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The plasma membrane calcium pump: recent developments and future perspectives

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Abstract. The Ca^{2+} pump of the plasma membrane (PMCA) is regulated by a number of agents. The most important is calmodulin (CaM), which binds to a domain located in the C-terminal portion of the pump, removing it from an autoinhibitory site next to the active site. The CaM-binding domain is preceded by an acidic sequence which contains a hidden signal for endoplasmic reticulum (ER) retention. Chimeras of the PMCA and endoplasmic reticulum (SERCA) pumps have revealed the presence of a strong signal for ER retention in the first 45 residues of the SERCA pump . Four gene products of the PMCA pump are known: two of them (1 and 4) are ubiquitously expressed, two (2 and 3) are specific for nerve cells and may be induced by their activation. Mutagenesis work has identified four residues in three of the transmembrane domains of the pump which may be components of the trans-protein Ca^{2+} path. The mutation of two of these residues alters the membrane targeting of the pump.
Key words. Calcium; ATPase; calmodulin; ion pumps.

Introduction

The plasma membrane Ca^{2+} adenosine triphosphatase (ATPase) was first described in erythrocytes in 1966 [1], and since then has been detected in all eukaryotic cells. Yeast cells contain a protein that has homologies to the Ca^{2+} ATPase of higher eukaryotes: its function as a Ca^{2+} pump, however, has not been confirmed [2]. The pump belongs to the family of P-type ATPases [3, 4], i.e. it forms a phosphorylated intermediate (an aspartyl-phosphate) during the reaction cycle. It is a target of calmodulin [5, 6]: the latter increases its affinity for Ca^{2+} by one order of magnitude, to a K_d of about 0.5 μM. In the absence of calmodulin, however, the pump can be activated by a number of alternative treatments: exposure to acidic phospholipids [7, 8], a controlled proteolytic treatment [8, 9], phosphorylation by at least two protein kinases, protein kinase A (PKA) [10] and protein kinase C (PKC) [11, 12], and an oligomerization process [13]. Not all these activating treatments have been documented in vivo, although those involving calmodulin, acidic phospholipids or the protein kinases have. Although the general consensus seems to be that calmodulin is the universal modulator of the pump, it is as well to remember that the pump in its natural habitat is surrounded by enough acidic phospholipids to be half-maximally activated [14].

The activation by calmodulin has been exploited to purify the pump using calmodulin columns [15], first from erythrocytes, and then from a number of other tissues. The purified enzyme is functionally competent and can be reconstituted in liposomes with optimal Ca^{2+} transport efficiency [14]: unlike the Ca^{2+} pump of sarcoplasmic reticulum, whose Ca^{2+}/ATP molar transport stoichiometry is 2.0, the plasma membrane Ca^{2+} (PMCA) pump transports only one Ca^{2+} per ATP hydrolysed.

A number of comprehensive reviews of the mechanism and general properties of the pump in vivo have appeared (see for example refs 16 and 17), and the reader is referred to them for in-depth discussion of the early findings. Following the purification of the pump, numerous studies have dealt with its molecular aspects, leading to significant advances in the understanding of the properties of this important enzyme: recent reviews offer detailed discussions [18–22]. This contribution will not attempt to be comprehensive: it will focus instead on recent developments which have emerged from the work on the purified enzyme, and which open interesting perspectives on the cell biology/physiology of the pump.

The membrane targeting of the PMCA pump

As will be discussed later, the human pump is the product of a multigene family: the first cloned human isoform was isoform 4, which is still the most intensively studied and the best known. Figure 1 shows its membrane topography, which is common to all other isoforms. It predicts 10 transmembrane domains, and four main units protruding into the cytoplasm: the first is the N-terminal portion of the pump, whose length varies between 90 and 100 residues in the various isoforms. The second, termed the β-strand domain, protrudes from transmembrane domain 2: its function is unknown. The third unit, which is the largest, protrudes from transmembrane domain 4: it contains the active site(s) of the pump, i.e. the site of aspartic acid phos-

