REVIEW

The Regulation of the Calcium Signal by Membrane Pumps

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Dedicated to Professor *Duilio Arigoni* on the occasion of his 75th birthday

Calcium may have a static, structure-stabilizing role in biological organs like the bones and the teeth, or may fulfill a dynamic function in cells as a regulator of signal-transduction pathways. This is made possible by the properties of the Ca^{2+} ion (*e.g.*, high dehydration rate, great flexibility in coordinating ligands, largely irregular geometry of the coordination sphere). Since Ca^{2+} is a universal carrier of signals, the control of its homeostasis is of central importance for the organism. It involves exchanges between the skeleton (which is the major calcium reservoir) and the extracellular and intracellular fluids. It also involves the intestine and the kidney, the organs of Ca absorption and release, respectively. The highly integrated homeostasis process consists of a number of hormonally controlled feedback loops, and an elaborate system of membrane channels, exchangers, and pumps that control the Ca^{2+} flux into and out of cells.

1. Introduction. – More than 100 years ago, the London physiologist *Sidney Ringer* serendipitously discovered that calcium is essential for the contraction of the heart muscle, *i.e.*, that Ca^{2+} is a carrier of signals [1]. This was a surprising result, since Ca was considered merely as a structural element responsible for the stability of bones and teeth. *Ringer*'s observation was not immediately acclaimed, but was the original milestone from which the all-encompassing area of Ca^{2+} signaling eventually developed. After a long period during which only a handful of occasional observations re-emphasized the messenger function of Ca^{2+} (see, *e.g.*, [2]), the turning point for the 'calcium concept' came with seminal findings by *Weber* [3], *Ebashi* and *Lipmann* [4], and *Hasselbach* and *Makinose* [5]. In the late 1950s and early 1960s, they demonstrated that Ca^{2+} is not only essential for the activation of actomyosin and to contract muscles, but is also actively accumulated in the vesicles of the sarcoplasmic reticulum from skeletal muscles by an ATP-consuming system. *Ebashi* and *Kodama* [6] then identified a Ca^{2+}-sensor protein, troponin C, the tropomyosin component of a protein complex essential for the Ca^{2+}-dependent activation of myofibrillar contraction. At the time of these early discoveries, the 'second messenger' concept had just been proposed by *Sutherland* and *Rall* [7] as a way to convey the information of extracellular stimuli to targets inside cells *via* signal carriers. In the case of *Sutherland*'s classical second messenger, cyclic AMP, the necessary reversibility of the signal transmission was made possible by the tight control of its synthesis and degradation by adenylate cyclases and phosphodiesterases. In the case of Ca^{2+}, the control of the signaling functions has, instead, demanded the development of a more-complex system based on proteins that

process the signal – the so-called Ca^{2+} sensor proteins – and on membrane-intrinsic proteins that regulate the flux of Ca^{2+} into and out of the cell as well as intracellular organelles (see *Fig. 1*). Ca^{2+} in the extracellular fluid and in the lumen of intracellular organelles is much more concentrated that in the cytosol of resting cells (100–300 nM), where most targets of the signaling function are located. To sense small changes of this steep transmembrane Ca^{2+}-concentration gradient, the Ca-binding proteins complex Ca^{2+} with high affinity and specificity, and do so in the presence of much higher concentrations of potentially competing cations (such as Mg^{2+}).

It has now become recognized that Ca^{2+} accompanies cells as a messenger from their birth at fertilization through the end of their life cycle by programmed death

