

Plasma Membrane Calcium Pump Isoform 4a Has a Longer Calmodulin-Binding Domain Than 4b*

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Alternate splicing of human plasma membrane calcium pump isoform 4 (hPMCA4) transcripts causes the expression of two variants, hPMCA4a and hPMCA4b, which have different downstream regulatory regions. Of the two, hPMCA4a has a lower affinity for calmodulin and a lower effective affinity for Ca^{2+} (Enyedi, A., Verma, A. K., Heim, R., Adamo, H. P., Filoteo, A. G., Strehler, E. E., and Penniston, J. T. (1994) *J. Biol. Chem.* 269, 41–43). Additional consequences of the alternate splice were studied by analyzing the characteristics of constructs (expressed in COS-1 cells) containing different portions of the carboxyl terminus of hPMCA4a. Our results show striking differences in the structure of the calmodulin-binding and autoinhibitory domains of the two variants. The calmodulin-binding region of hPMCA4b is a region of about 28 residues, whereas that of hPMCA4a is about 49 residues long and is probably interrupted by a region not involved in the binding. The autoinhibitory region of hPMCA4b (a part of the downstream region that keeps the molecule inactive in the absence of Ca^{2+} -calmodulin) is divided between the 28-residue calmodulin-binding region and a downstream region, whereas in hPMCA4a, all of it is contained within the 49-residue calmodulin-binding region.

The plasma membrane Ca^{2+} pump (PMCA)¹ is a calmodulin-regulated P-type ATPase that is an essential element in controlling intracellular Ca^{2+} concentration. The calmodulin regulation of the pump has been extensively studied. Most studies have been performed on red cell membrane preparations, which contain hPMCA4b plus a small amount of hPMCA1b. Ca^{2+} -calmodulin binds tightly to the pump and activates it by increasing its V_{max} and apparent affinity for Ca^{2+} . A similar activation of the pump can be achieved by removing about 15 kDa from its carboxyl terminus by proteolysis. After the calmodulin-binding domain had been located at the carboxyl terminus of the enzyme (James *et al.*, 1988; Verma *et al.*, 1988), a synthetic peptide containing the 28 residues believed to be

responsible for calmodulin binding was shown to bind calmodulin even more tightly than the intact enzyme (Enyedi *et al.*, 1989). This peptide also inhibited the pump that was activated by proteolytic removal of the calmodulin-binding domain and the whole carboxyl terminus. This was the first report that demonstrated that the calmodulin-binding domain itself may also serve as an internal inhibitor of the enzyme.

More direct evidence for the dual role of the calmodulin-binding domain was provided later utilizing an overexpression system in COS-1 cells. After the successful expression of the wild type hPMCA4b (Adamo *et al.*, 1992), a mutant of hPMCA4b called ct120 was made. This mutant, which lacked the calmodulin-binding domain and all residues downstream of it, was fully active and did not bind or respond to calmodulin (Enyedi *et al.*, 1993). Adding back all 28 residues of the calmodulin-binding domain, which made a construct called ct92 (Verma *et al.*, 1994; see also Fig. 1), induced a substantial inhibition of the pump in the absence of calmodulin. This inhibition, however, was only about 2% of the inhibition that was observed in the full-length pump, suggesting that other segments of the downstream region are also involved in self-inhibition of isoform 4b. ct92, on the other hand, had an apparent affinity for calmodulin just as high as that of the full-length enzyme, indicating that the portion of hPMCA4b downstream of the 28-residue calmodulin-binding domain played no role in calmodulin binding.

The upstream 19 residues of the calmodulin-binding domain are conserved in all the PMCA isoforms, but an alternate RNA splice affecting the middle of the sequence coding for this region changes the structure of the rest of the calmodulin-binding domain and the entire carboxyl terminus. The isoforms generated from transcripts containing an additional spliced-in exon are called “a” (or CII) and those produced from mRNA lacking this exon are called “b” (or CI). Because the change to the a forms involves the entire carboxyl-terminal regulatory region and produces a less basic calmodulin-binding domain, these forms are expected to have different regulatory properties. Indeed, studies using synthetic peptides corresponding to the calmodulin-binding domain showed that the peptide representing an a form had ten times lower affinity for calmodulin than the peptide representing a b form (Enyedi *et al.*, 1991). Subsequently, the full-length a and b forms of hPMCA4 were overexpressed in COS-1 cells, and their calmodulin-response curves were compared (Enyedi *et al.*, 1994). As expected, hPMCA4a showed lower apparent affinity for calmodulin than hPMCA4b. This change in the calmodulin affinity resulted in a reduced sensitivity of the pump isoform 4a to Ca^{2+} , which is probably the most important physiological consequence of the alternate splice.