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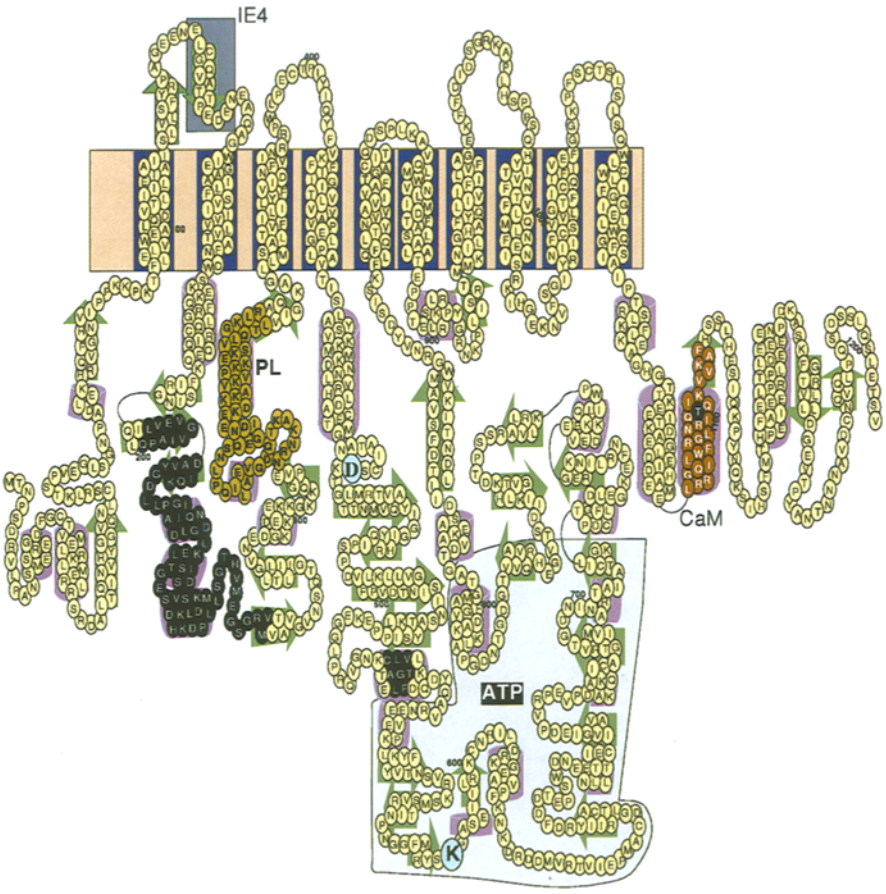

Figure 1. Sequence, predicted secondary structure and membrane topology of the plasma membrane Ca^{2+} pump. CaM is the calmodulin-binding domain, containing the Thr which is phosphorylated by PKC. PL is a predominantly basic sequence which interacts whith acidic phospholipids (the calmodulin-binding domain also interacts with them). The two black domains are the 'receptors' for the autoinhibitory calmodulin-binding domain. The shaded region (ATP) is the domain of the pump where ATP becomes bound: it contains a conserved Lys (K). D is the site of phosphorylation. The shaded area is the epitope for monoclonal antibody 1E4. The isoform shown is PMCA4, which does not contain the C-terminal consensus site for PKA.

phorylation, and the domain where ATP is bound, which includes a conserved lysine (K). The fourth unit protrudes from the last transmembrane domain and extends for about 150 amino acids to the C-terminus. It contains several domains which are important for the regulation of the pump: the calmodulin binding site, consensus sites for phosphorylation by PKA and PKC (the latter site is a threonine residue (Thr) within the calmodulin binding domain, while the former site is only present in some isoforms of the pump, see below), and sites which bind Ca^{2+}, possibly to modulate the activity of the pump [23]. The calmodulin binding site is one of the two domains that interact with acidic phospholipids, the other being a basic stretch of about 40 amino acids located in the second cytosolic unit [24]. The second and third cytosolic units also contain sites which interact with the calmodulin binding domain. This domain folds over the main body of the pump, locks together the second and third cytosolic units, and keeps the pump inhibited in the absence of calmodulin [25, 26]; the latter then interacts with its binding domain and swings it away from its receptor site, relieving the inhibition of the pump.

The proposal of an autoinhibitory function for the C-terminal cytosolic unit of the pump was strongly supported by experiments on its proteolysis by calpain [27], which showed that the activation by the protease was linked to the truncation of the pump at the beginning of the calmodulin binding domain. Attempts to reproduce the effects of calpain, i.e. to produce fully active, calmodulin-independent pumps by expressing truncated versions of them, led to the unexpected finding of a (masked) signal for endoplasmic reticulum (ER) retention in the C-terminal portion of the pump. A more detailed study of the membrane targeting of the PMCA pump was therefore undertaken [28]. The truncation by calpain exposes a very acidic sequence, which now becomes the C-terminal domain of the pump (fig.

2). Truncated pumps ending with this acidic sequence, whose last residue is Arg 1087 (i.e. the second residue of the calmodulin binding domain), were retained in the ER when expressed in Cos-7 cells (fig. 3), whereas versions of the pump truncated about 20 residues upstream, and thus not containing the acidic C-terminal sequence, were correctly delivered to the plasma membrane. A number of point mutations performed to neutralize negatively charged residues in the sequence Glu 1067–Arg 1087 showed strikingly that the replacement of one or more of the nine acidic residues of the stretch (Glu 1078 to Gln, Asp 1080 to Asn, Glu 1083 to Gln, Glu 1085 to Gln) restored the correct plasma membrane targeting of the enzyme (fig. 3). Selective retention and degradation within the ER are common in misfolded and mutated proteins, which exposes retention/degradation signals otherwise masked in properly folded molecules. Since the truncated PMCA pump would be fully and permanently active, its selective retention (and degradation) in the ER could have evolved as a defense mechanism to prevent the release to the plasma membrane of prematurely terminated pump versions with unwanted properties, i.e. with uncontrolled activity.