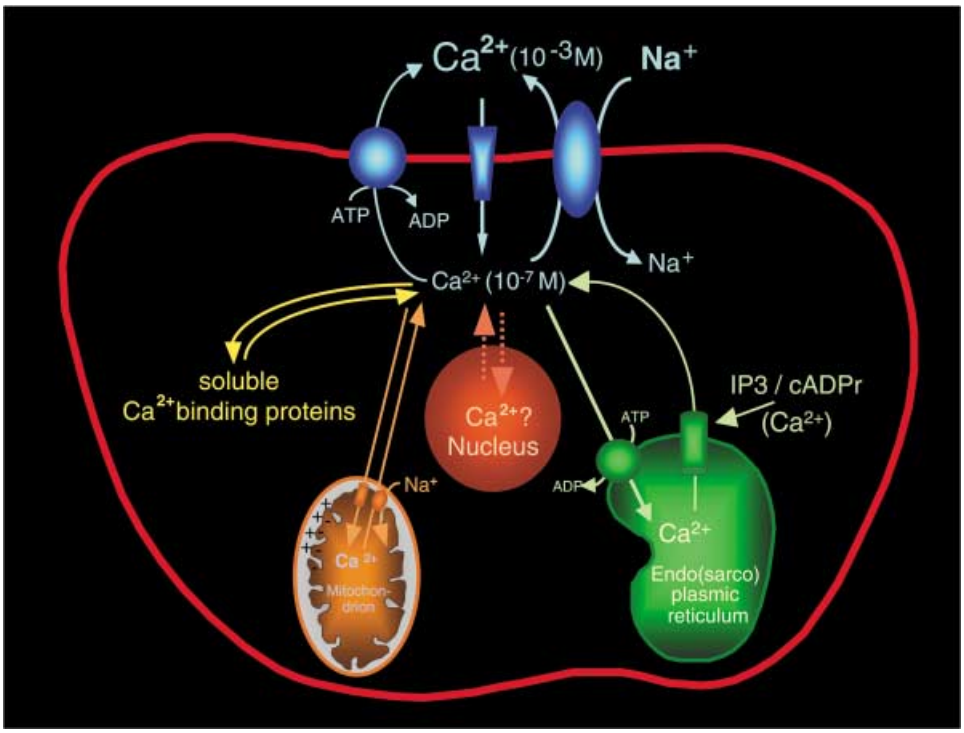

Fig. 1. *Cellular calcium homeostasis.* Control of the Ca^{2+} signal in cells: Ca^{2+} penetrates into cells *via* a channel (several types have been described) and is exported by means of a specific ATPase (the PMCA pump) and a Na^+/Ca^{2+} exchanger. The combined operation of the three plasma-membrane systems, coupled with the reversible sequestration of Ca^{2+} by internal, membrane-lined organelles, maintains the 10,000 fold gradient of Ca^{2+} concentration between the external space and the cytoplasm. Inside cells, Ca^{2+} may become bound to proteins (Ca^{2+} sensors) that process its message and transmit it to (enzyme) targets. Organelles that sequester Ca^{2+} reversibly are *1*) the mitochondrion, which takes up Ca^{2+} by means of an electrophoretic uniporter energized by the negative inner membrane potential generated by the operation of the respiratory chain, and releases it *via* a number of exchangers, the most important being a Na^+/Ca^{2+} exchanger; *2*) the endo (sarco)plasmic reticulum, which accumulates Ca^{2+} by means of a specific ATPase (the SERCA pump), and releases it by channels that are gated by Ca^{2+} itself, but demand the presence of effectors like inositol 1,4,5-triphosphate (IP3) or cyclic ADP ribose (cADPr); *3*) the nucleus, whose role in cellular Ca^{2+} homeostasis is still debated, since the nuclear envelope contains large pores that could be permanently open and could, thus, permit the rapid passive equilibration of cytoplasmic and nucleoplasmic Ca^{2+}.

(apoptosis). The succinct summary presented here will focus on the proteins involved in the control of the of Ca^{2+} concentration and in the processing of its message. A number of more-general reviews cover other aspects of the calcium concept as well [8–11].

2. Ca^{2+}-Ligation. – Ca^{2+}-Ligation usually occurs *via* (mono- or bidentate) carboxylates or neutral O-atom donors, the preferred type of ligands. Due to the great coordinative flexibility (the coordination number is usually 6–8, but can be as high as 12) and to the large variability of bond length and angles, Ca has a definite advantage in binding to proteins, which normally offer coordination sites of irregular geometry. This contrasts with Mg^{2+}, which demands a rigid octahedral geometry of the binding site, with six coordinating O-atoms at a fixed ionic-bond distance, due to the smaller ionic radius (0.64 Å) of Mg^{2+} compared to that of Ca^{2+} (0.97 Å). The greater flexibility in complexation geometry confers on Ca^{2+} greater versatility in coordinating ligands, leading to a higher exchange rate. This is reflected in a dehydration rate three orders of magnitude faster in the case of Ca^{2+} relative to Mg^{2+}, which makes the former much more suitable as a signal transducer.

3. Intracellular Calcium-Binding Proteins. – 3.1. *The EF-Hand Protein Family.* In resting cells, the steep concentration gradient across the plasma membrane allows for the intracellular level of Ca^{2+} to transiently increase significantly in response to the controlled opening of specific channels. However, intracellular Ca^{2+} can also increase due to the interaction of extracellular signals (first messengers) with membrane receptors, an operation that triggers the release of Ca^{2+} from membrane-enclosed intracellular stores. The increase of Ca^{2+} within the cells is not sufficient for the transduction of the signal. To function as a second messenger, the Ca^{2+} signal must be processed by a variety of reversibly interacting Ca^{2+}-binding proteins (see above). In the most important class of Ca^{2+}-sensing proteins, this occurs by the sequential arrangement of amino acids in a 12-membered loop flanked by two helical segments. This helix–loop–helix Ca^{2+}-binding motif, known as the 'EF-hand motif', can be present in proteins in several copies. The number of EF-hand proteins is steadily increasing: *ca.* 600 have been described so far.

The EF-hand Ca^{2+}-binding pattern was first described by *Kretsinger* and *Nockolds* in the early 1970s [12][13] in the crystal structure of parvalbumin. This protein consists of homologous Ca^{2+}-binding domains, each of which contains two α-helices roughly perpendicular to each other, which flank a Ca^{2+}-binding loop. Parvalbumin has become the structural prototype of numerous other Ca^{2+}-binding proteins of great importance, among them calmodulin, troponin C, recoverin, or the S100 proteins. All EF-hand domains in the more than 50 three-dimensional structures of EF-hand proteins that have been solved show a pentagonal-bipyramidal coordination of the Ca^{2+} ion in the loop flanked by the two helices. The residues that occupy loop positions 1, 3, 5, and 12 are highly conserved and contribute monodentate (1, 3, 5) or bidentate (12) ligands through side-chain O-atoms, whereas residue 7 contributes its backbone C=O O-atom. An invariant Gly in position 6 of the loop permits the sharp bend necessary to ligate Ca^{2+} through both the O-atom of the side chain of residue 5 and a backbone C=O group of residue 7. Residue 9 provides an additional ligand, either directly *via* an O-atom of its side chain or indirectly by means of a H_2O molecule. Position 1 of the loop is

usually occupied by Asp, whereas position 12 is occupied by an invariant Glu. This is so because both O-atoms of the side chain of Glu ligate Ca^{2+} and because Glu has the propensity to induce α-helices.