In this report, additional consequences of the alternate splice were analyzed by studying the characteristics of constructs containing different portions of the carboxyl terminus of hPMCA4a. 4a(ct56), a construct similar to 4b(ct92) containing

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¹ The plasma membrane calcium pump is referred to by a name such as hPMCA4b, where h indicates the human species, PMCA indicates the plasma membrane calcium pump, 4 indicates the product of the fourth gene, and b indicates which alternate splice was used. For each isoform we will also mention what the name would be using a recently proposed system (Carafoli, 1994), which describes alternate splices by a capital letter, denoting which site (A or C) is spliced, and by Roman numbers I and II, indicating which exon is inserted.

FIG. 1. Carboxyl-terminal sequences of hPMCA4a, hPMCA4b, and some of their truncated versions. The truncated mutants are named according to the number of residues cut off at the carboxyl terminus (*i.e.* 4a(ct56) is hPMCA4a lacking the last 56 residues). 4b(ct120) lacks the entire region shown here, so that it ends just before the sequence LRRG at position 1085 (Enyedi *et al.*, 1993). Because the alternate splice only changes the structure of the molecule downstream of residue 1104, all constructs are identical upstream of this residue. Basic amino acid residues are underlined, and acidic ones are boxed.

	1086	Alternate splice	1113	1134	1145
4a(ct56)	LRRGQILWFRGLNRIQTQIDVINTFQTG				
4a(ct44)	LRRGQILWFRGLNRIQTQIDVINTFQTGASFKGVLRNRM				
4a(ct35)	LRRGQILWFRGLNRIQTQIDVINTFQTGASFKGVLRNRMGQHLQVILV				
hPMCA4a	LRRGQILWFRGLNRIQTQIDVINTFQTGASFKGVLRNRMGQHLQVILVPSSSSYVAVAPV				
hPMCA4b	LRRGQILWFRGLNRIQTQIKVVKAFHSSLHESIQKPYNQKIHSMTHPEFAIEELPRTPT				
4b(ct92)	LRRGQILWFRGLNRIQTQIKVVKAFHSSL				
	1146	1169			1205
hPMCA4a	KSSPTTSVPAVSSPMGNQSGQSPV				
hPMCA4b	LLDEEEENPKASKFGTRVLLIDGQVTPYANTNNNAVDCNQVQLPQSDSSLSLETSTV				

the 28 residues believed to constitute the calmodulin-binding domain, had been used to show that the differences in the Ca^{2+} sensitivity between the isoforms originated from the differences in their respective calmodulin-binding domains. Closer inspection of 4a(ct56), however, showed that unlike 4b(ct92), it has lower affinity for calmodulin than its full-length parent and that like 4b(ct92) its activity is higher in the absence of calmodulin than the activity of its parent. To further study this region, more residues of the carboxyl terminus were added to 4a(ct56) and two additional constructs, called 4a(ct44) and 4a(ct35) were produced. The characteristics of 4a(ct35) appeared to be identical to those of full-length hPMCA4a, indicating that in hPMCA4a both calmodulin-binding and inhibitory characteristics reside in a region of about 49 residues at the carboxyl terminus.

MATERIALS AND METHODS

Chemicals— ^{45}Ca and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were purchased from DuPont NEN. Calmodulin was obtained from Sigma. The lipofectamine reagent was purchased from Life Technologies, Inc.

Construction of Full-length hPMCA4a and Its Truncated Mutants—A *Nsi*I and *Kpn*I fragment of hPMCA4a obtained from a partial clone was substituted for the 3' end of hPMCA4b to obtain a full-length hPMCA4a (Enyedi *et al.*, 1994). 4a(ct56), 4a(ct44), and 4a(ct35) were constructed by substituting smaller polymerase chain reaction-synthesized *Nsi*I and *Kpn*I fragments as described previously (Enyedi *et al.*, 1994; Verma *et al.*, 1994).

Transfection—Transfection was carried out by the lipofectamine method using the protocol described by the manufacturer. Essentially, 35×10^5 COS-1 cells were seeded in a 150-cm² flask. After 48 h, when cells were 80–90% confluent, transfection was started. DNA-lipofectamine complex was formed using 18.2 μg of DNA and 109 μl of 2 mg/ml lipofectamine reagent in 3.6 ml of serum-free medium for the transfection of each flask. Cells were incubated for 5 h at 37 °C with the DNA-lipofectamine complex in 14.5 ml of serum-free medium, then the medium was supplemented with serum, and incubation continued for a total of 24 h. The DNA-containing medium was then replaced by regular tissue culture medium with serum, and the cells were cultured for an additional 24 h.

