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Calcium – a universal carrier of biological signals

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Calcium is the most universal carrier of signals to cells. Chosen by evolution because of its peculiar flexibility as a ligand, it now regulates all important aspects of cell activity, beginning with the creation of new life at fertilization and ending with the dramatic event of apoptotic suicide at the end of the life cycle. The process of signal transduction by Ca^{2+} displays a number of properties that make it unique among all other carriers of signals: for instance, the ability to perform both a first messenger and a second messenger function, or the frequent activation of autoregulatory mechanisms. The aspect that distinguishes the Ca^{2+} signaling function most dramatically is ambivalence. Cells have an absolute dependence on the messenger function of Ca^{2+} in order to function properly and must control its homeostasis with precision to maintain its free concentration in their interior at the appropriate low level. Catastrophy, however, invariably follows whenever protracted failures of the control mechanisms lead to sustained Ca^{2+} overload.

Introduction

At the end of the 19th century it was common knowledge that Ca^{2+} was a structural element, essential to the stability of bones. As no other function of Ca^{2+} was imagined at that time, the serendipitous finding made by Ringer in 1883 [1], that Ca^{2+} transmitted to heart cells a signal that initiated contraction was probably considered a curiosity. This can be inferred from the fact that it was not seriously followed up for decades. It was only in the 1940s that it became clear that Ringer had actually made a landmark finding that showed that Ca^{2+} had a function in cell biochemistry that was completely unrelated to its structural role. Once the concept of a signaling role of Ca^{2+} became established, it rapidly gained momentum, aided by an impressive series of spectacular developments (recently reviewed in [2–4]). In time, (Table 1), Ca^{2+} became recognized as a universal and versatile carrier of information that guides cells from origin to death. Importantly, Ca^{2+} displays a number of properties that set it apart from all other signaling agents. It is peculiarly capable of autoregulating its messenger function, i.e. its production and movements inside cells, e.g. by membrane transporters, are regulated by Ca^{2+} itself. In addition, Ca^{2+} is not only a cell-produced second messenger that is active inside cells; it may also function as a bona fide first messenger that interacts on the outer side of the plasma membrane as if it were a hormone or a growth factor [5]. Finally, and most importantly, the signaling function of Ca^{2+} has a distinctive ambivalent character. While essential for the proper functioning of cells, as long as correctly produced and controlled, Ca^{2+} becomes a conveyor of doom,

Abbreviations
cADPr, cyclic ADP-ribose; CaMK II, calmodulin kinase II; CIF, calcium influx factor; InsP3, inositol-(1,4,5)-trisphosphate; NCX, Na^+/Ca^{2+} exchanger; NAADP, nicotinate derivative of $NADP^+$; NMDA(R), *N*-methyl-D-aspartate (receptor); PMCA, plasma membrane Ca^{2+}-ATPase; RyR, ryanodine receptor; SERCA, sarco(endo)plasmic reticulum ATPase; SOC, store-operated channels; TRP, transient receptor potential.

Table 1. Calcium-modulated cellular functions.

Cellular functions
Generation of fuels
Glycogenolysis (phosphorylase β kinase)
Lipases and phospholipases
Alpha-glycerophosphate dehydrogenase
Pyruvate dehydrogenase phosphate phosphatase
NAD-dependent isocitric dehyderogenase
Alpha-ketoglutarate dehydrogenase
NADH-dehydrogenase (plant mitochondria)
Beta-hydroxybutyrate dehydrogenase
Membrane-linked functions
Excitation-contraction coupling
Excitation/secretion coupling (e.g. neurotransmitters)
Some plasma membrane channels
Some action potentials
Tight junctions
Cell contact
Hormonal regulation
Formation/degradation of cyclic
Adenosine 5′-monophosphate and
Guanosine 5′-monophosphate
Release of several hormones from storage vesicles
Contractile and motile systems
Muscle myofibrils
Cilia and flagella
Microtubules and microfilaments
Cytoplasmic streaming
Pseudopod formation
Miscellaneous functions
Light emission
Cell cycle
Fertilization
Some proteolytic enzymes
Excitation-transcription coupling
Some protein kinases
One protein phosphatase (calcineurin)
Production of messengers (e.g. nitric oxide)
Vision
Generation and storage of memory
Apoptosis

precipitating apoptotic and/or necrotic cell death, when its concentration and movements inside cells fail to be properly controlled [6].

Why Ca^{2+}?

The evolutionary choice of Ca^{2+} as an intracellular messenger was probably made when the transition to multicellular life forced the division of labor among cells; a step that carried with it the necessity of transmitting regulatory signals. The choice of Ca^{2+} appears to have been dictated by its special flexibility as a ligand, that makes its reversible binding by complex molecules (i.e. proteins) particularly easy in the conditions prevailing in the intracellular ambient (see ref [7], for a detailed discussion of the topic). Complex formation is the means by which the concentration of free Ca^{2+} is kept low inside cells; as is self evident, Ca^{2+}, like all molecules that act as intracellular messengers, must be maintained inside cells at very low levels, to avoid prohibitive expenditures of energy to significantly change its concentration. Complex formation (coordination chemistry) is particularly easy as Ca^{2+} is able to bind to sites of irregular geometry, such as those normally offered by proteins. Sites that would optimally bind Ca^{2+} would, for instance, refuse Mg^{2+}, whose higher polarizing power demands geometrically regular octahedral sites, thus restricting greatly its binding possibilities. The proteins designed to specifically complex Ca^{2+} do so by using a number of structural modules, e.g. the annexin fold, the C-2 domain, and, most importantly, the EF-hand motif. The EF-hand proteins are the most important and best understood Ca^{2+}-binding proteins; their basic Ca^{2+}-binding module, first identified by Kretsinger in the molecule of parvalbumin [8], has now been found in hundreds of proteins. It consists of two roughly orthogonal helices flanking a 12-amino acid loop where Ca^{2+} is coordinated to side chains and carbonyl oxygens of five invariant residues. With the progress of structural information on the EF-hand proteins, variants on the basic Ca^{2+}-binding mode have become apparent. These include the possibility that protein domains outside the loop also contribute coordinating oxygens, but the basic general principles of Ca^{2+}-binding first extrapolated by Kretsinger from the X-ray structure of parvalbumin over 40 years ago have remained remarkably valid.

The decoding of the Ca^{2+} signal by Ca^{2+}-binding proteins

The EF-hand and other proteins mentioned above are important for the buffering of cell Ca^{2+}, as they contribute to reducing its ionic concentration to the sub-micromolar level prevailing in the soluble phase of cells. However, they perform another, possibly more important function related to Ca^{2+}-signaling. By binding Ca^{2+}, they decipher its information and pass it on in the decoded form to cellular (enzyme) targets. Therefore, these proteins are more aptly designated as Ca^{2+} sensors rather than as Ca^{2+} buffers. The molecular mechanism of the decoding process is only fairly well understood for the EF-hand proteins, and has actually been studied in great detail only for the

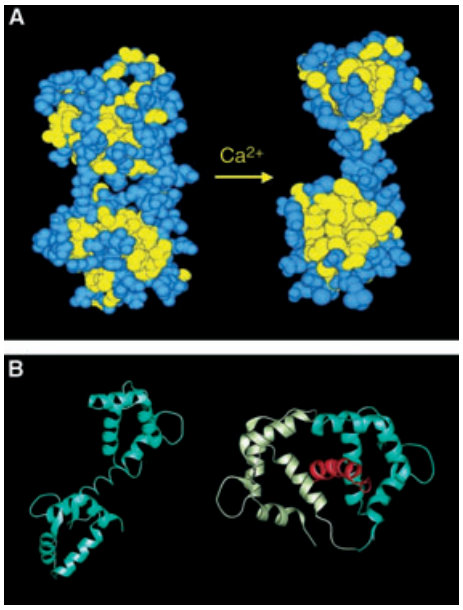

Fig. 1. Decoding of the Ca^{2+}-message by calmodulin. (A) Space filling model of the crystal structure of calmodulin that shows the hydrophilic residues in blue, and the hydrophobic residues in yellow. The binding of Ca^{2+} does not change the overall dimensions of the molecule (about 60 Å from top to bottom), but opens up hydrophobic pockets exposing a larger number of hydrophobic residues (methionines) on the surface of the protein. (B) Solution structure of Ca^{2+}-saturated calmodulin complexed to a 26-residue peptide (red) that is the calmodulin-binding domain of skeletal muscle myosin light chain kinase. Left panel, Ca^{2+} saturated calmodulin, showing the elongated dumbbell shape. Right panel, calmodulin complexed to the binding peptide. The complex has a globular shape, in which the length of the elongated form of calmodulin is reduced to about 47 Å. The collapse of calmodulin to engulf the binding peptide brings the two Ca^{2+}-binding lobes close to one another.