The EF-hand Ca^{2+}-binding domains usually occur in pairs stabilized by H-bond bridges between the central residues of adjacent loops that form a minimal antiparallel β-sheet. These pair-forming Ca^{2+}-binding domains enhance the Ca^{2+} affinity of the sites and their cooperativity. For instance, calmodulin and troponin C contain four Ca^{2+}-binding sites, conferring on these proteins Ca^{2+}-binding characteristics compatible with a 'pair of pairs' model of EF-hands, *i.e.*, the two N- and C-terminal globular domains each contain a pair of EF-hands. The two globular domains are connected by a long central helix, conferring to the crystallized protein a dumbbell-shaped appearance in which the N- and C-terminal moieties bind Ca^{2+} cooperatively, the latter with higher affinity. Multinuclear magnetic-resonance experiments on the Ca^{2+}-bound form of calmodulin [14] have confirmed the dumbbell-shaped structure also in solution, but have indicated a high degree of flexibility in the central part of the long helix [15]. This flexibility is important for the interaction of the molecule with targets to which the Ca^{2+} message must be transmitted. The transmission consists of two sequential conformational transitions. The binding of Ca^{2+} to calmodulin induces a first change, after which the bending of the central helix collapses, generating a more-compact form upon interacting with a target. At the end of the process, the two Ca^{2+}-binding domains are closer, engulfing the Ca^{2+}-binding peptide of the target protein (*Fig. 2*).

3.2. *Calmodulin and Its Targets.* The binding of Ca^{2+} to the EF-hand motifs of calmodulin results in the formation of deep hydrophobic clefts, a prerequisite for the interaction with targets. In the absence of a target, the N- and C-terminal halves of Ca^{2+}-bound calmodulin (CaM) in solution behave as independent structural units linked by a flexible tether. In approaching target proteins, CaM interacts with specific binding sequences that display similar properties (*Table*). They are composed of 20–30 amino acids that form a basic amphiphilic α-helix. Several three-dimensional structures of the complexes between calmodulin and target peptides of different enzymes, such as CaM-dependent kinases [16–18], the CaM-dependent protein kinase kinase (CaMKK) [19], the plasma-membrane Ca^{2+} ATPase (PMCA pump) [20], membrane channels such as the voltage-dependent K^+-channel [21], and adenylate cyclase [22] have now been solved either by NMR or X-ray crystallography. The structures of the complexes share the following common features: *1*) as mentioned above, upon collapse of the central helix of calmodulin, the resulting globular structure wraps around the target peptide; *2*) the binding peptide is located in a hydrophobic 'channel' passing through the center of the now globular calmodulin molecule; *3*) two hydrophobic residues of the binding peptide, separated by a defined number of residues, are essential for the interaction with CaM (see *Table*); and *4*) the binding peptides interact with CaM in an antiparallel manner, *i.e.*, the N-terminal half of CaM binds to the C-terminal moiety of the peptide, and *vice versa*. In *Fig. 2*, the structures of calmodulin are shown in its Ca^{2+}-bound form (*Fig. 2,a*) and in the complex with the CaM-binding domains of two target enzymes, *i.e.*, the plasma membrane calcium-pump (*Fig. 2,b*) and muscle-myosin light-chain kinase (MLCK; *Fig. 2,c*). Interestingly, recent small-angle X-ray and neutron-scattering experiments by *Trewhella* and co-workers [23] have provided evidence that CaM undergoes the conformational collapse observed

Table. *Aligned Sequences of CaM-Binding Domains of Different Enzymes.* The critical (hydrophobic) anchoring amino acids (single-letter code) of the corresponding CaM-binding domains are marked bold.

Enzyme	Relevant sequence
skMLCK (M13)	KRR**W**KKNFIAVSAANR**F**KKI
smMLCK (R20)	RRK**W**QKTGHAVRAIGR**L**SSM
CAMKI	KSK**W**KQAFNATA**V**VRH**M**RKL
CAMKII	RRK**L**KGAILTTM**L**ATRNFSA
CAMKIV	RRK**L**KAAVKAVVASSR**L**GSA
CAMKK	IPS**W**TTVILVKSMLRKRS**F**G
PMCA	●●●●●● QIL **W** FRGL**NR**IQ TQIR**V**VNA

with the peptide corresponding to its binding domain also when complexed to the intact enzyme (MLCK).

An exception to these principles of interaction has recently been reported by *Ikura* and co-workers [19], who solved the solution structure of the complex of CaM bound to the binding domain of CaM-kinase kinase (CaMKK). In this complex, the N-terminal half of the binding peptide forms the expected α-helix, whereas the C-terminal half folds back on itself in a hairpin loop. In addition, the peptide adopts a parallel orientation relative to the two halves of CaM.

