the pump is likely to be activated by them to about 50% of its maximal activity [32]. In animals, the pump is the product of a multigene family. Of the four genes, two (1 and 4) are expressed ubiquitously in tissues, whereas genes 2 and 3 are expressed only in brain and a few other cell types [33]. In addition to the four basic gene products, a complex pattern of alternative splicing, involving either a domain close to the phospholipid interacting site or a region involving the calmodulin binding domain, increases the number of pump isoforms. Of interest are truncated versions resulting from the inclusion of an insert encoded by an extra exon within the calmodulin binding domain. These truncated isoforms have very low calmodulin affinity and are at the opposite end with respect to PMCA2, which has the highest calmodulin affinity of all isoforms. One could also quote recent findings on cerebellar neurons, showing that during their development some PMCA isoforms (2 and 3, and the truncated version of PMCA1) are strongly upregulated, whereas PMCA4 is much more rapidly downregulated in a process mediated by the calmodulin-dependent phosphatase calcineurin [34; D. Guerini et al., unpublished]. The change in PMCA expression pattern is likely to be a response to the changing local demands of developing neurons in terms of Ca^{2+} signalling: i.e. as dendrites and the axon develop, the need may arise to either increase or decrease the local ability to extrude Ca^{2+}. It is likely that the PMCA pump is distributed inhomogeneously in the plasma membrane, particularly in cells of high structural complexity such as neurons: for instance, in Purkinje cells PMCA2 is specifically concentrated in the spines of dendrites [35].

At the end of this paper, it is important to mention that a sensor able to interact with extracellular Ca^{2+} has been recently identified in the plasma membrane of a number of cell types [36]. $[Ca^{2+}]$ of the extracellular fluid, at variance with intracellular $[Ca^{2+}]$, remains remarkably constant, and for this reason it had not generally been thought to serve as an intracellular messenger. The cloning of a G-protein-coupled Ca^{2+} [36] as well as the identification of inherited diseases resulting from mutations in this receptor [37] have provided strong support for the concept that external Ca^{2+} could be an important first messenger in addition to providing a potentially inexhaustible source of Ca^{2+} that penetrates into the cell to act as a second messenger (for a recent review see [38]).

Intracellular organelles

In the last few years, intracellular organelles such as the endoplasmic reticulum, the nucleus and the mitochondria have emerged as crucial to the generation and transduction of Ca^{2+} signals of high spatiotemporal complexity [39]. Ca^{2+} in the organelles is important not only in the control of general Ca^{2+} homeostasis in cells but also as a specific regulator of the function of the organelles. The following sections will discuss the cellular homeostasis of Ca^{2+} as a target of the compartments which are known to transport Ca^{2+}, namely the endoplasmic reticulum, the mitochondria and the nucleus.

Ideally, full knowledge of Ca^{2+} transport by the organelles should include quantitative information on both total and free Ca^{2+} levels. Ultrastructural techniques (e.g. secondary ion mass spectrometry microscopy, electron microprobe X-ray analysis, electron energy loss spectrometry and electron spectroscopy imaging) have provided useful information on total Ca^{2+} but not on free $[Ca^{2+}]$ within the organelle lumen. Since these methods are limited to fixed samples of tissues and cells, they are unsuitable to monitor the dynamic changes of $[Ca^{2+}]$. They thus need to be complemented with the methods described above which permit the monitoring of free $[Ca^{2+}]$: variants of the Ca^{2+} fluorophore dyes and chimeric recombinants of the Ca^{2+}-sensitive photoprotein aequorin.

The endoplasmic reticulum. Historically, endoplasmic reticulum [ER, or rather, its muscle cell counterpart, sarcoplasmic reticulum (SR), see below] has played the most important role in the development of the concepts of organellar Ca^{2+} homeostasis: the pioneering demonstration by Kumagai et al. [40] that the muscle-relaxing factor of Marsh [41] actually was a granular ATPase preparation opened the way for the landmark contributions by Hasselbach and Makinose, and Ebashi and Lipmann [42, 43], showing ATP-dependent Ca^{2+} uptake in muscle microsomal preparations. The original muscle work was later extended to other cell types: it is now recognised that the maintenance of Ca^{2+} homeostasis within the ER/SR is essential to a number of cell functions, ranging from cell growth, to protein synthesis and folding, to protein processing and transport. The ER and its tissue-specialised species such as

the SR are the main dynamic Ca^{2+} storage compartment of all eukaryotic cells. They contain a number of proteins which control Ca^{2+} accumulation and release, in particular pumps (SERCA-ATPases) for Ca^{2+} uptake, Ca^{2+}-binding proteins for Ca^{2+} storage (calsequestrin and calreticulin) and channels for Ca^{2+} release [the inositol 1,4,5-trisphosphate (IP_3) receptor, IP_3R, and the ryanodine-cADP-ribose (cADPr) receptor, RyR] [44–46].