case of calmodulin (Fig. 1). This elongated protein undergoes a first change in conformation upon binding Ca^{2+} that does not alter its overall dimensions, but 'opens up' its two Ca^{2+}-binding lobes, exposing hydrophobic pockets (essentially methionine residues). Calmodulin is usually rich in methionine, i.e. 6% of total residues, as opposed to about 1% in the proteome) that permit the interaction with the binding domain of target proteins. Thereafter, a second, more dramatic change takes place that collapses the elongated structure of the Ca^{2+}-sensor protein (calmodulin) to a hairpin conformation wrapped around the binding domain of the target enzyme [9]; at this point, the transmission of the Ca^{2+} signal is complete, i.e. the target protein now contains the Ca^{2+} information which was destined to modulate its activity. The description above refers to calmodulin, which is the most versatile EF-hand Ca^{2+}-sensor protein. It would be tempting to consider such a mechanism a paradigm for the transduction of the Ca^{2+} signal by all EF-hand proteins, of which, as mentioned, several hundred now exist. Whether this is so, however, cannot be said at the moment. EF-hand proteins other than calmodulin are generally committed, i.e. they only interact with a specific (enzyme) target. In addition, the structural information available so far indicates that the elongated dumbbell shape which is an essential ingredient of the processing of the Ca^{2+} signal by calmodulin, may only be peculiar to a few other EF-hand proteins.

The buffering of cell Ca^{2+} and the role of membrane Ca^{2+} transporters

The EF-hand Ca^{2+}-sensor proteins, and other sensor proteins like the annexins and gelsolin, are present within cells in finite amounts. These amounts can be very significant; those of calmodulin, for instance, can easily reach the micromolar range [10]. However, even if these amounts are large, they may be still inadequate to cope with the Ca^{2+}-buffering needs of cells, whose physiological cycle may periodically expose them, or at least portions of their cytoplasm, to large swings in the concentration of Ca^{2+}. To this, one may add the situations of abnormal cytosolic Ca^{2+} overload that may accompany the exposure to damaging agents. These quantitative limitations do not apply to another class of Ca^{2+}-binding protein, intrinsic to membranes and able to transport Ca^{2+} across them. They complex Ca^{2+} at one side of the membrane, transport it across, and discharge it to the other side. By continuously repeating the binding and transport cycle, they buffer large amounts of Ca^{2+} even if their concentration in the membrane is minute. As these proteins do not have any role in the processing of the Ca^{2+} message, evidently, they have been developed by evolution for the sole purpose of keeping the concentration of Ca^{2+} in the cytosol and in the organelles within the boundaries necessary for the proper execution of the signaling function. Membrane Ca^{2+}-transport proteins are numerous, and satisfy both the demands for the rapid and high affinity regulation of Ca^{2+}, and those for slower and lower affinity regulation (Fig. 2).

The plasma membrane Ca^{2+} entry channels

The plasma membrane, like all biological membranes, is impermeable to charged species like Ca^{2+}. It controls its influx into cells through a series of proteinaceous channels whose gating mechanism separates them in at least three main classes. The voltage-gated

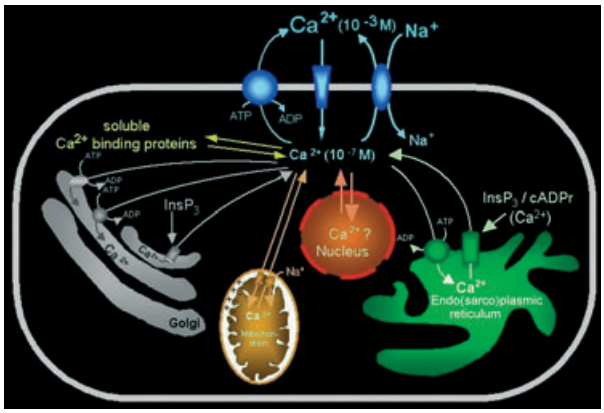

Fig. 2. The control of cellular Ca^{2+}. The Figure shows the transporters that control Ca^{2+} traffic across plasma and intracellular membranes. For simplicity, it only shows one plasma membrane Ca^{2+} channel type, although the text describes and discusses three. Calcium that has penetrated into the cell must eventually be re-exported, but prior to that it will be complexed by soluble Ca^{2+}-sensor proteins or may be temporarily and reversibly transported into organelles. Calcium is expelled from the cell by a high affinity, low capacity ATPase and/or a Na^+/Ca^{2+} exchanger with opposite properties. Calcium enters the sarco(endo)plasmic reticulum through the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (the SERCA pump) and is released through channels that require effectors like inositol-(1,4,5)-trisphosphate ($InsP_3$) or cyclic ADP ribose (cADPr). The Figure does not show the novel Ca^{2+}-mobilizing messenger NAADP because the store on which it functions is still undefined. The release route from the sarco(endo)plasmic reticulum creates Ca^{2+} hotspots (not shown in the Figure) which activate the low affinity electrophoretic Ca^{2+} uptake uniporter of neighboring mitochondria. Calcium exits mitochondria through a Na^+/Ca^{2+} exchanger (a H^+/Ca^{2+} release exchanger has also been described in some cell types). The Golgi vesicles accumulate Ca^{2+} using both an ATPase that differs from the SERCA and PMCA pumps (the SPCA1 pump), and a conventional SERCA pump. They release Ca^{2+} through channels of which so far only that gated by $InsP_3$ has been certified. Calcium can also be exchanged between the cytosol and the nucleus. The nuclear envelope is a genuine Ca^{2+} store that shares transporters and channels with the ER (not shown in the Figure). The question mark in the nucleoplasm highlights the debate on the nature of the Ca^{2+} traffic through the nuclear envelope (permanent passive permeability vs. reversible gating of the pores).

channels [11] open and close in response to changes in the transmembrane potential across the membrane. They are typical of the plasma membrane of excitable cells, e.g. heart and neurons, and are subdivided in several subtypes based on voltage and inhibitor sensitivity: L-, N-, T-, P/Q-, and R-type voltage-gated channels are presently recognized. Voltage-gated channels are tetrameric structures consisting of α1, α2-δ, β and γ subunits (Fig. 3). The Ca^{2+} channel proper is formed by the α1 subunits, while the other subunits have a regulatory function. The channel-forming α1

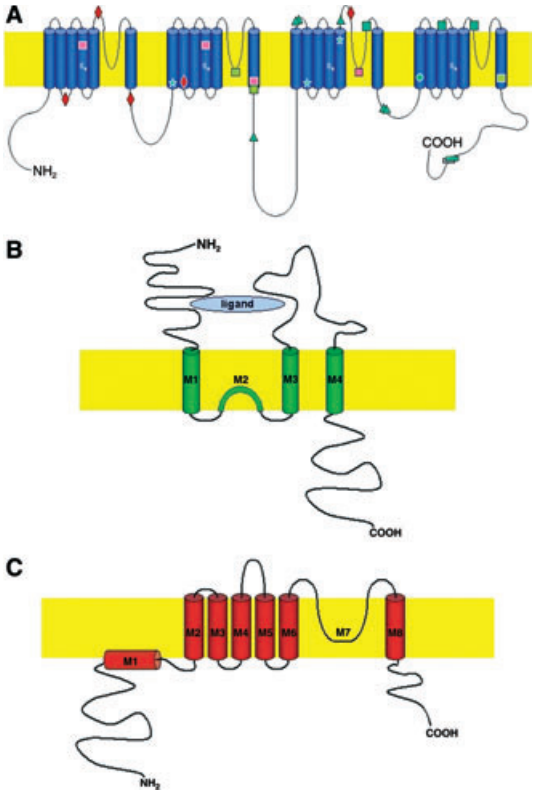

Fig. 3. The Ca^{2+} influx channels of the plasma membrane (A) The voltage gated channel. The membrane topography of the α1subunit, which forms the *trans*-protein Ca^{2+} channel proper, is shown. The Ca^{2+} pore is formed by the loop that folds within the membrane between transmembrane domains 5 and 6 in each of the four six-transmembrane domain repeats. The α1 subunit also contains a transmembrane domain (S4) which is rich in polar amino acids, and is assumed to be the voltage sensor of the channel. The Figure also shows the mutations in the channel responsible for a number of human diseases. Pink squares represent familial hemiplegic migraine, missense mutations. Light green squares represent familial hemiplegic migraine plus ataxia (missense mutations). Dark green squares represent episodic ataxia 2 (missense mutations). The dark green circle represents episodic ataxia 2 (missense mutations plus deletion). Light blue stars represent episodic ataxia 2 (aberrant splicing). Dark green triangles represent episodic ataxia 2 (truncations). The green rectangle represents episodic ataxia 2 (polyQ insertion). The brown rectangle represents spinocerebellar ataxia 6 (polyQ insertion). Red diamonds represent missense mutations in childhood absence epilepsy. (Adapted with permission from [6]). (B) A general membrane topography model of TRP channels (see text for details). (C) Membrane topography of the glutamate NMDA receptor (see text for details).

