Expression and Functional Characterization of Isoforms 4 of the Plasma Membrane Calcium Pump[†]

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ABSTRACT: PMCA isoforms 4CII (generated by splicing at the C-terminus) and 4BICI (a pump version lacking the 10th transmembrane domain) were expressed in Sf9 cells using the baculovirus system. The purified PMCA4CII had a 20-fold lower affinity for calmodulin than the PMCA4CI, the PMCA4 isoform of the erythrocytes' membranes, but had a higher activity in the absence of calmodulin. The amount of phosphoenzyme intermediate formed by PMCA4CII in the presence of Ca^{2+} alone was almost 3 times higher than in PMCA4CI and was increased by La^{3+} less than in the PMCA4CI. The isoform lacking the 10th transmembrane domain (PMCA4BICI) had no Ca^{2+}-dependent ATPase activity, but was still able to form the phosphoenzyme intermediate starting from phosphate. When expressed in COS cells, this isoform was retained in the endoplasmic reticulum; changes in membrane architecture apparently occurred during its expression; the C-terminal portion of the isoform was located in the cytosol, indicating that the deletion of the 10th transmembrane domain resulted in the loss of at least another transmembrane domain.

The plasma membrane Ca^{2+}-ATPase (or pump) (Carafoli, 1991, 1994) is crucial for the maintenance of the low cytosolic free Ca^{2+} concentration required for its second messenger function. It is regulated by a variety of mechanisms (Carafoli, 1994), among them the direct interaction with calmodulin (Jarret & Penniston, 1977; Gopinath & Vincenzi, 1977), which has been exploited to purify the pump (Niggli et al., 1979): calmodulin lowers the K_m of the enzyme for Ca^{2+} and increases its V_{max} (Carafoli, 1992). The pump, however, can also be stimulated by protein kinase C (Kuo et al., 1991) and by protein kinase A (James et al., 1989), and, at least in vitro, by acidic phospholipids (Zvaritch et al., 1990; Brodin et al., 1992). The primary sequence of the pump has been deduced from the corresponding cDNA: it is now known that four different genes code for the pump in humans (Carafoli & Guerini, 1993). Data from different groups have demonstrated that the number of isoforms is significantly increased by a complex pattern of alternative splicing (Adamo & Penniston, 1992; Heim et al., 1992a; Keeton et al., 1993; Stauffer et al., 1993; Brandt et al., 1992): the obvious problem of the physiological significance of the spliced isoforms is still entirely open. Since classical approaches are not suitable for the purification of single isoforms from their mixtures in plasma membranes, methods had be to developed to overexpress and purify them (Heim et al., 1992b; Hilfiker et al. 1994).

Three sites of alternative splicing have been demonstrated for the product of gene 4: A, B, and C (Strehler, 1991; Howard et al., 1993; Carafoli & Guerini, 1993). The influence of the splicing at site A, which is located near the phospholipid binding domain, had been previously investigated by overexpressing the PMCA2[1] isoforms, since the PMCA2 gene product has the highest number of splicing variants at the A-site (Heim et al., 1992a; Adamo & Penniston, 1992). However, no significant functional differences were detected (Hilfiker et al., 1994). The splicing at site C, located within the calmodulin binding domain, has been predicted, based on experiments with expressed C-terminal fragments of the PMCA1 pump, to alter the affinity of the pump for calmodulin (Kessler et al., 1992). Expression work on two PMCA4 splicing variants [PMCA4CI and PMCA4CII; for the nomenclature, see Carafoli (1994)], has supported this proposal (Enyedi et al., 1994). The splicing at the B-site, which is the third alternative splicing site found in PMCA4 and PMCA1, generates transcripts lacking the exon coding for the 10th transmembrane domain (Howard et al., 1993; Strehler, 1991): B-site splicing products have now been observed for both the PMCA4 and PMCA1 isoforms (Strehler et al., 1990; Howard et al., 1993), but their physiological role is still controversial (Carafoli & Guerini, 1993). PMCA4 and PMCA1 transcripts generated by alternative splicing at site B were nevertheless detected in the intestine, where they apparently represent an important fraction of the pump mRNA (Howard et al., 1993). Since the transport of Ca^{2+} is especially important in the intestine and since the PMCA pump has been proposed to play an important role in it (Van Os, 1987), a characterization of the protein product of this unusual splicing mode was needed.

PMCA isoforms 4CII (splicing at the C-terminus) and 4BICI (lacking the 10th transmembrane domain) have thus been expressed and purified and their properties characterized. The purified PMCA4CII isoform bound calmodulin 20 times less efficiently than PMCA4CI, in agreement with

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[1] Abbreviations: FITC, fluorescein isothiocyanate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMCA, plasma membrane Ca^{2+}-ATPase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; Tris, tris(hydroxymethyl)-aminomethane.

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previous observations on crude microsomal preparations of cells expressing it (Enyedi et al., 1994), but had a higher calmodulin-independent Ca^{2+}-ATPase activity; this was responsible for the formation of a larger amount of the phosphoenzyme intermediate in the presence of Ca^{2+} as compared with the PMCA4CI pump. PMCA4BICI, the isoform lacking the 10th transmembrane domain, was, on the other hand, inactive as an ATPase. When expressed in COS cells, it was retained in the endoplasmic reticulum, even if the portion of the protein preceding the deleted transmembrane domain appeared to be correctly folded.

