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Topical Review

Recent Advances in the Molecular Characterization of Plasma Membrane Ca^{2+} Pumps

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Introduction

Since its discovery some 25 years ago (Schatzmann, 1966) the plasma membrane Ca^{2+} pump (PMCA) has constantly gained in importance as a model membrane protein to study the structural, functional, regulatory and genetic basis of ATP-driven cation transport. The success in the characterization of this enzyme parallels that made in the molecular analysis of other complex low-abundance proteins spanning the lipid bilayer and reflects to a large extent the impressive technical progress made during the past two decades in all areas of modern biological research. It is not surprising, therefore, that several reviews have already been devoted mostly, if not exclusively, to the various aspects of the plasma membrane Ca^{2+} pump. Rather than trying to resummarize the contents of previous reviews, the present contribution will focus on those developments concerning the characterization of the Ca^{2+} pump which have only recently (i.e., during the past three to five years) been added to our existing views on this important enzyme. Accordingly, no attempt will be made to present a comprehensive picture of the subject; for more detailed information on different aspects of the plasma membrane Ca^{2+} pump the reader is referred to previously published reviews and references found therein. A recent summary of the general properties, and particularly of ion transport-related characteristics, of the pump can be found in Garrahan and Rega (1990), and a synopsis of the problems related to the mechanism of calcium pumping has been given by Jencks (1989). A limited selection of further reviews published during the past decade includes contributions by Sarkadi [1980 (emphasis on early results on the enzymatic properties of the erythrocyte pump)]; Schatzmann [1982 (comprehensive review of the discovery, enzymatic characterization and kinetic properties of the pump)], Carafoli and Zurini [1982 (purification and properties of the reconstituted erythrocyte enzyme)], Penniston [1983 (discussion of general properties and the possible regulation of the pump as well as an overview of its tissue distribution)], and Niggli, Zurini and Carafoli [1987 (efficient purification method, reconstitution results and refined properties of the pump)].

Key Words calcium homeostasis · membrane protein · ion transport · calcium ATPase · P-type pump · isoforms · structure-function relationships

The Plasma Membrane Ca^{2+} Pump: A Ubiquitous Eukaryotic P-Type ATPase Regulated by Direct Interaction with Ca^{2+}/Calmodulin

Most of the initial work on the characterization, isolation, purification and reconstitution of the plasma membrane Ca^{2+} pump has been performed on the enzyme present in human red blood cells. These cells are of obvious advantage for studies on a low abundance protein located in the plasma membrane. In addition to the lack of an extensive intracellular membrane network the absence of an active Na^+/Ca^{2+} exchange system further adds to the suitability of red cells as an in vivo model system for the study of the plasma membrane Ca^{2+} pump (e.g., *see* Schatzmann, 1982). However, shortly after its first demonstration in human erythrocytes it became clear that a specific ATP-driven calcium pump is found in the plasma membrane of other cell types as well, leading to the notion that this pump is probably a ubiquitous enzyme (*see*, e.g., Penniston, 1983). Considering the importance for all eukaryotic cells to maintain very low intracellular free Ca^{2+} concentrations in the presence of (normally) millimolar Ca^{2+} concentrations in the extracellular medium (Carafoli, 1987*a*), and the obvious importance

of the calcium pump in establishing and maintaining this large concentration gradient, it is fair to assume that plasma membrane Ca^{2+} pumps are not only found in all cell types of higher vertebrates but that they are in fact ubiquitous in all eukaryotic cells. The recent finding by Rudolph et al. (1989) of a putative plasma membrane Ca^{2+} pump in yeast as well as the description of a high affinity Ca^{2+} ATPase in the protozoan *Leishmania donovani* (Ghosh et al., 1990) lends further support to this notion. While plants have also been shown to contain a plasma membrane Ca^{2+} pump (Dieter & Marmé, 1980, 1981) displaying striking similarities to the mammalian enzymes (Robinson, Larsson & Buckhout, 1988; Briars, Kessler & Evans, 1988), the existence of such a protein in prokaryotes has not yet been unambiguously established although an ATP-driven Ca^{2+} pumping activity has been described a number of years ago in *Streptococcus faecalis* (Kobayashi, Brant & Harold, 1978).

The typical plasma membrane calcium pump of higher eukaryotes is a single polypeptide of M_r 130,000 to 150,000; however, the formation of homodimers (or oligomers) may be of relevance for the function of the pump in vivo (Cavieres, 1984; Kosk-Kosicka & Bzdega, 1988; Kosk-Kosicka, Bzdega & Johnson, 1990; Vorherr et al., 1991). In the classification scheme proposed by Pedersen & Carafoli (1987) for ion motive ATPases, the typical Ca^{2+} pump of the plasma membrane belongs to the P-type ATPases. The characteristic property of these enzymes is the formation of a covalently phosphorylated obligatory intermediate (hence the name *P-type ATPase*) that arises from the transfer of the γ-phosphate of ATP to a specific aspartate residue at the catalytic site of the polypeptide. Vanadate—which acts as a transition state analog of phosphate—inhibits all P-type ATPases (Pedersen & Carafoli, 1987), but no specific inhibitor acting selectively only on the plasma membrane Ca^{2+} pump has as yet been identified (*see*, e.g., Garrahan & Rega, 1990).

As early as 1977 it was discovered that the PMCA can be stimulated by a soluble cytoplasmic activator showing the properties of the Ca^{2+} binding modulator protein soon thereafter termed calmodulin (Gopinath & Vincenzi, 1977; Jarret & Penniston, 1977, 1978). Calmodulin (CaM) is itself a highly conserved ubiquitous Ca^{2+} binding protein involved in the regulation of a vast number of different enzymes and intracellular processes (Cheung, 1980; Means & Dedman, 1980; Klee & Vanaman, 1982). In the case of the PMCA the effect of CaM is due to its direct interaction, by binding to a specific target site(s), with the pump molecule (Niggli, Penniston & Carafoli, 1979; Graf & Penniston, 1981; Hinds & Andreasen, 1981; Guerini, Krebs & Carafoli, 1984). Although a regulation by direct interaction with CaM has also been found for other enzymes (for a recent review *see* O'Neil and DeGrado, 1990), the PMCA is the only ion transport ATPase known to be regulated by CaM in this way. The stimulation of the pump by CaM is Ca^{2+} dependent; Ca^{2+}-free CaM will not interact with the enzyme with high affinity (Foder & Scharff, 1981). Stimulation of the PMCA by Ca^{2+}/CaM is primarily due to an increase of the apparent affinity of the pump for Ca^{2+} (shift of the K_m from ≈10 μM Ca^{2+} in the absence of CaM to ≈0.5 μM Ca^{2+} in its presence) (Stieger & Luterbacher, 1981; Niggli et al., 1981).

In addition to its regulation by Ca^{2+}/CaM, the plasma membrane calcium pump has also been shown to be modified in its activity by interaction with acidic phospholipids and polyunsaturated fatty acids (Niggli et al., 1981; Carafoli & Zurini, 1982; Choquette et al., 1984; Enyedi et al., 1987; Missiaen et al., 1989), phosphorylation by the cAMP-dependent protein kinase (Caroni & Carafoli, 1981; Neyses, Reinlib & Carafoli, 1985) and by limited proteolysis (Sarkadi, Enyedi & Gardos, 1980; Niggli et al., 1981, 1987; Stieger & Schatzmann, 1981; for a review *see* Carafoli et al., 1987). Of particular interest is the specific activation of the pump by the protease calpain which is itself dependent on Ca^{2+} (Suzuki, 1987; Melloni & Pontremoli, 1989; Wang, Villalobo & Roufogalis, 1988; Wang, Roufogalis & Villalobo, 1989; James et al., 1989*b*). In all these cases an increased ATPase activity correlates with a shift towards a higher Ca^{2+} affinity of the enzyme. Phosphorylation of the PMCA by protein kinase C has also been reported in the literature (Smallwood, Giugi & Rasmussen, 1988).