subunit is a four-fold repeat of a six-transmembrane domain motif, in which the external loop connecting transmembrane domains 5 and 6 of each repeat folds back into the membrane to form the walls of the channel. Importantly, voltage-gated channels (L-type) are targets of the drugs called calcium antagonists

(dihydropyridines, phenyl-alkylamines, benzothiazepines) that are widely used in therapy, particularly for diseases of the circulatory system. An important development in the area of voltage-gated channels is the identification of a number of human genetic diseases, collectively called calcium channelopathies, linked to mutations in the channel-forming α1 subunit and/or in the regulatory subunits [12]. The channelopathies affecting the α1 subunit of several channel sub-types are indicated in Fig. 3. The second family of plasma membrane Ca^{2+} channels comprises those which are gated by ligands. The activation may be indirect, i.e. linked to the interaction of agonists with G-protein-coupled receptors that produce inositol-(1,4,5)-trisphosphate (InsP3) and thus empty the endoplasmic reticulum Ca^{2+} store (see below). The emptying of the store somehow activates plasma membrane Ca^{2+} entry channels. Although these channels could be effectively considered as ligand-activated channels, they are conventionally grouped in the separate family of capacitative or store-operated channels (SOC). Prominent among the channels that are gated directly by ligands are those activated by the neurotransmitter L-glutamate, which is the most widespread excitatory neurotransmitter (Fig. 3). Glutamate activates two classes of plasma membrane receptors: the ionotropic receptors, which are bone fide ion channels, and the metabotropic receptors, which are coupled to G-proteins that may generate InsP3 and thus empty the internal Ca^{2+}-store. They thus lead to the activation of the SOC channels as any other of the agonists mentioned above. The ionotropic receptors contain channels that are primarily permeable to Na^+ and K^+ (the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid and kainate receptors) or to Ca^{2+} (the NMDA receptors, which are activated by the glutamate agonist *N*-methyl-D-aspartate) [13]. The NMDA-receptors are heteropolymeric structures containing several subunits of the same family (NMDAR1, NMDAR2 and NMDAR3 subunits have been described); each subunit contains four hydrophobic stretches, of which three are membrane spanning domains (M1, M3, M4) and one (M2) a hairpin-loop that folds back into the membrane at the cytosolic mouth of the channel. The NMDA receptor/channel contains a calmodulin-binding site that also binds α-actinin 2 in the C-terminal tail of subunit 1. Incoming Ca^{2+} would activate calmodulin that displaces α-actinin 2 from the binding site, inactivating the channel; this activation/inactivation sequence is a striking example of the auto-regulative nature of the Ca^{2+} signal. The NMDA receptor is also a clear example of the ambivalent nature of the Ca^{2+} signal; normally operating NMDA receptors admit into the neurons the Ca^{2+} that is vital to their function, but abnormally functioning NMDA receptors increase cellular Ca^{2+} beyond manageable levels, leading to the generation of toxic reactive oxygen species and of deadly excess amounts of NO radical. As mentioned earlier, the SOC or capacitative (CCE) Ca^{2+} channels sense the level of filling of the intracellular Ca^{2+} store(s) [14]. They are homologous to the transient receptor potential (TRP) channels first identified in the retina of *Drosophila* [15] (Fig. 3). This family of cationic channels, which is presently attracting great interest, comprises nearly 30 members divided in six subfamilies with multiple members [16]. The seven members of the TRPC subfamily (the classical TRPs) are arranged in the membrane as tetramers with six transmembrane-spanning regions, with high homology to other ion-channels. They are all modulated by agonists that act on receptors linked to the activation of phospholipase C and to the production of InsP3. The mechanism by which the emptying of the intracellular Ca^{2+} store activates the TRPC Ca^{2+} entry pathway is still unknown; a 'calcium influx factor' (CIF) that is released from the emptied stores to gate the channel has been proposed, but the evidence for its existence is at best controversial. Alternatively, the store-operated channels would be gated by some form of direct interaction with the emptied endoplasmic reticulum Ca^{2+} store. An important development in the area of TRP channels is their involvement in different disease processes; abundant evidence shows that TRP channels are differently expressed in certain cancers [17], and relates mutations in TRPP, a member of another TRP channel superfamily, to the polycystic kidney disease [18].

The plasma membrane Ca^{2+} ejecting systems

Calcium that has entered cells via one or more of the channels described above must eventually be ejected, as the cytosolic Ca^{2+} concentration constantly oscillates around the 100–200 nM level during the normal life of most cells. This is accomplished using two systems: a Ca^{2+}-ATPase that is operating in all cells, and a $Na+/Ca^{2+}$ exchanger that is especially active in excitable cells. The Ca^{2+}-ATPase is a high affinity system (K_d for Ca^{2+} 0.2–0.5 μM) that has limited transport capacity, and thus functions as a housekeeping enzyme that controls cell Ca^{2+} even when its concentration remains at the level indicated above. In contrast, the Na^+/Ca^{2+} exchanger is a larger system that has low Ca^{2+} affinity (K_d for Ca^{2+} ≈1 μM) and thus requires that Ca^{2+} in its vicinity rises to the μM level, as one expects to occur in excitable cells as those in the heart.

The Ca^{2+} ejecting plasma membrane Ca^{2+}-ATPase (PMCA) pump

The plasma membrane Ca^{2+}-ATPase (PMCA) pump [19] enzyme is organized in the plasma membrane with 10 transmembrane domains, and has a long C-terminal tail which contains a calmodulin-binding domain; the pump is thus a further striking example of the autoregulation of the Ca^{2+} message, as it is activated to extrude Ca^{2+} from the cell by interacting with Ca^{2+}–calmodulin. The regulation of the PMCA pump by calmodulin has been studied in some detail. In the absence of Ca^{2+} and calmodulin, the C-terminal tail of the pump, including the calmodulin-binding domain, binds to two 'receptor' sites located in the first and second cytosolic protrusions of the pump [20,21]. This intramolecular interaction presumably hinders the access of Ca^{2+} and/or ATP to the active site, maintaining the pump in an inhibited state. Calmodulin removes its binding domain and the C-terminal tail that follows it, from the 'receptors', freeing the access to the active site and activating the pump (Fig. 4). The PMCA pump belongs to the family of P-type ATPases, characterized by the formation of a phosphorylated intermediate (an aspartyl-phosphate) during the reaction cycle. At variance with the sister pump of endo (sarco) plasmic reticulum to be discussed later, it only contains one Ca^{2+}-binding site, and indeed transports one Ca^{2+} as one ATP molecule is hydrolyzed. In higher eucaryotes, the PMCA pump is the product of four genes; two isoforms (PMCA1 and 4) are expressed in all tissues and are thus considered as housekeeping enzymes. The expression of the two other basic isoforms (PMCA2 and PMCA3), which have particularly high affinity for calmodulin, is restricted to some cells types, among which neurons are particularly important. Alternative splicing of the primary transcripts increases greatly the number of PMCA variants. The splicing occurs at two sites termed A and C, which are located next to a basic stretch of amino acids that binds activatory acid phospholipids in the first main cytosolic unit of the pump, and within the C-terminal calmodulin-binding domain, respectively. Interestingly, PMCA splice variants have a peculiar tissue distribution, in all likelihood reflecting (subtle) differences in functional properties that enable them to respond to special Ca^{2+} homeostasis demands of cells/tissues. One particularly striking case is that of a variant of PMCA2 that is truncated C-terminally as the result of an insert at site C, but also has a substantial insert (150 amino acids) at splice site A. This pump variant is the resident PMCA pump in the stereocilia of the hair cells of the organ of Corti, which have special Ca^{2+} homeostasis demands. The reason for directing special attention to this PMCA isoform is the recent discovery that inactivating mutations in the *PMCA2* gene produce hereditary deafness in two mice strains [22,23]. This is the only spontaneous disease condition so far discovered to involve the PMCA pump. The observation is interesting also because it could be a model for some forms of hereditary deafness in humans.