EXPERIMENTAL PROCEDURES

Materials

Chemicals and Enzymes. Egg yolk L-α-phosphatidylcholine was from Lipid Products (Nutfield, South Nutfield, Surrey, U.K.). Goat anti-rabbit alkaline phosphatase conjugate and FITC-swine anti-rabbit secondary antibody were from DAKO (DAKO, Glostrup, Denmark). Streptolysin O was from Sigma (Sigma Chemical Co. St. Louis, MO). ^{32}P (free phosphate), ^{125}I-labeled anti-rabbit Ig, and [γ-^{32}P]ATP were obtained from Amersham (Amersham International, Amersham, England). TNM-FH medium, gentamicin, fetal calf serum, and DMEM (Dulbecco's modified minimal Eagle's medium) were purchased from GIBCO-BRL (Life Technology AG, Basel, Switzerland). The vectors pVL1392 and pVL1393 for the baculovirus expression system were a gift of Dr. M. D. Summer (Department of Entomology, Texas A&M University, College Station TX). The pSG5 vector for expression in COS cells was obtained from Stratagene (Stratagene, La Jolla, CA).

Methods

Cell Culturing. The Sf9 cells (*Spodoptera frugiperda*) were grown in suspension at 29 ± 1 °C in TNM-FH medium supplemented with 10% fetal calf serum (FCS) and 50 μg/mL gentamicin. The COS-7 cells were grown at 37 °C, 6% CO_2, in DMEM that was supplemented with 5% FCS and 50 μg/mL gentamicin.

DNA Constructs. The different DNA constructs were prepared according to standard methods (Maniatis et al., 1982). The constructs were verified by extensive restriction mapping and DNA sequencing.

Preparation of Recombinant Baculoviruses. pVL1393 vectors carrying the cDNA encoding the PMCA4CII and PMCA4BICI isoforms were prepared as described earlier (Heim et al., 1992b). CsCl-purified DNA (Maniatis et al., 1982) was then cotransfected with wild-type AcNPV DNA according to Summer and Smith (1988). Recombinant baculoviruses were isolated by a combination of dot blot assay and visual inspection of viral plaques (Summer & Smith, 1988). The expression of the recombinant proteins was performed essentially as described earlier (Heim et al., 1992b).

Isolation of Crude Membranes of Sf9 Cells. At 48−72 h after infection, the cells from three 150 cm^2 plates [around $(5-8) \times 10^7$ cells] were washed twice with PBS (25 mM sodium phosphate, pH 7.2, 100 mM NaCl) and resuspended in 6 mL of 10 mM Tris-HCl, pH 7.4. After 20 min on ice, 1 mM dithiothreitol, 75 μg/mL PMSF (phenylmethanesulfonyl fluoride), and 5 μg/mL pepstatin, 5 μg/mL leupeptin, and 5 μg/mL antipain were added. After 40 strokes of a Dounce homogenizer, 6 mL of 10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 20% sucrose, and 10 mM EDTA was added, and the nuclei and cell debris were sedimented at 750*g* for 5 min at 4 °C. The supernatant was centrifuged for 45 min at 100000*g*. The pellet was resuspended with 12 mL of TNS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10% sucrose) and centrifuged again at 4 °C. The membranes were stored in TNS at 2−5 mg/mL at −70 °C. The protein concentration was determined according to the method of Lowry et al. (1951) with some modifications.

Mild Tryptic Digestion of the Proteins. Membrane proteins were resuspended to a concentration of 100 μg/mL in 20 mM HEPES−NaOH, pH 7.2, 1 mM $MgCl_2$, and 120 mM NaCl. The proteins were incubated with trypsin on ice for different times at a protein:trypsin weight ratio of 20:1. The digestion was stopped by the addition of SDS sample buffer. The digests were separated by SDS−PAGE, transferred to nitrocellulose, and stained with the monoclonal antibody 5F10.

Assay of the Ca^{2+}-ATPase. The Ca^{2+}-ATPase activity was measured by the method of Lanzetta et al. (1979). The assay buffer contained 20 mM HEPES−NaOH, pH 7.2, 100 mM KCl, 0.5 mM EGTA, and enough $CaCl_2$ to yield the desired free Ca^{2+} concentrations. The free Ca^{2+} concentration was calculated with the help of a computer program, written by Dr. T. Jean, modified and ported to ZBasic by Dr. W. Klee, on the basis of the program of Fabiato and Fabiato (1979). The reaction was started by the addition of 1 mM ATP to the solution containing between 100 and 250 ng of purified PMCA protein and stopped after 30 min. Other details are given in the legends of the figures.

Purification of the Overexpressed Pump Isoforms. The recombinant PMCAs were purified according to the basic protocol of Niggli et al. (1979) by affinity chromatography on a calmodulin−Sepharose 4B gel; 3 mM DTT and 15% glycerol were present throughout the procedure.

Western Blotting and Immunostaining. Proteins were electroblotted onto nitrocellulose sheets (Schleicher & Schuell, Dassel, Germany) after separation by SDS−PAGE gels (Laemmli, 1970) according to the procedure described by Towbin et al. (1979). After 1 h at room temperature (or overnight at 4 °C) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.5% Tween 20) containing 1% bovine serum albumin, the nitrocellulose sheets were incubated with the primary antibody for 1 h in TBST, followed by a 45−60 min incubation with the secondary goat anti-rabbit antibody alkaline phosphatase conjugate diluted 1:7000 in TBST. The staining reaction was carried out with nitro blue tetrazolium (NBT) and with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega, Madison, WI) as described by the manufacturer.

Quantification of the Purified Protein Isoforms. A calibration curve was prepared by transferring known amounts of purified erythrocyte Ca^{2+}-ATPase to nitrocellulose. After incubation with the 5F10 antibody as described above, the blot was treated with 0.5 μCi/10 mL of an ^{125}I-labeled anti-rabbit Ig antibody. The radioactivity associated with the PMCA bands was determined with a Phosphorimager.