N-terminal transmembrane domains are generally considered important for the membrane targeting of proteins [29]. The PMCA and the ER (SERCA) pumps are similar in functional properties and membrane topology, yet are strictly targeted to two different membranes. It was thus decided to study the targeting problem by constructing chimeras of the two pumps in which the N-terminal transmembrane domains were exchanged [30]. Five chimeric molecules were constructed (fig. 4): in four (chimeras A, B, C, D), different N-terminal portions of the SERCA pump were followed by the remainder of the PMCA pump molecule. In the fifth (chimera E), the N-terminal cytosolic unit and the first two transmembrane domains of the PMCA pump were followed by the remainder of the SERCA pump molecule. Surprisingly, none of the chimeras had Ca^{2+}-dependent ATPase activity (phosphoenzyme formation from ATP) when expressed in Cos-7 cells; a partial exception was chimera C, which still formed a weak phosphorylated intermediate. Immunohistochemistry work using SERCA or PMCA pump antibodies (fig. 5) revealed that a strong ER retention signal was evidently contained in the first 85 residues of the SERCA pump, since chimeras A–D were strictly re-

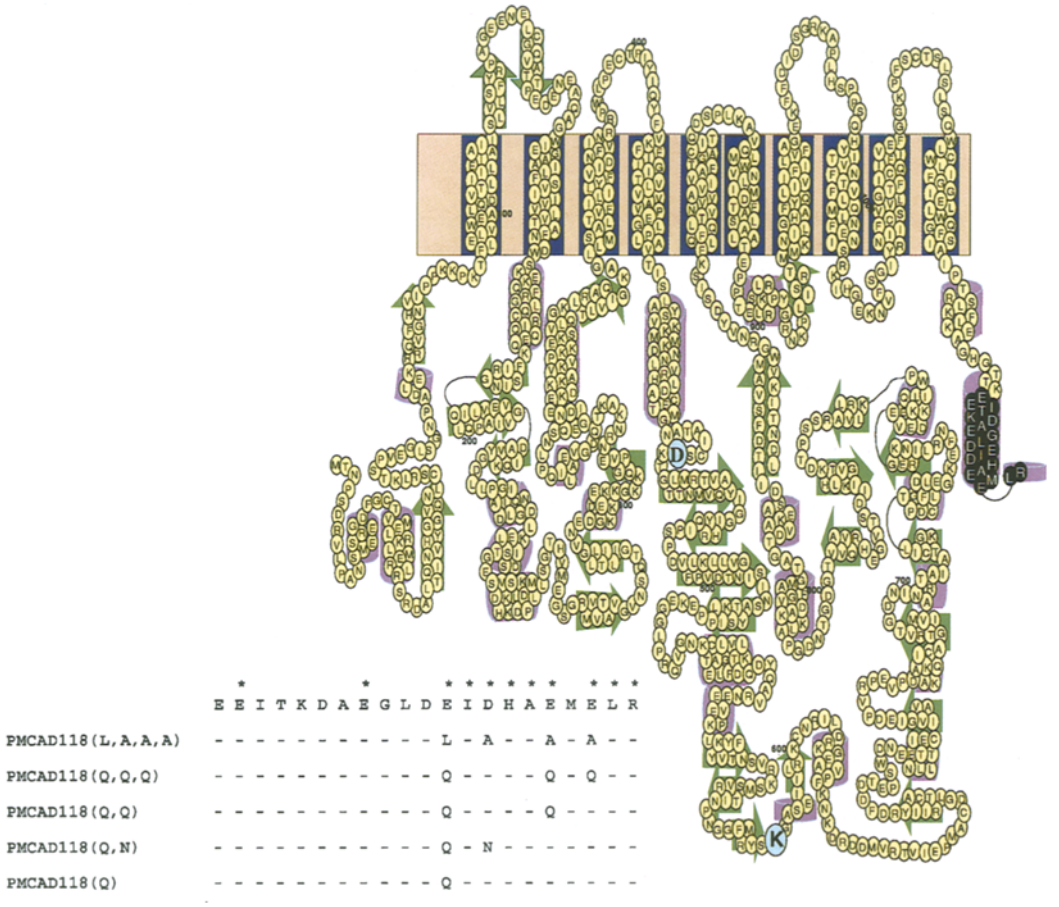

Figure 2. Truncation of the pump by calpain. The truncation exposes a C-terminal acidic sequence (black). The inset shows the mutations that have been introduced in the exposed acidic C-terminal sequence.