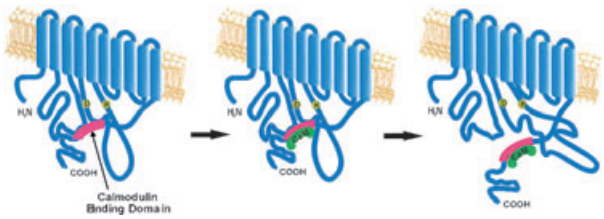

Fig. 4. The activation of the plasma membrane Ca^{2+} pump by calmodulin. The calmodulin-binding domain (red cylinder) interacts in the resting state with the first and second main cytosolic protrusions of the pump, hindering the access to the active site (indicated by the ATP-binding lysine and the phosphoenzyme-forming aspartic acid). Calcium-saturated calmodulin (green, CaM) complexes the binding domain, and removes it from the intramolecular 'receptor' sites.

The existence of so many isoforms of the PMCA pump suggests functional differences among them that could accommodate different Ca^{2+} homeostasis demands of cells in various tissues, but it could also reflect variations in the way Ca^{2+} signals must be regulated in different domains of a single cell, a suggestion that would in turn demand that PMCA isoforms, spliced or otherwise, are dishomogeneously distributed along the plasma membrane contour. Unfortunately, information on functional differences among PMCA isoforms is still very scarce, possibly due to the fact that the expected differences are likely to be small, reflecting (subtle) peculiarities in regulation and/or partnership with intracellular proteins. As mentioned, PMCA2 and PMCA3 have higher calmodulin affinity and, in the native (*in vivo*) environment, are much more efficient in extruding Ca^{2+} than the two housekeeping isoforms 1 and 4 [24]. Surprisingly, the C-terminal truncation of the pumps, by the insertion of a novel exon at splice site C – even if it removes about half of the calmodulin-binding domain – has no significant effect on the Ca^{2+} extrusion ability of the pumps *in vivo* [24]. Evidently, much must still be learned on the factors/conditions that determine the Ca^{2+} extrusion ability of the PMCA pumps.

The plasma membrane Na^+/Ca^{2+} exchanger (NCX)

The Na^+/Ca^{2+} exchanger (NCX) is the principle Ca^{2+} ejection system in excitable cells, e.g. heart [25]. As its affinity for Ca^{2+} is low, it is normally assumed that it only operates at full capacity when the concentration of Ca^{2+} in the subplasma membrane domain rises to the micromolar level; this occurs, for example, at peak activation in heart cells. The NCX should be expected to function very poorly, or even not at all, under normal resting cell conditions, when the overall cytosolic Ca^{2+} concentration (100–200 nM) is at least one order of magnitude lower than the K_m of the NCX for Ca^{2+}. Surprisingly, however, recent work [26] has shown the NCX to function at a significant rate in nonexcitable model cells overexpressing it. Evidently, the concentration of Ca^{2+} in the subplasma membrane layer at rest is (much) higher than in the bulk cytosol also in nonexcitable cells and is in any case adequate to activate the exchanger.

The exchanger operates electrogenically, exchanging 3 Na^+ against 1 Ca^{2+}. Its direction of transport therefore depends not only on the chemical gradients of Na^+ and Ca^{2+} across the plasma membrane, but also on the sign of the transmembrane potential: a typical case is that of the plateau phase of the action potential in heart, during which the NCX operates in the reverse mode, importing Ca^{2+} into the heart cell. The reverse mode of operation of the NCX is also used to explain the positive inotropic effect of ouabain (digitalis), and of other drugs that block the Na^+/K^+-pump. The reverse operation of the exchanger is in this case induced by the very large increase in the intracellular concentration of Na^+ that follows the inhibition of the Na^+/K^+-pump by ouabain.

The membrane topography of the exchanger had been originally proposed to consist of 11 transmembrane domains and a large cytosolic loop (about 500 residues) separating transmembrane domains 5 and 6. It has now been revised down to only 9 transmembrane domains [27] by eliminating two of the C-terminal domains. The path for Na^+ and Ca^{2+} across the protein is still not established, but it does not seem to involve the large cytosolic loop, which instead contains a Ca^{2+}-binding site with regulatory function. The loop also contains in its N-terminal portion a domain that binds calmodulin (termed XIP) but no evidence for the regulation of the exchanger by calmodulin has been provided as yet.

In higher eucaryotes, the exchanger is the product of three separate genes. Of the three basic isoforms, NCX1 is typical of heart, but is also expressed significantly in numerous other tissues, notably neurons. NCX2 and NCX3 have a more restricted pattern of tissue expression; they are expressed at high levels in neurons and to a lesser extent in skeletal muscle. Spliced variants of two of the exchangers have been described (NCX1 and NCX3), the splicing event involving the C-terminal portion of the large cytosolic loop.

The expression of NCX isoforms in neurons is regulated at the transcriptional level by Ca^{2+} [28]. This involves essentially NCX2 and NCX3; the former is rapidly down-regulated by Ca^{2+} in cultured cerebellar neurons in a process that is mediated by the Ca^{2+} calmodulin-dependent phosphatase calcineurin, the latter is instead more slowly up-regulated by Ca^{2+}. However, in the same neurons, NCX3 is also down-regulated by the Ca^{2+}-binding transcriptional repressor DREAM [29]. It is of interest that the same type of transcriptional regulation also affects the expression of the four basic PMCA isoforms [30,31] in cultured cerebellar granules. PMCA4 is rapidly down-regulated in a calcineurin-mediated process, PMCA2 and PMCA3 are more slowly up-regulated.

So far, no human condition has been described that could be related to genetic NCX defects. However, recent work (this laboratory) has shown that the excitotoxic death of neurons triggered by a Ca^{2+} overload may be due to the specific cleavage of NCX3 by the Ca^{2+}-dependent protease calpain.

Control of Ca^{2+} homeostasis by transport across intracellar membranes

Intake and ejection of Ca^{2+} at the plasma membrane level normally only accounts for a minor portion of the total Ca^{2+} used by cells for their functional cycle. Calcium entering from outside, however small in concentration, is nevertheless essential to cell function, as it sets in motion a number of important events, including the liberation of massive amounts of Ca^{2+} from the intracellular deposits of some cell types. Quantitatively, however, most of the Ca^{2+} used by cells is controlled by transporters located in the membranes of the organelles. Eventually, the Ca^{2+} that has entered must be ejected (or an amount equivalent to it), as cells do not normally experience cytosolic Ca^{2+} overload.

The sarco(endo)plasmic reticulum

The reticulum contains a sarco(endo)plasmic reticulum ATPase (SERCA) pump that has the same membrane topology and general mechanism of action of the PMCA pump. It is arranged in the membrane with 10 transmembrane domains (however, see below) and

forms an aspartyl-phosphate during the reaction cycle. It was purified from skeletal muscle in 1970 [32] and has been studied in great detail ever since ([33] for a recent review). At variance with the PMCA pump, it has two Ca^{2+}-binding sites, and indeed it transports two Ca^{2+} molecules per hydrolyzed ATP molecule. Three basic isoforms of the pump have been identified in animal tissues: SERCA1 is expressed at high levels in fast twitch muscles, and at lower levels in slow twitch muscles. SERCA2 has been detected in a number of tissues besides muscles, however, of the two spliced isoforms one, SERCA2A, is only expressed in muscles, e.g. heart, whereas SERCA2b is found in smooth muscles and in a number of other tissues as well.

One interesting aspect of isoform 2b is the replacement of the 4-C terminal residues of the 2a isoform with a 49 amino acid sequence that contains an 11th transmembrane domain [34]. Isoform 2b is thus peculiar in that its C- and N- termini are located at opposite sites of the reticulum membrane. Isoform 3 of the pump has been detected in the endoplasmic reticulum of a number of nonmuscle cells, most notably in platelets.