Transient Transfection of the COS-7 Cells. The cDNA encoding the PMCA4BICI was transferred to the pSG5 vector, using the strategy described previously (Heim et al., 1992b). Cells were seeded at about $(1.5-2) \times 10^4$ cells/cm^2. After 24 h, the cells were transfected with CsCl-

purified DNA using the calcium phosphate coprecipitation method (Chen & Okayama, 1987). The precipitate was removed after 24 h. The cells were analyzed 60−72 h after transfection.

Immunofluorescence after Permeabilization with Triton X-100. The cells were rinsed twice in PBS (150 mM NaCl, 20 mM NaH_2PO_4, pH 7.4, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$), fixed in 3% paraformaldehyde for 20 min, washed 5 times with PBS, and blocked for 30 min in 0.1 M glycine in PBS. After five washes with PBS, they were permeabilized in 0.1% Triton X-100 in PBS for 3 min, followed by five washes. Unspecific binding was prevented by incubation with the blocking buffer (5% FCS, 0.1% bovine serum albumin, 5% glycerol, and 0.04% NaN_3 in PBS) for 1 h at room temperature or overnight at 4 °C. The cells were incubated with the primary antibody diluted 1:50 in blocking buffer and 1 h later with a swine secondary antibody against rabbit fluorescein-conjugated antibody (1:50 dilution in blocking buffer). After the cells were mounted in a medium of 80% glycerol, 2.5% DAKO (an anti-fade reagent) in PBS, pH 8.0, the cells were analyzed with an AXIOVERT 10 microscope equipped with epifluorescence illumination and 25×, 40×, 63× and 100× oil immersion planneofluar objectives.

Permeabilization with Streptolysin O. After the cells were washed once with 25 mM HEPES−KOH, pH 7.4, 2.5 mM $Mg(CH_3COO)_2$, 25 mM KCl, and 250 mM sucrose, the activated streptolysin O (1.6 units/mL) was allowed to bind for 15 min on ice. The unbound toxin was removed by two short washes with PBS. The cells were then lysed in PBS containing 1 mM DTT for 30 min at 37 °C. After blocking with 3% paraformaldehyde, the coverslips were incubated as described above.

Formation of the Phosphoenzyme Intermediate from ATP. The reaction was carried out on ice in a final volume of 50 μl; 20−100 μg of crude membrane proteins or 20−200 ng of purified proteins was resuspended in 20 mM MOPS−KOH, pH 6.8, 100 mM KCl, 5 mM EGTA, and in or 50 μM $CaCl_2$ or in 50 μM $CaCl_2$ and 50 μM $LaCl_3$ as indicated in the legends of the figures. The reaction was started by the addition of 0.3 μM ATP [γ-^{32}P]ATP (100 Ci/mmol) and stopped after 40 s with 400 μL of 7% trichloroacetic acid and 10 mM sodium phosphate. The pellet was resuspended in sample buffer (6 M urea, 5% SDS, 0.5 M DTT, Tris−phosphate, pH 6.8, and 5 mM EDTA at room temperature). The proteins were separated by acidic SDS−PAGE (Sarkadi et al., 1986) and the gels dried and exposed for 12−96 h.

Phosphorylated Intermediate Starting from Phosphate. The reaction was carried out according to Chiesi et al. (1984) with some modifications; 50−100 μg of crude membrane proteins was resuspended in 100 μl of reaction buffer [50 mM Tris-HCl, pH 8.0, 30 mM NaCl, 40% dimethyl sulfoxide (DMSO), 0.05% Triton X-100, 0.5 mM DTT, 20 mM $MgCl_2$, and 5 mM EGTA]. The reaction was started by the addition of 100 μCi of ^{32}P (10 Ci/mmol), carried out for 10 min at room temperature, and stopped by trichloroacetic acid. The influence of vanadate was determined after preincubation of the crude membranes for 10 min at room temperature in the absence of phosphate, followed by the normal phosphorylation reaction. The concentration of vanadate varied from 0 to 20 μM. The influence of Ca^{2+} was determined by following the phosphorylation reaction in the absence and the presence of 1.2 mM $CaCl_2$.

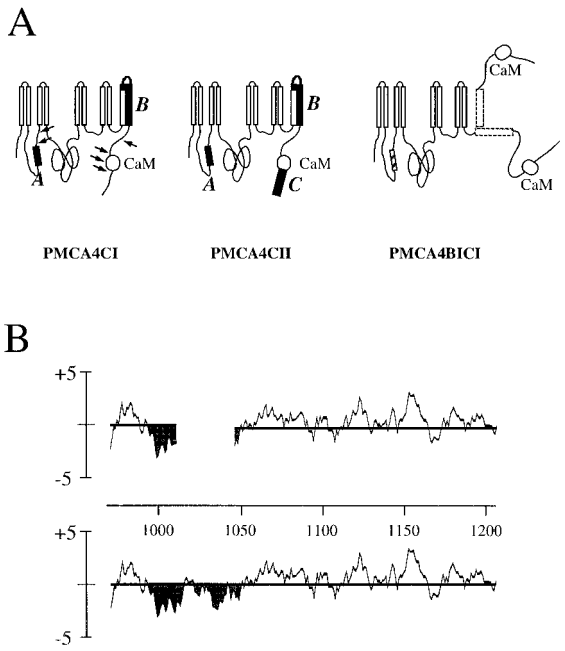

FIGURE 1: (A) Schematic representation of PMCA4 isoforms, with the results of alternative splicing at sites C and B. The splicing sites (the inserted or deleted sequences) are represented by black boxes. The sequenced cleavage sites for trypsin (Zvaritch et al., 1990) are indicated by the arrows in the model for PMCA4CI. The open boxes indicate the transmembrane domains; the two possible orientations for the 9th transmembrane domain of the PMCA4BICI protein are also shown. (B) Hydrophobicity plots of the C-terminal portion of PMCA4BICI (top) and PMCA4CI (bottom). The data for the region between amino acids 970 and 1205 were calculated according to Kyte and Dootlittle (1982).