Peptide Sequence Analysis and Molecular Cloning Reveal the Presence of Ca^{2+}-Pump Isoforms Generated from a Multigene Family and via Alternative RNA Splicing

With the development of an efficient and highly specific purification method (affinity chromatography on immobilized calmodulin (Niggli et al., 1979)), studies aimed at obtaining primary amino acid sequence information of the whole enzyme and of specific regions of interest finally became possible. The first short peptide sequences that were unambiguously derived from a PMCA (isolated from human erythrocytes) were reported in 1987 (Filoteo, Gorski & Penniston, 1987; James et al., 1987), and only a year later the first complete primary structures for two different rat as well as for a human PMCA were

published (Shull & Greeb, 1988; Verma et al., 1988). Slightly different approaches, both using molecular cloning techniques, led to this rapid success: Shull and Greeb (1988) used oligodeoxynucleotides corresponding to a highly conserved amino acid sequence of the ATP binding site of known P-type ATPases as probes to screen a rat brain cDNA library under low stringency conditions, whereas Verma et al. (1988) employed oligodeoxynucleotides designed on the basis of the amino acid sequence of two short specific peptides from human erythrocyte PMCA as probes to isolate the corresponding cDNA.

Extensive peptide sequencing efforts made on purified human erythrocyte PMCA suggest that at least two different isoforms of this enzyme are present in human red blood cells (Verma et al., 1988; Strehler et al., 1990). Screening of various rat, bovine and human cDNA libraries has so far led to the identification of at least five different mammalian PMCA isoforms that are encoded by separate genes (Brandt et al., 1988; Shull & Greeb, 1988; Verma et al., 1988; Greeb & Shull, 1989; Strehler et al., 1989*a*, 1990). The genes for two human isoforms, hPMCA1 and hPMCA4, have recently been mapped in the genome and were shown to be localized on chromosomes 12 and 1, respectively (S. Olson, M.G. Wang, E. Carafoli, E.E.S., O.W. McBride, *submitted*). Complete cDNA-deduced amino acid sequence data have been obtained for three rat (rPMCA 1,2,3) and for two human isoforms (hPMCA 1,4). A compilation of the currently available data on PMCA isoforms is shown in the Table. According to cDNA-deduced data mammalian PMCAs can vary considerably in size, containing between 1159 and 1258 residues with calculated molecular weights ranging from 127,300 (rPMCA3a) to 138,800 (hPMCA1d). "Corresponding" isoforms in different mammalian species (rat *vs.* human) are ≈99% identical, whereas the different gene products within a given species show only 75 to 85% identity and ≈85 to 90% similarity in their primary sequences. These values for the PMCA isoform variability are similar to those determined for the isoform variability among other ion-transporting ATPases (Brandl et al., 1986; Shull, Greeb & Lingrel, 1986; Sweadner, 1989). Figure 1 shows an optimal alignment of the primary structures of the presently known mammalian PMCA isoforms.

As in many other protein families (Breitbart, Andreadis & Nadal-Ginard, 1987), additional isoform variability of PMCAs appears to be generated via alternative RNA splicing of single gene primary transcripts (Shull & Greeb, 1988; Greeb & Shull, 1989; Strehler et al., 1989*a,b*; R. Heim, E. Carafoli and E.E.S., *unpublished results*) (*see* Table and Figs. 1 and 2). In the case of PMCA1, differential utilization of coding information contained within a single exon towards the 3′ end of the primary transcript can lead to the production of four isoforms differing only in their structure C-terminal to residue 1117 (Strehler et al., 1989*b*; *see also* Fig. 1). cDNA cloning results and comparisons of nucleotide and encoded amino acid sequences indicate up to four possible sites of alternative splicing in PMCA primary transcripts (Fig. 2). Although not all of these sites have formally been demonstrated to be "active" in generating isoform diversity from a given PMCA gene transcript, their location within the primary structure coincides with functionally, regulatory or structurally important domains (*see below* and Fig. 2).

Direct Protein Labeling and Sequencing Techniques Reveal the Primary Structures of Important Domains in the Pump Molecule

An impressive amount of work has been devoted to the study of functional, regulatory and structural properties of PMCAs, primarily using specific labeling, limited proteolysis and reconstitution techniques in combination with biochemical, immunological and biophysical methods as well as with enzyme activity and ion transport measurements (Schatzmann, 1982; Penniston, 1983; Niggli et al., 1987; Papp et al., 1989). Controlled proteolysis experiments have allowed a crude mapping of the location of some important domains such as the regions containing the ATP binding and the catalytic (phosphorylation) sites, the calmodulin binding domain and the site of interaction with acidic phospholipids/polyunsaturated fatty acids (Benaim, Zurini & Carafoli, 1984; Zurini et al., 1984; Sarkadi et al., 1986; Carafoli, 1987*b*; Niggli et al., 1987; Papp et al., 1989; Zvaritch et al., 1990). Direct labeling methods followed by proteolytic and/or CNBr cleavage, peptide purification and microsequencing have led to the identification of (partial) amino acid sequences corresponding to the phosphorylation domain containing the aspartate residue that serves as phosphate acceptor during the reaction cycle (James et al., 1987), the FITC binding site believed to represent a part of the ATP binding domain (Filoteo et al., 1987), a high affinity calmodulin binding domain (James et al., 1988) and the cAMP-dependent phosphorylation site (James et al., 1989*a*). Because the complete cDNA-derived primary structures of several PMCAs are available, the location of the different domains mentioned above could be precisely established (Shull & Greeb, 1988; Verma et al., 1988; Carafoli et al., 1989; James, 1990; Zvaritch et al.,