A recent important advance in the area of the SERCA pump has been the solution of its tertiary structure in both the Ca^{2+}-bound and Ca^{2+}-free states [35,36] (Fig. 5). The work has confirmed the predicted 10-transmembrane domain topology, and has shown that the cytosolic portion of the pump is arranged in three main domains. In the Ca^{2+}-bound state (the E1 state) they are well separated to permit ATP to penetrate the gap separating the nucleotide binding and the phosphorylation domains; this allows ATP to phosphorylate D351 in the latter domain. Large movements in the cytosolic domains, but also in the transmembrane helices, accompany the transition from the E1 to the E2 (Ca^{2+}-free) conformation, in which the cytosolic portion of the pump is more compact. The changes in the positions and tilting of the transmembrane helices, and of the loops that connect them, bring the Ca^{2+} ligands, which are well seperated in the E2 conformation, closer to each other to form two compact Ca^{2+}-binding sites. Site-directed mutagenesis work [37] had predicted the two sites to be formed by ligands located in transmembrane domains 4,5,6, and 8; the tertiary structure work has validated and extended the predictions, outlining the access and exit routes for Ca^{2+}.

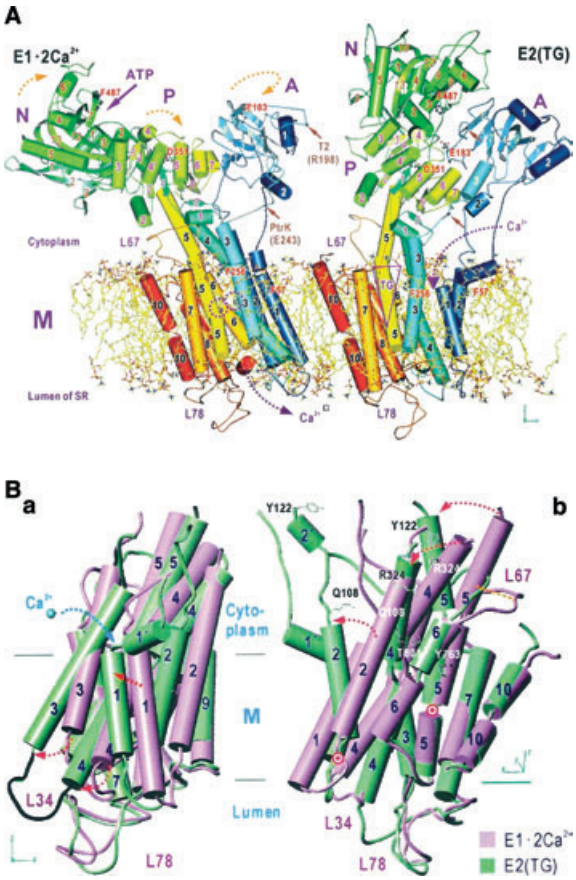

Fig. 5. Three dimensional structure of the SERCA pump. Ribbon representation of the ATPase in the E1 (Ca^{2+}) and E2 (Ca^{2+}-free) conformations. The E2 structure has been obtained in the presence of the pump inhibitor thapsigargin (TG). (A) The two forms of the ATPase in the lipid bilayer (M), generated by molecular dynamics simulation of dioleyl-lecithin. The transmembrane helices are numbered 1–10. The two magenta dotted-circles represent bound Ca^{2+} (sites I and II). The orange arrows in the E1 (Ca^{2+}) conformation indicate the direction of movement of the cytoplasmic domains during the E1–E2 transition. The large magenta triangle (TG) indicates the thapsigargin binding site (key residue, F256). The magenta arrows show the entry and exit paths for Ca^{2+}. Other critical residues are also indicated: the trypsin digestion site (T2, R198), the proteinase K digestion site (PrtK, around E243), the critical residue E183 in the A domain, the phosphorylation site (D351) in the phosphorylation domain, a key residue in ATPbinding (F487). K400 (the phospholamban interacting site) is at the bottom border of the N-domain (not shown in the figure). From [82]. (B) Rearrangement of the transmembrane helices (numbered 1–10) upon dissociation of bound Ca^{2+}. The E1-Ca^{2+} helices (violet) and the E2 (TG) form helices (green) are superimposed and viewed from the right (a) and the rear (b). Helices 8 and 9 are removed in (b) so that the movement of helices 5, 4 and 2 is seen more clearly. Double red circles show pivot position for helices 2 and 5. The orange dashed line shows a critical hydrogen bond between helix 5 and cytoplasmic loop 6/7, the red dashed arrows indicate the direction of movement in the E1–E2 transition, the blue dashed arrow the proposed entry path for the first Ca^{2+}. (Adapted and reprinted, with permission, from [82] the Annual Review of Biochemistry, Volume 73 © 2004 by Annual Reviews www.annualreviews.org)

The SERCA pump of cardiac, slow twitch, and smooth muscles is regulated by an accessory hydrophobic protein called phospholamban [38], which is composed by a hydrophilic N-terminal helix connected through a short flexible hinge to a very hydrophobic, transmembrane helix [39]. Phospholamban interacts with the SERCA pump both at the cytosolic nucleotide binding domain (Lys400 in the nucleotide binding domain, [40]) and with the transmembrane sector, maintaining the pump in an inhibited state. The inhibition is removed by the phosphorylation of residues 16 and 17 in the hydrophilic portion by protein kinase A and a calmodulin-dependent kinase. The phosphorylation is assumed to induce a conformational change of phospholamban that forces its detachment from the pump both at the cytosolic and the transmembrane interacting sites (Fig. 6). The formal similarity with the process of activation/deactivation described above for the PMCA pump is evident, even if the mechanisms by which pump inhibition is relieved differ; calmodulin-binding is at work in the case of the PMCA pump, kinase-mediated phosphorylation in that of the SERCA pump. It may be added that the calmodulin-binding domain of the PMCA pump and phospholamban have a significant degree of sequence similiarity.

Once taken up by the SERCA pump, Ca^{2+} is stored inside the reticulum as a complex with a number of low affinity Ca^{2+}-binding proteins, of which the most important is calsequestrin. Protein-bound Ca^{2+} is in equilibrium with free Ca^{2+} in the lumenal space, ready to be released when necessary to perform the cytosolic Ca^{2+} signaling function. Although a continuous, and minor, nonspecific leakage of Ca^{2+} is likely to occur, the release of Ca^{2+} from the reticular lumen is mediated by specific channels, of which two types have been recognized to date. Both channels require Ca^{2+} itself in order to be opened but their gating demands specific messengers. One messenger, inositol 1,4,5-tris phosphate ($InsP_3$) operates in cells other than striated muscles [41]. Its production by agonists acting on G-protein-linked receptors that activate a phospholipase C to cleave phospatidyl-inositol-bis-phosphate (PIP_2) has been studied in great detail. InsP3 acts on a tetrameric receptor characterized by a very long cytosolic N-terminal tail that contains the InsP3 binding site [42], which has been crystallized recently [43] (Fig. 7). The Ca^{2+} channel proper is located in the C-terminal portion of the molecule: it contains the 6-transmembrane domain motif, with the loop connecting helices 5 and helix 6 folding back into the membrane, as seen above in the plasma membrane voltage-gated channels. Three

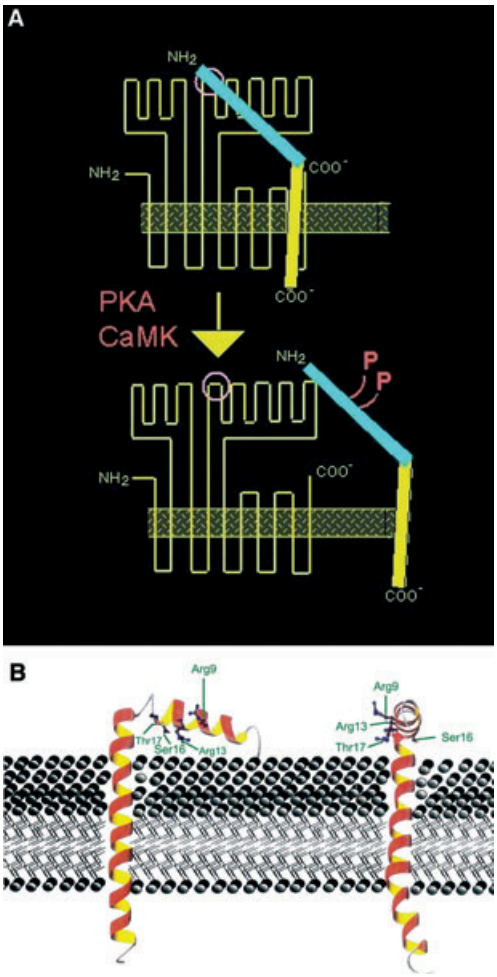

Fig. 6. Regulation of the activity of the SERCA pump by phospholamban. (A) The pump is represented with 10 transmembrane domains, with the intrinsic, hydrophobic protein phospholamban bound in the resting state to a site in the hydrophilic portion of the pump (K400 in the nucleotide binding domain [40]) and to sites in the transmembrane sector. In the phospholamban-bound state the pump is inhibited. Phosphorylation of phospholamban on S16 and T17 by one or two of the two kinases indicated in the text removes phospholamban from the pump, relieving the inhibited state. (B) Solution structure of phospholamban, ribbon representation [39]. The structure of the hydrophobic phospholamban has been obtained in chloroform/methanol, and shows two α-helices with a small unstructed portion around a proline (P21; not shown in figure) which may represent a flexible hinge between the cytosolic and intramembrane helices.