Historically, interest on Ca^{2+} storage by the ER started with work on skeletal muscle, where a complex of membrane cisternae was first described in landmark observations by Veratti in 1902 [47]. Only much later (1957), Porter and Palade [48] proposed that the SR could be the equivalent of the ER in nonmuscle tissues. Studies on skeletal muscle SR and also of its two specialised domains, the longitudinal and the junctional SR (the portion which is directly coupled to the T-tubules of the plasma membrane to form the triads), have revealed a high degree of specialisation. The SERCA type 1a Ca^{2+} ATPase is predominantly concentrated in the longitudinal portion [49], whereas the ryanodine receptor is abundant in the junctional membrane [50]. Within the SR lumen, the major Ca^{2+}-binding protein is calsequestrin [51].

Despite the differences in organisation (the rough-surface reticulum, RER, the smooth reticulum, SER, and the nuclear envelope, NE), evidently reflecting specialised functions (protein synthesis, transport, local protein degradation, nuclear segregation), the ER systems of cisternae and tubules are generally assumed to be a continuum. However, at least in some cells the situation may be more complex, e.g. the rapidly exchanging Ca^{2+} stores sensitive either to IP_3 or ryanodine-cADPr may not correspond to the entire ER but to specialised subcompartments. The heterogeneity of ER with respect to Ca^{2+} handling was first recognised by Volpe et al. [52], who proposed that nonmuscle cells may contain structures analogous to the SR (calciosomes) which are now indeed considered as a specialised subcompartment of the ER [53–55]. The heterogeneity depends on the distribution of the molecules governing Ca^{2+} uptake, release and binding within the ER subcompartments.

The SERCA pump isoforms have a high degree of sequence conservation (75–85%), and thus are assumed to have the same membrane topology [49]. All isoforms have 10 transmembrane domains, the first 5 extending into the cytoplasm to form a pentahelical 'stalk'. The ATP binding site and the essential aspartyl residue have been suggested to be located in a large globular domain between the fourth and fifth transmembrane sectors [56]. These suggestions have now been validated by the recent solution of the 3D structure of the pump at 8-Å resolution [57]. The study has made it possible to locate all 10 transmembrane domains and the cavity that forms the Ca^{2+} binding site.

Among the SERCA pump isoforms, only the cardiac type (which includes the smooth muscle and the slow-twitch muscle isoforms) is regulated by the small hydrophobic protein phospholamban [58], as briefly mentioned above. Dephosphorylated phospholamban binds to the SERCA pump, shifting its K_d for Ca^{2+} to a lower-affinity value and thus resulting in the inhibition of the pump activity. Phosphorylation of phospholamban by the cyclic adenosine monophosphate (cAMP) and Ca^{2+}/calmodulin-dependent protein kinases dissociates it from its binding site near the active site of the pump [59], relieving the inhibition.

While the IP_3 that will activate the IP_3 receptors is generated by phospholipase C acting on phosphatidylinositol 4,5-bisphosphate at the plasma membrane in response to a number of first messengers, activation of RyRs is triggered directly by plasma membrane depolarisation, through direct coupling (i.e. charge transfer) with L-type channels in the T-system in skeletal muscles. However, a process known as CICR (Ca^{2+}-induced Ca^{2+} release), which involves actual influx of external Ca^{2+} through the T-system L-type channels, is instead responsible for the activation of cardiac RyRs. It may be mentioned at this point that IP_3 receptors, even if they are activated by IP_3, are also sensitive to Ca^{2+}, which thus gates its release from all intracellular channels. Recently, the nicotinamide adenine dinucleotide (NAD^+) metabolite derivative cyclic ADP-ribose (cADPr), originally discovered as a potent Ca^{2+}-mobilising agent in sea urchin eggs, has been reported to stimulate Ca^{2+} release in higher eukaryotic systems as well (e.g. smooth and cardiac muscle cells, neuronal cells, adrenal chromaffin cells, macrophages, pancreatic acinar cells, T-lymphocytes). cADPr, which is synthesised by a plasma membrane ectoenzyme (CD38) [60] which also mediates its transfer inside the cytoplasm, is likely to act on the RyRs. An NADP metabolite, nicotinate adenine dinucleotide phosphate (NAADP), is the newest addition to the family of activators of intracellular Ca^{2+} channels [61]. It is also synthesised by CD38 [62], and it probably discharges a Ca^{2+} pool located in a membrane compartment different from those sensitive to IP_3 and cADPr.

