A Highly Active 120-kDa Truncated Mutant of the Plasma Membrane Ca$^{2+}$ Pump*  

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A truncated mutant (hPMCA4b(ct120)) of the plasma membrane Ca$^{2+}$ pump was expressed in COS-1 cells. The full-length pump (hPMCA4b) consisted of 1205 residues, and the mutant lacked 120 residues (including the 28-residue calmodulin-binding inhibitory domain) at the COOH terminus. To characterize this construct, Ca$^{2+}$ transport was determined in a microsomal fraction. Phosphate was added to increase Ca$^{2+}$ uptake, and specificity was enhanced by adding thapsigargin to inhibit the endoplasmic reticulum Ca$^{2+}$ pump. The mutant showed a similar level of expression as hPMCA4b and a Ca$^{2+}$ affinity and Ca$^{2+}$ transport activity about equal to that of hPMCA4b when hPMCA4b was activated. Addition of the synthetic peptide C28R2, corresponding to the calmodulin binding region of the pump, inhibited the mutant and restored the non-activated state of the enzyme. In these respects, the truncated mutant acted like hPMCA4b, when hPMCA4b had been proteolyzed to cleave a bond between the calmodulin binding region and the NH$_2$-terminal portion of the molecule. This indicates that the effects of proteolysis are due to removal of the COOH terminus and not to rearrangement of the two fragments. Since the truncated mutant was fully active and its tryptic digestion resulted in the appearance of the expected 81- and 76-kDa active fragments, we concluded that the COOH-terminal portion which is missing cannot be important in synthesis or proper folding of the enzyme.

The plasma membrane Ca$^{2+}$ pump is a calmodulin-modulated P-type ATPase which is essential for the precise control of intracellular Ca$^{2+}$ concentration. The protein has been cloned from several cell types (Verma et al., 1988; Shull and Greeb, 1988; Greeb and Shull, 1988; Strehler et al., 1990) and has been shown to be a product of a multigene family with a number of alternatively spliced mRNAs (Strehler et al., 1989; Khan and Grover, 1991; Brandt et al., 1992; Adamo and Penniston, 1992; Burk and Shull, 1992). Although its properties have been extensively studied (Carafoli, 1991; Penniston, 1983), alterations in its activity have so far been accomplished only by ordinary chemical and biochemical manipulation. The particular form of the Ca$^{2+}$ pump studied here is the 1205-residue hPMCA4b; in this name, "h" denotes that it is human, "PMCA" stands for plasma membrane Ca$^{2+}$ pump, "4" is the gene number and "b" denotes the alternate splicing pattern. Successful expression in our laboratory of hPMCA4b in COS-1 cells (Adamo et al., 1992b) has allowed us to glimpse the possibility of the study of structure-function relationships by site-directed mutagenesis, but the system initially appeared to have some serious problems. The main problems were the low activity to background ratio obtained, and the fact that a portion of the expressed hPMCA4b enzyme remained associated with intracellular membranes. Here we introduce a assay which improves the activity ratio, and we demonstrate that in spite of its localization, the expressed hPMCA4b isoyme is a functionally competent protein.

The biological activity of the plasma membrane Ca$^{2+}$ pump is regulated by calmodulin, acidic phospholipids, and cAMP-dependent phosphorylation. The regions which seem to be involved in regulation have been located (Enyedi et al., 1987; James et al., 1988; Zvaritch et al., 1990; Falchetto et al., 1991), and based on their sequences synthetic peptides have been made and used to study their interactions within the molecule (Enyedi et al., 1989b; 1991; Vorherr et al., 1990; Falchetto et al., 1991; Filoteo et al., 1992; Brodin et al., 1992). The most extensively studied region is the COOH terminally located high affinity calmodulin-binding domain which appears to be an internal inhibitor of the enzyme (Enyedi et al., 1989b). Addition of calmodulin or proteolytic removal of the calmodulin-binding domain activates the pump by increasing the maximum velocity and Ca$^{2+}$ affinity of the enzyme.