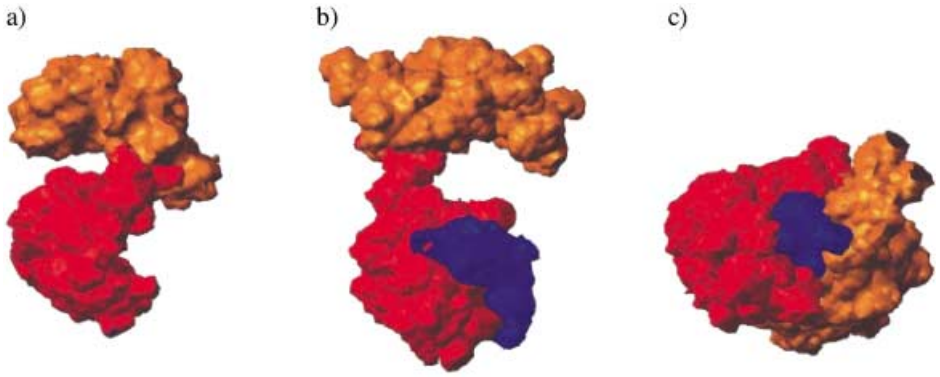

Fig. 2. *Surface representation of Ca^{2+}-bound calmodulin* (CaM) *and of its binding modes to peptides.* The N-terminal half of CaM is in orange, the C-terminal half is in red, and the peptides are in blue. The orientation of the C-terminal half of CaM is the same in all cases. *a*) *Crystal structure of Ca^{2+}-saturated CaM.* b) *Solution structure of the CaM/C20W complex.* The peptide C20W, corresponding to the N-terminal portion of the CaM-binding domain of the plasma membrane Ca^{2+}-pump, but lacking the C-terminal hydrophobic anchor residue, only binds to the C-terminal half of CaM. c) *The solution structure of CaM/M13 shows a compact globular complex in which both the C- and N-terminal halves of CaM are involved in the binding of peptide M13* (corresponding to the CaM-binding domain of MLCK) reproduced with permission from [20]).

The first crystal structure of a CaM-dependent enzyme (CaM-dependent kinase I; CaMKI) [24] in the absence of CaM, supports the view that the C-terminal regulatory domain of the kinase forms a helix–loop–helix segment that interferes with the two domains of the catalytic core, *i.e.*, with the binding site of peptide substrates and of ATP. An interesting feature of the structure is the accessibility of the N-terminal portion of the CaM-binding domain for the initial interaction with CaM. This portion comprises

the loop region of the regulatory domain of the kinase, including a conserved Trp (see the *Table*), which acts as a hydrophobic anchor for the C-terminal half of CaM. Furthermore, it has been suggested that the interaction between the C-terminal half of CaM and the CaM-binding domain of CaM-dependent enzymes is necessary – and sometimes sufficient – to release the autoinhibited state of the enzyme in the absence of CaM. This suggestion is based on the NMR structure of the complex between CaM and the N-terminal portion of the CaM-binding domain of the plasma-membrane Ca pump (*Fig. 2,b*), and has been supported by recent structural studies on MLCK [25], indicating that the initial binding of CaM occurs at substoichiometric Ca^{2+}/CaM concentrations, *i.e.*, at *ca.* 2 equiv. of Ca^{2+} per equiv. of CaM (saturation is achieved after binding of four Ca^{2+} ions).

4. The Plasma-Membrane Calcium Pump. – *Dunham* and *Glynn* first described a Ca^{2+}-dependent ATPase in the erythrocyte membranes in 1961 [26], but it was *Schatzmann*, who found in 1966 that Ca^{2+} is actually pumped out of erythrocytes with the energy of ATP against a steep gradient [27]. The enzyme belongs to the P-type pump family [28][29], *i.e.*, it is an ATPase characterized by the formation of a phosphorylated intermediate (an aspartylphosphate) during the reaction cycle. The enzyme exists in the two main conformational states E1 and E2, as documented in the recently solved structures of the Ca^{2+}-bound (E1) and the Ca^{2+}-free (E2) states of the homologous Ca^{2+}-pump of sarcoplasmic reticulum (*Fig. 3*) [30][31]. Similar Ca^{2+}-dependent $E1 \rightarrow E2$ conformational changes that could reasonably be expected to occur in the Ca^{2+}-pump of the plasma membrane (PMCA) have been explored in the molecular-modeling experiment shown in *Fig. 4*. Even if the sequence homology of the two enzymes is only *ca.* 45%, the similarity of the two structures is striking. Differences are nevertheless evident in the actuator (A) and nucleotide (N) domains (*Fig. 3*) in the luminal and extracellular loops, and in the large insert in the PMCA pump upstream of the M3 helix, which has been characterized as a phospholipid-binding domain [32]. Interestingly, this insert occurs at a position homologous to that surrounding Thr^{242} in the SERCA pump in the loop connecting domain A with transmembrane helix M3 (*Fig. 4*), which is one of the cleavage sites for proteinase K. *Ma et al.* [33] have recently shown that this site is protected from digestion when the SERCA pump is in the E1 (or Ca^{2+}-bound) form and when a nucleotide is bound to the N domain. It would now be of interest to investigate whether the phospholipid-binding domain within the corresponding loop region of the PMCA pump influences the cleavage of the pump by proteinase K.