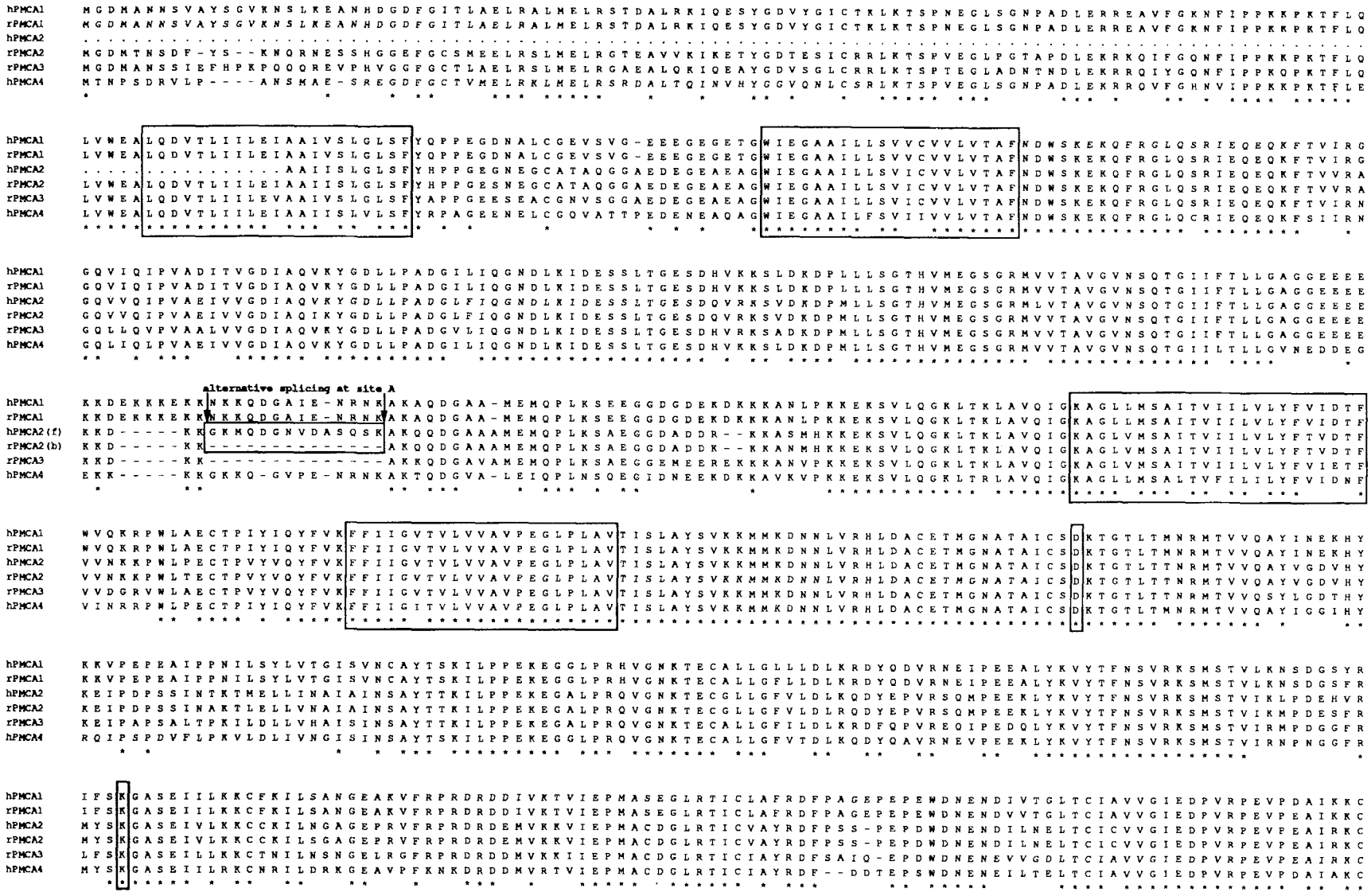

Fig. 1. Optimal amino acid sequence alignment of mammalian PMCA isoforms. cDNA-deduced amino acid sequences (*h*, human; *r*, rat; *b*, bovine) have been aligned using the program CLUSTAL of the University of Wisconsin Genetics Computer Group sequence analysis package (Devereux, Haeberli & Smithies, 1984), as well as by visual inspection. Gaps have been introduced to optimize the alignment. Residues identical in all isoforms are marked by an asterisk. The 10 hydrophobic sequence stretches forming putative transmembrane segments are boxed. *Legend continued on next page*

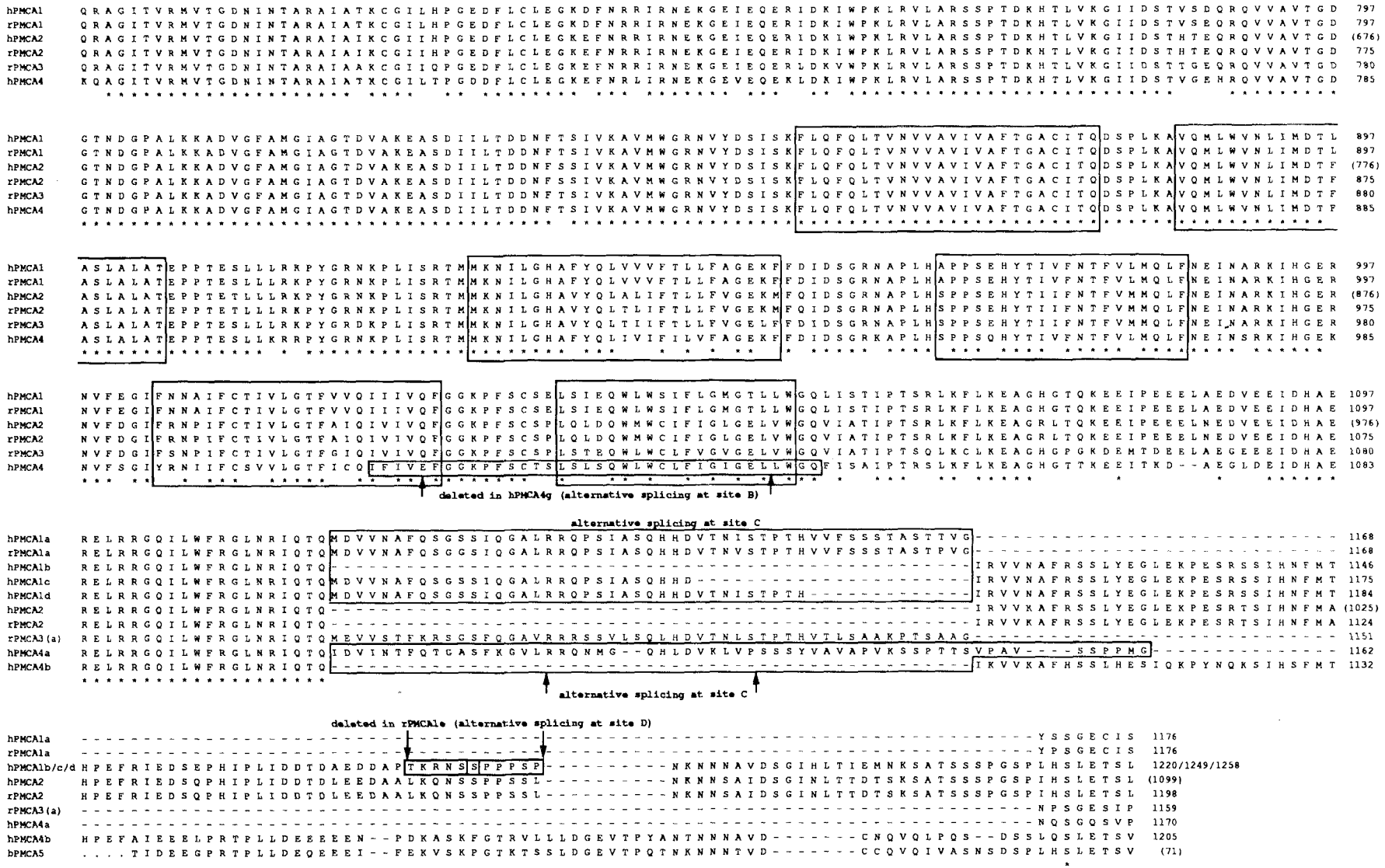

Fig. 1. *Continued.* Also boxed are the invariant aspartate residue where phosphorylation occurs during the reaction cycle, the lysine residue involved in FITC binding and a serine residue in isoform hPMCA1b/c/d that serves as phosphate acceptor upon phosphorylation by the cAMP-dependent protein kinase. Divergences between isoform subtypes that are probably due to alternative RNA splicing (at sites *A, B, C, D*; *see also* Fig. 2) are also indicated. Note that the sequences for human PMCA2 and bovine PMCA5 are incomplete (indicated by dotted lines), that cDNAs coding for a human PMCA3 and a rat PMCA4 have not yet been isolated and that the identity of the bovine PMCA as a separate isoform "5" has not yet been conclusively demonstrated

Table cDNA deduced data on mammalian plasma membrane Ca^{2+} pump isoforms

Isoform designation[1]	Source	Splicing variants[2]	Length in amino acid residues[3]	Calculated M_r
PMCA 1	Rat, human	1a[r,h]	1176	129,500
		1b[r,h]	1220	134,700
		1c[h]	1249	137,800
		1d[h]	1258	138,800
		1e[r,4]	1209	133,500
PMCA 2	Rat, human[5]	2 (b)[r]	1198	132,600
		2 (f)[h,5,6]	1099*	121,300*
PMCA 3	Rat	3a	1159	127,300
PMCA 4	Human	4a	1170	129,400
		4b	1205	133,900
		4g[7]	1169	130,300
(PMCA 5)	Bovine[8]	5 (?)	(71)†	(7,700)†

[1] For the rationale behind the general PMCA nomenclature, *see* Shull and Greeb (1988), Greeb and Shull (1989) and Strehler et al. (1990).
[2] The nomenclature for alternatively spliced isoform variants uses lower case letters added to the corresponding isoform gene number (1,2,3, etc.). The lettering follows a somewhat arbitrary "historical" pattern of the detection of alternative splicing variants.
[3] Full-length cDNA sequences have been published only for rPMCA1a, hPMCA1b, rPMCA2b, rPMCA3a, and hPMCA4b. All other isoform lengths are extrapolations assuming identity in the remaining regions with the corresponding known full-length sequence.
[4] Hypothetical isoform generated from an mRNA lacking putative alternatively spliced sequences at sites *C* and *D* (*see* Fig. 2).
[5] Human sequence incomplete (about 110 N-terminal residues missing; R. Heim, E. Carafoli & E.E.S., *unpublished*).
[6] Including putative alternatively spliced sequence at site *A* (Fig. 2).
[7] Lacking putative alternatively spliced sequence at site *B* (Fig. 2).
[8] Only C-terminal 71 residues known (Brandt et al., 1988).
[r] cDNA from rat source only; [h] cDNA from human source only; [r,h] cDNAs from both rat and human sources available.
* Footnote 5 above.
† Footnote 8 above.