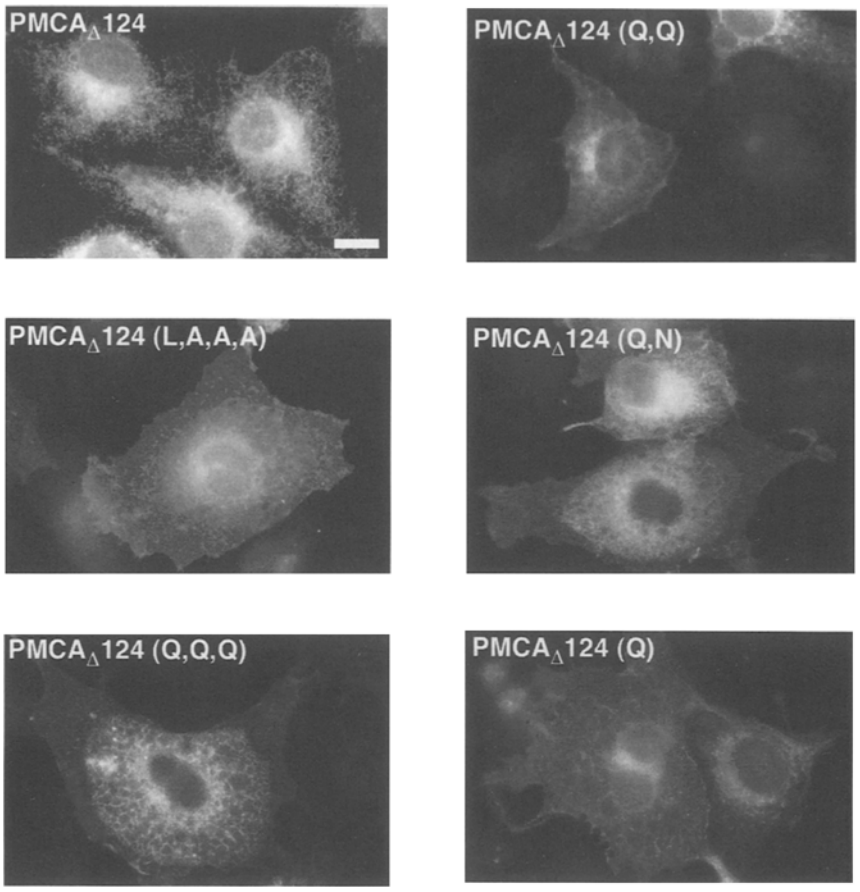

Figure 3. Membrane targeting of truncated/mutated versions of the pump expressed in Cos-7 cells. Two monoclonal antibodies and one polyclonal antibody against the N-terminal portion of the pump have been used. From ref. 28. Bar = 10 μm.

tained in the ER (chimera D, however, was peculiarly sensitive to proteolysis, and was degraded much faster than the others). Chimera E, in which the first two transmembrane domains were PMCA type, behaved differently: although it was still mostly retained in the ER, in 5–14% of the expressing cells a plasma membrane-staining pattern was visible instead. This indicated that this chimera had lost part of the ER retention signal, i.e. that located in the first 85 amino acids. However, it also indicated that additional, even if less efficient, ER retention signals exist in the SERCA pump molecule outside the region encompassing the N-terminal portion and the first two transmembrane domains.

Isoforms of the PMCA pump

Four genes for the PMCA pump are known in humans (and in rats). They have been mapped to chromosomes 12, 1, 3 and X (see ref. 31 for a recent review on isoforms and genes). Each of the four genes produces additional isoforms by alternative splicing of primary transcripts; more that 20 alternatively spliced transcripts have been described so far. Three of the four basic human gene products (1, 2, 4) have been cloned, expressed and studied functionally [32–34]. Table 1 summarizes their most important properties and also includes information on isoform 3, which has not been expressed as yet. Isoforms 1 and 4 are present (in large amounts) in all tissues, and can thus be considered as products of housekeeping genes. Isoforms 2 and 3, in contrast, are only found in significant amounts in brain: isoform 2 is typical of cerebellum, particularly of the Purkinje cells (fig. 6), and isoform 3 of the choroid plexuses. Functionally, the most striking difference among isoforms is the affinity for calmodulin, which is significantly higher in isoform 2. This is interesting, considering that this isoform is typical of Purkinje cells, which evidently have special Ca^{2+}-signalling requirements (these cells also contain unusually large amounts of the inositol-tris-phosphate receptor). Of interest is the different calpain sensitivity of the four basic isoforms (P. Bin, D. Guerini and E. Carafoli, unpublished), which suggests differences in their membrane topology, i.e. the exposure of different sites cleavable by calpain to the cytosol.

A particularly interesting aspect of the distribution of the isoforms is their developmental switch, which is also