InsP3 receptor subtypes have been identified: type I is prominently expressed in smooth muscle and brain, type II has a very low level of expression, type III in more widely distributed in tissues. Other receptor isoforms have also been described (IV and V) but their importance is unclear. The receptor isoform complexity is further increased by alternative splicing proces-

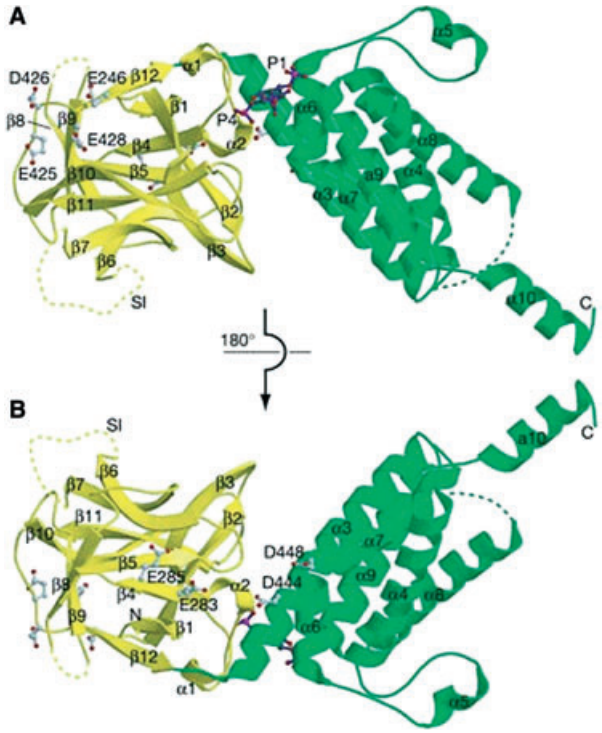

Fig. 7. Structure of the InsP3 binding domain of InsP3 receptor type 1 in complex with InsP3. Ribbon representation [43]. The domain consists of a β-subdomain (yellow) and a α-helical domain (green) with the InsP3 molecule at the interface. The protein fold in the β-domain is known as the β-trefoil (found in proteins as diverse as interleukins 1α and 1β, fibroblast growth factors, and mannose receptors. The α-domain has high homology to the armadillo repeat fold found in β-catenin, importins, and adenomatous polyposis coli. The structure in B is rotated 180° with respect to that in A. Adapted with permission from [43] © Nature 2002 (http://www.nature.com/).

ses. The receptor contains consensus sites for kinase phosphorylation and sites for the binding of a number of proteins and other factors with presumed regulatory roles. One protein of particular interest among these proteins is huntingtin; it may link the altered function of the InsP3 receptors to Huntington's disease [44].

The other ligand-activated receptor/channel is routinely referred to as the ryanodine receptor (RyR) – from the alkaloid that is commonly used to study it. (ryanodine can induce opening or closure of the channel depending on concentration.) The RyR is a tetramer of a very large protein (> 5000 amino acids per monomer). In analogy with the InsP3 receptor it also has a very large N-terminal cytosolic unit, and a transmembrane channel located in the C-terminal portion. Various claims have been made in the past as to the number of transmembrane helices forming the Ca^{2+} channel proper, but the matter now appears to be settled on a somewhat atypical variation on the canonical theme of six transmembrane helices with the loop connecting helices 5 and 6 on the luminal side folding into the membrane to form the walls of the channel [45]. As is the case for the InsP3 receptor, the RyR is activated by Ca^{2+} (however, in skeletal muscles Ca^{2+} appears to be unnecessary, as the channel is opened by a process of direct charge transfer from the neighboring T-tubular invaginations of the plasma membrane). The matter of the native messenger for the RyR is disputed; in a number of cell types the NAD metabolite cyclic ADP-ribose (cADPr) [46] has been shown to gate the channel but the data linking plasma membrane agonists to the production of cADPr are still very scarce. In addition, cADPr has been claimed to be produced extracellularly by an ectoenzyme [47] and must thus be imported into the cell to act. Three isoforms of the RyR have been described: RyR1 is expressed in several tissues, but most abundantly in skeletal muscles, RyR2 is found in heart and brain, RyR3 predominantly in some brain regions (but also in lymphocytes). As is the case for the InsP3 receptors, the RyRs also contain binding sites for a number of proteins with presumed regulatory function. A particularly interesting case is that of the immunophilin, FKBP12, which stabilizes the channel favoring either the opening or closed state, thus preventing irregular flickering. It is proposed that it does so by improving the cooperation among the four monomers. The spatial organization of the RyRs in tissues different from muscles is probably random along the endoplasmic reticulum network. In striated muscles, however, the RyR release channels are located in the terminal cisternae, next to the T-tubular system of the plasma membrane. The location is obviously convenient, as in this way the receptors are exposed immediately to the incoming Ca^{2+} that gates their channel.

An important development in the area of the RyR is the causative role of mutations in the *RyR1* gene in two human muscle diseases, malignant hyperthermia and central core disease [48]. The diseases are characterized by Ca^{2+} leakiness of the RyR, which produces contractures and extreme hyperthermia in the former condition, and degeneration of the central portion of the fiber in the latter. Defects in the RyR2 isoform cause two heart diseases characterized by arrhytmias and/or tachycardia that can lead to sudden death [49].

One last Ca^{2+} releasing messenger which has recently acquired prominence must be mentioned before leaving the topic of Ca^{2+} release from endo(sarco)plasmic reticulum. This is the nicotinic acid derivative of NADP+ (NAADP) [50]. It has been shown to release Ca^{2+} in a

number of cell types by acting on a store which is still undefined, but is not the endoplasmic reticulum [51]. NAADP appears to be synthesized by the same ecto-enzyme that also synthesizes cADPr and, thus, must also be imported in the cell, a necessity that opens a problem that is still not solved [46,52]. NAADP, at least in invertebrate oocytes, where is has been studied in particular detail, has characteristics that make it particularly interesting among Ca^{2+} mobilizing messengers. It appears to act on a store which is cortical, and somehow linked to the plasma membrane, where it gates a channel that carries Ca^{2+} into the cell [53].

The Golgi body

The Golgi system of membranes has only recently come to the forefront as a regulator of cellular Ca^{2+} homeostasis. It contains a conventional SERCA pump, but also a novel Ca^{2+}-ATPase that transports both Ca^{2+} and Mn^{2+} with high affinity. In higher eukaryotes this novel ATPase is orthologous to the Pmr1 pump in yeasts, and is termed SPCA1 (secretory pathway Ca^{2+} ATPase) [54], to differentiate it from a related protein (SPCA2) whose biological function is still unknown. SPCA1 is a P-type ATPase with 37% amino acid identity to the SERCA 2a pump. It is predicted to contain the conventional 10 transmembrane domains of Ca^{2+} pumps, but is smaller than the SERCA pump (about 900 residues) as residues are missing in the lumenal loops. One important development in the area of SPCA pump comes from the disease area; genetic loss of SPCA1 causes Hailey–Hailey disease, a serious autosomal dominant skin disease characterized by recurrent vesicles and erosions of the flexural areas. The defect is due to the dysregulation of intracellular Ca^{2+} in keratinocytes, which leads to the disruption of the epidermal Ca^{2+} gradient in the skin. Calcium is released from the Golgi store via a channel that is modulated by InsP3 [55] and is presumably identical to that of endoplasmic reticulum. So far, no ryanodine-type channel has been described in the Golgi system.