The genes that encode the IP_3R and the RyR have now been cloned [63–65] (fig. 4). The IP_3R/Ca^{2+} release channel is thought to be a complex of four subunits [66–68], organised in a square having a pinwheel appearance with fourfold symmetry (possibly, a channel pore). RyRs were purified from skeletal and cardiac muscles nearly 10 years ago and were also found to be multimeric complexes of very large protein subunits. Also in this case, electron microscopy and metal-shadowing techniques have revealed a fourfold symmetric

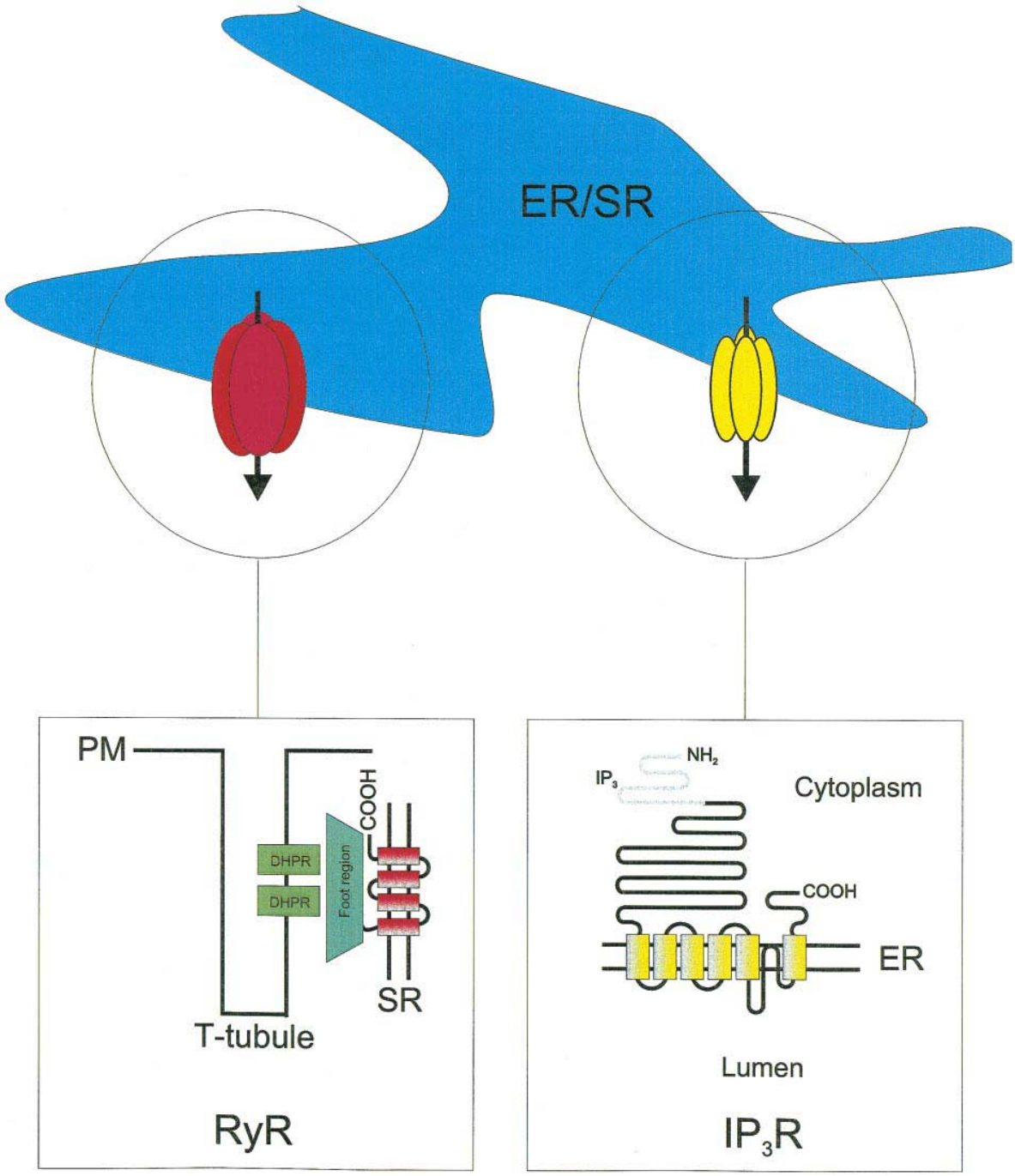


Figure 4. Transmembrane topology of the IP_3 and Ry channels. The RyR is proposed to contain four transmembrane helices (however, the number of transmembrane domains in this receptor is still uncertain) and a very large protruding cytoplasmic domain, the 'foot' region. In vertebrate skeletal muscles, E-C coupling seems to occur by a mechanical (charge-transfer) coupling mechanism involving protein-protein interaction between the L-type channel (here defined as the dihydropyridine receptor, DHPR) of the transverse tubule (T-tubule) membranes (PM) and the RyR of the terminal cisternae of the sarcoplasmic reticulum (SR) membrane. IP_3R has been proposed to traverse the membrane of intracellular Ca^{2+} stores such as the endoplasmic reticulum (ER) six times. The large N-terminal cytoplasmic region contains the ligand (IP_3)-binding domain and the modulatory and transducing domains. The short C-terminal region faces the cytoplasm. A complete IP_3R channel is a complex of four subunits.

