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The Ca^{2+} pumps and the Na^+/Ca^{2+} exchangers

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The Ca^{2+} ATPases or Ca^{2+} pumps transport Ca^{2+} ions out of the cytosol, by using the energy stored in ATP. The Na^+/Ca^{2+} exchanger uses the chemical energy of the Na^+ gradient (the Na^+ concentration is much higher outside than inside the cell) to remove Ca^{2+} from the cytosol. Ca^{2+} pumps are found in the plasma membrane and in the endoplasmic reticulum of the cells. The pumps are probably present in the membrane of other organelles, but little experimental information is available on this matter. The Na^+/Ca^{2+} exchangers are located on the plasma membrane. A Na^+/Ca^{2+} exchanger was found in the mitochondria, but very little is known on its structure and sequence. These transporters control the Ca^{2+} concentration in the cytosol and are vital to prevent Ca^{2+} overload of the cells. Their activity is controlled by different mechanisms, that are still under investigation. A number of the possible isoforms for both types of proteins has been detected.

Keywords: Ca^{2+} pumps, intracellular Ca^{2+} homeostasis, Na^+/Ca^{2+} exchangers

The Ca^{2+} transporters of the cells

The maintenance of a low free Ca^{2+} concentration (>= 0.2 μM) is vital to the correct functioning of the cells (Carafoli E 1987). In eucaryotic cells the opening of specific channels in the plasma membrane or in the sarco-endoplasmic reticulum allows Ca^{2+} to diffuse in the cytosol, down a 1,000–10,000 fold concentration gradient. It is important that increases in Ca^{2+} concentration are transient, since sustained increases of cytosolic Ca^{2+} would lead to mitochondrial overloading, activation of proteases, activation of DNA-fragmenting enzymes and finally to cell death. Efficient systems have therefore evolved to remove the Ca^{2+} from the cytosol (Carafoli E 1987). Two proteins perform this function in the plasma membrane: the Na^+/Ca^{2+} exchanger and the Ca^{2+} ATPase (pump), whereas a Ca^{2+} pump is responsible for the re-uptake of the Ca^{2+} in the sarco/endoplasmic reticulum.

The importance of mitochondria in this process has been recognized, after being neglected for a long time. This was due to the development of methods that allowed to measure the changes in the free Ca^{2+} concentration in the mitochondria (Rizzuto R *et al.* 1992). These experiments have also revived the interest in the proteins involved in the mitochondrial Ca^{2+} transport. It has been known that Ca^{2+} enters and leaves mitochondria through specific systems. The presence of a Na^+/Ca^{2+} exchanger has been suggested. It has, at least conceptually, similarities to that of bacteria. Unfortunately its biochemical and molecular characterization is still preliminary (Li *et al.* 1992) and will not be discussed further in this review. A general model of the systems involved in the movement of the Ca^{2+} in the cell is summarized in Fig. 1. The Na^+/Ca^{2+} exchanger couples the transport of Ca^{2+} to the 'downhill' cotransport of Na^+ (Philipson KD and Nicoll DA 1992). The Ca^{2+} pumps use ATP energy to transport Ca^{2+} against the chemical gradient (Carafoli and Stauffer 1994).

Although information on the Ca^{2+} homeostasis in prokaryotes is still fragmentary, systems have been described which extrude Ca^{2+} from their cytosol. In most bacteria Ca^{2+} is exported by Ca^{2+}/H^+ or Ca^{2+}/Na^+ antiporters, which are still poorly characterized (Rosen 1987). ATP driven Ca^{2+} transporting systems have also been identified in some bacteria: in one, *Flavobacterium odoratum*, the activity was found associated with a 60'000 Da protein which was

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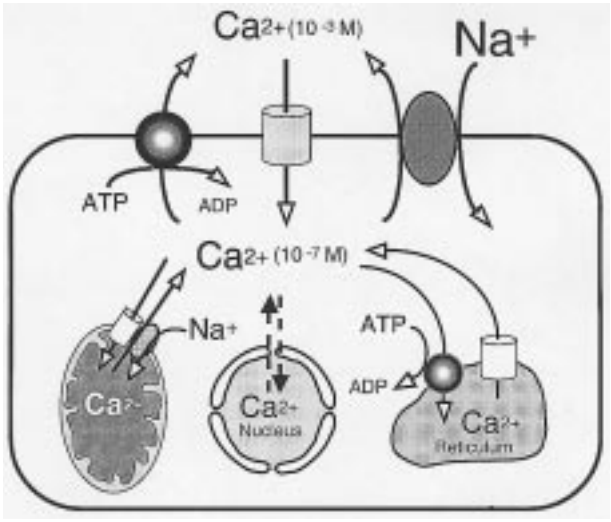


Figure 1 The proteins transporting the Ca^{2+} in a mammalian cell. The pumps are represented by circles, the Na^+/Ca^{2+} exchangers by ellipsoids and the channels by cylinders. It is not yet clear how Ca^{2+} transport in the nucleus is regulated (broken lines)

purified to apparent homogeneity (Desrosiers *et al.* 1996). The gene of a putative Ca^{2+}-transporting ATPase has been identified in the cyanobacterium *Synechocystis sp. PCC 6803* (Geisler *et al.* 1993). Its sequence had up to 30% identity with that of the SERCA3 cDNA, had a canonical phosphorylation site but no biochemical information on the product of this gene has been provided.

