Expression, Purification, and Properties of the Plasma Membrane Ca\textsuperscript{2+} Pump and of Its N-terminally Terminally Truncated 105-kDa Fragment*

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The plasma membrane Ca\textsuperscript{2+} ATPase is the largest of all P-type (Pedersen and Carafoli, 1987a; Pedersen and Carafoli, 1987b) ion motive pumps and has a molecular mass averaging 134 kDa. It is apparently present in all eucaryotic cells, and its properties have been summarized in a number of recent reviews (Carafoli, 1991; Carafoli, 1992; Strehler, 1991). The enzyme has now been cloned from several cell types, including brain (Shull and Greeb, 1988), porcine smooth muscle (De Jaegere et al., 1989), and human teratoma cells (Verma et al., 1988). The pump is the product of a multigene family: the expression of the pump in the COS cell system and the baculovirus system produced amounts of the pump sufficient to isolate it on calmodulin (CaM)\textsuperscript{2+} columns: the present contribution thus describes the first successful purification of an expressed P-type pump.

Early proteolysis work (Zurini et al., 1984) had suggested that a 40–50-kDa portion of the molecule could be removed leaving behind a 80-kDa fragment which was still active. However, the level of activity of the fragment isolated on a CaM column was very low, leaving open the possibility that a trace contamination of the preparation by the intact enzyme was responsible for the ATPase activity observed. Further work (Zvaritch et al., 1990) has shown that the N-terminal trypsin cut leading to the formation of the 90-kDa fragment occurred between the main body of the pump and a 30–40-kDa N-terminal portion. The problem of whether the N-terminally truncated pump is still active is important, since the portion missing from the N terminus contains a region that has been defined as the transducing domain (Green and MacLennan, 1989), i.e. a domain that should couple ATP hydrolysis to Ca\textsuperscript{2+} translocation. The 30–40-kDa missing portion of the molecule contains other domains that are highly conserved among P-type pumps. Even if no known role has so far been conclusively assigned to them, their conservation suggests their importance to the function of the enzyme. The missing N-terminal fragment, which contains the two first transmembrane domains of the pump, is relatively hydrophobic (Zurini et al., 1984) and tends to associate strongly after proteolysis with the remainder of the pump molecule, i.e. with the 90-kDa portion. For this reason, the purification of the latter has proven extremely difficult; expression in amounts sufficient for purification purposes has thus been successfully

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1 The abbreviations used are: CaM, calmodulin; DMEM, Dulbecco’s modified minimal Eagle’s medium; DTT, dithiothreitol; ER, endoplasmic reticulum; ERCA, endoplasmic reticulum Ca\textsuperscript{2+}-ATPase; FCS, fetal calf serum; HPMCA, human plasma membrane Ca\textsuperscript{2+}-ATPase; PBS, phosphate-buffered saline; PMSE, phenylmethylsulfonyl fluoride; MOPS, 3-(N-morpholino)propanesulfonic acid.

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attempted in the present study. Functional tests have shown the N-terminally truncated pump to be inactive.

**EXPERIMENTAL PROCEDURES**

**Materials**—The baculovirus AChNPV and the transfer vector pVL 1393, a derivative of pVL841 (Lucow and Summers, 1989), were obtained from Dr. Max Summers (Texas A & M University). The S. frugiperda cell line (Sf9) as well as purified AChNPV DNA were kindly provided by Dr. Salima Mathews (Hoffmann-La Roche, Basel, Switzerland). Other cell lines were obtained from American Type Culture Collection. The TNM-FH medium was from Sigma; Grace's insect medium, DMEM (Dulbecco's modified minimal Eagle's medium), nutrient mixture Ham's F-12, antibiotics, and FCS (fetal calf serum) were purchased from Gibco-BRL (Life Technology AG, Basel, Switzerland). 

The monoclonal antibody 5F10 was described previously (Borke et al., 1987, 1988). The polyclonal antibody directed against the pig endoplasmic reticulum Ca$^{2+}$-ATPase isoform 2b was a kind gift of Dr. F. Wuytack and was described by Eggermont et al. (1986).