1990). In Fig. 2 the location of major domains is schematically indicated within the linear representation of the PMCA primary structure.

A Model for the Overall Structure of Plasma Membrane Ca^{2+} Pumps can be Deduced from the Available Data

Sequence comparisons of PMCAs with other members of the P-type class of ion pumps (e.g., Na^+/K^+ pump α-subunits, H^+ pumps, Ca^{2+} pumps of the SR/ER, bacterial K^+ pumps) reveal similarities in the overall arrangement of important functional and structural domains (Serrano, 1988; Shull & Greeb, 1988; Verma et al., 1988; Green, 1989*a*; Green & MacLennan, 1989; Taylor & Green, 1989). Although the degree of amino acid sequence identity averaged over the entire length of the molecules is generally rather low between members of different P-type pump classes (25% or less except for Na^+/K^+ *vs.* H^+/K^+ pumps which share about 65% sequence identity (Serrano, 1988)), there are several highly conserved amino acid sequence stretches found in all P-type pumps that correspond in all likelihood to crucial functional/structural domains (Brandl et al., 1986; Serrano, 1988; Shull & Greeb, 1988; Verma et al., 1988; Green & MacLennan, 1989). Among them are the region surrounding the Asp-residue that forms the phosphorylated intermediate and several sequences contributing to the ATP-binding domain (Serrano, 1988; Green & MacLennan, 1989). Computer-aided secondary structure predictions, hydropathy calculations and amino acid sequence comparisons with known protein structural domains have been used to design planar models for the possible topology of ion-motive ATPases (Brandl et al., 1986; Ovchinnikov et al., 1988*a*; Serrano, 1988; Green & MacLennan, 1989; James, 1990). From these studies it is apparent that all P-type ATPases are built according to a common general principle (Serrano, 1988; Green & MacLennan, 1989; Silver et al., 1989). The corresponding model, as applied to the plasma membrane calcium pump, is illustrated

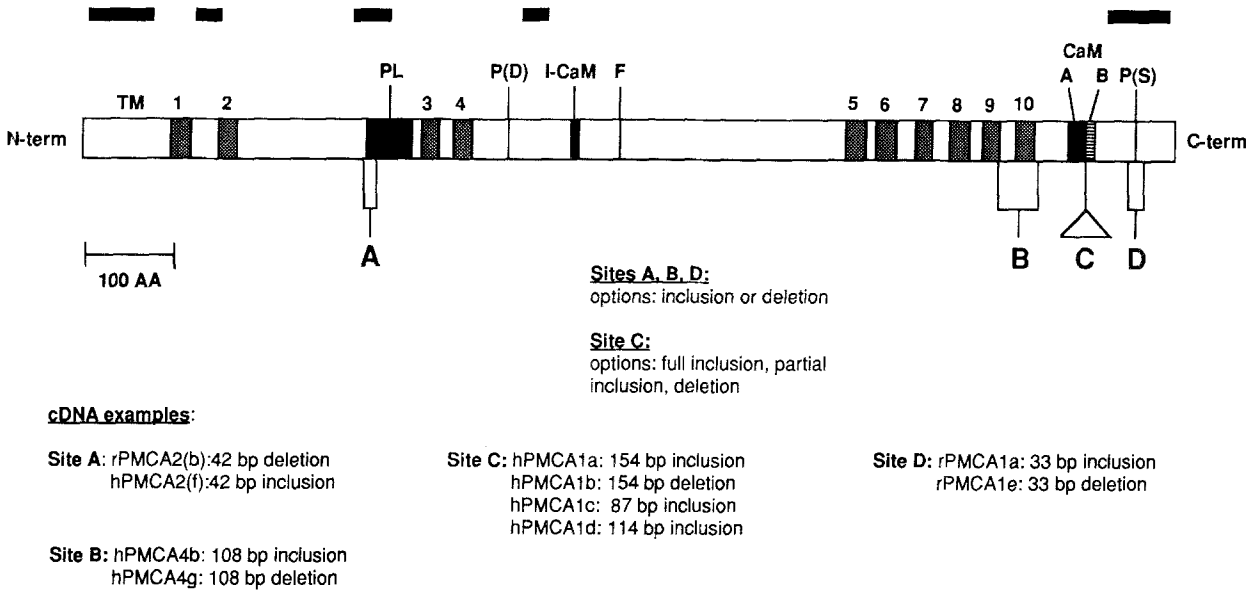

Fig. 2. Domain assignment in the plasma membrane calcium pump and location of isoform variable regions. *Top:* Scheme of a PMCA (isoform type hPMCA1b), showing the location of known important domains, the areas of particularly high sequence divergence (black bars) between isoforms encoded by different genes and the sites (*A, B, C, D*) of possible alterations generated by translation of alternatively spliced mRNAs. *TM*, putative transmembrane domain; *PL*, putative acidic phospholipid-sensitive region; *P(D)*, site (aspartate residue) of acylphosphate formation; *I-CaM*, "inhibitory" region that interacts with the calmodulin binding region in the absence of Ca^{2+}/calmodulin; *F*, fluorescein isothiocyanate (FITC) binding site (= part of the ATP binding region); *CaM*, calmodulin binding region, separated into subdomains *A* and *B*; *P(S)*, site (serine residue) of phosphorylation by the cAMP-dependent protein kinase. The different alternative splicing options at sites *A, B, C, D* are also indicated. Note that, depending on the splicing pattern, some isoforms may be much shorter at their C-terminus and may lack certain domains (e.g., the site for phosphorylation by the cAMP-dependent protein kinase). *Bottom:* cDNA examples (isolated from either rat (rPMCA) or human (hPMCA) cDNA libraries) for the possible alternative splicing variants at each of the sites *A, B, C* and *D*

in Fig. 3. Some of its characteristic features are briefly outlined below.

1) The bulk of the hydrophilic sequences is located intracellularly (or, generally for P-type ATPases, on that side of the membrane where high affinity binding of the specifically transported cation occurs). In PMCAs, these sequences are concentrated in three major blocks. The first corresponds to the intracellular loop between transmembrane regions 2 and 3 and contains the "transduction" domain (Green & MacLennan, 1989). The second and largest contiguous cytoplasmic sequence is located between transmembrane segments 4 and 5 and contains the "hinge" region and the phosphorylation and nucleotide binding domains. The third block corresponds to the C-terminal "regulatory" domain that contains the calmodulin binding site and the site of cAMP-dependent phosphorylation (Fig. 3). This sequence block is specific for PMCAs and accounts for the bulk of the additional molecular mass present in PMCAs as compared to all other P-type ATPases (Shull & Greeb, 1988; Verma et al., 1988). In contrast, the tranduction, catalytic and nucleotide-binding domains are conserved even in bacterial P-type cation transporters (Green & MacLennan, 1989; Silver et al., 1989).