In the yeast *Saccharomices cerevisiae* two genes corresponding to the Ca^{2+} pumps of mammalian cells have been identified (Rudolph *et al.* 1989, Cunningham KW and Fink GR 1994). The first (PMR1) encodes a protein that is 30% similar to the SERCA pump but 50% similar to a P-type pump of unknown function for which transcripts have been detected in rat (Gunteski-Hamblin *et al.* 1992). The protein encoded by the second gene (PMC1) has a 40% identity to the PMCA pump (Cunningham KW and Fink GR 1994). Deletion of both genes resulted in loss of the viability of the cells, indicating that at least one Ca^{2+} pump is required for yeast survival (Cunningham and Fink 1996). A H^+/Ca^{2+} exchanger in yeast that seems to be related to the mammalian exchangers has also been described (Cunningham and Fink 1996). The homology is significant in the transmembrane domains, but low in the others. The H^+/Ca^{2+} exchanger is located in the vacuoles and as in the case of the pumps, it is important for tolerance of the yeast cells to high Ca^{2+} (Cunningham and Fink 1996).

The Na^+/Ca^{2+} and the Na^+/Ca^{2+},K^+ exchanger

The mammalian Na^+/Ca^{2+} exchanger was discovered in heart sarcolemma by Reuter and Seitz in 1968 (Reuter and Seitz 1968) and in axonal plasma membrane by Baker *et al.* in 1967 (Baker *et al.* 1967). Many of the properties of this protein have been studied on membrane (sarcolemma) isolated from the dog heart (Philipson and Nicoll 1992). A Na^+/Ca^{2+} dependent exchanger activity was identified in the retinal rods. In contrast to the cardiac exchanger, the retinal protein also co-transports K^+ with Ca^{2+} (Cervetto *et al.* 1989; Schnetkamp *et al.* 1989). Two families have been identified functionally: the Na^+/Ca^{2+} (NCX, typified by the cardiac protein) and the Na^+/Ca^{2+}-K^+ (NCXK, typified by the retinal protein) exchangers. While the cardiac exchanger was resistant to purification, the retinal exchanger could be obtained in a relatively pure form and reconstituted in artificial liposomes (Cook and Kaupp 1988). This permitted to confirm the observation made by Cervetto *et al.* (Cervetto *et al.* 1989; Friedel *et al.* 1991) that the retinal exchanger cotransported Ca^{2+} and K^+. The stoichiometry of transport of the two exchanger types is different, the retinal enzyme exchanges $4Na^+$ for $1Ca^{2+}$ and $1K^+$, whilst the cardiac type exchanges $3Na^+$ for $1Ca^{2+}$ (Philipson and Nicoll 1992). The cloning of the cDNA for both exchangers (Nicoll *et al.* 1990; Reilander *et al.* 1992) confirmed that the exchangers belonged to two different gene families. Despite the low homology at the primary sequence level the predicted membrane topology of the two exchangers is very similar. A model for the NCX and NCXK exchangers is shown in Fig. 2. The N-terminal region in front of the first transmembrane domain (TM1) is much longer in the NCXK (Fig. 2B). The NCX exchanger has a cleavable signal peptide at its N-terminus that does not seem to be essential for the activity (Hryshko *et al.* 1993; Furman *et al.* 1995; Loo *et al.* 1995). The suggested presence of an additional transmembrane domain at the amino terminus of the NCXK exchanger (TM0 of Fig. 2B) is still a matter of debate (Reilander *et al.* 1992; Tsoi *et al.* 1998).

Three genes for the cardiac type exchanger (termed NCX1, NCX2, and NCX3 (Nicoll *et al.* 1990; Li *et al.* 1994; Nicoll *et al.* 1996)) are present in mammalian cells. Two NCXK exchangers genes have been cloned (Reilander *et al.* 1992; Tsoi *et al.* 1998). The expression of the NCXK1 is restricted to the retina, while the NCXK2 exchanger has been found to be expressed at high level throughout the