Isolation of Microsomes from COS-1 Cells—Crude microsomal membranes were prepared essentially as described (Enyedi *et al.*, 1993) with some modifications. Cells from one 150-cm² flask were washed three times by gentle agitation with chilled phosphate-buffered saline containing 0.1 mM phenylmethylsulfonyl fluoride, 9 $\mu\text{g}/\text{ml}$ aprotinin, 2.2 $\mu\text{g}/\text{ml}$ leupeptin, and 1 mM EGTA. By this procedure we removed cells that were loosely attached to the surface of the flask. The cells that remained attached to the surface were then harvested in the same medium and were collected by centrifugation at $2000 \times g$ for 20 min and resuspended in an ice-cold hypotonic solution containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl_2 , 0.5 mM EGTA, 4 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, and 4 mM dithiothreitol. Preparation of membranes was then continued as described (Enyedi *et al.*, 1993).

Ca^{2+} Transport Assay—Calcium uptake by microsomal vesicles was measured at 37 °C for 5 min by rapid filtration through Millipore membrane filters (pore size, 0.45 μm ; type HA) as described (Enyedi *et al.*, 1993). The transport medium contained 100 mM KCl, 25 mM TES-

triethanolamine² (pH 7.2), 40 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH 7.2), 200 nM thapsigargin, 5 mM NaN_3 , 4 $\mu\text{g}/\text{ml}$ oligomycin, 7 mM MgCl_2 , 100 μM CaCl_2 (labeled with ^{45}Ca), and sufficient EGTA to obtain the desired free Ca^{2+} concentration. Microsomes at 4–7 $\mu\text{g}/\text{ml}$ concentrations were preincubated for 3 min at 37 °C before initiating calcium uptake by the addition of 6 mM ATP.

Phosphorylated Intermediate Formation, Separation by Electrophoresis, and Autoradiography—10 μg of microsomal membrane protein was phosphorylated on ice in a medium containing 100 mM KCl, 20 mM TES-triethanolamine (pH 7.2), 400 nM thapsigargin, 50 μM CaCl_2 , and 50 μM LaCl_3 . The reaction was initiated by the addition of 1 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. After 30 s of incubation, the reaction was terminated by the addition of 1 ml of ice-cold 6% trichloroacetic acid containing 1 mM ATP and 10 mM phosphate. After the addition of 50 μg of bovine serum albumin, the precipitated proteins were collected by centrifugation and washed three times with the 6% trichloroacetic acid solution and once with distilled water. The precipitates were dissolved in the electrophoresis sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5 mM EDTA, 125 mg/ml urea, and 100 mM dithiothreitol. An aliquot containing 2 μg of the phosphorylated microsomal membrane protein was applied on a 7.5% acidic SDS-polyacrylamide gel according to Sarkadi *et al.* (1986). After electrophoresis, the gels were dried and exposed to X-OMAT film at -70 °C overnight.

Immunoblotting—2 μg of microsomal membrane proteins were dissolved in the same electrophoresis sample buffer described above and loaded on a 7.5% acrylamide gel following Laemmli's procedure (1970). After electrophoresis, the samples were blotted as described (Enyedi *et al.*, 1993). The blots were immunostained with monoclonal antibody 5F10.

RESULTS

To analyze the calmodulin-binding regulatory region of hPMCA4a, constructs containing different portions of the carboxyl terminus have been synthesized. The differences in the carboxyl-terminal sequences of the mutants are shown in Fig. 1. 4a(ct56) contains 28 residues of the putative calmodulin-binding domain but lacks everything (56 residues) downstream of it. 4a(ct44) and 4a(ct35) are 12 and 21 residues longer than 4a(ct56) (lacking only 44 and 35 residues at the carboxyl terminus), respectively. As judged by SDS gel electrophoresis followed by immunoblotting (Fig. 2A), each construct was expressed at a high level similar to that of the full-length enzyme and migrated with the expected size, which was in between full-length hPMCA4a and ct120. ct120 is only 28 residues shorter than 4a(ct56), but it has been called ct120, indicating that it lacks 120 residues from the carboxyl terminus of full-length hPMCA4b, which is 36 residues longer than hPMCA4a (Enyedi *et al.*, 1993).

As shown in the autoradiogram of Fig. 2B, each construct had the capability to form the phosphorylated intermediate (EP) of the enzyme from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of Ca^{2+} . The phosphorylation of the mutants was greatly enhanced by La^{3+} (the $\text{Ca}^{2+} + \text{La}^{3+}$ -dependent EP formation is shown in

² The abbreviation used is: TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-ethane sulfonic acid.