2) Only short stretches of the protein make up the loops between adjoining transmembrane segments and are thus facing the outside milieu. This property may account, at least in part, for the difficulties encountered in obtaining specific antibodies recognizing extracellular parts of P-type pumps (Ovchinnikov et al., 1988*b*; Caride, Gorski & Penniston, 1988).

3) Invariably, there seem to be four transmembrane segments between the N-terminus and the bulk intracellular catalytic/ATP binding domain (Serrano, 1988). This places the N-terminus of P-type pump molecules on the same (cytoplasmic) side as the major hydrophilic domains (*see* Hennessey and Scarborough (1990) for a recent experimental confirmation of this hypothesis for a *Neurospora crassa* H^+ ATPase). Since in PMCAs the regulatory domain containing the calmodulin binding and cAMP-dependent phosphorylation sites must be located on the cytoplasmic face of the plasma membrane, an even number of membrane-spanning regions should be present in the C-terminal half of these molecules (Shull & Greeb, 1988; Verma et al.,

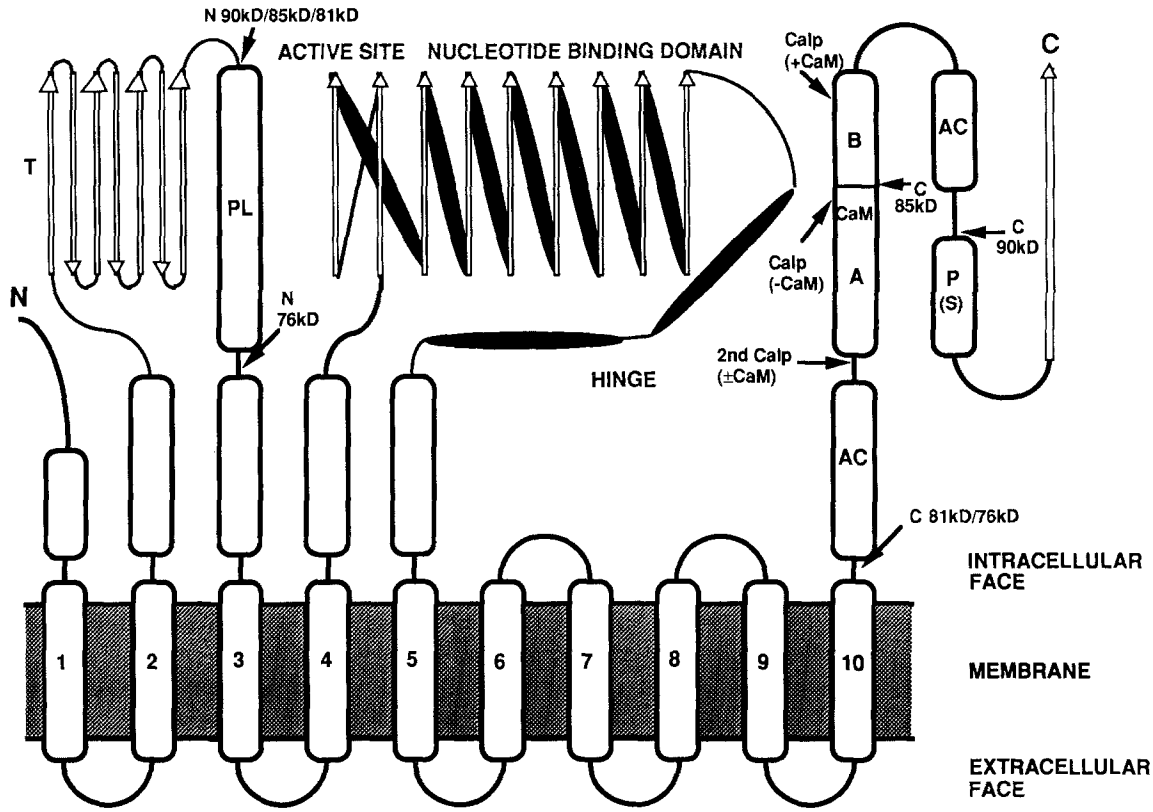

Fig. 3. Proposed model for the overall topology of PMCAs. A planar representation of the PMCA is shown, including the putative transmembrane topology (TM 1 to 10) and the assignment of important domains. Open rods and black cigar-shaped bars correspond to putative alpha-helices, and arrows denote beta-sheet secondary structural elements. The N-terminal (*N 90kD/85kD/81kD*, *N 76kD*) and C-terminal location (*C 90kD*, *C 85kD*, *C 81kD/76kD*) of the tryptic cleavage sites leading to the production of major proteolytic fragments is also indicated, as are the sites of calpain attack in the presence (*Calp/ + CaM*) and absence (*Calp/ − CaM*) of calmodulin. The site of secondary, calmodulin-independent, calpain attack is labeled 2^{nd} *Calp*(±*CaM*). *AC*, acidic regions flanking the calmodulin binding domain; *C*, C-terminus; *CaM*, calmodulin-binding domain consisting of subdomains *A* and *B*; *N*, N-terminus; *T*, transduction domain; *P(S)*, region containing the serine residue susceptible to phosphorylation by the cAMP-dependent protein kinase; *PL*, phospholipid-sensitive region

1988). According to the model proposed for the SR Ca^{2+} ATPase (Brandl et al., 1986) and adopted for the PMCA (Fig. 3), six transmembrane segments may be present in the C-terminal portion of these ion transporters, but evidence obtained for other P-type pumps suggests that the number of membrane-spanning regions may probably only be two to four in other members of this class (Serrano, 1988; Silver et al., 1989). The C-terminal transmembrane topology of Na^+/K^+-ATPase α-subunits seems to differ significantly from that of other P-type pumps (at least of PMCAs) because experimental evidence indicates an extracellular location of the C-terminus in these pumps (Ovchinnikov et al., 1988*a*,*b*) and implies an odd (three) number of transmembrane segments in their C-terminal region. The precise transmembrane topology, particularly in the C-terminal portion, has not yet been solved for any of the P-type ATPases, but recent developments using antibodies raised against synthetic peptides promise to shed more light on this important question in the near future (Ovchinnikov et al., 1988*b*; F. Hofmann, T. Vorherr and E. Carafoli, *personal communication*).

Several of the structural details presented in the model of Fig. 3 have been discussed previously for the SR Ca^{2+} pump (Brandl et al., 1986; Green & MacLennan, 1989). This applies mostly for the "stalk," the transduction, the phosphorylation and the nucleotide-binding domains. Some recent experimental data on these domains (inasmuch as they may be applicable to PMCAs) as well as on the "regulatory," PMCA-specific C-terminal domain will be discussed in the following sections.

Towards a Better Understanding of Structure-Function Relationships in Plasma Membrane Ca^{2+} Pumps