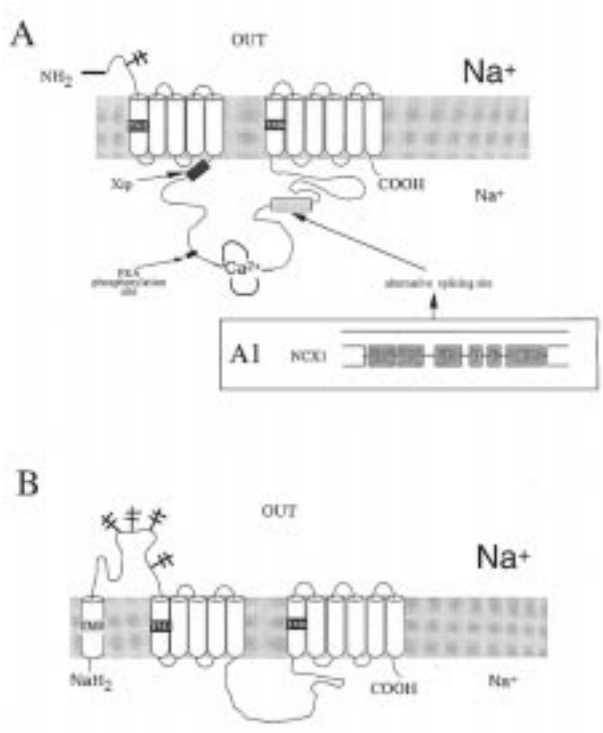


Figure 2. A) A model of the topology of the NCX exchanger. The transmembrane domains are indicated by cylinders. The glycosylation site is indicate by the little tree. Three other regions possibly involved in the regulation of the exchanger are indicated, the cytosolic high affinity Ca^{2+} binding site, the autoinhibitory peptide Xip and a potential c-AMP dependent protein kinase (PKA) phosphorylation site. The location of the alternative splicing site is also indicated. A simplified structure of the six exons involved in the alternative splicing are depicted in the A1 panel.
B) A model of the topology of the NCXK exchanger. The transmembrane domains are indicated by cylinders. The putative glycosylation sites are indicate by little trees. Notice the presence of a putative transmembrane domain (TM0) at the N-terminus of the protein.

brain, but at very low level in the retina and outside neural tissues. No cDNAs for the non-neuronal NCXK exchanger type have been cloned so far, despite that Na^+/Ca^{2+}-K^+ activity has been measured in platelets (Kimura *et al.* 1993). More that four homologous genes for the NCXK exchanger have been found in *Caenorhabditis elegans* (Tsoi *et al.* 1998) suggesting that more than two members of this family may exist in mammalian cells.

Surprisingly, the cardiac exchanger is relatively resistant to deletions. A large portion of the cytosolic loop, a fragment of 440 amino acids between residues 240 and 679 of NCX1 sequence, could be deleted without loss of exchanger activity (Matsuoka *et al.* 1993). The same was true for deletions where portions of the C-terminal sequence were removed before expression in embryonic kidney cells (Gabellini *et al.* 1995).

A high affinity binding site for Ca^{2+} located in the large cytosolic loop (Li *et al.* 1991; Matsuoka *et al.* 1993; Levitsky *et al.* 1994) was suggested to regulate the activity of the exchanger. When the reverse mode of action of the retinal exchanger was analyzed, a strong dependence on the free cytosolic Ca^{2+} concentration has also been demonstrated (Rispoli *et al.* 1995). This lead to an inhibition of the retinal exchanger following the increase of the cytosolic Ca^{2+}. Such an effect was not observed for the cardiac Na^+/Ca^{2+} exchanger, although regulation by internal Ca^{2+} has been described (Matsuoka *et al.* 1993).

A number of tissue-specific variants of the exchanger protein have been identified (Furman I *et al.* 1993; Kofuji *et al.* 1994; Lee *et al.* 1994; Reilly and Lattanzi 1996) that arise from the differential splicing of several small exons (Fig. 2 A1, (Kofuji *et al.* 1994)). The resulting proteins differ in a small portion of the main cytoplasmic loop, close to the domain that binds Ca^{2+} with high affinity (Matsuoka *et al.* 1993). The complete gene structure of the human NCX1 gene confirmed the complex structure of the cassette exons involved in the alternative splicing (Kraev *et al.* 1996).

Independent experiments indicated that the cardiac type exchanger may be regulated by phosphorylation (Caroni and Carafoli 1983, Iwamoto *et al.* 1995). A direct phosphorylation has been demonstrated for cardiac exchanger (Iwamoto *et al.* 1995; Iwamoto *et al.* 1996) and for the exchanger of the giant axon of the squid (DiPolo and Beauge 1994). Another puzzling property of the cardiac exchanger is its regulation by ATP. This was reported by Hilgemann in giant patches of guinea pig myocytes (Hilgemann 1990). This effect was only very slowly reversed, indicating that it was related to a long lasting modification. In agreement with this observation, the cardiac exchanger protein overexpressed in the CHO was not phosphorylated under a variety of conditions (Condrescu *et al.* 1995), but showed an ATP dependence. Recent experiments indicated that the ATP effect was the consequence of the increase of the synthesis of PIP2. The activation of the exchanger was a direct consequence of the increase of this phospholipid (Hilgemann and Ball 1996). The cloning of the Na^+/Ca^{2+} exchanger from frog heart muscle has led to the discovery of an alternatively spliced isoform that contains a P-loop motif