complex having the shape of a square prism with a bump projecting from the center of one face [69, 70]. The location of the transmolecule Ca^{2+} path in the RyRs is unknown, and even the number of transmembrane domains in the molecule is controversial. Each monomer of the tetrameric IP_3R, instead, is generally accepted to be organised, in analogy with the voltage-gated channels of the plasma membrane, with six transmembrane domains and a large loop between transmembrane domains 5 and 6 folding within the membrane to form the Ca^{2+} channel (see [71], for a recent and comprehensive review).

Within the lumen of the ER, Ca^{2+} is buffered by Ca^{2+}-binding proteins, which ensure the storage of large amounts of the cation. Several have been described, e.g. the chaperone BiP and the protein disulfide isomerase (PDI), which are needed for the proper folding of the newly synthesised ER proteins and for the maintainance of their optimal conformation. Other proteins have higher Ca^{2+}-binding capacity (25–50 mol/mol) and low Ca^{2+} affinity (K_d in the millimolar range): among them calsequestrin (CSQ) and calreticulin (CR) are often expressed at high levels in specific cell types to dynamically store Ca^{2+}. Calsequestrin predominates in striated muscles, calreticulin in non-muscle cells. Calsequestrins are the products of two different genes, one typical of fast-twitch muscles, the other of heart; calreticulin is also expressed in multiple isoforms. The two proteins have poor sequence homology, but conformational similarities may exist. They are not EF-hand proteins: clusters of acidic residues at their COOH termini are responsible for the Ca^{2+} binding.

The monitoring of free $[Ca^{2+}]$ in the ER lumen ($[Ca^{2+}]_{er}$) is difficult, and has used the ER targeting of aequorin [18, 72] and of green fluorescent protein (GFP)-based indicators [73, 74], or trapped low-affinity fluorescent indicators, such as mag-fura-2 [75, 76]. Whereas several direct or indirect measurements have indicated millimolar values for the total Ca^{2+} content (for reviews see [77, 78]), discrepant results have been reported for luminal free $[Ca^{2+}]$, varying from the low micromolar range [79], to 60–200 μM [80, 81] or 400–700 μM [18, 73, 82]. Whereas the reasons for the discrepancies are most probably methodological, the most reliable estimates of free lumenal $[Ca^{2+}]$ are in the millimolar range [78].

The mitochondria. The uptake of Ca^{2+} by mitochondria was discovered in 1962 [83], and for more than a decade thereafter mitochondria were thought to be of central importance in the control of cytosolic calcium [84]. Originally, Ca^{2+} handling by mitochondria was simply described in terms of active uptake and passive release [85]. Experimental measurements of the internally negative membrane potential predicted by the chemiosmotic theory [86] across the inner membrane showed that the gradient was the driving force for Ca^{2+} accumulation, which would be mediated by an electrophoretic uniporter [87]. However, if Ca^{2+} would reach electrochemical equilibrium, its concentration within the mitochondrial matrix would become about 10^6-fold higher than in the cytosol, i.e. 0.1 M, a concentration incompatible with the osmotic properties of mitochondria and with existing data on total mitochondrial Ca^{2+} in situ. Conveniently, then, it was discovered that, in addition to the electrogenic uptake uniporter mitochondria also possessed an electroneutral antiporter that extruded Ca^{2+} from the matrix in exchange for Na^+ [88]. Later on, a Ca^{2+}:H^+ exchange reaction was also discovered.