**Construction of Recombinant Vectors**—A full-length cDNA coding for the human plasma membrane Ca$^{2+}$-ATPase 4b (hPMCA4b) has been assembled from overlapping partial cDNA clones (Strehler et al., 1990) introducing unique artificial SalI and KpnI restriction sites adjacent to the 5'- and 3'-ends of the coding region, respectively, using standard methods of recombinant DNA technology including the polymerase chain reaction. The cDNA was cut with SalI and KpnI and with a blunt-ended SalI site cloned into pVL 1393 vector. A deletion mutant of the hPMCA4b cDNA coding for a 105-kDa fragment of the pump (replacement of the first 942 base pairs by an artificial start codon and a preceding BamHI site), but extending to the regular C-terminus of the pump, was introduced in a BamHI/KpnI-treated pVL 1393 vector. The 105-kDa fragment has the N-terminus of the previously described 90-kDa fragment (Zurini et al., 1984; Zvaritch et al., 1990), but extends to the C-terminus of the intact pump. The exact boundaries of the baculovirus transfer vector constructs pVL-PMCA and pVL-PMCA105 are displayed in Fig. 1. The inserts of the final constructs were double-strand sequenced using gene-specific primers. The endoplasmic reticulum Ca$^{2+}$-ATPase (ERCA) transfer vector construct was obtained by cutting out the cDNA coding for the pig ERCA-isoform 2b (Eggermont et al., 1989) from a pGEM7-Zf(+) vector with EcoRI and cloning it into the EcoRI site of a pVL 1393 vector. The two possible orientations yielded both the correct construct pVL-ERCA and the nonsense vector pVL-Inf, the source of the control virus used for some negative control infections.

The plasmid construct used for the transfection of COS cells was composed of the full-length cDNA of the plasma membrane Ca$^{2+}$-ATPase 4b isoform (PMCA4b) excised from pVL-PMCA with BamHI and KpnI (Fig. 1), ligated into the vector pSG5 at BamHI and KpnI restriction sites. The KpnI restriction site was introduced into the EcoRI site of a pVL 1393 vector. The two possible orientations yielded both the correct construct pVL-ERCA and the nonsense vector pVL-Inf, the source of the control virus used for some negative control infections.

**Cell Culturing and Transfection**—COS-7 cells were grown to 90% confluence in DMEM with 10% FCS, and 100 μg/ml gentamicin. The cells were then incubated for 2.5 h at 37 °C in 5 ml of DMEM/FCS, containing 100 μM chloroquine, followed by treatment with 10% dimethyl sulfoxide in PBS for 2 min at room temperature, 48-60 h later the cells were harvested by scraping. S. frugiperda (Sf9) cells were grown in monolayer or suspension cultures in a humidified incubator at 29 ± 1 °C using TNM-FH medium supplemented with 10% FCS and 100 μg/ml gentamicin. The culturing of Sf9 cells and all procedures involving them including routine subculturing, transfections, production of high titer viral stocks, and production of recombinant proteins were performed as described in the “Manual of Methods for baculovirus Vectors and Insect Cell Culture Procedures” (Summers and Smith, 1987).

Cells other than the Sf9 or COS-7 were grown to 90% confluence in an humidified incubator at 37 °C, 6% CO$_2$ for 3 days before collection and membrane preparation. All cell lines, including the Ptkt1 cells maintained in DMEM, 10% FCS, and 100 μg/ml gentamicin. For Ptkt1 cells Ham's F-12 was used in place of DMEM.