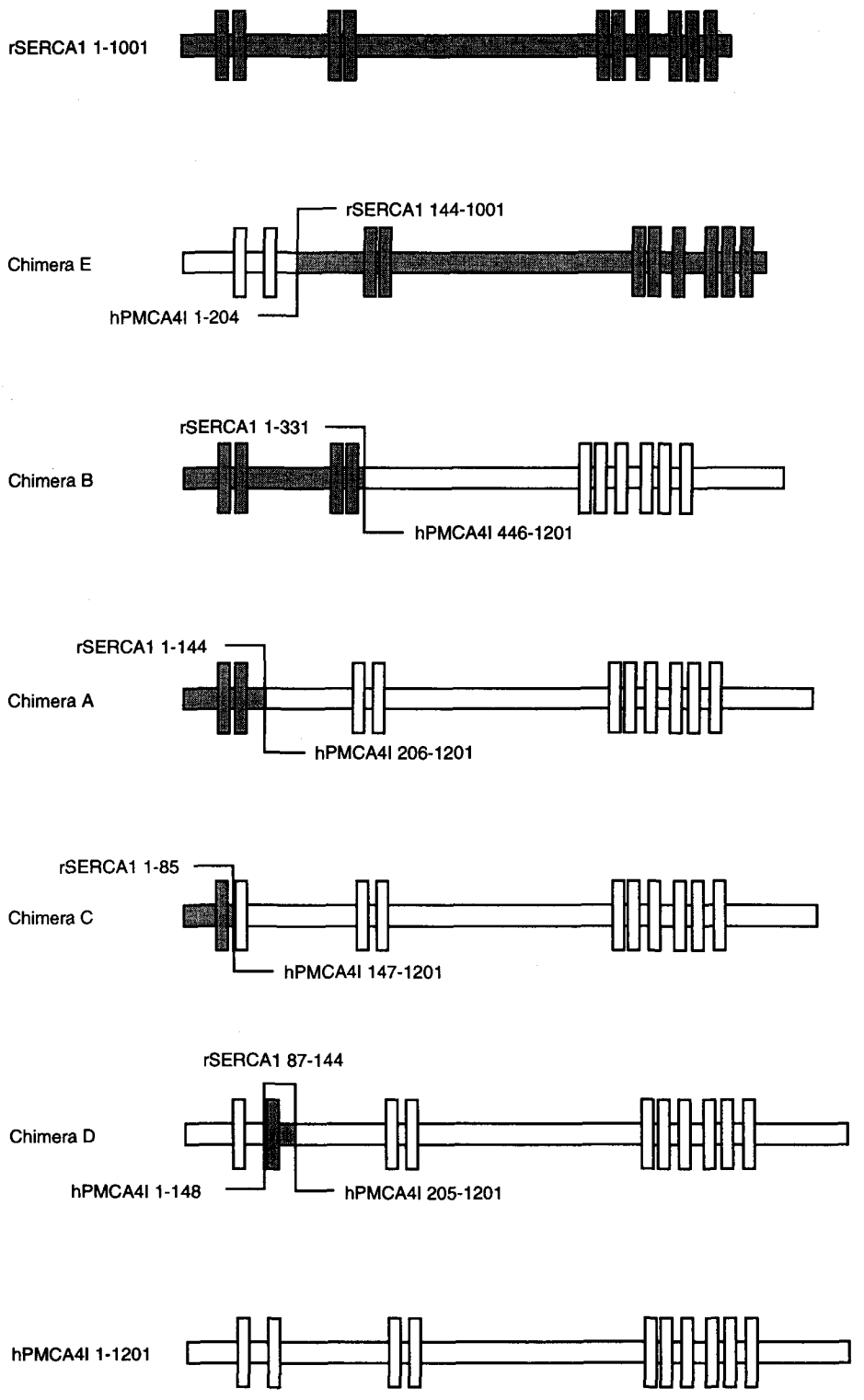

Figure 4. Chimeras of the PMCA and SERCA pumps. The isoform of each pump that has been used is shown. From ref. 30.

mentioned in table 1. An important development in the understanding of PMCA isoform has recently been made by using primary cultures of granular cells of rat cerebellum. These cells have often been used as models for neuronal cell development. They are very homogeneous in culture (more than 90% are granular neurons), and their survival has a peculiar dependence on the presence of potassium or other depolarizing agents [35, 36]: the treatement has been shown to produce a sustained increase of cytosolic Ca^{2+}, and to