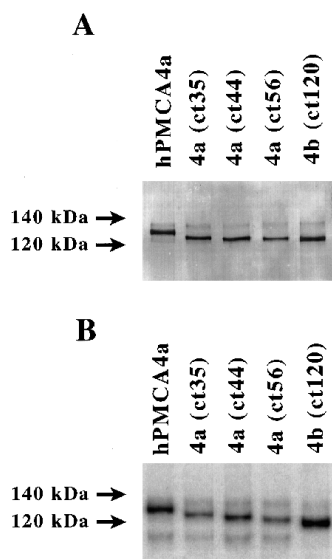


FIG. 2. Demonstration of expression of functionally active hPMCA4a and its truncated versions 4a(ct56), 4a(ct44), and 4a(ct35) in COS-1 cells. **A**, immunoblots of microsomes from cells transfected with cDNA encoding hPMCA4a and the mutants. 2 μ g of membrane protein was applied on each lane of a 7.5% SDS-polyacrylamide gel according to Laemmli. After the proteins were separated on the gel they were immunoblotted. The slower migrating faint band seen on each lane corresponds to the endogenous calcium pump of COS-1 cells. **B**, formation of the phosphoenzyme intermediate of hPMCA4a constructs. 10 μ g of each of the membrane preparations shown in **A** was subjected to phosphorylation from [γ - 32 P]ATP on ice in the presence of 50 μ M Ca^{2+} and 50 μ M La^{3+} as described under "Materials and Methods." After trichloroacetic acid precipitation, 2 μ g of each phosphorylated sample was applied on a 7.5% acidic SDS-polyacrylamide gel according to Sarkadi *et al.* (1986). An autoradiography of the phosphorylated proteins is shown after overnight exposure of the dried gel.

the figure) as is characteristic of this pump. That the strong phosphorylated bands correspond to the mutants can be seen from their characteristic migration pattern (compare with Fig. 2A). The slower migrating faint radioactive bands also seen on the immunoblots of Fig. 2A are due to the endogenous Ca^{2+} pump of COS-1 cells, whereas the faster migrating faint radioactive bands are most probably due to the incomplete inhibition of the EP formation of the endoplasmic reticulum Ca^{2+} pump by thapsigargin. Each construct showed high ATP-powered, phosphate-enhanced Ca^{2+} uptake into microsomes from the transfected COS-1 cells when measured in the presence of calmodulin. The Ca^{2+} transport activity varied within the range of 6–12 nmol/mg membrane protein/min depending on the level of expression. In order to compare the maximum activities of the mutants and the wild type, each activity was corrected for expression level, as determined by a scanned Western blot. When this was done, no significant differences were found between the activities. Both the phosphorylation and Ca^{2+} transport experiments prove that each mutant was functionally active. This was expected because the mutations affected only the regulatory region, and no mutations were done at the active sites of the molecule.

To study the characteristics of the mutants, their Ca^{2+} transport activities were tested as a function of calmodulin concentration at a relatively high, fixed Ca^{2+} concentration. In Fig. 3, the calmodulin response curves of the constructs are compared with that of the full-length hPMCA4a. 4a(ct56), containing all 28 residues of the putative calmodulin-binding domain, showed lower sensitivity to calmodulin than full-length hPMCA4a; it required about 3 times higher calmodulin concentration for half-maximal activation. This result was rather surprising because ct92, a similar construct of hPMCA4b, had