Subsequent work on isolated mitochondria showed that the electrogenic uniporter had low Ca^{2+} affinity and became activated to appreciable levels only when cytosolic Ca^{2+} reached about 0.5 μM [77]. Since this concentration was out of the physiological range in resting cells, interest in mitochondria as cytosolic Ca^{2+} regulators gradually faded, shifting the emphasis on its functional significance to the newly discovered Ca^{2+} dependence of some matrix dehydrogenases. Ca^{2+} modulated three matrix dehydrogenases, i.e. the NAD^+-dependent isocitrate dehydrogenase, the 2-oxoglutarate dehydrogenase and the pyruvate dehydrogenase [89]. A number of results quickly showed that changes in mitochondrial Ca^{2+} concentration occurring as a consequence of extramitochondrial Ca^{2+} changes modulated the dehydrogenases. However, the extrapolation of these results to the in vivo situation was prevented by the difficulty of measuring intramitochondrial Ca^{2+} in living cells. Although a number of studies had appeared in which fluorescent dyes were specifically loaded into mitochondria, only the targeting of aequorin to mitochondria eventually permitted the reliable measurement of mitochondrial Ca^{2+}, $[Ca^{2+}]_m$, in mitochondria in living cells. Mitochondrial calcium spikes occurred, in response to receptor agonists, or plasma membrane channel activation, which increase the cytoplasmic Ca^{2+} concentration [16]. $[Ca^{2+}]_m$ rapidly reached the micromolar range, i.e. the concentration required for the optimal activation of the matrix dehydrogenases. As a result of these findings interest in mitochondrial Ca^{2+} enjoyed a robust renaissance, whereas the possibility of studying it in intact cells led to the redefinition of some of its properties. An important finding was that, in contrast to isolated mitochondria, mitochondrial Ca^{2+} accumulation in intact cells was unexpectedly rapid. This was rationalised with the proposal, supported by a number of results, that these organelles 'sense' microdomains of high Ca^{2+} close to the source of the Ca^{2+} signal. In permeabilized cells perfused with Ca^{2+} concentrations similar to those which can be measured in the cytosol upon cell stimulation (about 1–2 μM), the uptake by mitochondria was negligible. However, when Ca^{2+} was released from the

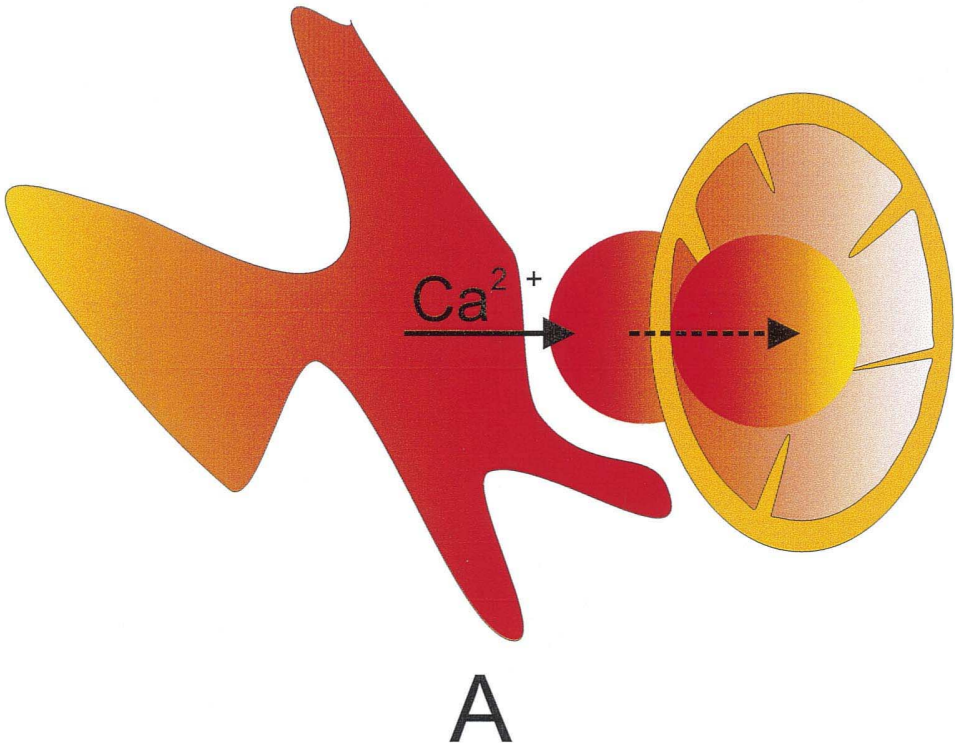
intracellular ER stores by IP_3, a rapid and large increase in mitochondrial Ca^{2+} was observed [90]. A second observation showed that a recombinant aequorin chimera, protruding in the mitochondrial intermembrane space, i.e. in the presumed external microenviroment of the mitochondrial uniporter, reported higher $[Ca^{2+}]$ than a cytosolic Ca^{2+} probe. Lastly, close contacts (80 nm) could be demonstrated between the mitochondria and the ER in HeLa cells [91]. Thus cell stimulation may generate microdomains of high $[Ca^{2+}]$ in close proximity to the ER sites of Ca^{2+} release, which mitochondria will rapidly accumulate and dissipate before the signal spreads to the entire cytoplasm (fig. 5). This mechanism would on one hand efficiently modulate the mitochondrial metabolism in phase with the cell needs, and on the other prevent the nonspecific activation of all Ca^{2+}-sensitive cell processes.