RESULTS

Expression of the PMCA4 Isoforms. The splicing at sites B and C generates two PMCA4 isoforms (Figure 1), which should be reasonably expected to have substantial functional differences with respect to isoform PMCA4CI, which is found in red blood cells. The alternative splicing occurring in PMCA4CII results in the complete alteration of the sequence C-terminal to the calmodulin binding domain, that of PMCA4BICI in the deletion of the 10th putative transmembrane domain (Figure 1A). Inspection of the hydrophobicity plot of isoform PMCA4BICI revealed that deletion of the region encompassing the 10th transmembrane domain failed to influence the predicted hydrophobicity value of the surrounding regions and in particular that of the 9th transmembrane domain which was similar to that of the PMCA4CI isoform (Figure 1B). One should thus in principle predict that the C-terminal region containing the calmodulin binding domain of isoform PMCA4BICI would lie on the external side of the plasma membrane (see Figure 1A).

To study the properties of PMCA4CI, as compared to those of the two spliced isoforms PMCA4CII and PMCA4BICI (see Figure 1), the full-length cDNAs of the proteins were expressed in Sf9 cells using the baculovirus system, which had been previously shown to produce amounts of PMCA protein suitable for purification work (Heim et al., 1992b; Hilfiker et al., 1994). Recombinant viruses were generated by homologous recombination of the AcNPV wild-type virus with the pVL1393 vectors containing the PMCA cDNA (Heim et al., 1992b; see Experimental Procedures). Membranes prepared from Sf9 cells infected with the recombinant baculoviruses contained in each case

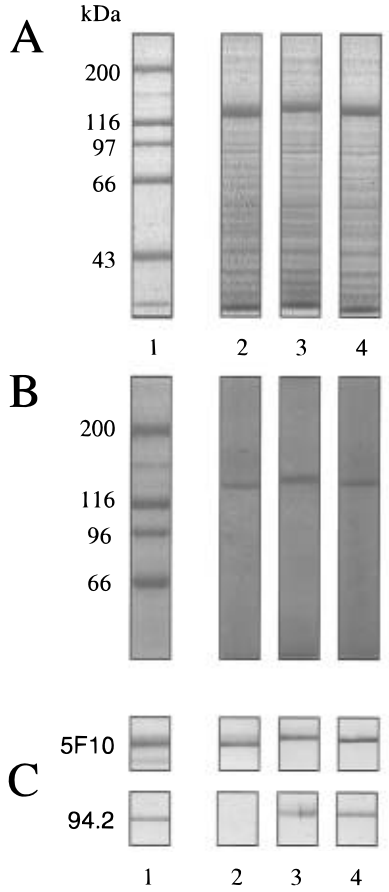

FIGURE 2: Expression and purification of the PMCA4CI, PMCA4CII, and PMCA4BICI isoforms. (A) 25 μg of total membrane proteins of Sf9 cells infected with recombinant baculoviruses for PMCA4CII (lane 2), PMCA4CI (lane 3), and PMCA4BICI (lane 4) was separated by SDS−PAGE and stained with Coomassie Blue. The major bands at 130−135 kDa corresponded to the expressed pump isoforms. Markers are given in lane 1. (B) The three expressed PMCA4 isoforms were purified on a calmodulin affinity column. 100−200 ng was separated by SDS−PAGE and silver-stained (Merryl et al., 1981). (C) 100−200 ng of purified PMCA4CII (lane 2), PMCA4CI (lane 3), and PMCA4BICI (lane 4) was transferred to nitrocellulose and probed with the monoclonal antibody 5F10 (upper lanes) and the polyclonal antibody 94.2 (lower lanes). 50 μg of human erythrocyte membrane proteins was run in parallel as a control (lane 1).

a major band at 130−135 kDa corresponding to the PMCA4CII protein (Figure 2A, lane 2), PMCA4CI (Figure 2A, lane 3), and PMCA4BICI (Figure 2A, lane 4). These were not present in noninfected Sf9 cells or in Sf9 cells infected with the wild-type virus (not shown). The three pump isoforms were solubilized with Triton X-100 and purified by affinity chromatography on calmodulin columns (Figure 2B). After elution with EGTA, only a major band with the expected size was present in the respective gels (Figure 2B, lanes 2−4). Western blotting confirmed the identity of the overexpressed protein (Figure 2C). The PMCA4CII protein was recognized by monoclonal antibody 5F10, whose epitope is located in the major cytosolic loop of the pump (Adamo et al., 1992), but not by polyclonal antibody 94.2 (Figure 2C, lane 2), which recognizes the last 40−50 amino acids of the PMCA4CI protein (Guerini, unpublished observations). This was in agreement with expectations, since the 66 C-terminal amino acids of the PMCA4CII isoform are different from those of the other two. The expression levels for the three isoforms in Sf9 cells

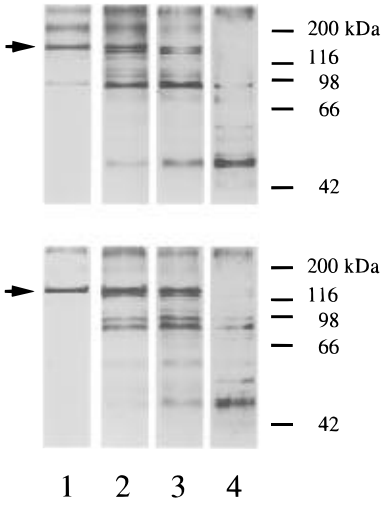

FIGURE 3: Mild tryptic digestion of the membrane containing the expressed isoforms. Membrane proteins were incubated on ice with trypsin (20:1 protein:trypsin weight ratio) for 0 min (lane 1), 1 min (lane 2), 2 min (lane 3), and 10 min (lane 4). After stopping the digestion with sample buffer, 3−4 μg of the mixture (PMCA4CI, upper panel; PMCA4BICI: lower panel) was separated by SDS−PAGE electrophoresis and blotted to nitrocellulose, and the pump fragments were visualized with antibody 5F10. The arrow indicates the position of the undigested pump. The locations of the sequenced trypsin cleavage sites are given in Figure 1A.