The PMCA Ca^{2+} pump is essential to the fine tuning of the intracellular free Ca^{2+} level. It is a low-abundance protein (representing less than 0.1% of the total membrane proteins of the human erythrocytes), but is present in the plasma membranes of all cells. Calmodulin activates it by direct interaction, lowering the K_M value for Ca^{2+} by one order of magnitude and increasing V_{max} of Ca^{2+} transport two- to threefold. The ability of the Ca^{2+}-pump to interact with CaM was exploited to purify it from detergent-solubilized erythrocyte membranes by calmodulin-based affinity chromatography [34]. The pump can also be activated by alternative treatments, *e.g.*, by acidic phospholipids [35], long-chain polyunsaturated fatty acids [35], phosphorylation by different protein kinases (PKA/PKC; [36][37]), oligomerization [38] or a controlled proteolytic

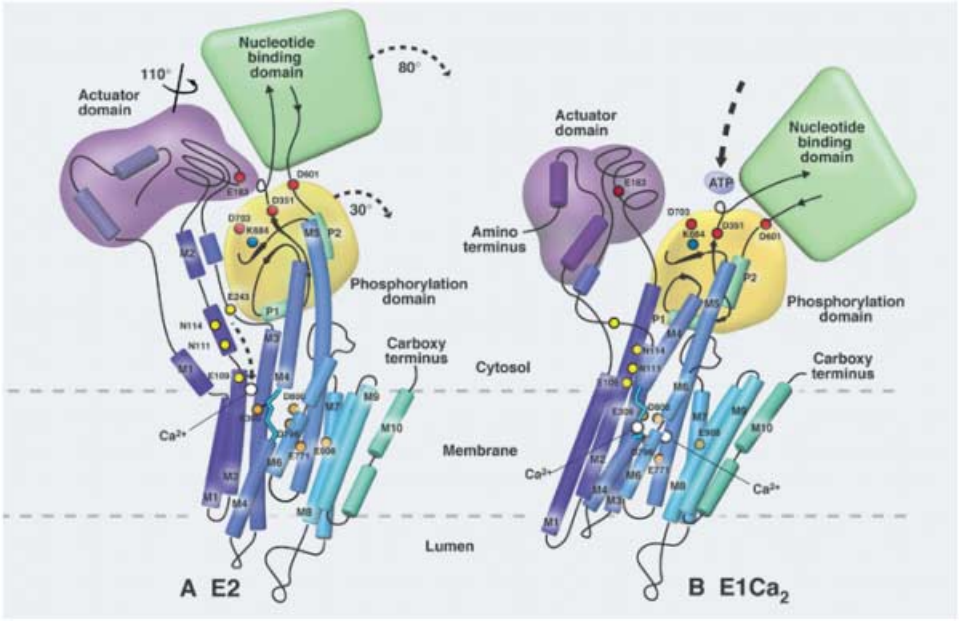

Fig. 3. *Structures of the SERCA pump in* a) *the* Ca^{2+}*-free* (thapsigargin-bound; thapsigargin is a pump inhibitor) *and* b) Ca^{2+}*-bound forms.* The three cytosolic domains are indicated as A (actuator), P (phosphorylation), and N (nucleotide-binding) domains. The P domain (with N inserted) connects the transmembrane helices M4 and M5. Asp^{351} within the P domain becomes phosphorylated during the reaction cycle. A number of residues important for the catalytic center or for the Ca^{2+}-binding sites within the membrane are also shown (reproduced with permission from [39]).

treatment [35][40]. The last procedure has helped identifying a number of functional domains of the enzyme [40]. The PMCA pump was cloned as a single polypeptide (1200 amino acids, *ca.* 135 kDa [41][42]), which spans the membrane ten times. Most of the pump mass protrudes into the cytosol, with very short loops connecting the proposed transmembrane domains on the extracellular side. A significant difference between the PMCA and the SERCA pumps is the Ca^{2+}/ATP stoichiometry, which is 1 for the PMCA pump, but 2 for the SERCA enzyme. Therefore, the PMCA enzyme has only one Ca^{2+}-binding site, whereas the SERCA pump has two. It is of interest that one of the residues, predicted (and confirmed in the atomic structure, see [30]) to participate in Ca^{2+} binding and transport within the SERCA pump (a glutamate in transmembrane-domain 5), is missing in the PMCA pump. The replacement of Ala^{854}, which occupies the place of the essential Glu residue in transmembrane domain 5 of the PMCA pump with a charged residue, apparently reconstituted the second Ca^{2+}-binding site, conferring to the PMCA pump properties similar to those of the SERCA pump, including the Ca^{2+}/ATP stoichiometry [43].

In analogy to the structural principles that have emerged from the three-dimensional structure of the SERCA pump, the cytosolic portion of the PMCA enzyme can also be divided into the following three different units:

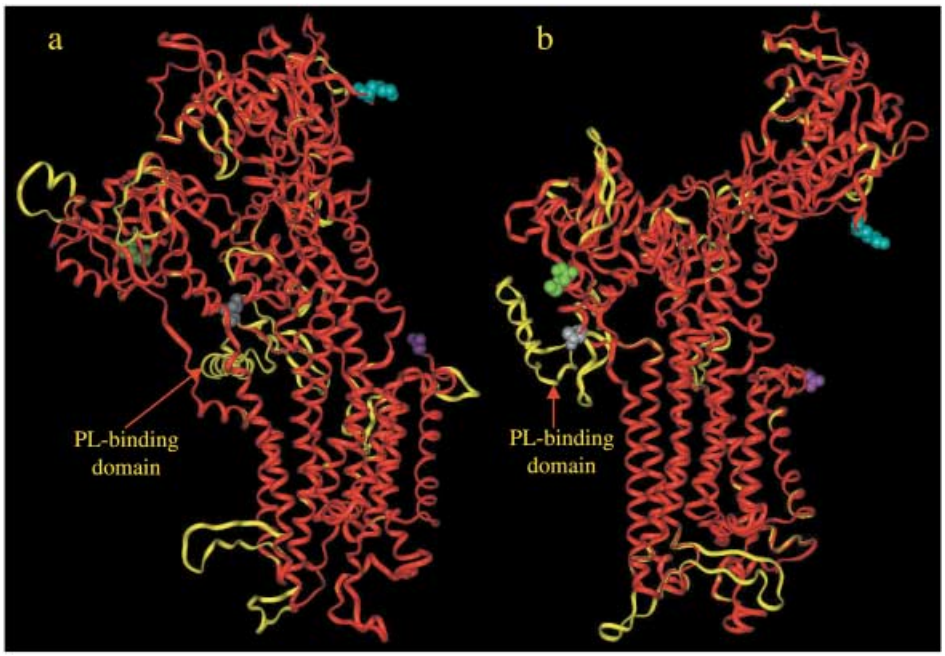

Fig. 4. *Homology modeling of the PMCA pump based on the structures of the SERCA pump* [30][31] *in* a) *the* Ca^{2+}*-free* (E2) *and* b) Ca^{2+}*-bound forms* (E1). The models were generated with the automatic SWISS-MODEL server [44]. The sequence alignments between the SERCA isoform 1A, ending with Gly^{994} [30][31], and the PMCA (human PMCA1 [42]) pumps used as input were obtained by applying the multiple-sequence-alignment software CLUSTAL W [45], using the PMCA and SERCA amino acid sequences contained in the SWISSPROT database (omitting the 37 N-terminal and the 144 C-terminal amino acids of PMCA, which have no correspondence in the SERCA pump). The two structures are shown as an overlay of the backbones in ribbon representation, SERCA in red, PMCA in yellow. In case of a perfect match between the two structures, one sees only the red color of SERCA (the width of the ribbon for SERCA is somewhat wider than that for PMCA). The following amino acids of the SERCA pump are depicted as CPK models: N-terminal Met^{1} (green), Thr^{242} (gray), Lys^{400} (blue), and the C-terminal Gly^{994} (purple). The structures of the SERCA pump have been obtained from the PDB platform. The Ca^{2+}-free or E2 conformation is 1IWO, the Ca^{2+}-bound or E1 conformation is 1EUL. The orientation of the structures is comparable with those in *Fig. 3*.

a) *Actuator* (A) (or transduction) *Domain*. This domain comprises the portion of the pump protruding between transmembrane-domains 2 and 3. In agreement with the structure of the SERCA pump, it should also contain the N-terminal sequence upstream of transmembrane-domain 1. The domain is proposed to couple the hydrolysis of ATP to the transport of Ca^{2+}, but in the PMCA pump, it also contains a phospholipid-binding domain that is unique to the plasma-membrane Ca^{2+}-pump [32]. This domain might play a role in the tissue-specific expression of isoforms. It lies close to splice-site A, where an insert of *ca.* 40 amino acids determines the membrane targeting of the pump [46].

b) *Catalytic Domain*. This domain spans the cytosolic portion of the pump between the 4th and the 5th transmembrane domain (see *Fig. 5*), and contains both the phosphorylation (P) (aspartyl phosphate) and the nucleotide-binding (N) sites. The

Fig. 5. *A model of the complex between PLN and the SERCA pump obtained by molecular mechanics* (AMBER force field). The SERCA pump is in the E2 conformation. The interacting partners of PLN (yellow) are the M6 (green) and M4 (blue) helices of SERCA. The cytosolic loop of SERCA, containing Lys^{400} as interacting partner of the N-terminal portion of PLN, is shown in green. The orientation of the structures is comparable to those in *Fig. 3* (reproduced with permission from [47]).

P domain also contains the so-called 'hinge' region, a sequence highly conserved among ion-pumping ATPases, which is essential to bring the phosphorylation site close to the bound ATP. In sharp contrast to the SERCA pump, the catalytic unit of the PMCA pump also contains a 'receptor' for the autoinhibitory calmodulin-binding domain. This receptor is composed of two distinct sites [48][49], one of them located between the P and the N sites, the other N-terminal to the phospholipid-binding sequence in the A domain. The interaction with the CaM-binding domain 'bridges' the transduction and the catalytic domains, presumably limiting the access of substrates to the catalytic center of the enzyme.

c) *Regulatory Domain*. The sequence protruding into the cytosol from the last transmembrane domain contains several sites that are important for the regulation of the Ca^{2+} pump, namely the CaM-binding domain, which was originally identified by cross-linking experiments [50], and consensus sequences for two protein kinases, PKA and PKC. The PKA-site, which is present in only one of the isoforms of the pumps, is C-terminal to the CaM-binding domain, whereas the PKC-site is located within the CaM-binding domain. Phosphorylation of the latter site is prevented by CaM.