The nucleus

The matter of independent Ca^{2+} homeostasis in the nucleus is controversial (see [56] for a comprehensive review). This is so because the nuclear envelope, which is a continuation of the endoplasmic reticulum, contains a large number of pores. These are complex structures containing a channel whose diameter is large enough to be permeable to molecules having masses as high as 20–30 kDa. In principle, then, Ca^{2+} in the nucleoplasm should be in continuous passive equilibrium with Ca^{2+} in the cytosol, as suggested by a number of studies in which nuclear and cytosolic Ca^{2+} have been monitored simultaneously [57,58]. On the other hand, other studies with a number of fluorescent probes have shown a clear attenuation of the signal at the nuclear envelope [59,60]. Perhaps more importantly, patch-clamp experiments on isolated nuclei, and even on nuclei within cells, routinely show single channel current events even if the patches contain dozens of pores [61,62]. This suggests that the pores may be gated, admitting or excluding even species as small as Ca^{2+}, depending on their opening state. However, irrespective of the controversy over whether Ca^{2+} is (or isn't) admitted passively into the nucleus, the nuclear envelope is a bona fide Ca^{2+} store; this is not surprising considering its continuity with the endoplasmic reticulum. It contains a Ca^{2+} pump which is identical to that of the endo(sarco)plasmic reticulum [63] and channels sensitive to InsP3 and cADPr [64]. Recent reports also indicate the presence of a Na^{+}/Ca^{2+}-exchanger [65] and results in exocrine pancreas cells indicate that the nuclear envelope contains a store that is sensitive to NAADP [66]. The question at this point is that of reconciling the documented presence of Ca^{2+} transporters in the envelope with that of the pores, which, even if they were opened only part of the time, will still exchange massive amounts of Ca^{2+} between the nucleus and the cytosol. One conciliatory view [2] proposes that the pores could be large capacity Ca^{2+} buffers for the whole nucleoplasm, whereas the Ca^{2+} store in the envelope, and its channels and transporters, would fine-tune Ca^{2+} in selected subenvelope domains. The nucleus is now known to contain the full array of the enzymes of the phosphoinositide signaling pathway, which is another indication of the ability of the nucleus to carry out independent Ca^{2+}-signaling functions. Perhaps the most distinctive of them is the regulation of gene transcription, which now concerns a rapidly increasing number of genes. Most of the observations have been made on immediate-early genes and have involved CREB, a transcription factor of the bZIP family [56,67]. Its Ca^{2+}-mediated activation involves calmodulin-kinases II and IV, the latter being much more effective. The Ca^{2+}-dependent protein phosphatase calcineurin also intervenes in the modulation of nuclear gene expression, most strikingly by dephosphorylating the transcription factor NFAT and transporting it into the nucleus, where it regulates a number of genes of T-lymphocytes (and other cells as well) [68]. More recently, another Ca^{2+}-dependent factor (a protein of the EF-hand family that has been called DREAM) [69] has been shown to silence a number of genes by

binding the DRE sites in their promoter regions. Calcium binds to DREAM, removing it from the DRE sites and reinitiating transcription. Interestingly, one of the genes controlled by DREAM is that for the Na^+/Ca^{2+} exchanger 3 (NCX3) which is of particular importance to neuronal Ca^{2+} homeostasis [29]. This is another striking example of the autoregulative character of the Ca^{2+} signal.

Apart from the controversial role of the envelope pores, the idea of independent Ca^{2+}-signaling in the nucleus faces another problem. This is the transmission of the signals originating at the plasma membrane to the nuclear envelope, for example to initiate the events of the phosphoinositide signaling pathway. Some promising leads are starting to appear, but this still is a significant obstacle to the concept of autonomous Ca^{2+} regulation in the nucleus.

Mitochondria

The story of mitochondria as cellular Ca^{2+} buffers has unusual aspects (for a recent review see [70]). When it was first found in the 1950–1960s that mitochondria could actively accumulate Ca^{2+}, a period of very active research ensued, during which the mechanism of the transport reaction was clarified. At that time, a role of mitochondria as important regulators of cytosolic Ca^{2+} seemed obvious. However, a number of findings soon quelled the enthusiasm. Chief among them was the discovery that the affinity of mitochondria for Ca^{2+} (the apparent K_m is in the micromolar range) was too low to permit them to efficiently control Ca^{2+} in cells, considering its low bulk concentration in the cytosol. Thus, even if it was shown that the active transport process did nevertheless occur in mitochondria *in vivo* [71], the belief that mitochondria were important in the regulation of cytosolic Ca^{2+} rapidly lost momentum. Later on, the discovery of the Ca^{2+} releasing function of InsP3 further decreased interest on mitochondria and Ca^{2+}, shifting emphasis on the regulation of cytosolic Ca^{2+} to the endoplasmic reticulum. The process of mitochondrial Ca^{2+} transport as an important player in Ca^{2+} homeostasis in the cell only emerged from oblivion about 10 years ago, thanks to the ingenious use of targeted Ca^{2+} indicators [72], which showed that mitochondria became exposed to 'hotspots' of high Ca^{2+} concentration created in their vicinity by the opening of InsP3-gated channels in the neighboring vesicles of endoplasmic reticulum. Ca in the 'hotspots' was high enough to activate the low affinity mitochondrial uptake system; from that point on, mitochondria were re-installed in the important role of regulators of cellular Ca^{2+} homeostasis which had been suggested when their ability to accumulate Ca^{2+} was discovered by the pioneering work in the 1960s.

The transport reaction consists of separate uptake and release legs. The energy favorable uptake leg, uses an as yet still unidentified electrophoretic uniporter energized by the negative membrane potential maintained inside mitochondria by the respiratory chain. The release route is mediated by a neutron-neutral Na^+/Ca^{2+} exchanger (replaced by a H^+/Ca^{2+} exchanger in some mitochondrial types) that, acting in concert with the uptake leg, generates an energy dissipating Ca^{2+}-cycle [73]. The uptake of Ca^{2+} from the vicinal high Ca^{2+} concentration 'hotspots' is rapid, and does not only efficiently regulate cytosolic Ca^{2+} it also efficiently regulates intramitochondrial Ca^{2+}. This is a point of great importance, as the mitochondrial matrix contains three dehydrogenases of crucial importance to the citric acid cycle [74] that are exquisitely sensitive to Ca^{2+} (Fig. 8). The delivery of reducing equivalents to the respiratory chain, and thus in the end the synthesis of ATP, depend on their correct functioning. A number of elegant recent experiments have now documented the synchrony between the uptake of Ca^{2+} by mitochondria and the production of NADH [75,76].

One finding made in the early days of mitochondrial Ca^{2+}-transport was the simultaneous accumulation of inorganic phosphate to precipitate Ca^{2+} as an insoluble salt deposit in the matrix [77] (Fig. 9). This

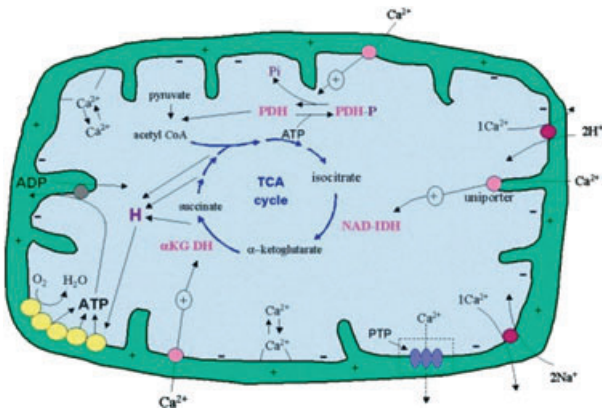

Fig. 8. Regulation of dehydrogenases in the mitochondrial matrix by Ca^{2+}. The regulated enzymes are two dehydrogenases of the citric acid cycle (the NAD-dependent isocitric dehydrogenase (NAD IDH) and the α-ketoglutarate dehydrogenase (αKG DH) and the phosphatase that dephosphorytes pyruvate dehydrogenase to make it active. The figure also shows (the violet structure, lower right) the mitochondrial permeability transition pore (PTP) which may also be involved in the movements of Ca^{2+} but which has not been discussed in the text.