(Iwata *et al.* 1996), a sequence characteristic of many ATP binding proteins. Although the significance of the spliced-in insert is still not yet clear, the finding has important implications for the electrophysiology of frog sarcolemma, where the Na^+/Ca^{2+} exchanger is thought to play a predominant role in the muscle relaxation. The effect of ATP on the exchangers may be therefore the result of a direct effect on the protein (= phosphorylation, others?) or an indirect effect, which may explain the difficulty to interpret the results obtained on this protein in vivo

The P-type pumps

The cells contain different types of pumps, i.e. enzymes that transport charged substances through the membranes. These enzymes normally use the chemical energy stored in the ATP to carry out their function. Ions are transported by F, V and P-type ATPases (Pedersen and Carafoli 1987a, 1987b) The F-type (like the F_1F_0 ATP synthase of mitochondria) and V-type (located in the vacuoles) pumps are multi-subunits hetero-oligomeric complexes. The P-type pumps are normally composed of one or at most two subunits, the catalytic function being associated with a large polypeptide chain of 100–130'000 kDa. The common property of P-type pumps is the formation of an enzyme intermediate from ATP: the γ-phosphate of ATP is transferred to an Asp residue located in the active site resulting in the formation of a high energy acyl-phosphate (Figs. 3 and 4). The reaction is coupled to the transition of the enzyme between two conformational states, called E1 and E2 (Jencks 1992).

The SERCA pump

A reticulum Ca^{2+} dependent ATPase was discovered well over 30 years ago (Hasselbach and Makinose 1961). Experiments with the specific inhibitor of the SERCA pump thapsigargin and its specific activator phospholamban (Luo *et al.* 1994) have clearly shown that the SERCA pump is the Ca^{2+} extruding system controlling cardiac muscle relaxation.

Biochemical work on the pump concentrated on vesicles prepared from skeletal muscle, where it represents about 70% of the proteins of the reticulum membrane (MacLennan 1970). The pump, a 100 kDa integral membrane protein, could be isolated in active form from the same tissue. Cloning of its cDNAs from muscle and non-muscle cells confirmed that all intracellular Ca^{2+} pumps belong to the same gene family. The protein binds Ca^{2+} with high affin-

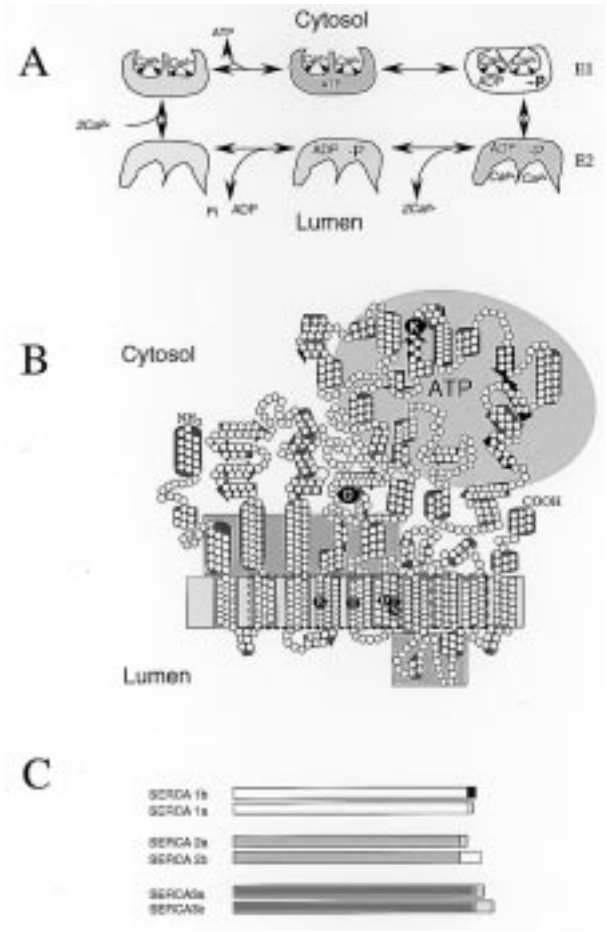
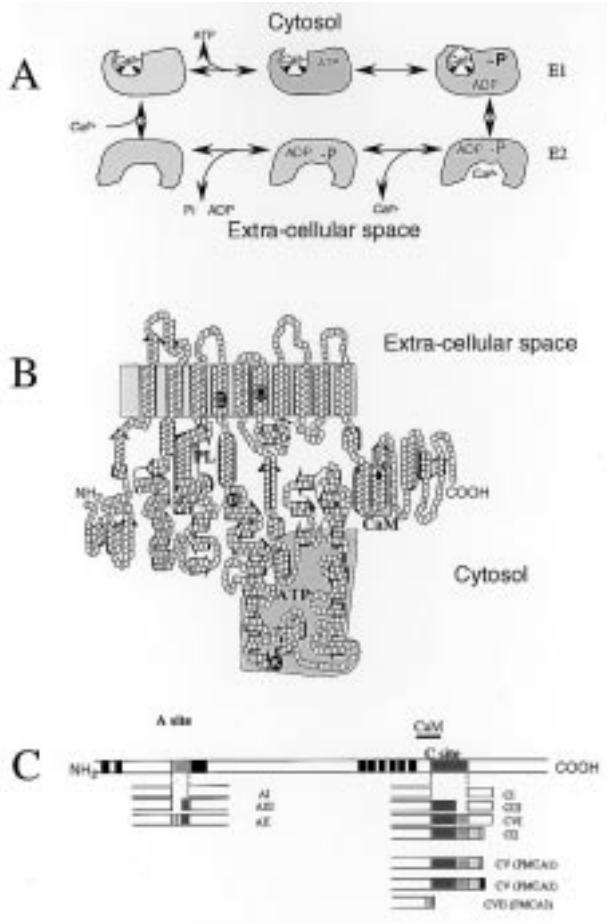