(Figures 2A) and other systems (not shown, see below for COS-7 cells) were equivalent.

In the case of the PMCA4BICI pump (Figure 1), it was important to establish whether the deletion of the 10th transmembrane domain would interfere with the proper folding of the protein. Membrane fractions containing the different expressed isoforms were thus washed with carbonate to remove extrinsic proteins (Fujiki et al., 1987). All three expressed proteins behaved identically; i.e., the carbonate treatment failed to remove them from the membrane (not shown). A mild treatment with trypsin was used as a means to probe the folding state of the PMCA isoforms in the membrane. The experiments showed that the digestion patterns of the PMCA4CI and PMCA4BICI proteins were very similar (Figure 3). Apparently, thus, the lack of the 10th transmembrane domain in the PMCA4BICI isoform did not result in the gross misfolding of the portion of the protein that precedes the deletion.

Activity Measurements on the Expressed Isoforms. The Ca^{2+}-dependent formation of the phosphoenzyme intermediate from ATP, which is characteristic of P-type pumps (Pedersen & Carafoli, 1987), is specifically enhanced by lanthanum in the PMCA pumps (Carafoli & Guerini, 1993). Membranes containing PMCA4CI and PMCA4CII showed a strong phosphorylated band at the expected size upon treatment of Ca^{2+} and La^{3+} , which was not detected in PMCA4BICI. The same experiment was repeated with the purified protein: a phosphorylated band was observed for PMCA4CII (Figure 4A, lanes 2 and 3) and PMCA4CI (Figure 4A, lanes 5 and 6), but not for PMCA4BICI (Figure 4A, lane 7): the phosphorylated bands migrated to the same position of the PMCA band of erythrocytes (Figure 4A, lane 8). As expected, the phosphorylated band was not visible in the presence of EGTA (Figure 4A, lanes 1, 4) and was much less evident when La^{3+} was omitted (Figure 4A, lanes 2, 5). In the case of PMCA4CII, the Ca^{2+}-dependent phosphoenzyme intermediate was 2−4 times stronger in the

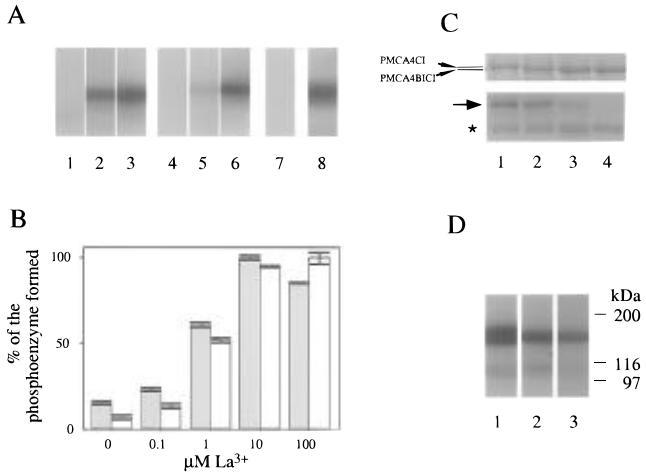

FIGURE 4: Phosphoenzyme intermediate formation by the PMCA4 isoforms. (A) 200 ng of purified PMCA4CII (lanes 1−3), PMCA4CI (lanes 4−6), and PMCA4BICI (lane 7) and 50 μg of erythrocyte membrane proteins (lane 8) were phosphorylated in the presence of 3 mM EGTA (lanes 1 and 4), of 400 μM $CaCl_2$ (lanes 2 and 5), and of 400 μM $CaCl_2$ and 400 μM $LaCl_3$ (lanes 3, 6, 7, and 8), separated by acidic SDS gels, and exposed to X-ray films for 2−5 days. (B) 15 μg of total membrane proteins expressing the PMCA4CI (white bars) and PMCA4CII (gray bars) proteins was incubated in the presence of 0.3 μM ATP (300 Ci/mmol), 10 μM $CaCl_2$, and different concentrations of $LaCl_3$. After the formation of the phosphorylated intermediate, the mixtures were separated by acidic SDS gels. The radioactive bands were quantified with a Phosphorimager. The amount of phosphoenzyme intermediate formed was calculated as a percent of the maximal amount, obtained in the presence of 100 μM La^{3+} and 10 μM Ca^{2+}. (C) The formation of the phosphoenzyme intermediate from ATP was studied on membrane proteins obtained from Sf9 cells infected with PMCA4CI (lane 1) or PMCA4BICI (lane 4) or coinfected with PMCA4CI and PMCA4BICI recombinant viruses (lanes 2 and 3). The ratio of the two viruses (PMCA4CI:PMCA4BICI) was 1:1 (lane 2) or 1:9 (lanes 3). 30−40 μg of membrane proteins was incubated in the presence of about 0.3 μM [γ-^{32}P]ATP (150 Ci/mmol), 200 μM $CaCl_2$, and 200 μM $LaCl_3$ under conditions promoting the formation of the phosphoenzyme intermediate of the plasma membrane pump (see Experimental Procedures). The upper panel represents Coomassie Blue stained gels (the two thin arrows indicate the migration of the PMCA4CI and PMCA4BICI isoforms); the lower panel is the autoradiogram of the same gel. The thick arrow indicates the PMCA4CI specific radioactive band and the asterisk the endogenous SERCA pump (migrating at around 100 kDa). (D) The phosphoenzyme intermediate of 50 μg of membrane proteins containing the PMCA4CI pump was formed from phosphate (as described under Experimental Procedures) in the absence (lane 1) or in the presence of 5 μM (lane 2) and 20 μM (lane 3) vanadate. The radioactive band at 135 kDa was not observed in uninfected Sf9 cells.