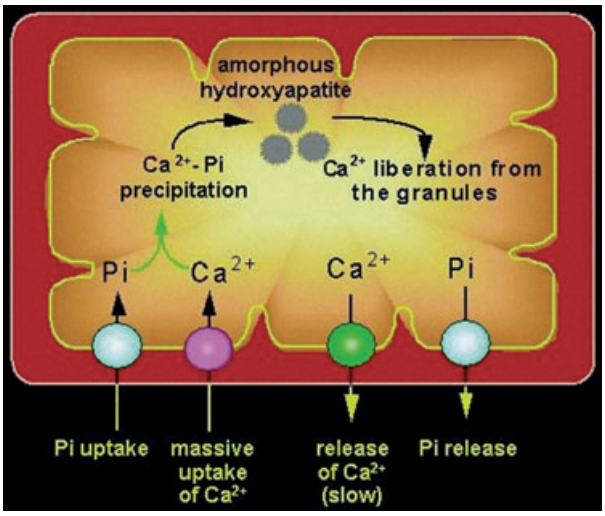

Fig. 9. A cartoon showing the uptake and storage of massive amounts of Ca^{2+} and inorganic phosphate in mitochondria. Details in the text.

enabled purified mitochondria exposed to Ca^{2+} and phosphate to store massive amounts of Ca^{2+}. The deposits which were seen easily as electron-dense granules under the electron microscope [78], were isolated, and found to have the molar stoichiometric Ca/P_i ratio of hydroxyapatite. However, they failed to become crystalline, and remained amorphous even if observed after protracted periods of time. What makes these observations interesting was the finding that a number of disease conditions affecting various tissues were also characterized by the presence of large numbers of dense granules, identical to those seen in isolated mitochondria, within the mitochondrial profiles [79] (Fig. 10). As a result of all these observations, the accumulation of massive amounts of Ca^{2+} and P_i became recognized as a useful means to control situations of abnormal Ca^{2+} overload in the cytoplasm. In other words, mitochondria became essential safety devices that enabled cells to successfully confront, albeit necessarily only for a limited time, situations of Ca^{2+} emergency. While the importance of mitochondria as regulators of cellular Ca^{2+} under normal conditions only became fully accepted a short while ago (see above), the role of mitochondrial Ca^{2+} (and P_i)-storage in cell pathology was consistently considered as a function of primary importance. The finding that the hydroxyapatite deposits remain (undefinitely) amorphous may be important, as it could facilitate the dissolution of the granules once the Ca^{2+} storm has abated, and cytosolic Ca^{2+} has fallen back to the normal low resting concentration.

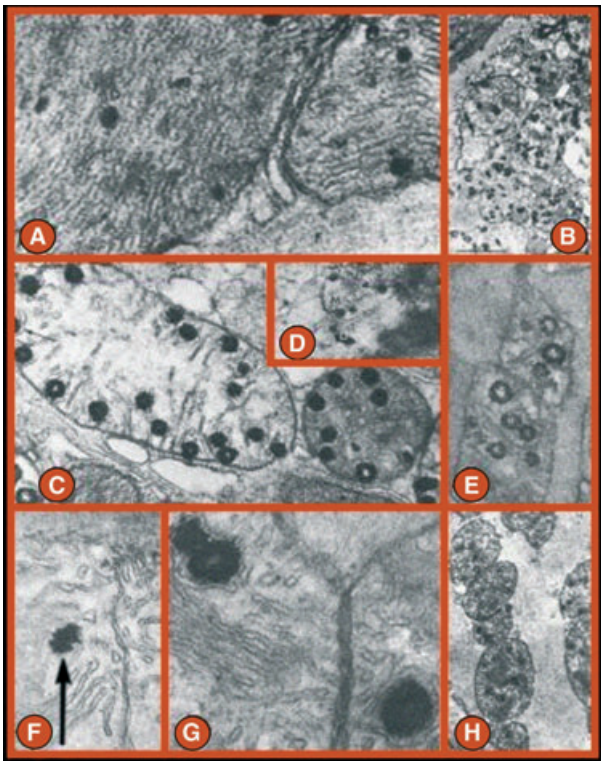

Fig. 10. Electron opaque masses (calcium–phosphate hydroxyapatite deposits) in mitochondria of variously injured cells. The hollow core seen in some of the granules is an artefact due to damage to the granules by the electron beam. Adapted with permission from [78]. (A) Muscle of a mouse treated with tetanus toxin. (B) Kidney tubule of a rat after the injection of sublimate. (C) Kidney tubule of a mouse treated with parathyroid hormone. (D) Dog myocardium injured by 40 min ischemia followed by 20 min of arterial blood reflow. (E) Myocardium of a Mg^{2+}-deficient, cold-stressed rat. (F) Dog myocardium injured by 40 min ischemia. (G) Dog myocardium injured by 40 min ischemia, followed by 20 min arterial flow reperfusion. (H) Rat myocardium intoxicated with isoproterenol. The arrow in F indicates the calcium phosphate deposit.

Spatiotemporal aspects of cellular Ca^{2+} control

Calcium signaling normally starts with an initial transient Ca^{2+} increase that is restricted to a localized area. This localized increase may be dissipated by uptake into neighboring stores, and/or by buffering by soluble proteins. However, more frequently, the initial Ca^{2+} transient 'activates' vicinal Ca^{2+} stores triggering further Ca^{2+} release from them which spreads in an autocatalytic process. This is the phenomenon known as Ca^{2+}-induce Ca^{2+} release. The propagation of the signal may occur in the form of a wave that eventually occupies the entire cell, or of repetitive spikes of different frequency and amplitude. However, no initial elementary signaling event occurs unless the

second messengers mentioned in the sections above are produced to gate the release channels in the stores. The initial release events may be shaped differently depending on the release channels involved; they have been termed 'puffs' if generated by the InsP3-sensitive channels and 'sparks' when generated by the ryanodine channels [80]. The repetitive (oscillatory) pattern, for the case of the InsP3-mediated release, may be generated by two mechanisms: either an oscillatory production of InsP3, or an oscillatory inactivation of the InsP3 receptors. Both mechanisms could be mediated by feedback inhibition by Ca^{2+}.

Calcium oscillations have now become a favored research topic, as they are assumed to have physiological advantages over the static long-lasting Ca^{2+} elevations alluded to in the sections above. The oscillatory behavior would in other words be a means to transmit signals to targets that require the protracted presence of Ca^{2+} while at the same time avoiding the frequently lethal, or at least deleterious, effects of the sustained, static elevation of Ca^{2+}. Calcium oscillations are now known to be involved in the control of a number of important cell processes. A particular interesting case is that of the regulation of gene transcription, where the efficacy of the control varies with the amplitude and the frequency of the oscillations. Some transcription factors sense the amplitude of the oscillations, others are activated by rapid Ca^{2+} oscillations, still others by lower frequency oscillations [81].

Important insights on the molecular mechanism by which the cell decodes the message contained in the frequency of the oscillations have come from studies on the activity of calmodulin kinase II (CaMK II) as a determinant of memory formation and storage in the hippocampus [81]. CaMK II is a calmodulin-dependant multimeric enzyme which is transformed into a Ca^{2+}-independent state by autophosphorylation induced by a brief exposure to Ca^{2+}. The Ca^{2+}-independent activity could then self-perpetuate, a property which is now widely assumed to be the basis of memory formation and storage.

Conclusions

At the end of this review covering Ca^{2+} signaling in a succinct yet comprehensive way, it seems worthwhile to stress again the aspects that make Ca^{2+} unique among carriers of signals to cells. The most impressive aspect, which was alluded to in some detail in the sections above, is perhaps ambivalence. Cells cannot survive if they have no way to exchange Ca^{2+} with the external ambient, where Ca^{2+} is so abundant that cells never run the risk of not finding Ca^{2+} to import. On the other hand, if the Ca^{2+} import operation is not carefully controlled, disasters are bound to occur. This is the tightrope-walking concept; unfortunately, the line that separates cells from Ca^{2+} catastrophy is not as well defined as one would like.

Another aspect of the signaling operation which is unique to Ca^{2+} is autoregulation, which occurs at different steps of the operation. Evidently, the precise regulation of Ca^{2+} signaling is so important to cells that a wealth of autoregulatory mechanisms have been developed. Some have been mentioned in the sections above, but cells use many more.

Then, there is the matter of defining Ca^{2+} only as a second messenger. In an increasing number of cell types it is now clear that Ca^{2+} also acts on the surface of cells as a first messenger, interacting with a G-protein linked receptor [75] that may even affect the liberation of Ca^{2+} from intracellar stores. At this point, one could even formally consider Ca^{2+} as a bona fide hormone.

One last key question could be considered in closing, and this is the reason for the evolutionary choice of Ca^{2+} as a carrier of information. As mentioned the choice was probably made at the time of the transition between monocellular and pluricellular life, when the division of labor among cells generated the necessity of transmitting information from cell to cell. Evolution evidently singled out Ca^{2+} for the task because of the great flexibility in its ligation properties, which made it possible to maintain it at the very low concentrations in the cytosol demanded by the messenger function. An important dividend of the ability to keep Ca^{2+} at a very low level was the possibility of orienting the energetic metabolism of cells towards P_i. Had cells been unable to maintain their own Ca^{2+} at a very low level, the low solubility of P_i–Ca^{2+} compounds would have prevented the use of phosphate as the bioenergetic currency.

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