In the present study, we have used deletion mutagenesis to analyze the function of the calmodulin-binding domain. We have deleted 120 amino acids from the COOH terminus of the hPMCA4b isofom. This deletion began at the NH$_2$ terminus of the calmodulin-binding domain and continued through the end of the molecule. Our aim was to produce a fully active, calmodulin-insensitive Ca$^{2+}$ pump which might be an especially useful construct for further mutations. Our success demonstrates that this COOH-terminal portion of the molecule is not required for proper folding. We call this mutant ct120, which stands for C-truncated 120 residues. Its full designation is, therefore, hPMCA4b(ct120).

MATERIALS AND METHODS

Construction of the hPMCA4b(ct120)-truncated Mutant—To facilitate the assembly of mutated fragments, the full-length hPMCA4b was cloned into the small vector pSP72 (Promega). The truncated mutant was made by using the polymerase chain reaction and two oligonucleotide primers with the following sequences: 1) H4MA1-GGGCCATGCAATCTCATTGC and 2) H4MA2-GGCCGG-TACCCTATGCTGACTTCGAT. Primer H4MA1 contained an NsiI site, which occurs naturally in hPMCA4b. Primer H4MA2 contained an artificially introduced stop codon and KpnI site right after the amino acid sequence IDHAEME of sequence IDHAEMEL.RRGQIL, which is the beginning of the calmodulin-binding site of hPMCA4b. Polymerase chain reaction-produced DNA was cloned into the TA cloning vector pCR1000 (Invitrogen). A few clones from this vector were inserted into M13mp18 for single strand
sequencing. After locating a correct-sequence clone, its DNA was cut out of the pCR1000 vector with restriction nucleases SalI and KpnI. This was cloned into pSP72-hPMCA4b from which the original NsiI-KpnI fragment had been removed. Subsequently, the full cDNA insert of ct120 was removed with restriction nucleases SalI and KpnI and transferred to the pMM2 vector. This is the vector we previously used (Adam et al., 1992a). It is the pMT2 vector (Kroon et al., 1990) with a modified polylinker. Three DNA preparations (pMM2 vector, pMM2-hPMCA4b, and pMM2-hPMCA4b(ct120)) were prepared according to the Qiagen maxipreparation procedure.

**Transfection—** COS-1 cells were transfected with the three DNA preparations as described in a previous publication (Adam et al., 1992b). The amount of DNA (10 μg/75-cm² flask, or 20 μg/150-cm² flask), transfection time (3 h), and posttransfection time (48 h) used in the previous study were found to be optimal.

**Isolation of Microsomes from COS-1 Cells—** Crude microsomal membranes were prepared by a modified version of the procedure described by Clarke et al., 1989. Cells from four 150-cm² flasks were washed once with 20 ml of phosphate-buffered saline containing 1 mM EDTA and harvested in 10 ml of phosphate-buffered saline containing 0.1 mM phenylmethylsulfonyl fluoride and 9 μg/ml aprotinin. Cells were centrifuged (2,000 × g, 10 min) at 4°C. The supernatant was collected and added to 6 ml of a hypotonic solution of 10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 9 μg/ml aprotinin, 1 μl/ml leupeptin, and 2 mM dithiothreitol. The cells were swollen for 15 min on ice and then homogenized with 40 strokes in a Dounce homogenizer. The homogenate was diluted with 20 ml of 100 mM KCl, 2 mM dithiothreitol, and harvested in 10 ml of phosphate-buffered saline containing 0.25 M sucrose, 0.15 M KCl, 10 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, and 20 μM CaCl₂ at a protein concentration of 1–3 mg/ml and stored in liquid N₂ until needed.