The correlation between $[Ca^{2+}]_m$ changes and the activation of the matrix dehydrogenases has been directly demonstrated in living cells by measuring simultaneously $[Ca^{2+}]_m$ and mitochondrial NADH production [92]. Further studies on single cells showed that mitochondrial Ca^{2+} oscillations occurred in parallel with cytosolic Ca^{2+} oscillations, and that the $[Ca^{2+}]_m$ oscillations, which evidently influenced matrix dehydrogenase, translated into an increase in NADH concentration. High-frequency oscillations were translated by mitochondria into a sustained metabolic stimulation, since the duration of the NADH increase was longer than that of the $[Ca^{2+}]$ changes [93]. A correlation between $[Ca^{2+}]_m$ changes and pyruvate-dehydrogenase activity was recently demonstrated in CHO cells [94] and in primary cultures of hepatocytes [95].

The nucleus. Although most of the traditional targets of Ca^{2+} signalling are located in the cytosol or at the plasma membrane (calmodulin, protein kinase C, ion channels and so on), recent evidence has indicated that several nuclear processes (e.g. the breakdown of the nuclear envelope, transcription activation, DNA metabolism) are modulated by changes of $[Ca^{2+}]$ in the nucleoplasm [96–98]; (see [99, 100] for recent comprehensive reviews). It has also been shown that nuclear $[Ca^{2+}]$, $[Ca^{2+}]_n$, may have a role that is distinct from that of cytosolic $[Ca^{2+}]$, $[Ca^{2+}]_c$, the effects being mediated by the translocation of transcription factors [101] and/or protein kinases [102] into the nucleus. That nuclear Ca^{2+} activates a number of immediate early genes, through the action of calmodulin-dependent kinases (CaMKIV), has been shown in a number of reports by Greenberg et al. [103]. More recently, Bading et al. (1997) have demonstrated that different cis-acting elements with the proximal promoter region of the c-*fos* gene respond selectively to increases in either $[Ca^{2+}]_c$ or $[Ca^{2+}]_n$ [104].

The issue of the control of nuclear calcium has traditionally been controversial. The nuclear envelope has been viewed by some either as a structure that offers no barrier to the movements of calcium, which would thus freely and immediately equilibrate between the cytosolic and the nuclear compartments, or as an effective sieve that instead limits its traffic between the two compartments. Unfortunately, fluorescent indicators are not adequate for the study of the relationship between the cytosolic and nuclear Ca^{2+} concentration, since their successful loading into the nucleus depends on their permeability through the nuclear pores and on the presence of esterases in the nucleoplasm. Problems are also encountered in distinguishing the signals coming from the cytosol (where the fluorescent indicators are easily loaded) and the nucleoplasm. Not surprisingly, then, the literature in this area is riddled with controversies. Since the nuclear pores are generally assumed to be permeable to molecules as large as 40 kDa, the low M_r fluorescent dyes ($M_r \sim 1000$ Da) should diffuse freely. Nevertheless, local accumulation or exclusion of dyes has been noticed [105, 106], and it has even been reported that isolated nuclei hydrolyse fura2/AM and trap the acid form inside, suggesting that, in living cells, they could be impermeable to the acid dyes generated in the cytoplasm [107]. The nuclear trapping of dyes was particularly striking in oocytes [108] which retained the injected fura2 for minutes. At variance with this, it has been shown that the permeabilisation of the plasma membrane (with digitonin, streptolysin O or electroporation) led to the loss of most of the trapped dye, including nuclear dye, to the medium, whereas the minor fractions accumulated in the ER, lysosomes and mitochondria remained trapped.

Aequorin has also been targeted to the nucleus by fusing its cDNA to the cDNA coding for a portion of the glucocorticoid receptor (GR) containing the nuclear localisation signals (NLSs) [17, 109]. The probe revealed that, in different embryonic cell types, the nuclear membrane was not a significant barrier to the diffusion of Ca^{2+}, which increased in the nucleus in rapid equilibrium with the cytosolic compartment. In agreement with this, Meyer et al. targeted a fluorescent Ca^{2+} indicator to the nucleus by coupling an NLS peptide to Calcium Green dextran, concluding that nuclear Ca^{2+} was not independently controlled [110]. At variance with these findings, others have reached different conclusions from experiments using aequorin targeted to the nuclear compartment by fusing it with a resident protein, nucleoplasmin [111]: under certain conditions, rises in cytosolic Ca^{2+} failed to result in the elevation of nuclear Ca^{2+}. Evidence that the nuclear envelope membrane contains ion channels [112] which produce single-channel opening events in patches that contain numerous pores has also been repeatedly provided, suggesting that the pores under some circumstances could somehow be sealed.