**Production and Isolation of the Recombinant AChNPV Virus**—2.5 μg of CsCl-purified recombinant transfer vector DNA and 1 μg of wild-type AChNPV viral DNA were used for cotransfection of Sf9 cells by the calcium phosphate precipitation technique (Summers and Smith, 1987). Recombinant viruses, the result of in vivo homologous recombination between the polyhedron sequences of the wild type viral DNA and the recombinant transfer vectors, were amplified by serial dilutions of transfection mixtures and by infecting Sf9 cells in 96-well microtiter plates. After 5 days of incubation, the supernatants were transferred to new plates, and the cells were lysed and transfected to Zeta-probe blotting membranes (Bio-Rad) using a slot blot apparatus. The membranes were hybridized with gene-specific DNA probes according to published procedures (Sambrook et al., 1989) to identify wells containing recombinant viruses. Supernatants from three wells infected with the maximally diluted transfection mixture which were still positive, were assumed to be enriched in recombinant viruses and were used for subsequent plaque purification by screening for occlusion-negative phenotypes or, if recombinant viruses were unsufficiently abundant, by plaque hybridization (Summers and Smith, 1987). One or two rounds of amplification and two to three rounds of plaque assays were necessary to obtain pure recombinant viruses AChPMCA and AChPMCA105. The control viruses AChERCA and AChInf were obtained in a similar way.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting**—Proteins were separated by SDS-polyacrylamide gels essentially according to Laemmli (1970). The sample buffer (2x) was composed of 60 mg of DTT, 50 μg of SDS, 5 mM EDTA, and 0.005% bromphenol blue per ml of stacking buffer. In addition, it contained 400 μg of urea per ml. For immunoblotting analysis the proteins were transferred to nitrocellulose (Towbin et al., 1979). Nonspecific binding was blocked by 5% bovine serum albumin in TBST (10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.05% Tween 20). Primary antibodies were applied in TBST for 1 h at room temperature. After washing the nitrocellulose sheets three times in TBST, the blots were incubated with alkaline phosphatase-coupled secondary antibody (1:7500; Promega) for 40 min, rinsed three times in TBST and developed with the Proteoblot system (Promega).

**Immunofluorescence Experiments on Sf9 Cells**—Sf9 monolayer cells were collected 48 h after the infection, washed once in PBS, and placed on Alcian Blue-coated slides. The cells were then fixed in methanol at −20 °C for 15 min and air-dried. The glass slides were
rehydrated in PBS and blocked for unspecific binding by incubating them in blocking buffer (5% FCS, 0.1% bovine serum albumin, 5% glycerol, 0.04% azide in PBS) for 45 min at 37 °C. The cells were then incubated with primary antibody diluted in blocking buffer (1:1000 for monoclonal antibody SF10, 1:200 for the polyclonal antibody against human CaM) in blocking buffer (0.1% gelatin, 0.04% azide in PBS) for 2 h at 4 °C. The preparations were exhaustively washed in PBS prior to mounting in a medium containing 70% glycerol, 0.33 M Tris-HCl, pH 9.5, and 0.5% propyl gallate as a bleaching agent. Laser scanning confocal microscopy was performed on a Zeiss Axioscope fluorescence microscope equipped with a Bio-Rad MRC 600 confocal laser scanning unit and a Silicon Graphics workstation computer. Pictures were taken with Ilford FP4 black and white film.

Preparation of Crude Membranes from COS and SF9 Cells—Crude membrane deficient in CaM were prepared according to the protocol described by Scully et al. (1982) with some modifications. Infected SF9 cells were harvested at about 61 (AcPMCA) or 68 h (AcPMCA105) after infection by centrifugation at 1600 rpm for 10 min (4 °C) and were resuspended in washing buffer (50 mM Tris-HCl, 130 mM NaCl, pH 7.4, at room temperature), once with washing buffer containing 2 mM EDTA and once again with washing buffer. The cells were swollen in 5 mM Tris-HCl, pH 7.4, 0.2 mM PMSF at a density of 4.2 × 10^6 cells/ml for 15 min on ice, then supplemented with 3 mM DTT and homogenized with 25 strokes in a tightly fitting Dounce homogenizer. The homogenate was diluted five times with 5 mM Tris-HCl, pH 7.4, 0.05 mM MgCl₂, 0.5 mM EDTA, 3 mM DTT, 0.2 mM PMSF, and centrifuged for 20 min at 5000 × g to sediment nuclei and unbroken cells. The pellet was resuspended once again in half the initial homogenization volume by applying the same procedure. The two supernatants were combined and centrifuged at 20,000 × g for 20 min. This pellet was defined as the crude membrane fraction and was washed once with membrane buffer (10 mM Hepes, pH 7.2, 130 mM KCl, 0.5 mM MgCl₂, 0.05 mM CaCl₂, 3 mM DTT, 15% glycerol), recovered at 30,000 × g, and finally resuspended in membrane buffer at a protein concentration of 0.6 mg/ml. The membranes were frozen in liquid nitrogen and stored at −70 °C. The procedure for COS cells was essentially the same as described above except that the storage buffer for the final membrane fraction did not contain Ca²⁺ and Mg²⁺.