Ca²⁺ Transport Assay—**Calcium influx into microsomal vesicles was measured at 37°C by rapid filtration through Millipore membrane filters (0.45-μm pore size, type HA) as described by Bond et al., 1989. The transport medium contained 0.3 M sucrose, 50 mM KCl, 2 mM dithiothreitol, 10 mM Tris-HCl, pH 7.5, 10 mM EGTA, 100 μM CaCl₂, and 100 μM aprotinin. The suspension was centrifuged at 100,000 × g for 15 min. The supernatant was made 0.6 M KCl and in order to remove calmodulin, an excess of EDTA (1.5 mM) was also added. The suspension was centrifuged at 100,000 × g for 40 min to sediment the microsomal fraction. The final pellet was resuspended in a solution containing 0.25 mM sucrose, 0.15 M KCl, 10 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, and 20 μM CaCl₂ at a protein concentration of 6–15 μg/ml were preincubated for 3 min at 37°C before initiating calcium uptake by the addition of 6 mM ATP. The incubation time was 5 min unless otherwise stated. The transport activity was determined in the presence and absence of 240 nM calmodulin.

Proteolytic Digestion of Microsomes—**This was carried out on ice for 2, 5, and 10 min, as indicated. The proteolysis medium contained 0.5–1 nmol microsomal membrane protein, 0.1 mM EGTA, 40 mM Tris-EGTA, 100 μM aprotinin, 10 μM CaCl₂, and 100 μM dithiothreitol. The digestion was stopped with 10-fold excess of trypsin-chymotrypsin inhibitor. Ca²⁺ uptake measurements or SDS-gel electrophoresis followed by immunoblotting were performed immediately.

**Quantitation of Ca²⁺ Uptake in COS Cell Membrane Preparations—** The amount of Ca²⁺ pump expressed in COS cells was determined by sandwich ELISA as described by Leberer and Pette (1986) with some modifications. Antibodies raised against the human erythrocyte Ca²⁺-ATPase (which contains predominantly the hPMCA4 isoform) were used. For the first antibody, which binds directly to the plates, a highly specific, high affinity monoclonal antibody (5F10) (Adam et al., 1992a) was used. For the second or top antibody, a polyclonal antibody (Verma et al., 1982) was used, and for ease of detection that antibody was conjugated to horseradish peroxidase (Nakan and Kawai, 1974).

The membrane preparations were washed free of dithiothreitol before incubation in a modified buffer system where the detergent concentration was 0.5% Triton X-100, 0.5% deoxycholate, and 0.05% SDS and to which 1 μM leupeptin was added. The samples were incubated for 15 min on ice before introducing to 5F10-treated plates. For standards, purified erythrocyte Ca²⁺-ATPase and/or erythrocyte ghosts were used. Absorbance was read at 492 nm using an SLT-Lab Instruments EAR 400 plate reader.

Ct120 has significantly lower cross-reaction than hPMCA4b with the polyclonal antibody used in ELISA (data not shown). Hence, the amount of pump in mutants as compared to wild type was determined on immunoblots using monoclonal antibody 5F10. The blots were scanned digitally using MacIntosh Software Image 1.44.

Peptide—**The peptide C28R2, representing the calmodulin-binding domain, was made as previously described (Enyedi et al., 1991).

**RESULTS**

**Selective Determination of Ca²⁺ Transport Activities in Microsomes from pMM2-** and hPMCA4b-transfected COS-1 Cells—** To detect the total Ca²⁺ transport activity of hPMCA4b expressed in microsomal membranes, and to improve the activity to background ratio, changes in the assay conditions were made. Ca²⁺ uptake by microsomal vesicles derived from transfected COS-1 cells was determined. This was measured in the presence of calmodulin (unless otherwise stated), and the activity of the endoplasmic reticulum Ca²⁺ pump was inhibited by addition of thapsigargin. The potential of Ca²⁺ uptake by phosphate is shown in Fig. 1. Panel B shows that, in the absence of phosphate, maximum Ca²⁺ accumulation was reached after about 5 min, and the apparent Ca²⁺ pump activity was only about 50% higher in microsomes derived from hPMCA4b-transfected cells than in microsomes from controls sham-transfected with pMM2. As shown in panel A, the accumulation of calcium, particularly in microsomes isolated from hPMCA4b-transfected cells, was much higher when 40 mM phosphate was present in the incubation medium. In the presence of phosphate, Ca²⁺ uptake by the vesicles was nearly linear for at least 15 min. Under these circumstances, the Ca²⁺ pump activity of the hPMCA4b-transfected microsomes was about four to six times higher than that of the control membranes. 5 mM oxalate, a precipitating anion widely used for Ca²⁺ uptake in the endoplasmic lumen, was added to the assay.