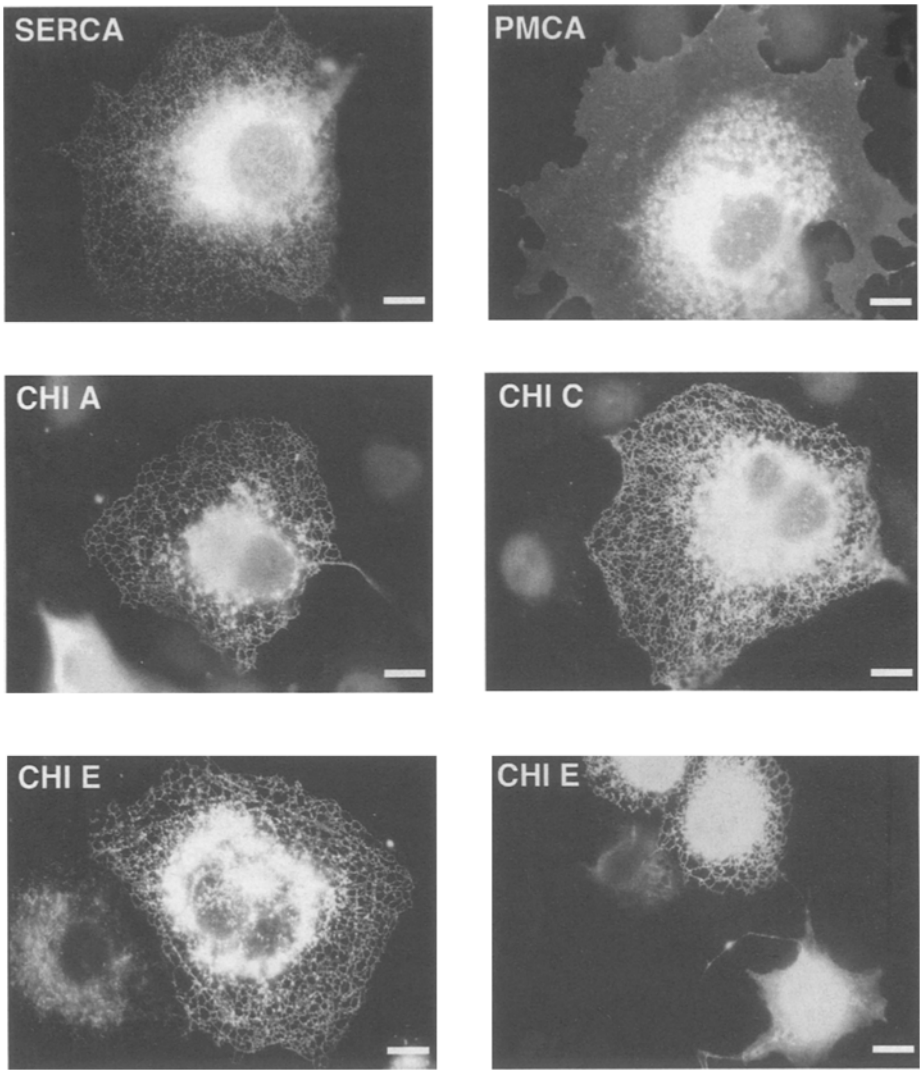

Figure 5. Membrane targeting of the PMCA/SERCA chimeras expressed in Cos-7 cells. Bar = 30 μm. Chimera B is not shown, because its targeting pattern was identical to that of chimera A. Chimera D was very rapidly proteolysed and yielded no clear immunofluorescence pattern. Isoform-specific antibodies have been used (ref. 38).

influence the expression of several genes. Membrane proteins from cells that had been cultured in the presence of 25 mM KCl for 2 or 5 days were probed with antibodies specific for the various PMCA isoforms. Isoform PMCA1 increased in level, most evidently in its 130-kDa component, which migrated in gels with the molecular mass expected of the PMCA1CII alternatively spliced isoform (fig. 7). Interestingly, the latter

Table 1. Properties of the PMCA isoforms.

	PMCA1	PMCA2	PMCA3	PMCA4
Tissue distribution	ubiquitous	restricted (brain)	restricted (brain)	ubiquitous
Level of expression in rat and human tissues	high	high	low	medium
Developmental expression/switch	isoform switch fetal/adult	isoform switch fetal/adult	downregulated in adult tissues	isoform switch fetal/adult
K_dCaM*	40–50 nM	8–10 nM	NA	40–50 nM
K_dATP†	0.1 μM	0.2–0.3 μM	NA	0.7 μM
$T_{1/2}$ of the degradation by calpain	2–4 min	50–55 min	NA	50–65 min

NA = data not available.
* The isoform analysed was CI, i.e. without insert at hot spot C, which encompasses the calmodulin binding domain.
† determined from the phosphoenzyme formation.

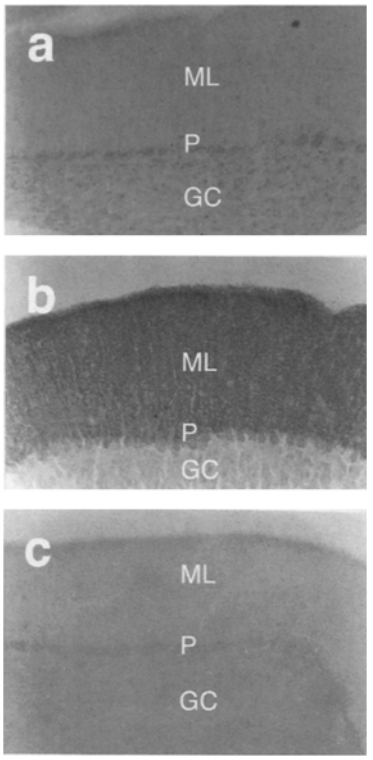

Figure 6. PMCA pump isoforms in rat cerebellum. (a) PMCA1; (b) PMCA2; (c) PMCA3. ML = molecular layer, P = Purkinje cells (bodies), GC = granular cells. The isoform-specific antibodies described in ref. 38 have been used.