absence of La^{3+} than that of PMCA4CI (Figure 4A, compare lanes 2 and 5). The amount of phosphoenzyme intermediate formed by the purified PMCA4CII in the absence of La^{3+} was 45 ± 20% (SD, three independent protein purifications) of that formed in its presence, while in the case of PMCA4CI it was only 16 ± 10% (SD, three independent purifications). As indicated in Figure 4B, the difference between the two isoforms was also detected on the crude membrane, although in this case the difference was less dramatic. Since the PMCA pump can oligomerize (at least in vitro, Kosk-Kosika & Bzdega 1988; Vorherr et al., 1991) and since transcripts for PMCA4CI have been found in the tissues where also PMCA4CI was present (Howard et al., 1993), the possibility that the formation of oligomers between PMCA4CI and PMCA4BICI could rescue the activity of the latter pump could not be ruled out. To verify this hypothesis, the two

Table 1: Inhibition of the Phosphoenzyme Intermediate Formed from P_i[a]

	5 μM VO_4^{3-}	20 μM VO_4^{3-}	1.5 mM $CaCl_2$
PMCA4Cl	65 ± 5	22 ± 2	11 ± 1
PMCA4Cll	ND[b]	40 ± 5	26 ± 6
PMCA4BlCl	60 ± 1	10 ± 1	12 ± 2

[a] The reaction (see Experimental Procedures and the legend for Figure 4D) was done in the presence of different concentrations of VO_4^{3-} or in the presence of 1.2 mM Ca^{2+}. After separation, the gels were dried and the radioactive bands quantified with the help of a Phosphorimager. The values reported in the table represent the percent of the maximal phosphoenzyme intermediate obtained without inhibitors and are the average of three experiments. [b] Not determined.

isoforms were coexpressed in Sf9 cells, and the formation of the phosphoenzyme intermediate from ATP was studied (Figure 4C). Although the two isoforms had similar mobility in acidic gels, the PMCA4BICI migrated slightly faster than PMCA4CI. This could be seen when similar amounts of the two isoforms were present in the membranes (Figure 4C, lane 2, upper panel, the broadening of the PMCA-specific band was reproducible). Under the conditions used (a 20-fold excess of virus over the Sf9 cells), both isoforms should have been coexpressed in a high percentage of the cells. Nevertheless, no shifting or broadening of the PMCA-specific phosphoenzyme intermediate (Figure 4C, lower panel) was observed. Although a small increase in the activity of the PMCA4BICI isoform could not be ruled out, the experiment was consistent with the conclusion that coexpression of PMCA4CI could not rescue the activity of the PMCA4BICI.

The PMCA pump (in this case the PMCA4CI isoform) can also form a phosphorylated intermediate starting from phosphate (Figure 4D, lane 1), in a reaction that is inhibited by micromolar concentrations of vanadate (Figure 4D, lanes 2 and 3; Pedersen & Carafoli, 1987) or by Ca^{2+} (Table 1). The same behavior was observed also in the case of PMCA4CII (although the inhibition in this case was weaker, see Table 1) and, unexpectedly, also for that of the PMCA4BICI isoform (Table 1). The amount of phosphorylated intermediate formed from phosphate by the PMCA4BICI protein was 64 ± 13% (5 different experiments) of that observed for the two other PMCA4 isoforms. The level of inhibition by Ca^{2+} and by vanadate, and the phosphate concentration dependence of PMCA4BICI, was the same as in the case of PMCA4CI (Table 1).

The Ca^{2+}-dependent ATPase activity was also measured on the purified proteins. In agreement with the observations mentioned above, the PMCA4BICI isoform could still perform part of the catalytic cycle (phosphorylation from phosphate), but was incapable of ATPase activity (Table 2). Remarkably, however, the PMCA4CII protein had higher basal activity in the absence of calmodulin than PMCA4CI (Table 2). The Ca^{2+} dependence of the activity is shown in Figure 5A. To demonstrate that the Ca^{2+} dependence of the basal activity of the two isoforms was identical, it has been expressed in arbitrary units and set to similar values (Figure 5A). PMCA4CII showed the typical shift of the K_m after addition of calmodulin, which, however, was less dramatic than in the case of PMCA4CI. This was due to the lower affinity of the PMCA4CII pump for calmodulin as compared to PMCA4CI (Figure 5B): 50% stimulation of PMCA4CII by calmodulin needed 20 times more calmodulin then in the

Table 2: Ca^{2+} and Calmodulin ATPase Activity of the Purified PMCA4 Isoforms[a]

	PMCA4Cl	PMCA4Cll	PMCA4BlCl
$+Ca^{2+}$	412 ± 35	495 ± 38	0
$+Ca^{2+}$ and CaM	1740 ± 120	1214 ± 83[b]	0
stimulation by CaM	4×	2.4×	0

[a] The activity was determined as described in the legend to Figure 5, in the presence of 1 μM free Ca^{2+} or in the presence of 1 μM free Ca^{2+} and 2×10^{-7} M calmodulin. The concentration of the proteins was determined by transferring the proteins to nitrocellulose and probing them with the 5F10 antibody and a ^{125}I-labeled secondary antibody (see Experimental Procedures). [b] In this experiment, the same concentration of calmodulin was used as that in Figure 5A. In the presence of higher amounts of calmodulin, the activation factor was close to 4 (compare also Figure 5B).