**FIG. 1.** Effect of phosphate on Ca²⁺ uptake by microsomal vesicles isolated from COS-1 cells sham-transfected with the empty plasmid containing the full-length Ca²⁺ pump hPMCA4b (circles). Time course of Ca²⁺ uptake by the vesicles in the presence (A) or absence (B) of 40 mM phosphate and in the presence of 0.2 μM thapsigargin and 240 nM calmodulin. Free Ca²⁺ concentration was 1.2 μM. All data points represent the average values of four independent determinations. Error bars show the standard deviation.
reticulum, also enhanced Ca\(^{2+}\) uptake in microsomes from hPMCA4b-transfected cells; however, it was less effective than phosphate (data not shown). Since the permeability to oxalate is much higher for the endoplasmic reticulum than for the plasma membrane (Raeymaekers et al., 1983; Enyedi et al., 1989a; DeSmedt et al., 1983), the oxalate stimulation was most probably due to Ca\(^{2+}\) uptake by the portion of hPMCA4b which was expressed in endoplasmic reticulum. Thus, that portion of the expressed hPMCA4b protein remaining in the endoplasmic reticulum was also active. In subsequent Ca\(^{2+}\) uptake experiments, 40 mM phosphate was used because both endoplasmic reticulum and plasma membrane are permeable to this precipitating anion, and a larger potentiating effect on Ca\(^{2+}\) uptake was obtained.

As is mentioned above, all experiments were performed in the presence of thapsigargin, a potent and selective inhibitor of the endoplasmic reticulum Ca\(^{2+}\) pump (Thastrup et al., 1990). Fig. 2 shows that thapsigargin specifically inhibited calmodulin-insensitive calcium uptake, due to the endoplasmic reticulum Ca\(^{2+}\) pump, with an IC\(_{50}\) of about 0.4–0.5 nM. Thapsigargin had no effect on the calmodulin-dependent component of Ca\(^{2+}\) uptake, which is mediated by the plasma membrane Ca\(^{2+}\) pump. This figure also shows that Ca\(^{2+}\) uptake measured in the presence of phosphate and thapsigargin was highly dependent on the presence of calmodulin, characteristic of the plasma membrane pump.

The amount of the expressed protein recovered in the microsomal fraction was determined by a sandwich ELISA procedure. This showed that about 2 µg of hPMCA4b was expressed per milligram of microsomal protein. Combining this value with the observed rate of Ca\(^{2+}\) uptake, we find that the estimated specific activity of the expressed protein was about 1 µmol of Ca\(^{2+}\)/ (mg Ca\(^{2+}\) pump, min), which is comparable to that of the erythrocyte Ca\(^{2+}\) pump in inside-out red cell membrane vesicles (2-4 µmol/(mg Ca\(^{2+}\) pump, min). Thus, it is apparent that a functionally competent plasma membrane Ca\(^{2+}\) pump protein was expressed in the COS-1 cell system.

**Expression and Characterization of a 120-kDa-truncated Mutant of the Plasma Membrane Ca\(^{2+}\) Pump—**Previous studies on both the purified (James et al., 1989) and the membrane-bound (Papp et al., 1989) erythrocyte Ca\(^{2+}\) pump had shown that chymotrypsin or calpain digestion of the enzyme produced a fragment (apparent size 125 kDa) which was activated and had lost calmodulin dependence. Even without calmodulin, this fragment acted like the native enzyme did in the presence of calmodulin. Recent sequencing work (Zvaritch et al., 1990) has suggested that hPMCA4b is cleaved upstream of the calmodulin-binding domain to produce the activated molecule. Based on these studies, a truncated mutant, hPMCA4b(ct120) starting at the NH\(_{2}\) terminus of the enzyme and extending to the NH\(_{2}\) terminus of the calmodulin-binding domain was designed and expressed in the COS cell system. This construct ended with the sequence .DEIDHAEME at its COOH terminus, and its molecular mass was expected to be 120,228 with 1,085 residues.