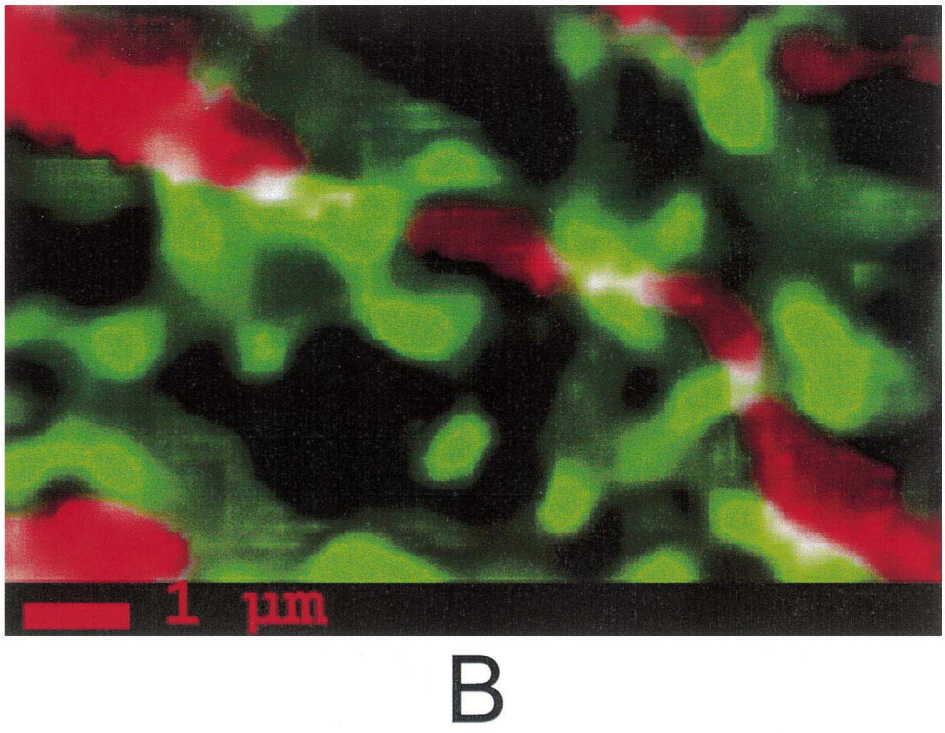


Figure 5. Close contacts between the ER and mitochondria. (*A*) A schematic model of the high [Ca^{2+}] microdomains generated at the mouth of IP_3-sensitive Ca^{2+}-release channels which are sensed by the neighboring mitochondria; (*B*) high-resolution 3D image of ER and mitochondria in a HeLa cell transiently transfected with two different mutants of the green fluorescent protein (GFP) specifically targeted to the two organelles by the introduction of localisation sequences. The close contacts (within the resolution limit of the image, i.e. < 80 nm) between the two organelles can be appreciated by the superposition of the 3D images of the two organelles. (Reproduced with permission from [91].)

A Ca^{2+}-ATPase identical to the SERCA enzyme [113] and IP_3R and RyR [114–117] have now been documented in the nuclear envelope, again suggesting autonomous regulation of nuclear Ca^{2+} (fig. 6). Several groups have shown that IP_3 and cADP-ribose may provoke Ca^{2+} release from the nuclear membrane into the nucleoplasm, [115, 117, 118]. These findings rationalise earlier work by Divecha et al. showing that the β-1-isoform of phospholipase C (PLC) is largely localised to the inner side of the nuclear membrane, where it may mediate IP_3 generation [119, 120]. As for cADPr, evidence has recently also so far been provided for the presence of its synthesising enzyme (CD38, see above) in the nuclear envelope [121]. Once released into the nucleoplasm, however, Ca^{2+} must eventually be again eliminated from it. It would be logical to assume that the SERCA-type pump of the envelope plays a role in the process: the envelope is an extension of the ER and would be expected to contain the pump in both of its leaflets, as shown in the cartoon of figure 6. Somewhat unexpectedly, however, in a recent report in which the two leaflets of the envelope have been separated [116], the pump has only been detected in the outer leaflet. In addition, its total Ca^{2+} pumping capacity is presumably low [115]: thus, the rapid decrease in $[Ca^{2+}]_n$ seen, for instance, after stimulation of pancreatic acinar cells, may also be due to the rapid leakage of Ca^{2+} through the nuclear pores. Thus the pores and the transporting systems of the envelope would cooperate in the exchanges of Ca^{2+} between the nucleoplasm and the cytosol. An important problem which is still open in nuclear Ca^{2+} signalling is that of the transmission of the signals generated at the plasma membrane to the nuclear envelope to activate, for instance, the IP_3-producing machinery. Although contributions in this area have begun to appear [122, 123], the field is still largely unknown.