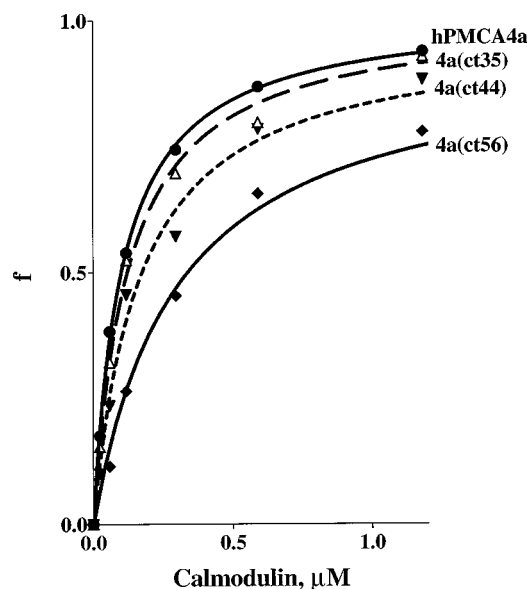


FIG. 3. 4a(ct56) is less sensitive to calmodulin stimulation than is full-length hPMCA4a. Calmodulin concentration dependence of Ca^{2+} uptake by microsomal vesicles isolated from COS-1 cells transfected with hPMCA4a (circles) and the truncated mutants 4a(ct56) (diamonds), 4a(ct44) (filled triangles), and 4a(ct35) (open triangles) is shown. The membrane vesicles were incubated at 37 $^{\circ}\text{C}$ in the presence of the appropriate concentration of calmodulin at a free Ca^{2+} concentration of 1.2 μM for 3 min, after which Ca^{2+} uptake was initiated by the addition of ATP. After subtracting the values of control membrane vesicles, Ca^{2+} uptake was expressed as $f = (v - V_o)/(V_c - V_o)$, where V_o is the activity at 1.2 μM free Ca^{2+} in the absence of calmodulin, v is the activity in the presence of the appropriate calmodulin concentration, and V_c is the maximum Ca^{2+} uptake at 1.2 μM Ca^{2+} , which was determined by fitting the data of activity versus calmodulin concentration to the Michaelis-Menten equation. In all cases, the highest activity measured was at least 90% of the V_c calculated. V_c varied from one transfection to the next, because of variation in the transfection level ranging from 5 to 10 nmol/mg membrane protein $^{-1}$, min $^{-1}$. The expression level of each membrane preparation was judged by digital scanning of the immunoblots. Data points are averages of two to three independent determinations on two to three different preparations. The $K_{1/2}$ values \pm standard deviation were: hPMCA4a, 0.102 ± 0.004 μM ; 4a(ct35), 0.115 ± 0.012 μM ; 4a(ct44), 0.180 ± 0.032 μM ; and 4a(ct56), 0.303 ± 0.098 μM .

been shown to have the same sensitivity to calmodulin as full-length isoform 4b (Verma *et al.*, 1994). Thus in hPMCA4b no residues beyond the 28 residue region are involved in calmodulin binding. In contrast, hPMCA4a required an additional 21 residues downstream of the 28-residue region for full calmodulin affinity; the calmodulin response curve of 4a(ct35) was indistinguishable from that of the full-length enzyme, whereas 4a(ct44) still had slightly lower calmodulin affinity. To put this finding in context, it is important to remember that full-length hPMCA4a has about a 5-fold lower apparent affinity for calmodulin than full-length hPMCA4b (Enyedi *et al.*, 1994).

In Fig. 4, the Ca^{2+} transport activities of the mutants without calmodulin are compared with that of full-length hPMCA4a. The procedure used for preparation of the membranes removed calmodulin and avoided the activation of the pump protein by proteolysis as much as possible so that the activities measured in this experiment would represent the activity of the intact mutant without calmodulin. The maximum activity of each mutant was also determined in the presence of saturating concentrations of Ca^{2+} and calmodulin, and the activities measured in the absence of calmodulin were expressed as a percentage of this maximum. Thus, the possibility that the observed differences in the activities were due to a variation in the expression level of the different mutants in the COS cell membranes could be avoided. When measured in

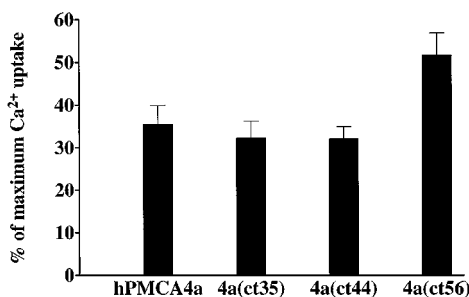


FIG. 4. The activity of 4a(ct56) is higher than that of full-length hPMCA4a when measured in the absence of calmodulin. Ca^{2+} uptake by microsomal vesicles isolated from COS-1 cells transfected with hPMCA4a, 4a(ct35), 4a(ct44), and 4a(ct56) was measured in the absence of calmodulin at $7 \mu\text{M}$ free Ca^{2+} concentration. Maximum Ca^{2+} uptake was also determined for each construct at $7 \mu\text{M}$ free Ca^{2+} and at a saturating calmodulin concentration, and the activities shown were expressed as a percentage of this maximum. Ca^{2+} uptake by control vesicles has been subtracted from all data points. The data points represent the average of three independent determinations on three different preparations \pm standard deviation.

the absence of calmodulin, 4a(ct56) showed higher activity than full-length enzyme. These data were consistent with results obtained with a similar construct of hPMCA4b (Verma *et al.*, 1994). Maximal self-inhibition was achieved by adding 12 more residues to 4a(ct56); in the absence of calmodulin, 4a(ct44) had the same low activity as full-length enzyme. It is worth mentioning that the activity of hPMCA4a in the absence of calmodulin was measured at several Ca^{2+} concentrations and was always higher than the activity of hPMCA4b (data not shown). The greater self-inhibition observed in hPMCA4b is under investigation.