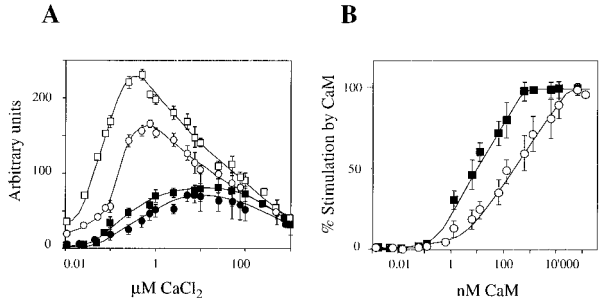

FIGURE 5: Ca^{2+}-dependent ATPase activity of the purified PMCA4CI and PMCA4CII isoforms. (A) The Ca^{2+}-dependent ATPase activity was measured as described under Experimental Procedures and expressed in arbitrary units. The activity of PMCA4CI (squares) and PMCA4CII proteins (circles) was determined in the absence (closed symbols) or in the presence of 200 nM calmodulin (open symbols). The basal activity of the two pump isoforms was set to similar values and the calmodulin-dependent activity related to them. (B) Calmodulin dependence of the Ca^{2+}-ATPase activity of the PMCA4CI pump (squares) and PMCA4CII (circles), determined in the presence of 1 μM free $CaCl_2$. The points are the average of two to five determinations, obtained with three independently purified batches of proteins.

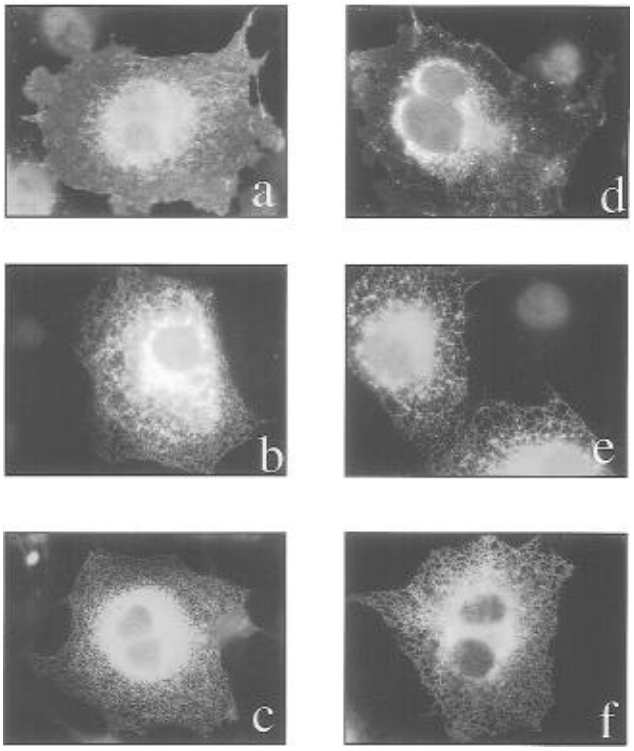

FIGURE 6: Targeting of the PMCA4BICI isoform and localization of its C-terminus. COS-7 cells grown on coverslips were treated and prepared for immunocytochemistry as described under Experimental Procedures. The cells were transfected with cDNA encoding the PMCA4CI (panels a and d), PMCA4BICI (panels b and e), and SERCA1b proteins (panels c and f). The cells were then permeabilized with Triton X-100 (panels a−c) or with streptolysin O (panels d−f) and stained with antibodies 94.2 (specific for the C-terminus of the PMCA4 isoform, panels a, b, d, and e) and A52 (specific for the major cytosolic loop of the SERCA pump; panels c and f).

case of PMCA4CI. The experiments in Figure 5A were performed at 0.2 μM calmodulin, a concentration adequate for maximal stimulation of PMCA4CI but not of PMCA4CII (see Figure 5B and Table 2). The pH dependence of the Ca^{2+}-dependent ATPase of the two isoforms was also investigated and found to be identical in the presence or in the absence of calmodulin (not shown).

The finding that the PMCA4BICI isoform was inactive could have been due to the external location of the C-terminus, consistent with the uneven number of transmembrane domains. This possibility was tested by performing immunocytochemistry experiments in COS-7 cells, since the large size of the nucleus of the Sf9 cells makes them unsuitable for these experiments. Isoform PMCA4BICI was expressed in COS-7 cells in amounts similar to the those of the other two isoforms used as controls (not shown). To verify the stability of the PMCA4BICI construct and of its transcript in COS-7 cells, total RNA was prepared from the transfected cells and reverse-transcribed. A PCR (polymerase chain reaction) with PMCA4-specific oligonucleotides spanning the 10th transmembrane domain resulted in the amplification of a band of 880 bp for the PMCA4CI cells and a band of 770 bp for the PMCA4BICI COS-7 cells: this was expected since a 108 bp DNA is missing in PMCA4BICI (results not shown). These bands were not detected in COS-7 cells transfected with the control vector. Immunocytochemistry after permeabilization of the cells expressing PMCA4CI (or PMCA4CII) with Triton X-100 yielded the expected plasma membrane staining (Figure 6, panel a). Surprisingly, a typical ER staining was instead observed in the case of PMCA4BICI (Figure 6, panel b), i.e., the same staining pattern as that of cells expressing the SERCA1b pump (Figure 6, panel c). More than 99% of the cells expressing the PMCA4BICI protein showed this pattern. To establish whether the C-terminus of the PMCA4BICI protein was located in the cytosol or in the lumen of the endoplasmic reticulum, the experiments were repeated on cells permeabilized with streptolysin O, known to specifically permeabilize the plasma membrane, but not the endoplasmic reticulum (Campbell et al., 1992). The 94.2 antibody (see Figure 2) was used for these experiments. The results on the PMCA4CI and SERCA1b proteins did not differ from those after Triton X-100 solubilization (Figure 6, panels d through f): this was expected because the epitopes for the respective antibodies are located in the cytosolic portion of the protein [the C-terminal domain for the PMCA4CI pump and the second cytosolic loop for the SERCA1b protein (Zubrzycka-Gaarn et al., 1984)]. The same staining pattern was observed after solubilization with Triton X-100 and with streptolysin O, also in the case of the PMCA4BICI protein (Figure 6, panels b and e), strongly suggesting that the C-terminus of the isoform retained in the endoplasmic reticulum was located in the cytosol: i.e., it would be located in the cytosol if the protein were to be correctly targeted to

the plasma membrane. It is thus likely that the topology of the PMCA4BICI isoform in which the 10th transmembrane domain is missing is similar to that of the PMCA4CI protein.