4.1. *Isoforms of the PMCA Pump*. Four genes coding for the PMCA pump have been identified in mammals. The structure of the gene for human PMCA1 has shown 22

exons and 21 introns, accounting for more than 10^5 base pairs [51]. Additional pump-isoform variability is produced by alternative splicing of the primary transcripts, which occurs close to the phospholipid-binding sequence, and within the calmodulin-binding domain [52]. Splicing produces tissue specific variants of the pump [53], which differ in the affinity to calmodulin [54]. The four PMCA genes have been localized on human chromosome 12q21 (PMCA1) [55], 3p24 (PMCA2) [56], Xq28 (PMCA3) [57], and 1q25 (PMCA4) [55]. PMCA1 and PMCA4 are distributed ubiquitously in tissues, whereas PMCA2 and PMCA3 are mainly found in the brain [53].

4.2. *The Interaction of the PMCA Pump with Calmodulin*. At variance with all other CaM targets, the plasma-membrane Ca^{2+} pump is activated by the separate C-terminal half of CaM, but not by the N-terminal half [58]. In other cases of CaM targets, each isolated N- and C-terminal half of CaM may bind the target, but *both* halves are necessary for activation. This raises the question of the mode of CaM interaction with the binding domain of the pump, and of which part of the pump domain interacts with the C-terminal half of CaM. Previous experiments have shown that a splicing site is located in the middle of the CaM-binding domain of the pump [52], *i.e.*, only the N-terminal half of the binding domain is identical in all isoforms. Therefore, it appeared interesting to examine the formation of the complex between CaM and the shorter synthetic peptide C20W, which stops just before the splicing site upstream of the putative second residue that anchors CaM. The solution structure of the complex, solved by multidimensional, heteronuclear NMR spectroscopy [20], has provided evidence that CaM in the complex still displays the extended conformation, since the peptide became bound to only the C-terminal half of CaM (*Fig. 2*,*b*). Interestingly, the CaM/C20W structure is, halfway through the folding pathway of CaM, complexed with other targets (*i.e.*, the binding domain of myosin light-chain kinase M13), in which both domains of CaM become eventually wrapped around the peptide (*Fig. 2*,*c*). The structure of the CaM/C20W complex may help understanding why the Ca^{2+}-pump is peculiarly activated by the isolated C-terminal half of CaM (and not by the N-terminal). This, together with the significantly higher affinity of the C20W peptide for the C-terminal than for the N-terminal half of CaM [59], is likely to reflect the finding that only the C-terminal half of CaM interacts with the CaM binding domain of the PMCA pump (peptide C20W).

The structure of the CaM/C20W complex may have special significance for the function of the PMCA pump. Alternatively spliced isoforms of the pump, differing in the C-terminal half of the CaM-binding domain [51] also differ in the activation profile by CaM, a finding that may be relevant to the fine-modulation of the activity of the pump [60][61]. Since the Ca^{2+}-loaded C-terminal half of CaM is sufficient for pump activation [58][62], it is reasonable to expect that CaM loaded with just two Ca^{2+} ions in the C-terminal lobe would bind and activate the pump as well. It has long been known that Ca^{2+} binds first to the C-terminal half of CaM, as corroborated by recent experiments on skMLCK [25]. It would, thus, follow that the complex between CaM and the pump could already become formed at substoichiometric Ca^{2+} concentrations, suggesting that the pump might be active (albeit at lower efficiency) even at the low Ca^{2+} concentrations prevailing in resting cells, at which only the Ca^{2+}-binding sites of the C-terminal half of CaM could be occupied. Possibly, the interaction between the

Ca^{2+}-loaded C-terminal half of CaM and the CaM-binding domain of the pump bound to its 'receptors' in the pump molecule would be sufficient for the release of the autoinhibited state of the enzyme, but not for the full cooperativity typical of the system.

5. The Ca^{2+} Pump of Sarcoplasmic Reticulum. – The principal protein component of the sarcoplasmic reticulum (SR) is the Ca^{2+} pump SERCA, which can represent as much as 90% of the total SR membrane protein in skeletal muscles. Even in heart cells, the pump can still make up as much as 50% of the total SR membrane protein. Three genes have been identified that encode the SERCA pump in mammals. SERCA1 is mainly expressed in fast-twitch skeletal muscles, whereas SERCA2 is the major form of cardiac, smooth, and non-muscle tissues. SERCA3 has been cloned from a kidney library and is prominently present in platelets. Several spliced variants have been described for SERCA1 and SERCA2. The pump is also present, albeit in much lower concentrations, in the membrane of endoplasmic reticulum of non-muscle cells.