Problems related to structure-function relationships in P-type pumps are being investigated by a number

of different approaches. These include the use of specific antibodies against synthetic peptides or defined epitopes of the pump molecule, the use of synthetic peptides to study intramolecular interactions as well as interactions of parts of the pump with its specific regulators, the expression of the pump from its cDNA modified by site-directed mutagenesis, and the generation of truncated forms of the protein for studies of defined properties and, ultimately, for complete structural resolution. In the case of PMCAs, the molecular properties of the "extra" regulatory domain are of particular interest. Shortly after its direct identification by sequencing of the corresponding labeled peptide, the calmodulin binding domain has been studied in more detail by using synthetic peptides either corresponding to the "natural" or to specifically altered sequences (Enyedi et al., 1989; Vorherr et al., 1990). Studies with other calmodulin-regulated enzymes have shown that the primary sequences of calmodulin binding domains are not well conserved; rather, these domains share secondary and tertiary structure features such as the propensity to form an amphiphilic α-helix in their N-terminal region (James et al., 1988; O'Neil & DeGrado, 1990). Using a variety of biophysical techniques it has recently been shown (Enyedi et al., 1989; Vorherr et al., 1990) that the minimal-length peptide that still shows high affinity CaM binding comprises only about 20 residues (corresponding to the N-terminal subdomain A) and that a conserved aromatic (tryptophan) residue in the N-terminal half of the CaM binding region may play a crucial role in the binding process. A large body of evidence accumulated over the last years indicates that the CaM binding region in the PMCA serves as an "inhibitory" domain that prevents high affinity Ca^{2+} binding of the pump in the absence of CaM (Benaim et al., 1984; Zurini et al., 1984; Carafoli et al., 1987; James et al., 1989*b*). A similar mode of regulation by CaM has also been suggested for other CaM-dependent enzymes such as the myosin light chain kinase and the protein kinase II (Buschmeier, Meyer & Mayr, 1987; Kennelly et al., 1987; Payne et al., 1988; Pearson et al., 1988; Fong & Soderling, 1990). Accordingly, the CaM binding domain not only interacts with Ca^{2+}/CaM (in the case of the PMCA, this interaction occurs preferentially with the C-terminal half of CaM (Guerini et al., 1984; Vorherr et al., 1990)) but also with a specific region in the enzyme itself (Enyedi et al., 1989). Although it has originally been suggested that in the PMCA this internal domain might correspond to a highly negatively charged region in the pump (residues 1079 to 1094 in hPMCA1; *see* Figs. 1 and 3) located just N-terminal to the CaM binding domain and perhaps involved in "channeling" Ca^{2+} ions towards their high affinity site close to the membrane (Verma et al., 1988; Carafoli et al., 1989; Vorherr et al., 1990), very recent data indicate that a labeled synthetic peptide corresponding to the CaM binding domain specifically interacts with a region (residues 537 to 544 in hPMCA4) located between the phosphorylation and the FITC-(ATP-) binding sites of the erythrocyte PMCA (Falchetto et al., 1991). Thus, in the absence of Ca^{2+}/CaM the CaM binding domain in the C-terminal protruding sequence of the PMCA appears to be involved in a long-range interaction with the central cytoplasmic catalytic/ATP binding domain, thereby preventing Ca^{2+} from binding to its specific site(s) with high affinity.

Further aspects of the structure and function of separate domains in PMCAs are currently being investigated by analyzing truncated forms of the enzyme corresponding to the fragments generated by limited proteolysis (90, 85, 81, and 76 kDa) and previously found to still show essential ATPase and/or Ca^{2+} transport function (Carafoli et al., 1987; Zvaritch et al., 1990). For these studies, over-expression of the proteins from their corresponding cDNA fragments in suitable systems such as *Xenopus* oocytes, the *Baculovirus* expression system, or even in *Escherichia coli* has been initiated (Meili et al., 1990; Heim et al., 1990).

Site-Directed Mutagenesis Promises to Yield Valuable Information Concerning the Molecular Properties of Plasma Membrane Ca^{2+} Pumps: A Preview

The conceptual "dissection" of the large pump molecules into smaller, relatively well-defined domains represents an important first step in the design of experimentally testable hypotheses concerning the molecular properties of these molecules. For example, the relative importance for ion transport and ATPase activity of specific amino acid residues in specific "modules" of the pump model in Fig. 3 can be tested by site-directed mutagenesis of the corresponding region in the coding *c*DNA, followed by expression of the altered protein and enzymatic measurements. Although no such study has as yet been performed on a PMCA, some conclusions drawn from similar experiments carried out with other P-type pumps are almost certainly valid for PMCAs too.

In the SR Ca^{2+} pump, changing the conserved Asp-351 residue that forms the phosphorylated intermediate to Glu, Ser, Thr, His or Ala led to the abolishment of Ca^{2+} transport and phosphorylation (Maruyama & MacLennan, 1988), whereas mutations of the corresponding residue (Asp-378) in the yeast H^+ pump (to Asn, Glu or Thr) caused a somewhat less drastic result and even suggested that the

Asp → Asn change still produced a functional enzyme (Portillo & Serrano, 1988). Interestingly, a mere switching of the positions of Asp-351 and Lys-352 already eliminated Ca^{2+} transport in the SR pump (Maruyama & MacLennan, 1988). Similarly, the importance for the proper function of P-type pumps of the Lys residue that reacts specifically with FITC (Lys-515 in the SR Ca^{2+} pump, Lys-474 in the H^+ pump) has been demonstrated by site-directed mutagenesis (Maruyama & MacLennan, 1988; Portillo & Serrano, 1988). Here, too, changes to Glu (H^+ ATPase) or to Arg, Glu or Gln (SR Ca^{2+} ATPase) clearly diminished ion transport activity. Specific mutations introduced in the transduction domain (also called phosphatase domain (Serrano, 1988)) of the yeast H^+ pump (Portillo & Serrano, 1988) and the SR Ca^{2+} pump (Andersen et al., 1989) blocked the turnover of the enzyme intermediate (while still allowing the formation of the phosphorylated intermediate E1 ~ P). Mutations of conserved residues believed to participate in nucleotide binding and located in the C-terminal part of the large intracellular hydrophilic loop (*see* Fig. 3) have also been made for both the yeast H^+ pump (Portillo & Serrano, 1988) and a mammalian SR Ca^{2+} pump (Maruyama et al., 1989), demonstrating the relative contributions of these residues to nucleotide binding and specificity. An interesting mutation characterized for the *Schizosaccharomyces pombe* H^+ pump concerns the change of Gly-268 (a conserved residue present in the transduction domain of all P-type pumps) to Asp: The mutated pump shows a remarkable resistance to vanadate while still functioning as a proton pump, albeit with a reduced ATPase activity (Ghislain, Schlesser & Goffeau, 1987).

One of the most crucial problems that has to be solved to better understand the mechanism of ion transport concerns the location and chemical nature of the amino acid residues involved in specifically and selectively binding the cation(s) to be moved across the lipid bilayer. This problem has recently begun to be addressed in a series of elegant studies on the SR Ca^{2+} ATPase, again using the site-directed mutagenesis approach (*see* Green, 1989*b*, for a short review). The picture, as it emerges from these studies, clearly demonstrates the importance of charged residues found in the putative transmembrane segments M4, M5, M6 and M8, as well as of proline residues present in M4 and M6, for Ca^{2+} binding, Ca^{2+} transport and Ca^{2+}-dependent phosphorylation of this pump (Clarke et al., 1989*a,b*; Vilsen et al., 1989). Given the overall similarities in the topology of P-type pumps and, presumably, of the general mechanism of cation transport, it seems reasonable to assume that many of the findings described above for other pumps will also be applicable to the PMCAs. In Fig. 4, those conserved residues whose counterparts have already been altered in the SR Ca^{2+} and in H^+ pumps have therefore been marked for the human PMCA1. While many of the conclusions drawn from the above studies will have to be confirmed for the PMCA, the results obtained on the other P-type pumps greatly help in identifying the specific mutations that will now have to be made and tested in PMCAs.

What is the Functional Significance of Isoform Variability?

The availability of a growing number of primary amino acid sequences for different isoforms within each class of (mammalian) P-type pumps, together with the sequence comparisons and computer-assisted model predictions outlined above, provides an ideal basis for the preliminary assignment of putative domains. Highly conserved sequences are likely to represent domains essential for the basic catalytic and transport function and may also reflect specific constraints imposed upon structural elements of the enzyme. In contrast, highly divergent sequences probably specify isoform-specific regulatory and functional specializations of the pump that are adapted to the physiological needs of the tissue/cell type in which the corresponding enzyme is expressed. Although a rigorous study of the pattern of expression has not yet been carried out for the PMCAs, Northern analysis of RNA isolated from different rat tissues indicates that at least one PMCA isoform (PMCA 1) may be ubiquitous whereas others may be expressed in a tissue-dependent manner (Greeb & Shull, 1989). From the differentially spliced hPMCA1 mRNAs the one corresponding to hPMCA1c seems to be particularly abundant in adult skeletal muscle (Strehler et al., 1989*b*), whereas peptide analysis studies show that hPMCA4 is the major isoform expressed in human erythrocytes (Strehler et al., 1990). The isoform predominantly expressed in (rat) liver appears to have distinct properties, e.g., with respect to its affinity for CaM and its larger molecular weight of about 150,000, that set it apart from the major (human) erythrocyte (hPMCA4) enzyme (Kessler et al., 1990). Although this protein has recently been purified to near homogeneity by antibody-affinity chromatography (Kessler et al., 1990), not enough primary amino acid sequence information is as yet available to unequivocally determine to which of the known genes and splicing variants it should be assigned, or whether it even corresponds to the product of a "novel" gene.