Figure 3. A) The catalytic cycle of the SERCA pump. The signs '~' and '-' indicate the high and low energy content of the complex between the enzyme and the phosphate atom. The arrows indicate high affinity Ca^{2+} binding.
B) A model of the membrane topology of the SERCA pump. The model is based on predictions from the sequence of the SERCA1a protein (the fast twitch SERCA pump (Brandl *et al.* 1986)). α-helices are represented by black cylinders, β-strand by large black arrows, transmembrane helices are shaded in dark grey. The aspartate (D = D351) in the catalytic centre (which becomes phosphorylated during the reaction cycle) and the lysine (K = K515) labeled by FITC are in bold.
The two boxes in the transmembrane region enclosed in rectangles identified by the bold dashed contours identify the transmembrane helices that were predicted to form compact structural units (Toyoshima *et al.* 1993).
The putative ATP binding (ATP) domain is indicated. The amino acids shown by the single letter code in the transmembrane are the amino acids involved in the transfer of Ca^{2+} across the protein.
C) A scheme of the three SERCA genes and their alternative splicing. The sequence indicated by different shades of grey are those that are generated by the alternative splicing.

ity at the cytosolic site, a step that does not require ATP (Inesi *et al.* 1992). The γ-Pi of ATP is transferred to Asp 351 (Allen G and Green MN 1976, MacLennan DH *et al.* 1985) resulting in the formation of the high energy acyl phosphate. Subsequently, the enzyme undergoes a conformational change from the so called E1 state to the E2 state. The E1 state can be imagined as the enzyme state in which the high affinity Ca^{2+} binding sites are open towards the cytosol, whereas in the E2 state, the low affinity Ca^{2+} binding sites are accessible from the lumen of the reticulum (Fig. 3A). The process is reversible, i.e., the pump can synthesize ATP from phosphate in the absence of Ca^{2+}. Under optimal conditions the SERCA pump transports up to 2 Ca^{2+} ions per hydrolyzed ATP (Fig. 3A) (Inesi G *et al.* 1992, Vilsen B 1995).



A structural model of the pump is presented in Fig. 3B. The protein is anchored in the membrane by 10 transmembrane domains, which are predicted to have α-helical conformation. The first large cytosolic loop protruding between transmembrane domains 2 and 3 contains an extended β-sheet region. The 2nd large cytosolic loop between the transmembrane domains 4 and 5 is the site of ATPase activity (MacLennan DH *et al.* 1985). This loop contains the aspartic acid, Asp 351, which forms the phosphoenzyme intermediate, and the residues that form the ATP binding site (Lys515, 492 and 684). A structure of the pump has been obtained using vanadate as a crystallisation inducer and electron microscopy as tool. Although the resolution of the crystals was low and secondary structures of the pump could not be distinguished, the domains were clearly visible (Toyoshima C *et al.* 1993). This structure has confirmed general features of the model presented in Fig. 3B. A striking feature of the crystal structure was the long tilted transmembrane 7, which had been predicted to be the longest transmembrane domain of the SERCA pump. A lumenal domain, likely made up by the loop between transmembrane domains 7 and 8 was discernible, consistent with

Figure 4. A) The catalytic cycle of the PMCA pump. The signs '~' and '-' indicate the high and low energy content of the complex between the enzyme and the phosphate atom. Notice that only one high affinity Ca^{2+} binding site is present (indicated by the arrows).

B) A model of the membrane topology of the PMCA pump. This model is based on the sequence of the PMCA4CI protein (Strehler *et al.* 1990). α-helices are represented by dark cylinders, β-strand by large black arrows, transmembrane helices are shaded in darker grey. D465 (aspartic acid 465), the residues phosphorylated during the catalytic cycle and K591 (lysine 591), the residue labeled by FITC in the ATP binding domain are in bold. T1102, the residue located in the calmodulin binding domain, which is phosphorylated by protein kinase C is in black.