The protein consists of a single polypeptide chain of *ca.* 100 kDa, which was first purified from skeletal muscles by *MacLennan* in 1970 [63]. The predicted secondary structure and membrane topography of the enzyme were confirmed by the solution of the three-dimensional structure of the Ca^{2+}-bound and the Ca^{2+}-free forms of the enzyme [30][31] (see *Fig. 3*). The structural properties of the pump, as established by X-ray analysis, can be summarized as follows: *1*) the hydrophobic portion of the protein consists of 10 transmembrane helices; *2*) transmembrane helix M5 reaches into the cytosolic portion of the pump (60 Å total length), coupling the membrane portion of the enzyme to the cytosolic domains; *3*) the cytosolic portion of the pump is divided into the actuator (A), phosphorylation (P), and nucleotide-binding (N) domains (see *Fig. 3*), and the P and N domains, which connect transmembrane helices 4 and 5, contain the catalytic center of the enzyme, where the conserved aspartyl residue (ASP_{351}) is phosphorylated during the reaction cycle; *4*) two Ca^{2+}-binding sites are located within the transmembrane portion of the pump formed by residues in helices M4, M5, M6, and M8 (predicted by mutagenesis experiments), and by additional residues, site 1 being located in the space between M5 and M6, whereas site 2 is formed essentially on M4; *5*) large-scale movements of the A, P, and N domains occur during the reaction cycle (documented by comparing the Ca^{2+}-free with the Ca^{2+}-bound structures [30][31]) and are coupled to changes in the orientation and position of helices M1 – M6.

The SERCA pump of cardiac and smooth muscle is regulated by a hydrophobic protein called phospholamban (PLN) [64], which is composed by hydrophilic N-terminal helix connected to a very hydrophobic, intramembrane C-terminal helix by a short, flexible hinge region [65]. The PLN interacts with the SERCA pump at a small loop near Lys^{400} in the P domain, but also with the transmembrane region [66][67], maintaining the pump inhibited. The inhibition is relieved by the phosphorylation of PLN by two kinases, PKA and a CaM-dependent kinase [68][69]. Thus, the unphosphorylated form of phospholamban is an endogenous inhibitor of the SR pump, analogous to the autoinhibitory CaM-binding domain of the PMCA pump.

The detailed mechanism by which PLN modulates the activity of the SERCA pump is not yet fully understood. We have used the three-dimensional structures of the two

proteins to model their complex by applying energy-minimizing calculations *in vacuo* using the AMBER force field [47]. The results have provided evidence that transmembrane helix M6 of the pump, as suggested by mutagenesis studies [70], is the energetically favored binding partner for the intramembrane C-terminal helix of PLN, still permitting the necessary contact between the N-terminal residues of PLN and the critical cytosolic loop of the pump containing Lys^{400} (see *Figs. 4* and *5*). One suggestion emerging from this study was that PLN may not traverse the membrane completely. The C-terminal residues of PLN are all hydrophobic, but transmembrane domains are normally 'fixed' on both sides of the membrane by polar residues. For instance, sarcolipin, a modulator protein of skeletal muscle SR, a phospholamban analog [71], which is predicted to transverse the full width of the membrane [72], contains polar residues on both sites of the transmembrane region. Our molecular-modeling study, thus, suggests that hydrophobic residues of the N-terminal portion of domain II of PLN are in contact with hydrophobic residues of the cytosolic domain of helix M4, protecting them from solvent exposure. This may contribute to the stabilization of the intramembrane location of PLN.

Recently, *MacLennan*, *Toyoshima*, and co-workers. [73] also published a molecular-modeling study of the interaction between PLN and the SERCA pump based on the structure of the thapsigargin-bound E2-form of the latter [31]. Some of the conclusions of the study differ somewhat from those discussed above, *e.g.*, PLN is claimed to cross the membrane completely, stabilized by interactions with the M2 transmembrane helix of SERCA. To permit the simultaneous interaction of PLN with the Lys^{400} cytoplasmic loop of the SERCA pump, the authors concluded that the N-terminal portion of the C-terminal helix of PLN (between Pro^{21} and Asn^{30}) is unwound. This is an interesting suggestion: one possible problem, here, is that the binding of thapsigargin to the SERCA pump has been claimed to be incompatible with the binding of PLN [74]. Furthermore, thapsigargin may induce conformational changes of the SERCA pump[1]) in addition to stabilizing one of its conformations [31].

6. Conclusions. – Calcium plays a central role in biological systems, controlling numerous cellular processes. It fulfills a static function by stabilizing structures, and a dynamic function by participating in signal-transduction pathways as a second messenger.

The Ca^{2+} homeostasis in the organisms is carefully controlled, involving a variety of systems in the skeleton, the extracellular fluid, and the intracellar ambient. Depending on its function, Ca^{2+} can be complexed in different forms: *1*) as hydroxyapatite in the skeleton; *2*) by acidic, low-affinity proteins in the extracellular fluid; and *3*) by high-affinity binding proteins inside cells.

The extracellular and intracellular concentrations of Ca^{2+} differ by several orders of magnitude. Therefore, cells are exposed to a steep Ca^{2+} gradient across the plasma membrane, which makes it possible for small changes of membrane permeability to lead to substantial changes in intracellular free Ca^{2+} concentration. Ca^{2+}-dependent signals can be converted from an extracellular analog to an intracellular digital form.

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[1]) *D. Stokes*, personal communication.

The control of cellular Ca^{2+} is performed by an elaborate system of channels, exchangers, and pumps located both in the cellular and intracellular membranes.

The EF-hand proteins are the most-important intracellular Ca^{2+}-binding proteins. They play a central role in permitting Ca^{2+} to function as a second messenger, binding Ca^{2+} with high affinity, selectivity, and cooperativity, and guiding the interaction with targets. More than 600 EF-hand proteins have so far been identified. They fulfill the different demands of Ca^{2+}-dependent processes (*e.g.*, glycogen metabolism, muscle contraction, excitation-secretion coupling, cell-cycle control, gene expression, apoptosis, *etc.*).

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