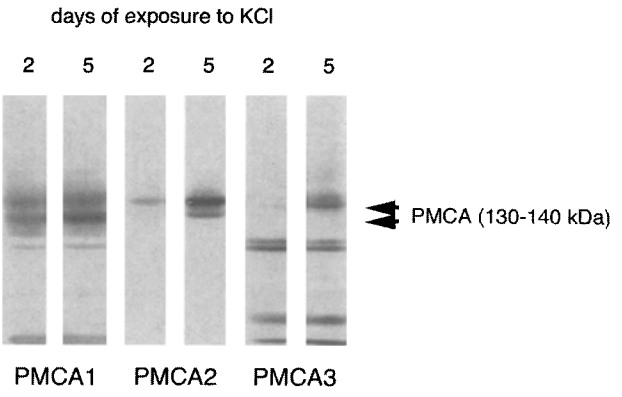

Figure 7. PMCA isoforms in granular cells of rat cerebellum. Twenty-five to thirty micrograms of membrane proteins, prepared cultures of rat cerebellum granular cells, were incubated for 2 or 5 days in the presence of 25 mM KCl. Membranes were isolated and their proteins separated by SDS-PAGE electrophoresis as described in ref. 38. The nitrocellulose sheets were stained with affinity purified antibodies against the N-terminal portion of the PMCA1, PMCA2 and PMCA3 pump isoforms essentially as described in ref. 38.

isoform is typical of nerve cells [37, 38]. After 2 days in culture hardly any PMCA2 protein, and no PMCA3 protein at all, was detected. After 5 days in the presence of 25 mM potassium, however, large amounts of both isoforms appeared in the cells (fig. 7). These striking results suggest that the expression of the PMCA2 and PMCA3 may be controlled by the cytosolic Ca^{2+} concentration. Since the antibody against the human PMCA4 protein does not recognize the corresponding rat protein [38], the presence of this isoform in the granular cells could not be explored. It is interesting, however, that the other housekeeping isoform, PMCA1, was also increased by the depolarizing treatement (although, as specified above, the increase only concerned the 130 kDa nervous tissue-specific spliced isoform) (fig. 7).

The path of Ca^{2+} across the PMCA molecule

The Ca^{2+}-binding sites in the C-terminal portion of the PMCA pump which were mentioned above [23] are not involved in the translocation of Ca^{2+} across the pump molecule. This follows from the finding [27] that the removal of the C-terminal cytosolic tail of the pump by calpain does not impair the ability of the latter to transport Ca^{2+}: as discussed above, it actually leads to its permanent activation. The channel for the transport of Ca^{2+} across the molecule must evidently be formed by amino acids located in (some of) the transmembrane domains of the pump. Site-directed mutagenesis of selected amino acids in these domains of the SERCA pump [39] has led to the conclusion that the wall of the Ca^{2+} channel through that pump is formed by six residues, four of them acidic, located in transmembrane domains 4, 5, 6 and 8. These 6 residues are shown in table 2, which aligns them with residues in equivalent positions in other P-type pumps, including the PMCA pump. Some of these residues are conserved, but others are not. In particular, there is no (acidic) residue equivalent to Glu 771 of transmembrane domain 5 of the SERCA pump in the corresponding domain of the PMCA pump. Furthermore, Glu 908 in the eighth

Table 2. Conserved residues in the transmembrane domains of the SERCA, PMCA, H^+/K^+- and Na^+/K^+-ATPases.

	TM4	TM4	TM4	TM5	TM6	TM6	TM6	TM8
SERCA	P308	E309	P312	E771	N796	T799	D800	E908
H^+/K^+-ATPase	P342	E343	L46	E795	E820	T823	D824	E936
H^+/K^+-ATPase	P326	E327	L330	E779	D807	T807	D808	V924
PMCA	**P**422	**E**423	**P**426	A854	**N**879	M882	**D**883	**Q**971

Taken from ref. 40, reprinted with permission of the American Chemical Society.
The PMCA residues which have been mutated are in bold.