Three domains are indicated by shadowed or black boxes, one of the two phospholipid binding domains (PL) (the other phospholipid binding domain is the C-terminal calmodulin binding site), the calmodulin binding domain (CaM) and the ATP binding domain (ATP).

The amino acids that form the receptor site for the calmodulin binding domain are grey. The two amino acids indicated by the single code in the transmembrane domains are likely to be involved in the transport of Ca^{2+} (Guerini et al. 1996)

C) Generation of PMCA isoforms by alternative splicing. All alternative splicing variants so far found to be generated by the insertion or the omission of exons at sites A or C are shown. The domain distribution of the PMCA's is shown in the upper part of the Figure. CaM is the calmodulin binding domain. The nomenclature of the isoforms is described in details by Carafoli (Carafoli 1994).

work with antibodies (Clarke *et al.* 1990, Matthews *et al.* 1990). Transmembrane domains 2 to 5, 6 and 8 form a compact transmembrane structure that is likely to contain the Ca^{2+} channel of the pump. The cytosolic head had an asymmetric conformation, and the refined structure of the pump allowed to distinguish subdomain, in particular the ATP binding pockets and the region involved in the phosphorylation (Zhang *et al.* 1998).

Starting from the structural model derived from the primary structure and other available information (MacLennan 1970; Brandl *et al.* 1986) mutations of polar amino acids in the transmembrane domains were performed. After expression in mammalian cells it was possible to show that mutation of Glu309, Glu771, Asn769, Thr799, and Asp800 abolished the ability of the pump to transport Ca^{2+} and to form the phosphoenzyme from ATP (Clarke *et al.* 1989; Clarke *et al.* 1990). The mutants, however, retained the ability to form the phosphoenzyme from phosphate, a reaction of the catalytic cycle that does not require high affinity binding of Ca^{2+} (Clarke *et al.* 1989), suggesting that these 5 residues are involved in the high affinity binding sites of Ca^{2+}. The five residues were located in the middle of transmembrane domains 4, 5 and 6 (Fig. 3B). The activity of an additional mutant that had lost high affinity Ca^{2+} binding sites (Glu908Ala, 8th transmembrane domain) was partially rescued at mM concentrations of Ca^{2+}. This suggested that Glu908 is probably not directly involved in the binding of the Ca^{2+} (Andersen and Vilsen 1994).

The regulation of the pump by phospholamban has been described for the heart sarcoplasmic reticulum more than 20 years ago. Dephosphorylated phospholamban binds to the SERCA pump, shifting its Kd for Ca^{2+} to a lower affinity value. This results in the inhibition of the pump activity. Phosphorylation of phospholamban by the c-AMP and the Ca^{2+}/calmodulin-dependent protein kinases dissociates the protein from the SERCA pump, relieving the inhibition. Studies on phospholamban knock-out mice have demonstrated that the regulation of the pump by phospholamban is critical to the positive inotropic effect of cathecolamines (Luo *et al.* 1994).

The isoforms of the SERCA pump

The SERCA pump is encoded by three genes (Grover AK and Khan I 1992), termed SERCA1, SERCA2 and SERCA3 (Fig. 3C). The SERCA1 pump isoform is expressed in high amounts in fast twitch skeletal muscles (Brandl *et al.* 1986) and in lower amounts in slow-twitch muscles (Brandl *et al.* 1987). Two alternatively spliced isoforms have been described. While the SERCA1a is present predominantly in adult muscles, the SERCA1b, which is 7 amino acids longer at the C-terminus, is transcribed at high level in neonatal tissues (Brandl *et al.* 1987). Transcripts of the SERCA2 pump have been found in slow-twitch, cardiac and smooth muscles (MacLennan *et al.* 1985; Brandl *et al.* 1987). Alternatively splicing has been described also for the SERCA2 pump. The SERCA2b differs from the SERCA2a in that the last 4 amino acids are substituted by 49 amino acids (Gutenski-Hamblin *et al.* 1988; Lytton and MacLennan 1988; Eggermont *et al.* 1989; Lytton *et al.* 1989). The insertion of these amino acids has no influence on the activity of SERCA2b pump but the 49 C-terminal amino acids were suggested to be associated to the lipid bilayer, thereby forming an additional (the 11th) transmembrane domain (Campbell *et al.* 1992). The third isoform, SERCA3, had been cloned from a rat kidney library (Burk *et al.* 1989) and has only been demonstrated to be functional after expression in COS cells. The corresponding protein is the major SERCA isoforms present in platelets (Bobe *et al.* 1994; Wuytack *et al.* 1994). A possible up regulation of this isoform in spontaneously hyperactive rats was described (Bobe *et al.* 1994). In Jurkat cells, a human lymphoma cell line, however, activation of the cells by ionomycin and phorbol ester resulted in the down regulation of the SERCA3 pump (Launay *et al.* 1997). Recently alternative splicing for the SERCA3 was demonstrated, as in the case of the other SERCA genes it occurred at the foremost C-terminus and generated two isoforms (Bobe *et al.* 1998).