The amounts and the size of the truncated mutant were judged by SDS-gel electrophoresis followed by immunoblotting, using 5F10 monoclonal anti-Ca\(^{2+}\) pump antibody. As shown in Fig. 3, lanes 1 and 5, the mutant was expressed at a level similar to the wild type and migrated with the expected molecular mass. Since 5F10 also reacted with the calcium pump native to the COS-1 cells, the amount of this latter could be estimated from lane 5; it was less than 20% of the total expressed mutant or wild type enzyme. It is worth mentioning that the COS-1 cell PMCA ran with a slightly lower mobility than hPMCA4b, while hPMCA4b migrated with the same velocity as the erythrocyte Ca\(^{2+}\) pump (not shown).

As a test of native topology, microsomes from transfected cells were exposed to proteolysis with trypsin. Immunoblots of the proteolyzed samples are shown in lanes 2-4 and 6-8 of Fig. 3. The antibody used (5F10) recognizes all the active fragments discussed in this report (Papp et al., 1989; Adamo et al., 1992a). The hPMCA4b enzyme had the proteolytic pattern previously observed in erythrocyte vesicles, with the 90-, 81-, and 76-kDa fragments, the latter of which accumulated after 10 min of digestion. Tryptic digestion of ct120 produced only two major fragments of 81 and 76 kDa, as expected. When membranes expressing ct120 were proteolyzed, a substantial portion of the plasma membrane Ca\(^{2+}\) pump native to COS-1 cells (apparent molecular mass 140 kDa) remained intact after proteolysis. Because of the low amount of this pump present, the bands representing undigested COS-1 pump are hard to discern in lanes 6-8 of Fig. 3. However, other experiments consistently showed the presence of this band. Its presence indicated that a large proportion of right-side-out plasma membrane vesicles were present.

**Fig. 2. Selective inhibition of Ca\(^{2+}\) transport activity of the endoplasmic reticulum Ca\(^{2+}\) pump by thapsigargin.** Ca\(^{2+}\) uptake by microsomal vesicles isolated from hPMCA4-transfected COS-1 cells was determined in the presence of 40 mM phosphate and in the presence (circles) or absence (triangles) of 240 mM calmodulin. The arrows show that the calmodulin-dependent Ca\(^{2+}\) uptake was not affected by thapsigargin. Free Ca\(^{2+}\) concentration was 1.2 µM. Representative data from one of three similar experiments are shown.
such vesicles trypsin cannot cleave the protein but the antibody reacts with the electrophoretically separated bands. The presence of right-side-out vesicles would account for the undigested portion of the hPMCA4b and ct120 proteins evident in Fig. 3.

The capacity of ct120 to function as a calcium pump was compared to that of hPMCA4b; Fig. 4 shows the time course of calcium uptake into microsomes isolated from transfected COS-1 cells. The mutant showed the same high Ca\(^{2+}\) transport activity as the wild type, but in contrast to the wild type its activity was totally independent of the presence of calmodulin.

As shown in Table I, in the absence of calmodulin the Ca\(^{2+}\) transport activity of microsomes isolated from cells transfected with ct120 was more than 10 times higher than the Ca\(^{2+}\) transport activity of control microsomes. Thus, this mutant should be especially suitable for further mutations.

A critical parameter for the correct functioning of the plasma membrane Ca\(^{2+}\) pump is its affinity for calcium. Therefore, Ca\(^{2+}\) transport activities of microsomes isolated from the transfected cells were tested as a function of free Ca\(^{2+}\) concentration. As shown in Fig. 5, hPMCA4b had low Ca\(^{2+}\) affinity and low activity in the absence of calmodulin. Addition of calmodulin greatly enhanced the activity and shifted the $K_{1/2}$ for Ca\(^{2+}\) from about 8 to 0.3 $\mu$M. A similar activation pattern was obtained by proteolysis of hPMCA4b with chymotrypsin (Fig. 6). It is clear from Fig. 6 that the Ca\(^{2+}\) affinity and maximum activity of the truncated mutant were like those of the chymotryptically activated wild type enzyme. Synthetic peptide C28R2 (corresponding to the calmodulin-binding region of the rPMCA2b isoform, Enyedi et al., 1991) inhibited the mutant by decreasing the maximum activity and Ca\(^{2+}\) affinity and restored the inhibited state of the enzyme. Based on these data, we can conclude that the deletion of the COOH-terminal, calmodulin-binding region from the hPMCA4b Ca\(^{2+}\) pump by mutagenesis resulted in the expression of a fully active, calmodulin-insensitive Ca\(^{2+}\) transport protein.