Preparation of Total Membranes from Different Cell Lines—The cells were collected by scraping, rinsed once in PBS and once in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl), and resuspended in 50 mM Tris-HCl, pH 7.0, 500 mM NaCl, 75 µg/ml PMSF, 1 mM DTT at 1 to 2 × 10^6 cells/ml. The cells were disrupted by four cycles of freezing and thawing at −70 °C. The pellet was spun down and resuspended in 50 mM Tris-HCl, pH 7.0, 1 mM DTT, 75 µg/ml PMSF at 2-4 mg/ml protein.

Calmodulin Affinity Chromatography—Solubilization of crude SF9 membranes containing CaM affinity chromatography was performed according to Niggli et al. (1987) except that the solubilization was performed for 20 min at a protein concentration of 4 mg/ml and that all column buffers contained 3 mM DTT and 15% glycerol. The column was washed with 9 bed volumes prior to the EDTA elution step. In the case of the purified 105-kDa product, a second purification step was applied by washing the CaM column with 100 bed volumes of the Ca²⁺ buffer prior to the elution step with EDTA. Phosphatidylcholine was used as the stabilizing lipid. The control erythrocyte ATPase was isolated as described (Niggli et al., 1987).

ATPase Activity—The Ca²⁺-ATPase activity was followed by the cold trypsin assay as described by Vorhorst et al. (1991). In the measurements on the membranes, 5 µg/ml of oligomycin was included to eliminate the interference by ATPases in the mitochondrial membranes, and the Ca²⁺-ATPase was assumed to be the fraction of the activity which was sensitive to 3 mM EGTA. In the Ca²⁺ affinity experiments, the release of inorganic phosphate from ATP was measured by the colorimetric method described by Lanzetta et al. (1979). The reaction was performed at 37 °C for 30 min with 250 ng of purified hPMCA in 30 mM Hepes, pH 7.2, 120 mM KCl, 1 mM ATP, 2.42 mM MgCl₂, 1 mM EDTA, 1 mM EGTA and various amounts of Ca²⁺ to produce the desired free Ca²⁺ concentration as calculated by the software program described by Fabiato (1986). ATPase Activity

Experiments on the Phosphorylated Intermediate of the Reaction Cycle—The phosphorylation of membrane samples was carried out at 4 °C in 100 µl of reaction buffer containing 20 mM MOPS, pH 6.8, and 100 mM KCl, in the presence of either 50 µM CaCl₂, 50 µM CaCl₂ plus 50 µM LaCl₃, or 4 mM EGTA. The samples purified by CaM affinity chromatography contained EDTA, and therefore the Ca²⁺ and La³⁺ concentrations were increased to 400 µM. The reaction was started by the addition of [γ-³²P]ATP to a final concentration of 0.1 µM under vigorous stirring and was stopped after 10 with 1.2 ml of ice-cold 8% trichloroacetic acid. The precipitates were spun down in a Microfuge and washed once with 800 µl of distilled water. For the hydroxylamine treatment, the washed pellets were resuspended in 0.5 ml of 0.6 M hydroxylamine, pH 5.2, and kept for 10 min at room temperature prior to precipitation with 300 µl of cold 24% trichloroacetic acid. Control samples were treated in the same way, but 0.6 M sodium acetate, pH 5.2, replaced hydroxylamine. The washed precipitates were suspended in the acidic sample buffer containing urea and separated by acetic SDS-polyacrylamide gel electrophoresis (Sarkadi et al., 1986).

RESULTS

Expression of the PMCA4b Isoform cDNA in the COS Cell System—The activity levels of the expressed PMCA4b cDNA in COS cells were judged from experiments on the catalytic phosphorylation of the ATPase by ATP. In a series of experiments of the type shown in Fig. 2 the highest levels of expressed PMCA, as estimated from densitometric tracings, were 2-4 times higher than those of the arbitrary endogenous pump (densitometric values for the experiment shown in Fig. 2: control, 12.7, expressed, 33.9). Two other phosphorylated protein bands can be seen in Fig. 2 at about 200 and 90 kDa; they most probably represent aggregated (dimerized) forms of the plasma membrane Ca²⁺-ATPase (Niggli et al., 1979) and the catalytic subunit of the Na/K-ATPase, respectively. In these experiments Na⁺ ions in the phosphorylating medium were substituted for K⁺, because separate experiments had indicated that the former reduced the extent of phosphorylation of the ER Ca²⁺-ATPase in the COS cell membranes and improved the overall resolution of the gels.