DISCUSSION

In principle, alternative splicing could generate more than six different PMCA4 isoforms, if splicing site B were also considered (Strehler, 1991; Carafoli & Guerini, 1993). Alternative splicing at site A has been already studied in detail for the PMCA2 isoform, revealing no major functional differences in the expressed pumps (Hilfiker et al., 1994). On the other hand, work with expressed peptides encompassing the C-terminal portion of the pump (Kessler et al., 1992) and with the pump spliced at the C-site expressed in COS cells (Enyedi et al., 1994) has shown that C-site splicing had an important effect on the binding of calmodulin. The splicing at site B (Howard et al., 1993; Strehler, 1991), was, on the other hand, predicted to have a dramatic impact on the membrane topology of the pump. The expression of the three PMCA4 proteins in Sf9 cells using recombinant baculoviruses has allowed their purification and has thus made it possible to measure parameters that could not be measured in expressing COS cells: e.g., the Ca^{2+}-dependent ATPase activity could be studied in well-defined detergent−lipid mixtures.

The PMCA4CII protein bound calmodulin with lower affinity than PMCA4CI; i.e., this pump would interact with calmodulin in vivo only under conditions in which high amounts of it are available. At variance with what was observed with the C-terminal peptides of the PMCA1 pump expressed in bacteria (Kessler et al., 1992), the pH had no influence on the binding of calmodulin to the expressed isoform: this was not unexpected since the C-terminal spliced sequences are different in PMCA1 and PMCA4. Most importantly, the His residues of PMCA1CII and PMCA1CIV, which have been shown to be instrumental in mediating the peculiar pH sensitivity of calmodulin binding, are absent in the PMCA4CII isoform. The increase of the basal Ca^{2+}-dependent ATPase activity of the PMCA4CII protein as compared to that of PMCA4CI was probably related to less autoinhibition of the pump in the absence of calmodulin. This would be consistent with the suggestion that in addition to the calmodulin binding domain other amino acids C-terminal to them are involved in the autoinhibition phenomenon (Verma et al., 1994). The amount of phosphoenzyme formed by the PMCA4CII pump in the absence of La^{3+} was higher than in the case of PMCA4CI. This was probably not related to the difference in affinity for calmodulin since the experiments were performed at 100 μM Ca^{2+}, a concentration which eliminated the effect of calmodulin on the activity of the pump. Thus, this finding could reflect differences in the high-affinity Ca^{2+} binding sites located in the acidic domain C-terminal to the calmodulin binding site (Hofmann et al., 1993). These acidic regions differ significantly in the two pumps.

Although the PMCA4BICI isoform was inactive as an ATPase, it was still capable of forming the phosphoenzyme intermediate from phosphate. This was consistent with the finding that no gross misfolding of this protein had probably occurred. Surprisingly, however, PMCA4BICI was retained in the endoplasmic reticulum of expressing COS-7 cells. Since the experiments with streptolysin O indicated that the C-terminus of this isoform was still in the cytosol, the isoform evidently still had an even number of transmembrane domains (eight); i.e., the deletion of transmembrane domain 10 caused a reorganization of the pump topology leading to the elimination of the 9th transmembrane domain, as well. The exposure of a portion of the protein normally inserted in the bilayer (or at least "shielded" by the 10th transmembrane domain) would thus cause retention of PMCA4CI in the ER. Retention of modified or not properly shielded transmembrane domains has been observed in other proteins (Bonifacino & Lippincott-Schwartz, 1991). This could indicate that transmembrane domains 9 and 10 interact with each other in a very specific way: the removal of one of them would necessarily cause the improper folding of the other. A similar effect was observed when the second transmembrane domain of PMCA4CI was replaced by that of the SERCA pump (Foletti et al., 1995).

A final word is in order on the physiological significance of the two isoforms described. Very likely, isoform PMCA4BICI is a result of aberrant splicing. The sequence of the PMCA4 gene is not yet available, but a comparison with those of PMCA3 and PMCA1 indicates that the spliced-out region corresponded exactly to exon 23 in the rat PMCA3 gene (Burk et al., 1992) and to exon 21 in the human PMCA1 gene (Hilfiker et al., 1993). Thus, during the maturation of pre-mRNA, a certain amount of PMCA4BICI could be possibly generated. Even if the same would be translated to a protein, it would however never reach the plasma membrane and would be inactive.

In the case of the PMCA4CII isoform, transcripts had been detected in brain, muscle, and stomach (Brandt et al., 1992; Keeton et al., 1993; Stauffer et al., 1993), where they represented up to 50% of the PMCA-specific transcripts (Stauffer et al., 1993). In selected regions of the brain, a band with the expected molecular mass of PMCA4CII was seen using PMCA4 antibodies (Stauffer et al., 1995). These results indicate that large amounts of the PMCA4CII pump are present in these tissues. Since the PMCA4CII pump has higher basal activity and is less sensitive to calmodulin, it is likely that it will significantly influence the transport of Ca^{2+} across the plasma membrane at very low Ca^{2+} concentrations and in cases when insufficient calmodulin would be available to activate the pump.

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