Finally, the characteristics of each mutant were analyzed by measuring the dependence of Ca^{2+} uptake on free Ca^{2+} in the presence and the absence of calmodulin (Fig. 5). In the absence of calmodulin, at all Ca^{2+} concentrations tested, 4a(ct56) was more active than full-length hPMCA4a, whereas 4a(ct44) had a low activity similar to that of its parent enzyme. When measured in the absence of calmodulin, the 12 residues added to 4a(ct56) affected only the maximum velocity without having any effect on the apparent affinity for Ca^{2+} (for all three mutants, $K_{1/2}$ for Ca^{2+} was in the range $0.5\text{--}0.6 \mu\text{M}$). In the presence of a high calmodulin concentration ($4.7 \mu\text{M}$), the picture was quite different. The maximum velocity of the constructs was the same (it varied only somewhat with the expression level of the mutants), whereas the apparent Ca^{2+} affinity was different. When measured in the presence of high calmodulin, 4a(ct56) was less responsive to Ca^{2+} stimulation, with a $K_{1/2}$ of $0.55 \pm 0.05 \mu\text{M}$, than the full-length enzyme ($K_{1/2}$ of $0.35 \pm 0.04 \mu\text{M}$), and a slight difference in the Ca^{2+} response curves was observed even between 4a(ct44) and full-length hPMCA4a. This difference in the apparent Ca^{2+} affinities came from the difference observed in the apparent calmodulin affinity of the constructs, similar to the difference previously observed between full-length hPMCA4b and hPMCA4a (Enyedi *et al.*, 1994). The Ca^{2+} curve of 4a(ct35) was indistinguishable from the parent enzyme, as expected (not shown). Because 4a(ct35) had the same characteristics as the full-length hPMCA4a, we conclude that the calmodulin-binding and inhibitory domains in hPMCA4a reside in the same region of about 49 residues (Fig. 6), with the inhibitory domain being slightly shorter than the calmodulin-binding domain.

DISCUSSION

All of the results concerning the calmodulin binding and inhibitory properties of the calmodulin-binding domain of PMCA isoform 4a must be considered in the context of its

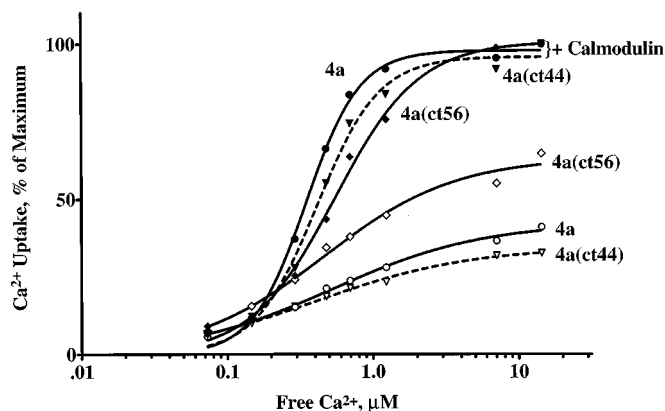


FIG. 5. Characteristics of hPMCA4a constructs as a function of free Ca^{2+} concentration. Ca^{2+} uptake by microsomal vesicles from hPMCA4a (circles), 4a(ct44) (triangles), and 4a(ct56) (diamonds) was measured in the absence (open symbols) and in the presence (filled symbols) of $4.7 \mu\text{M}$ calmodulin. Maximum activities of the constructs were determined at a saturating free Ca^{2+} and calmodulin concentration, and Ca^{2+} uptake activities were expressed as a percentage of this maximum activity. The control values were subtracted from each data point. The data points represent the average of three independent determinations on three different preparations. The lines represent the best fit to the data given by the Hill equation. In the absence of calmodulin, V_{\max} for 4a(ct56) was $63 \pm 3\%$, whereas V_{\max} for 4a(ct44) was $34 \pm 4\%$ and for hPMCA4a was $42 \pm 1.5\%$ of the corresponding V_{\max} in the presence of calmodulin. The characteristics of 4a(ct35) were indistinguishable from those of the full-length hPMCA4a, but this is not shown in the figure.