Conclusions

The development of concepts in the area of cellular Ca^{2+} signalling has followed a peculiar course, advancing for 6 or 7 decades in quantum steps separated by long intervals of quiescence. Originally confined to muscle research, Ca^{2+} as a carrier of information laboriously invaded other areas, to eventually permeate, beginning in the 1960s and 1970s, all corners of biology from biochemistry to cell biology to biophysics. In looking at it today, the famous statement by Otto Loewy—"ja, Kalzium, das ist alles…" ("Well, calcium—that's everything!")—actually appears all the more remarkable because it was uttered in 1959, at a time when the curtain hiding the entrance to the Ca^{2+} 'eldorado' had just begun to be lifted. But once the ball started rolling, it all happened at a very rapid pace. It is hard to believe that membrane transport of Ca^{2+}, Ca^{2+}-binding proteins, fluorescent and targeted Ca^{2+} indicators, calmodulin, Ca^{2+}-dependent kinases and

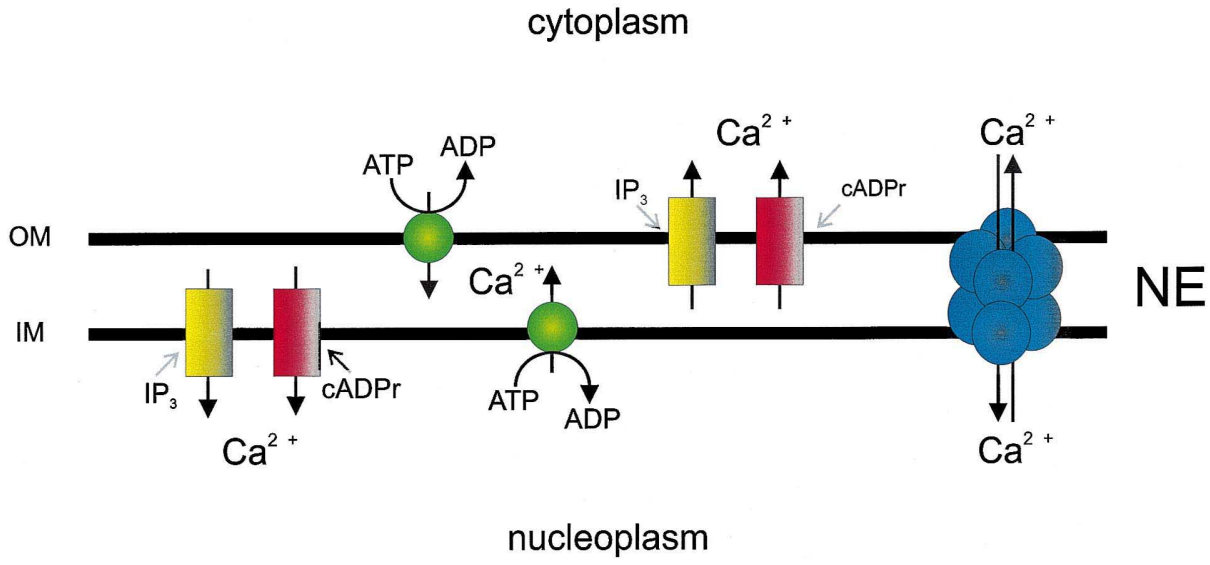


Figure 6. The nuclear envelope in the regulation of nuclear calcium. The model shows the calcium-transporting systems of the envelope (NE): the Ca^{2+}-ATPase, the IP_3-sensitive Ca^{2+}-release channel and the cyclic ADP-ribose (cADPr)-sensitive channel (ryanodine receptor), which are visualised in both the inner (IM) and outer (OM) membranes. A receptor sensitive to IP_4 has also been described [124]. Nuclear pores are represented in a calcium-permeable state.

phosphatases, as well as countless other Ca^{2+}-modulated enzymes, were all but unknown as recently as 20 or 25 years ago. Knowledge, in fact, continues to grow exponentially: topics such as regulation of gene expression, numerous aspects of neuronal function and quality control of proteins trafficking between membranes are but a few new additions to the list of Ca^{2+}-modulated functions, which includes last but not least, processes linked to the suffering and eventual demise of cells. It may be unpleasant to end the discussion of the signalling role of Ca^{2+} on a somber note, but it is important to stress again that the essence of the messenger role of Ca^{2+} is the ability of a cell to keep its swings under tight control. Should this control fail, the dark side of Ca^{2+} will take over, transforming it into a conveyor of doom.

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