**DISCUSSION**

Our previous work has demonstrated that the hPMCA4b isoform of the plasma membrane Ca\(^{2+}\) pump can be expressed in COS-1 cells but that a portion of the expressed protein remained associated with the endoplasmic reticulum (Adamo et al., 1992b). In that study, the activity of the pump in the transfected cells appeared to be only about 1.5–3.0-fold higher than in controls, and no estimate of the specific activity was made. The low activity to background ratio made it seem possible that only the pump protein which reached the plasma membrane was active and that the newly synthesized protein located in the endoplasmic reticulum was misfolded and inactive. We have now substantially increased the activity to background ratio, by use of thapsigargin and phosphate. Thapsigargin selectively inhibited the activity of the endogenous endoplasmic reticulum Ca\(^{2+}\) pump, thus lowering the background. Phosphate allowed us to better measure the initial activity of the pump by increasing the capacity of the vesicles. The activity under these conditions became four to six times higher than that of the PMCA native to COS cells. This activity was stimulated about 5-fold by calmodulin and was reproducible between different transfections of COS-1 cells. In addition to this improved activity, we present here three types of evidence supporting the idea that the Ca\(^{2+}\) pump protein is fully active and correctly folded in both plasma membrane and endoplasmic reticulum.

The first type of evidence is based on the effects of permeant anions. Incorporation of a precipitating anion into a system for assaying Ca\(^{2+}\) uptake has the effect of greatly increasing the total amount of Ca\(^{2+}\) which can be accumulated. Oxalate and phosphate can both cause this effect, but their relative effectiveness depends on the rate at which they penetrate the membrane. In the case of endoplasmic reticulum membranes, oxalate is more effective than phosphate at stimulating Ca\(^{2+}\).
uptake (DeSmedt et al., 1983). In plasma membranes, the opposite is true, and oxalate is nearly ineffective at stimulating Ca\(^{2+}\) uptake (Raeymaekers et al., 1983; Enyedi et al., 1989a). Our observation that oxalate caused a substantial stimulation of the Ca\(^{2+}\) uptake due to hPMCA4b expressed in COS cells indicated that active (and, therefore, correctly folded) hPMCA4b was expressed in the endoplasmic reticulum.

The second kind of evidence was based on the specific activity of hPMCA4b. By use of the ELISA described above, we were able to measure the Ca\(^{2+}\) uptake activity per milligram of pump protein and to compare that with its activity in inside-out erythrocyte membrane vesicles. The specific activity was estimated to be about 1 \(\mu\)mol of Ca\(^{2+}\)/(mg pump, min). This was certainly an underestimate since about 35-45% of the pump protein was trapped in right-side-out plasma membrane vesicles (see Fig. 3), and an unknown amount of the pump was in leaky vesicles. Reported activities of the purified Ca\(^{2+}\) pump in mixed micelles of lipid and detergent, range from 2-6 \(\mu\)mol/(mg protein, min) and varied depending on the composition of the medium. The value of the Ca\(^{2+}\) transport rate in erythrocyte inside-out vesicles was measured in this study (data not shown) and was 2-4 \(\mu\)mol/mg Ca\(^{2+}\) pump/min. The ELISA assay described above showed that the Ca\(^{2+}\) ATPase makes up about 0.5% of the total membrane protein in erythrocyte inside-out vesicles. The good specific activity of the expressed hPMCA4b showed that most (or perhaps all) of it was fully active.