overall lower calmodulin affinity and less effective autoinhibition when compared with isoform 4b. The lower affinity of the full-length hPMCA4a for calmodulin has already been described (Enyedi *et al.*, 1994), and its less effective autoinhibition is currently under study and has been described in an abstract (Enyedi *et al.*, 1995). The unexpected characteristics of the calmodulin-binding domain of hPMCA4a described here show the presence of a region that is less efficient in both of its functions, a property that must be desirable in certain kinds of cells. Studies utilizing polymerase chain reaction techniques have shown that transcripts for hPMCA4a are present in substantial amounts in brain, in tissues containing smooth muscle, (uterus, stomach, small intestine, and colon), and in the pancreas. PMCA4a mRNA is also present in skeletal muscle, heart, and lung (Keeton *et al.*, 1993; Stauffer *et al.*, 1993). Because the lower calmodulin sensitivity of 4a leads to a lower Ca^{2+} sensitivity (Enyedi *et al.*, 1994), it is probable that pump isoform 4a occurs in cells that have more intense Ca^{2+} spikes and higher sustained levels of intracellular Ca^{2+} .

Because the calmodulin-binding site of isoform 4b is restricted to the 28 residues originally envisioned for this role (Verma *et al.*, 1994), the novel properties of the 4a calmodulin-binding site are interesting. Inspection of the sequences of these two forms (Fig. 1) discloses the probable structural basis for the differences observed here. In isoform 4b, the 20 residues downstream of the 28-residue calmodulin-binding domain constitute a region with a net charge of +1 that contains few hydrophobic residues. Such a region would not be expected to make a significant contribution to calmodulin binding. The calmodulin-binding domain of isoform 4a shows quite a different structure, because it is interrupted at its 20th residue by a negatively charged aspartic acid. The nine residues from this aspartic acid to the next glycine downstream have no positively charged amino acids so that this region has a net charge of -1 . This contrasts with the net charge of $+2$ for the corresponding residues in 4b, which are combined with an appropriate number of hydrophobic residues.

The next region immediately downstream in 4a appears to be

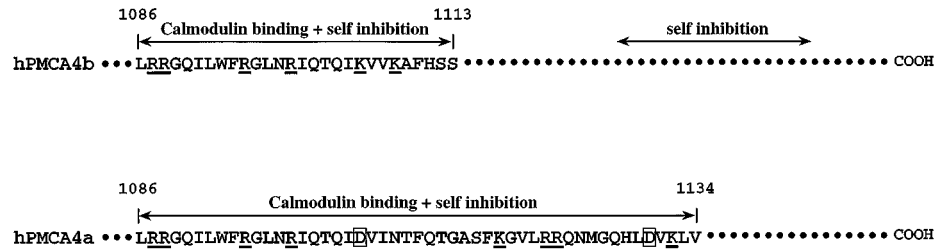


FIG. 6. **Structural differences between regulatory regions of hPMCA4a and hPMCA4b.** In hPMCA4a both calmodulin binding and self-inhibition reside within a 49-residue region. In hPMCA4b the calmodulin-binding domain is only 28 residues long, but additional residues contributing to self-inhibition reside in a region further toward the carboxyl terminus. The location of this second inhibitory region has not been determined yet. Basic and acidic residues are marked as in Fig. 1.

much more favorable for binding of calmodulin. The next 16 residues (1114–1129) have three positively charged side chains and a substantial number of hydrophobic residues. Indeed, motifs that appear at the beginning of the calmodulin-binding domain are repeated here. The first seven residues of the calmodulin-binding domain are LRRGQIL; the LRR is repeated in residues 1120–1122, and the GQIL is echoed by GQHL in residues 1126–1129. Its positive charge and content of hydrophobic residues render this region a probable contributor to calmodulin binding, in contrast with the relatively hydrophilic nature and low positive charge present in the corresponding region of isoform 4b. In the similar isoforms 1a, 1c, and 1d, the presence of an analogous second putative calmodulin-binding region was noted by Kessler *et al.* (1992), who suggested that it might contribute to calmodulin binding.

This analysis of the structure suggests that the calmodulin-binding domain of hPMCA4a consists of two shorter calmodulin-binding regions interrupted by the region containing the aspartic acid at position 1105. It is interesting to speculate that the region surrounding this aspartic acid may not be in direct contact with calmodulin, but rather may be folded out. If so, the calmodulin-enzyme complex would consist of two helical regions interacting directly with calmodulin separated by a hairpin that is folded out of the interaction surface. Thus, it appears that isoform 4a has a two-part calmodulin-binding domain, which is less effective than the domain in isoform 4b but still binds calmodulin tightly, so that calmodulin remains a physiologically important regulator for this pump.