The catalytic phosphorylation experiments on the expressed pump revealed that its mobility in SDS-gels coincided with that of the endogenous Ca²⁺-pump of COS cells, but was significantly lower than that of the erythrocyte Ca²⁺-ATPase (Fig. 2). This is of interest, since partial protein sequencing of the erythrocyte pump has unambiguously shown it to consist mainly of the PMCA4b isoform originally cloned from a human teratoma cDNA library (Strehler et al., 1990).

Expression of the 105-kDa Fragment in the COS Cell System and Study of Its Activity—The previous trypsin proteolysis work mentioned in the Introduction section on the purified erythrocyte Ca²⁺ pump had suggested that a tryptic fragment of 90 kDa identified in SDS gels retained the basic properties of the pump, i.e. ATP hydrolysis coupled to Ca²⁺ transport. However, all attempts to isolate the fragment in a functionally active state from the complex mixture of other tryptic fragments have so far failed. Expression of the fragment in COS

![FIG. 2. Catalytic phosphorylation of the plasma membrane Ca²⁺-ATPase in the COS cell system. Membrane fractions (20 µg of total protein) of erythrocytes (1) and of COS cells transfected with either a PMCA4b cDNA plasmid construct (2) or with a control plasmid (3) were treated with [γ-³²P]ATP in the presence of 50 µM Ca²⁺ and 50 µM La³⁺. The proteins were resolved on SDS gel under acidic conditions and were subjected to autoradiography. Other experimental details are found under "Experimental Procedures."](image-url)
cells was thus attempted to establish whether the fragment acts as an active Ca\(^{2+}\)-transporting ATPase.

A fragment of the PMCA4b cDNA coding for the sequence starting at the N terminus of the 90-kDa polypeptide (Zvaritch et al., 1990) and extending to the C terminus of the pump was cloned into the pSG5 vector at BamHI and KpnI restriction sites. The molecular mass of the designed polypeptide was expected to be 105 kDa. Since attempts were planned to isolate the expressed, truncated version of the pump by CaM affinity chromatography (see below), the presence of the C-terminal portion containing the CaM binding domain was essential. The expression of the cDNA coding for the 105-kDa fragment was monitored by immunoblotting experiments: total lysates of cells expressing cDNAs of either the PMCA4b or the 105-kDa fragment were subjected to gel electrophoresis, and the blotted proteins were stained with affinity-purified polyclonal antibodies against the human erythrocyte membrane Ca\(^{2+}\) pump (Fig. 2a). As judged from these experiments, the levels of the expression of the 105-kDa fragment were comparable to those of the full-length pump.

In disrupted cells the expressed polypeptides were found associated with the membranous fractions. However, phosphorylation experiments with \(\gamma\)-\[^{32}\text{P}\]\text{ATP in the presence of Ca\(^{2+}\), and of Ca\(^{2+}\) plus La\(^{3+}\), failed to reveal any activity in the 105-kDa fragment (Fig. 2b). A phosphorylated band in the molecular mass region of the 105-kDa fragment was observed when Ca\(^{2+}\) ions were present in the phosphorylating medium. However, the intensity of phosphorylation was the same in the controls and in the samples from the cells which had expressed the 105-kDa fragment, suggesting that the phosphorylation was due to the activity of the endogenous ER Ca\(^{2+}\)-ATPase. Indeed, in the presence of Ca\(^{2+}\) and La\(^{3+}\), which specifically favor the accumulation of the phosphorylated intermediate of the plasma membrane Ca\(^{2+}\)-ATPase, while degrading that of the ER pump, no phosphorylation was observed in the molecular mass region around 105 kDa.