A close look at the areas of unusually high sequence divergence among PMCAs (summarized in

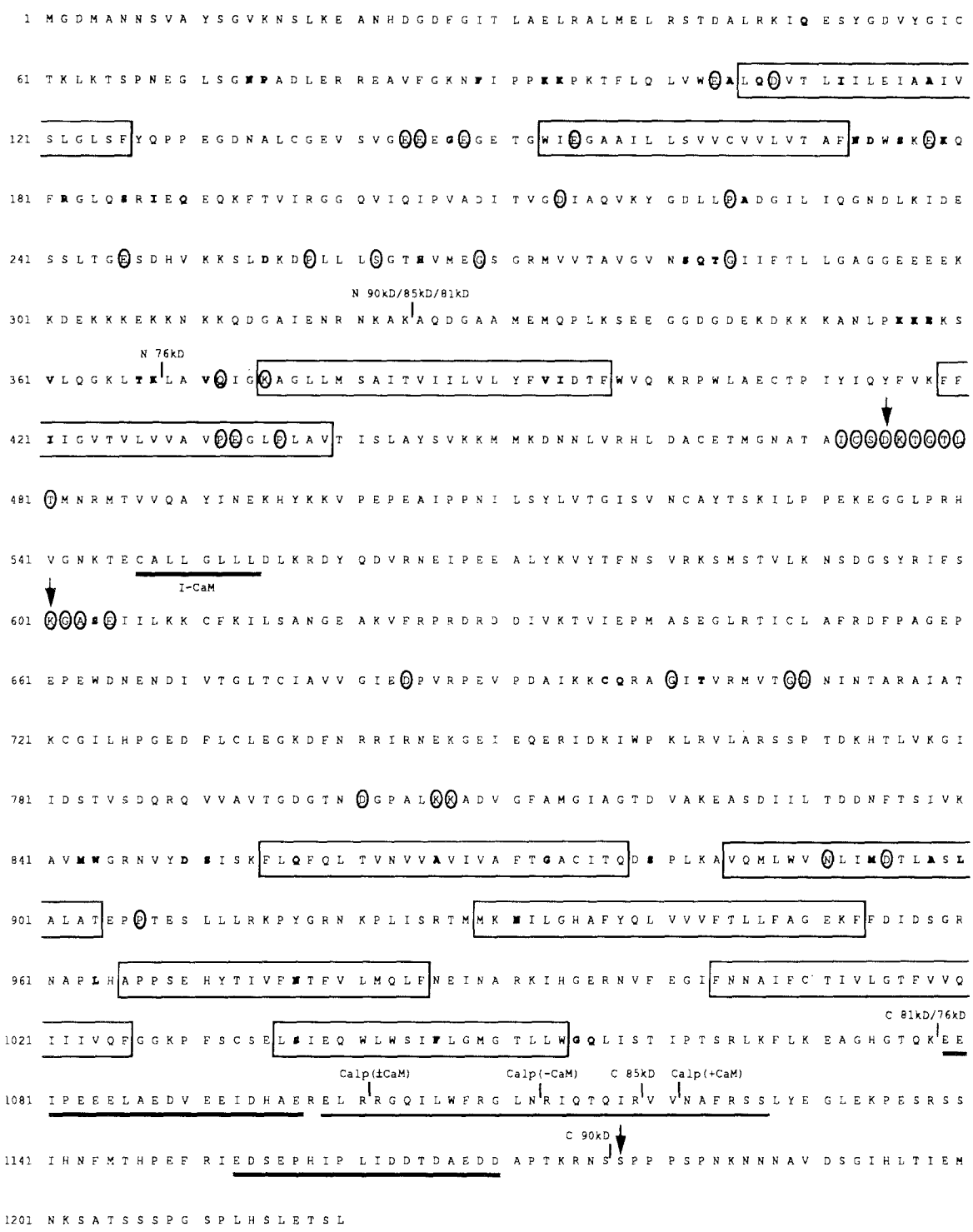

Fig. 4. Conserved amino acid residues in the PMCA that have been altered by site-directed mutagenesis in other P-type pumps. The amino acid sequence of a representative plasma membrane Ca^{2+} pump (hPMCA1b isoform) is shown. Residues conserved in other P-type ATPases and already altered by site-directed mutagenesis in one or more of these related pumps (essentially the rabbit sarcoplasmic reticulum Ca^{2+} pump and the yeast proton pump) are circled. Residues whose (nonidentical) counterparts at the corresponding positions have been mutated in the SR Ca^{2+} pump and/or the yeast H^+ pump are printed in boldface. The site (aspartate residue) of formation of the phosphorylated intermediate, the FITC binding site (lysine residue) and the site (serine residue) of phosphorylation by the cAMP-dependent protein kinase are highlighted by arrows. The 10 putative transmembrane segments are boxed, the calmodulin-binding domain is underlined, the putative "inhibitory" sequence interacting with the calmodulin-binding region in the absence of Ca^{2+}/calmodulin (*I-CaM*) is double underlined and the two acidic domains flanking the calmodulin-binding region are also double underlined. Proteolytic cleavage sites (by trypsin and calpain) are also indicated (abbreviations as in the legend to Fig. 3)

Fig. 2; *see also* Fig. 1) indicates that the putative extracellular loop between transmembrane segments M1 and M2 is rather poorly conserved. It may be speculated that this loop defines an area of specific recognition by some as yet unknown extracellular factor. Alternatively, this region—corresponding to a surface loop—may not be of functional importance and would therefore not have to be conserved during evolution. The (intracellularly located) N-termini are also poorly conserved among PMCA isoforms, but nothing is known yet about their function. A highly charged stretch in the transduction domain (around residue 300) is neither conserved in primary sequence nor in absolute length; in addition, alternative splicing (at site A in Fig. 2; *see also* Fig. 1) may lead to mRNAs coding for isoforms with considerable length differences in this region. This region has been implicated in the phospholipid sensitivity of PMCAs (Zvaritch et al., 1990), and different isoforms may thus vary significantly in this property. The region suggested to be responsible for interacting with the C-terminal, calmodulin-binding domain and shown to be located between the phosphorylation and the FITC-binding domain (Falchetto et al., 1991) also varies between isoforms. Differential interaction with the "inhibitory" domain may thus constitute a further means to fine-tune the regulatory properties of different PMCA isoforms.

Of high interest are isoforms generated from mRNAs with a spliced-out exon at site *B* in Fig. 2; if such proteins were actually made they would lack one of the predicted transmembrane segments (Figs. 1 and 2). As mentioned earlier, the assignment of membrane-spanning regions in the C-terminal half of the pump has not yet been possible with certainty. Proof of the existence of PMCA isoforms of the type hPMCA4g (Table, *see also* Strehler et al., 1989*a*) could therefore force us to change the model to one with (most likely) only eight transmembrane segments.