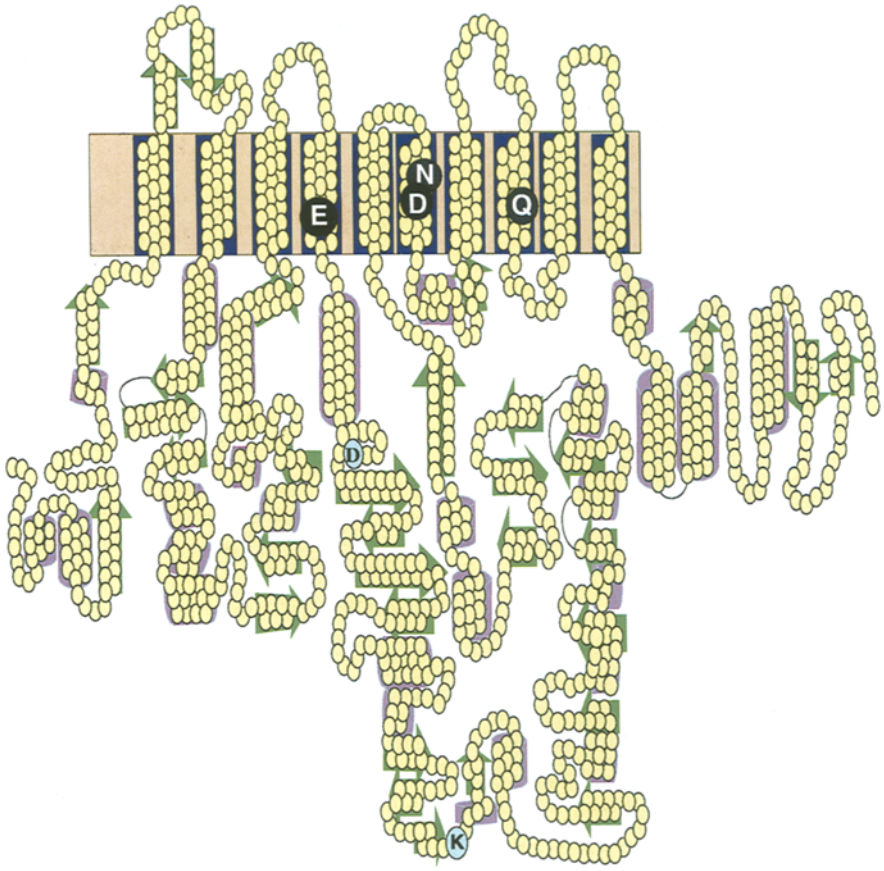

Figure 8. A scheme of the membrane topography of the PMCA showing the residues of the transmembrane domains that have been mutated.

transmembrane domain of the SERCA pump is replaced by a Gln (971) in the PMCA enzyme.

The three conserved residues in transmembrane domains 4 and 6 and Gln 971 of domain 8 were thus mutated [40] (fig. 8). In all cases, the mutations abolished the uptake of Ca^{2+} by the pump expressed in Cos-7 cells, a result which would prima facie be consistent with the conclusions reached from the mutagenesis results on the SERCA pump [39]: i.e. the mutated residues in the three transmembrane domains would line the wall of the trans-protein Ca^{2+} channel. Unfortunately, however, immunohistochemistry controls showed that two of the point mutations, of Asn 879 and of Gln 971, caused retention of the mutated PMCA pumps in the ER (fig. 9). Thus, irrespective of whether these two residues are components of the trans-protein Ca^{2+} path, their mutation has structural effects. These effects are not dramatic, as established by a number of controls performed and discussed in [40], but they are evidently sufficient to interfere successfully with the plasma membrane-targeting signal(s) of the pump. Obviously, point-mutation experiments of this type not accompanied by appropriate controls of nonspecific (structural) effects may lead to erroneous interpretations. This comment, which need not be confined to the case of ion pumps, is made here to underline the statement that the path of Ca^{2+} across the PMCA pump molecule is still unidentified.

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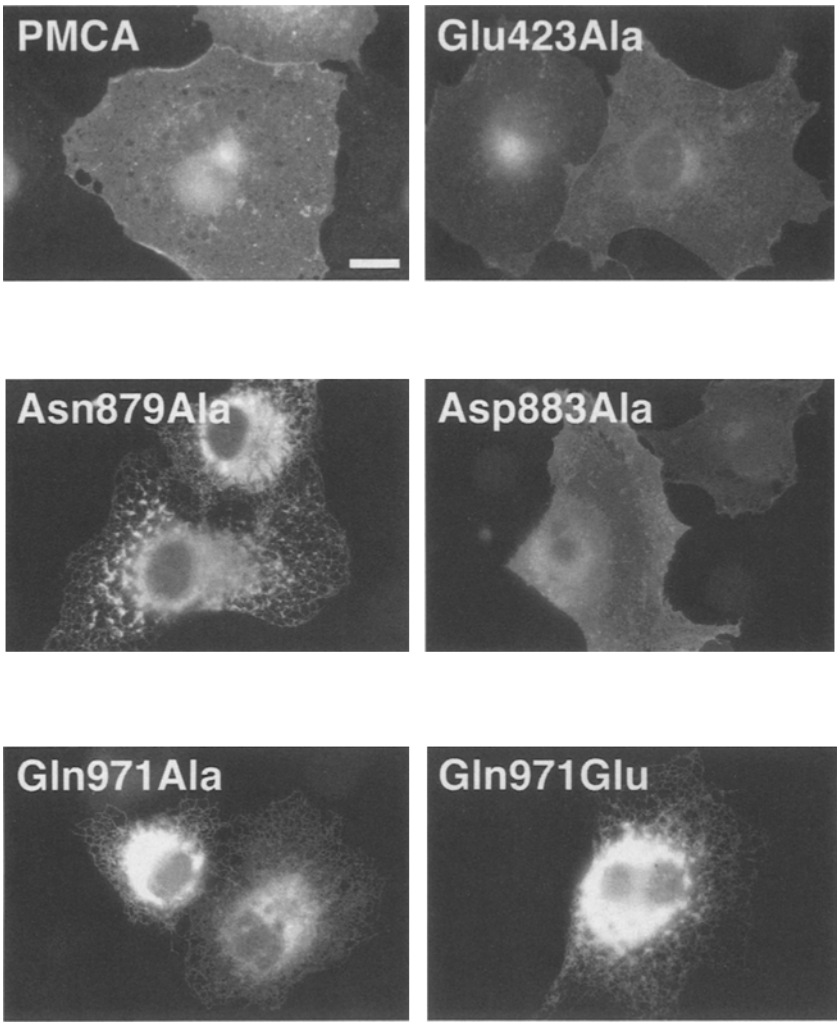

Figure 9. Membrane targeting of the mutants of the PMCA pump shown in figure 8 after expression in Cos-7 cells. (From ref. 40.)

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