The third evidence for correct folding and insertion into the membrane was given by tryptic proteolysis. Trypsin treatment produced fragments from the COS cell-expressed hPMCA4b of the same size as previously observed in erythrocyte vesicles, showing that the same parts of the molecule were accessible to trypsin. Taken altogether, our data indicate that the expressed protein is functionally active and the endoplasmic reticulum localization was not due to accumulation of misfolded protein. Recently, a similar observation on the expression of the plant plasma membrane H\(^{+}\)-ATPase has been reported (Villalba et al., 1992). In that case, a fully active plasma membrane protein was retained at the endoplasmic reticulum of yeast. Having established the validity of this system, we used it to express a calmodulin-insensitive, highly active, 120-kDa truncated mutant (ct120). Previous proteolysis work had indicated that cleavage of a bond 19 residues upstream or 2 residues downstream of the NH\(_2\) terminus of the calmodulin-binding domain activated the enzyme. We made ct120 to explore the effect of the total removal of the calmodulin-binding domain and to provide us with a more active pump to be used in future mutagenesis studies.

Immunohistochemistry experiments (not shown) revealed that both mutant (ct120) and wild type Ca\(^{2+}\) pumps were present within the same cellular compartments as expressed in COS-1 cells. This is consistent with their similar levels of expression in the same microsomal fraction and their similar phosphate-dependent Ca\(^{2+}\) transport properties. The mutant exhibited more than 10 times higher calmodulin-independent Ca\(^{2+}\) transport activity than the pump native to these cells, and this activity was comparable to that of hPMCA4b in the presence of calmodulin. In addition, the trypsin fragmentation pattern of the mutant indicated a native topology. Therefore, deletion of the COOH-terminal portion did not affect synthesis and targeting of the newly expressed protein, nor did it result in a misfolding of the molecule. Since the truncated mutant showed the same Ca\(^{2+}\) transport characteristics as the wild type when the latter had been digested with chymotrypsin or calpain, we concluded that activation of the pump by proteolysis was due to the removal of the COOH terminus and to rearrangement of the fragment produced.

### Table I

Comparison of Ca\(^{2+}\) transport activities of microsomes isolated from pMM2, hPMCA4b, or ct120-transfected COS-1 cells

<table>
<thead>
<tr>
<th>Type of transfection</th>
<th>Ca(^{2+}) transport</th>
<th>Ca(^{2+}) transport with calmodulin</th>
<th>Ca(^{2+}) transport without calmodulin</th>
</tr>
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<tbody>
<tr>
<td>pMM2</td>
<td>0.13 ± 0.03</td>
<td>0.50 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>hPMCA4b</td>
<td>0.40 ± 0.16</td>
<td>2.26 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>ct120</td>
<td>1.61 ± 0.14</td>
<td>1.79 ± 0.17</td>
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**Fig. 5.** Ca\(^{2+}\) concentration dependence of Ca\(^{2+}\) uptake by microsomal vesicles isolated from hPMCA4b transfected COS-1 cells: effect of calmodulin. Membrane vesicles were preincubated for 2 min at 37 °C with or without calmodulin at 5 \(\mu\)g of calmodulin/mg membrane. Calcium uptake was then started by addition of the vesicles to the transport medium. After this addition, the calmodulin concentration was adjusted to 0.24 \(\mu\)M (circles) or remained zero (triangles). Ca\(^{2+}\) uptake by vesicles made from pMM2 transfected cells has been subtracted from all data points. Representative data from one of three different experiments are shown.
Finally, ct120 is expected to be of great value in further mutations aimed at investigating structure-function relationships in the plasma membrane Ca\(^{2+}\) pump. Its value will derive in part from its high activity to background ratio, but an additional important factor is the ability to measure the true Ca\(^{2+}\) affinity of the fully activated pump. In the full-length hPMCA4b, measurements of activity versus Ca\(^{2+}\) concentration in the presence of calmodulin reflect both the binding of Ca\(^{2+}\) to calmodulin and the activation of the transport site of the pump by Ca\(^{2+}\). In the ct120 mutant the interaction of Ca\(^{2+}\) with the fully active pump can be measured without interference from calmodulin. This property will be necessary to assess the effects of mutants which alter the pump's Ca\(^{2+}\) affinity.

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REFERENCES