A further region of high PMCA isoform variability is the C-terminal "regulatory" region starting immediately after the conserved calmodulin-binding subdomain A (Figs. 1 and 2). Alternative splicing variants (at site *C* in Fig. 2) of the type "a," "c" or "d" contain a CaM binding subdomain B that differs considerably from that present in the corresponding "b" subtype (Figs. 1 and 5), leading to the conjecture that this may influence (weaken) the CaM-binding properties of their corresponding isoforms (Strehler et al., 1989*b*). Recent studies with a synthetic peptide (14mer) corresponding to a B subdomain indicate that this region alone does not have a major effect on the interaction with CaM (Vorherr et al., 1990); however, synthetic 28mers corresponding to the entire CaM-binding sequence (domains A and B), but containing either the hPMCA1b or the hPMCA1a/c/d subdomain B, differ significantly in affinity towards CaM (J.T. Penniston, *personal communication*). Considering the dual role of the CaM-binding region in PMCAs, involving interaction with CaM one one hand and with an internal enzyme-specific sequence on the other (Enyedi et al., 1989; Falchetto et al., 1991), the differences among PMCA isoforms arising from alternatively spliced mRNAs may well be of functional significance. It is interesting to note that isoforms such as "1c" and "1d" would contain a duplication of the CaM-binding subdomain B, with a spacing between the two subdomains of 29 and 38 residues, respectively (*see* Fig. 1). The site of phosphorylation by the cAMP-dependent protein kinase, identified as Ser-1178 in the sequence of hPMCA1b, is also differently spaced in the PMCA 1b *vs.* 1c and 1d subtypes, and it is missing altogether in the 1a isoform (James et al., 1989*a*; Strehler et al., 1989*b*). The cAMP-dependent phosphorylation site may also be absent in other isoforms, most notably in the major enzyme (hPMCA4) present in human erythrocytes (James et al., 1989*a*; James, 1990). Alternative splicing at site *D* in Fig. 2 would provide an additional means to generate PMCA isoforms that lack the consensus cAMP-dependent phosphorylation sequence. Studies using crude cardiac sarcolemmal vesicles and purified erythrocyte PMCA indicate that cAMP-dependent phosphorylation leads to an about twofold increase in Ca^{2+} ATPase activity (affecting both V_{max} and $K_m(Ca^{2+})$), a value which is, however, considerably smaller than the full activation of the pump achieved in the presence of Ca^{2+}/calmodulin (Neyses et al., 1985; Dixon & Haynes, 1989; James et al., 1989*a*; James, 1990). Recent work with a synthetic peptide encompassing the hPMCA1b phosphorylation sequence supports the notion (James, 1990) that a possible role of this modification may consist in protecting the calmodulin binding, "regulatory" domain from being attacked by the protease calpain. Addition of a phosphate group could lead to the formation of a negatively charged amphiphilic helix in the corresponding protein region, and this structure might then interact with the positively charged amphiphilic calmodulin-binding domain (James, 1990), thereby shielding it from proteolytic cleavage (and concomitant constitutive activation of the pump) by calpain (James et al., 1989*b*). It is tempting to speculate that, depending on their tissue distribution, different PMCA isoforms may or may not "need" this type of regulation by cAMP-dependent phosphorylation.

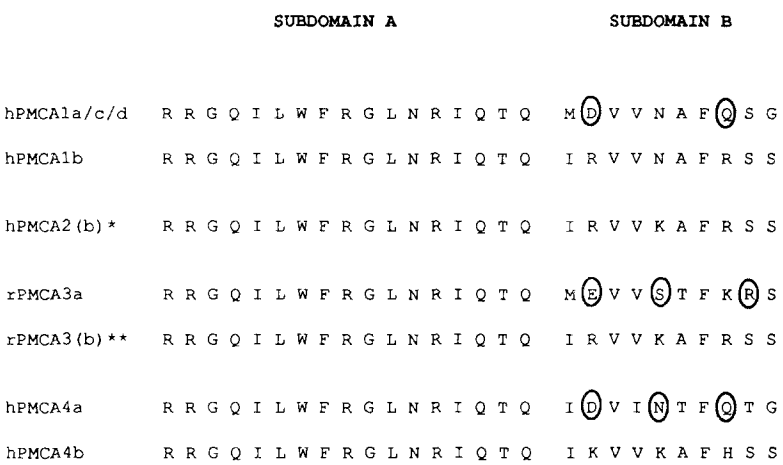

Fig. 5. Sequence comparison of the calmodulin binding region of plasma membrane calcium pump isoforms. Amino acid residues in splicing variants of the subtype "a" (as well as "c" and "d" in the case of hPMCA1) that deviate in their charge character (basic *vs.* neutral/acidic) from the corresponding residues in the subtypes "b" are encircled. Note that the sequence of the calmodulin-binding subdomain *A* is fully conserved in all PMCA isoforms. *No splicing variant encoding a subtype "a" isoform of PMCA2 has as yet been isolated. **Hypothetical "b" subtype of rPMCA3 obtained by translating a rPMCA3a mRNA from which 154 nucleotides have been removed at a position corresponding to alternative splice site *C* (*see* Fig. 2)

Conclusions and Perspectives

Refined purification methods and the technical advances of protein and nucleic acid molecular biology are mainly responsible for the recent success in the molecular characterization of the plasma membrane Ca^{2+} pump. Within less than three years since the first determination of partial amino acid sequences complete primary structures have become available for several isoforms of this pump as deduced from their coding DNA. This has opened up a wealth of new possibilities to study this largest of all P-type pumps on a molecular level. Previously performed experiments on whole membranes, on the isolated protein and on its proteolytic subfragments can now be combined with the new amino acid sequence data to propose testable models for the topology of this pump. Sequence comparisons among different PMCA isoforms and of PMCAs with other members of the class of P-type ion pumps have allowed the construction of a model predicting the location and possible secondary and even tertiary structural arrangement of functional and regulatory domains. Direct labeling and protein sequencing studies are yielding important clues concerning the interplay between different subdomains of the molecule. These interactions appear to be the basis for many functional properties of the pump and for its specific regulation by Ca^{2+}/calmodulin, acidic phospholipids and cAMP-dependent phosphorylation. Genetic data show that a large number of PMCA isoforms exists and that this diversity, at least in mammals, is due to the presence of a multigene family as well as to alternative RNA splicing occurring at several crucially located sites within the primary transcript. Results obtained from site-directed mutagenesis studies on other P-type pumps, particularly on the Ca^{2+} pump of the sarcoplasmic reticulum, are likely to be valid for the PMCA as well and help to pinpoint specific amino acid residues in the primary sequence that are involved in phosphorylation, nucleotide binding, Ca^{2+} binding and ion transport. The stage is now set for similar experiments on the various forms of the PMCA. Besides further attempts to improve our understanding of the precise nature of ion binding and transport the molecular mechanism of regulation of this pump will be one of the focal points for future research. Such studies will require the establishment of efficient expression systems either for overproduction of the protein and subsequent analyses in vitro, or for functional studies of the enzyme in the proper membrane environment. The presence of multiple isoforms for the PMCA that may be co-expressed in the same tissue makes detailed studies of the molecular properties of these enzymes more difficult. For the PMCAs (and for many other P-type pumps as well) it is therefore imperative that each specific isoform can be expressed and purified in sizeable quantities in a suitable system. Expression in mammalian cells in culture, in frog oocytes, in the *Baculovirus*-dependent system, in yeast and even in *E. coli* are possibilities that will be explored in the near future. Questions related to the physiological significance of the PMCA isoform diversity and to the evolutionary relationship among PMCAs and between PMCAs and other P-type pumps will also have to be addressed. Methodological improvements such as PCR technology are expected to help solve problems related to the tissue distribution of particular PMCA mRNAs and to qualitative and quantitative aspects of alternative RNA splicing in different cell types. Antibodies generated against the whole PMCA as well as against specific peptides will be useful tools

to study the abundance and tissue distribution of PMCA isoforms on the protein level, as well as to gain a better insight into their native topology. There can be no doubt that the next few years will see a continuous and exciting progress in the further characterization of these important proteins responsible for active transmembrane Ca^{2+} transport.

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