The PMCA pump

The plasma membrane calcium ATPase is essential to the control of the cytosolic Ca^{2+} concentration in non muscle cells. The amount of PMCA pump is normally very low and it never exceeds 0.1–0.3% of the total membrane protein. This value may be higher in nervous cells (Stauffer *et al.* 1995) consistent with a more prominent role of the PMCA pump important in the homeostasis of Ca^{2+} in neurons. (Carafoli *et al.* 1996).

The PMCA pump was originally isolated from human red blood cells by affinity chromatography to calmodulin (Niggli *et al.* 1979), but it is now clear that all mammalian cells studied so far contain this pump (Carafoli and Guerini 1993). Reconstitution of the active pump in liposomes demonstrated that the 135 kDa peptide was sufficient for pumping activity (Niggli *et al.* 1981).

The reaction mechanism of the PMCA pump is similar to that of all other P-type pumps, with the difference that the Ca^{2+} pump of the plasma membrane can transport only 1 Ca^{2+} per hydrolyzed ATP (Niggli *et al.* 1982; Hao *et al.* 1994). As for the SERCA, the PMCA pump oscillates between a E1 and E2 state (Fig. 4A). The existence of the two conformations was also demonstrated experimentally (Krebs *et al.* 1987). The step responsible for the translocation of the bound Ca^{2+} across the protein has not been conclusively identified, but it is likely to correspond to the E_1~P to E_2~P transition.

As all other P-type pumps the PMCA pump is inhibited by μM concentrations of the phosphate analogue orthovanadate $[VO_3(OH)]^{2-}$. The other general inhibitor of P-type ATPases, La^{3+}, acts on the PMCA pump in a peculiar way: although La^{3+} inhibits the Ca^{2+} pumping activity, the same ion enhances significantly the amount of the phosphorylated intermediate of the plasma membrane Ca^{2+} pump.

The pump in the resting state has low Ca^{2+} affinity ($K_m > 10$ μM) and would be inactive at physiological cytosolic Ca^{2+} concentrations. Calmodulin increases its Ca^{2+} affinity to a K_m that can be as low as 0.2 μM (Carafoli 1992). Acidic phospholipids have also been shown to activate the pump at a concentration range similar to that found in plasma membranes (Niggli *et al.* 1981; Brodin *et al.* 1992).

The PMCA pump can be phosphorylated by the cAMP-dependent protein kinase (Caroni and Carafoli 1981; Neyses *et al.* 1985; James *et al.* 1989) and by protein kinase C (Wang *et al.* 1991). The cAMP-dependent protein kinase activates the pump by lowering its K_m for Ca^{2+} to about 1μM (James *et al.* 1989). The protein kinase C has been claimed to activate the pump, although the magnitude of the effect has varied in different reports (Smallwood *et al.* 1988). Experiments with a synthetic peptide corresponding to the calmodulin binding domain of the pump phosphorylated on Thr 1102 have shown that the phosphorylation weakens the interaction of the calmodulin binding domain with the binding ('receptor') site in the pump (Hofmann *et al.* 1994). Phosphorylation by protein kinase C has been shown to occur at different sites downstream the calmodulin binding domain of the PMCA4 (Enyedi *et al.* 1996). Tyrosine phosphorylation of PMCA4 was shown to partially down regulate the pump (Dean *et al.* 1997). Unfortunately, most of these studies are based on in vitro experiments, and still need in vivo verification.

Experiments on partial trypsin proteolysis of the purified enzyme provided information on the domain structure of the pump. The C-terminally truncated pump gradually looses the ability to respond to calmodulin (Zvaritch *et al.* 1990; Carafoli 1994). The intracellular Ca^{2+}-dependent neutral protease calpain also attacks the pump and makes it calmodulin-insensitive. Calpain generates a fully active 124 kDa pump, which is completely calmodulin independent (James *et al.* 1989). The calpain activated pump was used to determine the sequence of the 'receptor' site for the calmodulin binding domain of the pump, i.e., the site which is involved in the autoinhibition of the pump (Falchetto *et al.* 1991, 1992). (Fig. 4B). Two 'receptor' sites are present in the pump, a short one located just downstream of Asp(465) residue involved in the formation of the phosphorylated intermediate, a second less well defined located in the first large cytosolic loop.

The calmodulin binding-domain has the propensity to form a basic amphiphilic helix (James *et al.* 1988). Extensive work with synthetic versions of the domain showed its tight interaction with calmodulin (Vorherr *et al.* 1990) that led to the collapse of the elongated structure of calmodulin (Kataoka *et al.* 1991).