The differences between the structures of the calmodulin-binding domains and inhibitory domains of isoforms 4a and 4b are summarized in Fig. 6. In isoform 4b, a substantial part of the inhibition contributed by the carboxyl terminus is due to a region in an as yet undefined position beyond the end of the 28-residue calmodulin-binding domain. In isoform 4a, the calmodulin-binding domain is much longer, and we have shown here that the inhibitory domain is included within the calmodulin-binding domain. Although the inhibitory region is slightly shorter, most of the determinants of calmodulin binding also contribute to inhibition, and there is no inhibitory region beyond the end of the calmodulin-binding domain in isoform 4a. These interesting structural differences provide a framework that in large part accounts for the differences in calmodulin

affinity and basal activity between isoforms 4a and 4b, a pattern that is probably followed by the a and b isoforms of the other three gene products of the plasma membrane Ca^{2+} pump.

Other calmodulin-regulated enzymes also have calmodulin-binding and autoinhibitory regions. In most cases these regions overlap only partially, if at all; in smooth muscle myosin light chain kinase (Krueger *et al.*, 1995) and in calmodulin-dependent protein kinases I (Yokokura *et al.*, 1995) and II (Brickey *et al.*, 1994), the autoinhibitory region is upstream of the calmodulin-binding region. In calmodulin-dependent protein kinase I, there is little or no overlap between the regions. Thus isoform 4a of the plasma membrane Ca^{2+} pump is unusual because all of its inhibitory determinants are contained within the calmodulin-binding domain.

REFERENCES

- Adamo, H. P., Verma, A. K., Sanders, M. A., Heim, R., Salisbury, J. L., Wieben, E. D., and Penniston, J. T. (1992) *Biochem. J.* **285**, 791–797
- Brickey, D. A., Bann, J. G., Fong, Y. L., Perrino, L., Brennan, R. G., and Soderling, T. R. (1994) *J. Biol. Chem.* **269**, 29047–29054
- Carafoli, E. (1994) *FASEB J.* **8**, 993–1002
- Enyedi, A., Vorherr, T., James, P., McCormick, D. J., Filoteo, A. G., Carafoli, E., and Penniston, J. T. (1989) *J. Biol. Chem.* **264**, 12313–12321
- Enyedi, A., Filoteo, A. G., Gardos, G., and Penniston, J. T. (1991) *J. Biol. Chem.* **266**, 8952–8956
- Enyedi, A., Verma, A. K., Filoteo, A. G., and Penniston, J. T. (1993) *J. Biol. Chem.* **268**, 10621–10626
- Enyedi, A., Verma, A. K., Heim, R., Adamo, H. P., Filoteo, A. G., Strehler, E. E., and Penniston, J. T. (1994) *J. Biol. Chem.* **269**, 41–43
- Enyedi, A., Verma, A. K., Filoteo, A. G., and Penniston, J. T. (1995) *Biophys. J.* **68**, A318 (abstr.)
- James, P., Maeda, M., Fischer, R., Verma, A. K., Krebs, J., Penniston, J. T., and Carafoli, E. (1988) *J. Biol. Chem.* **263**, 2905–2910
- Keeton, T. P., Burk, S. E., and Shull, G. E. (1993) *J. Biol. Chem.* **268**, 2740–2748
- Kessler, F., Falchetto, R., Heim, R., Meili, R., Vorherr, T., Strehler, E. E., and Carafoli, E. (1992) *Biochemistry* **31**, 11785–11792
- Krueger, J. K., Padre, R. C., and Stull, J. T. (1995) *J. Biol. Chem.* **270**, 16848–16853
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Sarkadi, B., Enyedi, A., Foldes-Papp, Z., and Gardos, G. (1986) *J. Biol. Chem.* **261**, 9552–9557
- Stauffer, T. P., Hilfiker, H., Carafoli, E., and Strehler, E. E. (1993) *J. Biol. Chem.* **268**, 25993–26003
- Verma, A. K., Filoteo, A. G., Stanford, D. R., Wieben, E. D., Penniston, J. T., Strehler, E. E., Fischer, R., Heim, R., Vogel, G., Matthews, S., Strehler-Page, M., James, P., Vorherr, T., Krebs, J., and Carafoli, E. (1988) *J. Biol. Chem.* **263**, 14152–14159
- Verma, A. K., Enyedi, A., Filoteo, A. G., and Penniston, J. T. (1994) *J. Biol. Chem.* **269**, 1687–1691
- Yokokura, H., Picciotto, M. R., Nairn, A. C., and Hidaka, H. (1995) *J. Biol. Chem.* **270**, 23851–23859