It has been possible to determine the sequence of two regions involved in the formation of the phosphoenzyme intermediate (Asp465) (James *et al.* 1987) and in the binding of ATP, (Lys591) (Filoteo *et al.* 1987). Hydropathy analysis of the sequence of the pump obtained by cDNA cloning and a comparison with the model proposed for the SERCA pump (Verma *et al.* 1988) led to the suggestion that the two pumps have a very similar architecture. 10 transmembrane domains have been identified in the PMCA that are connected on the external side by 5 short loops. On the inner side, the pump protrudes into the cytoplasm forming 4 main domains. The first one encompasses the first 80–90 N-terminal amino acids, the second contains the phospholipid interacting site (Zvaritch *et al.* 1990), the third, (and the largest) the catalytic site. The fourth domain comprises the C-terminal portion of the pump, where a number of regulatory sites are located, among them the calmodulin-binding domain (James *et al.* 1988) and the substrate domains for protein kinases (James *et al.* 1989; Wang *et al.* 1991). The model has been supported by work on antibody binding coupled to proteolysis and to the analysis of peptide fragments (Carafoli 1992).

The isoforms of the PMCA pump

Four genes of the PMCA pump (PMCA1, PMCA2 PMCA3 and PMCA4) are now recognized in mammals (Carafoli E and Guerini D 1993, Guerini D 1998) but the situation is less well defined in lower

organisms. Plasma membrane Ca^{2+} pumps of different types have been inferred from experiments on liver tissues (Pavoine *et al.* 1987), but no information on their sequence is yet available. Sequence differences among the isoforms are found mostly in the N-terminal and the C-terminal regions of the protein (Fig. 4B).

The regions that are involved in the alternative splicing show considerable differences. Alternative splicing is theoretically responsible for the generation of more than 30 PMCA pump isoforms (Carafoli & Guerini 1993). Alternative splicing occurs at two sites one located in the N-terminus and the other at the C-terminus, just after the calmodulin binding domain (Fig. 4C). An additional alternative splicing site, that would generate a pump with only 9 transmembrane domains was likely to be a cloning artefact (Seiz-Preianò *et al.* 1996). A maximum of three different introns is inserted or omitted at site A (Adamo & Penniston 1992, Heim *et al.* 1992, Keeton *et al.* 1993, Stauffer *et al.* 1993). In the case of PMCA2 up to three (possibly four in the rat) different isoforms may be generated by the alternative splicing process, whereas for PMCA3 and PMCA4 only two splicing products are generated. In the case of PMCA1 no alternative splicing at site A has been detected (Hilfiker *et al.* 1993; Stauffer *et al.* 1993). The highest number of alternative spliced products at the C site was found for the rat PMCA3: up to 7 different isoforms are possible (Keeton *et al.* 1993). The insertion or the omission of the exons at site C involves a portion of the calmodulin binding domain (Carafoli & Guerini 1993) modifying it substantially. Insertion at this site generally causes a dramatic decrease of the affinity of the pump for calmodulin, but also an increase of the basal activity (Seiz-Preianò *et al.* 1996). A summary of selected PMCA pumps isoform is presented in Table 1.

Conclusions

The Ca^{2+} pumps and the Na^{+}/Ca^{2+} exchangers are responsible for the removal of the Ca^{2+} from the cytosol. The cloning of the corresponding cDNA's provided information of their primary structure and allowed some structural predictions to be made. In the case of the SERCA pump, and its highly homologous PMCA pump, the structural predictions have been supported by recent cryo-electron microscopic and crystallographic works (Toyoshima *et al.* 1993, Zhang *et al.* 1998). The development of expression systems for the Ca^{2+} transporters has provided information on their biochemical properties (see for example Table 1). It has been established that exchangers and pumps are encoded by multigene families, and the number of possible isoforms is increased by alternative splicing. Nevertheless, despite recent efforts, relatively little is known on the physiological significance of the two transporter isoforms, at which stage of the cellular development they are expressed and which specific mechanisms are controlling their transcription. These aspects are important since pumps and exchangers influence the kinetic of the cytosolic Ca^{2+} fluctuations: the set of isoforms expressed may match to the needs of a certain cell type. Changes in the isoforms composition could therefore affect the Ca^{2+} homeostasis of this particular cell.

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Table 1. The properties of the PMCA isoforms

	PMCA1	PMCA2	PMCA3	PMCA4CI	PMCA4CII
Tissue distribution	ubiquitous	restricted	restricted	ubiquitous	restricted
Level of expression	high	high	medium-low	medium-high	medium-high
Susceptibility to calpain	high	low	NA	low	NA
acdid phospholipids stimulation	NA	yes	NA	yes	NA
KdCaM	30–40 nM	3–7 nM	NA	30–40 nM	700–800 nM
KdATP†	0.1 μM	0.2–0.3 μM	NA	0.7 μM	NA

NA = data not available; † analyzed as phosphoenzyme formation

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