Expression of PMCA4b in the Baculovirus System—To achieve the higher expression levels of the PMCA4 pump necessary for studies on the isolated expressed enzyme, the baculovirus system was tried. The recombinant baculoviruses AcPMCA and AcPMCA105, containing the cDNA coding for the full-length or the truncated version of the human ATPase isoform 4b, respectively, were isolated as described under “Experimental Procedures.” Infection of Sf9 monolayer cells with AcPMCA (multiplicity of infection of 10) resulted in the time-dependent expression of a protein of about 142 kDa that was well visible on a Coomassie Blue-stained gel and that was found to bind the erythrocyte Ca\(^{2+}\)-ATPase-specific monoclonal antibody 5F10 in Western blots. As is typical for the baculovirus system, the expression product appeared after 22 h, reached a maximum level at 70 h, and decreased thereafter, coinciding with the appearance of degradation products due to increased cell lysis. Interestingly, as the expression times became longer, an increasing fraction of the expression product only entered the gel when a sample buffer containing urea (see “Experimental Procedures”) was used. The time course for the expression of the mutant virus AcPMCA105 was found to be somewhat faster. Sf9 suspension cultures generally yielded smaller expression levels, but were nevertheless useful to produce large amounts of recombinant protein.

Targeting of the hPMCA Protein in Sf9—To establish whether the expressed hPMCA protein was targeted to the plasma membrane, laser scanning confocal microscopy was carried out in combination with immunofluorescence staining of AcPMCA- and AcPMCA105-infected Sf9 cells with monoclonal antibody 5F10, directed against the erythrocyte Ca\(^{2+}\) pump (Fig. 4, a and b). Control infected cells (AcInf) yielded only very faint signals with the antibody (Fig. 4c) showing that the endogenous Ca\(^{2+}\) pump is apparently very scarce in Sf9 cells. Alternatively, the antibody did not cross-react with the endogenous insect cell pump: this seems a likely possibility, since the antibody failed to detect any protein in Western blots with 40 \(\mu\)g of uninfected Sf9 cell membranes. Because Sf9 cells contain very large nuclei, which became even enlarged after infection (Currie et al., 1991), it was difficult to assign signals to specific subcompartments in the very small region of the cell left free by the nucleus under the cell surface. To validate the interpretation of the hPMCA expression signals, Sf9 cells were thus infected with a virus containing the cDNA coding for the pig endoplasmic reticulum Ca\(^{2+}\)-ATPase isofrom 2b (AcERCA) (Eggermont et al., 1989), and a polyclonal antibody directed against the SERCA2b pump protein (Wuytack et al., 1989) was used to detect and locate the expressed protein (Fig. 4d). Control infected cells (AcInf) yielded no signal (Fig. 4e), confirming the specificity of the antibody. On comparing the three expressed pumps, it is clear that the full-length and the truncated version of the hPMCA protein accumulated in the cell periphery. By contrast, in the case of the ERCA2b pump, strong reaction was observed around the nucleus of Sf9 cells and only a slight reaction in the cell periphery. Membrane preparations of the cells infected with the AcERCA virus yielded high amounts of expressed active pump.

Isolation of the Expressed hPMCA Proteins—The relatively high expression level of the recombinant hPMCA pump in insect cells permitted experiments aimed at isolating the expressed enzyme. The CaM affinity chromatography procedure initially developed for the isolation of the erythrocyte ATPase was used. The first step was the preparation of crude membranes from infected Sf9 cells as described under “Experimental Procedures.” “Crude” membranes were separated from the nuclei and the cytosol, but no other subfractionation was applied. In Fig. 5a, the proteins from 20-\(\mu\)g membranes from uninfected (lane 1), AcPMCA-infected (lane 2), and AcPMCA105-infected (lane 3) Sf9 cells are shown. The
Ca\textsuperscript{2+} Pump Expression

**Fig. 4. Targeting of the expressed PMCA proteins.** Sf9 cells were infected with either AcPMCA (a), AcPMCA105 (b), AcERCA (d), or with the nonsense virus AcInf (c, e), fixed in methanol and incubated with the following antibodies: a monoclonal antibody against the erythrocyte ATPase (5F10) was used for the cells displayed in a, b, and c, and a polyclonal antibody against the endoplasmic reticulum Ca\textsuperscript{2+}-ATPase was used for the cells displayed in d and e. Fluorescein isothiocyanate-conjugated secondary antibodies were used to produce the fluorescence visualized in the laser scanning confocal microscope.

Expression products of about 142 and 105 kDa (arrows) were absent in the control cell lane. The membranes of infected cells were then solubilized and used for the CaM column chromatography step. EDTA eluates of the Ca\textsuperscript{2+}-washed column were subjected to gel electrophoresis and silver-stained as shown in Fig. 5b: the expression products from AcPMCA (lane 2) and AcPMCA105 (lane 3)-infected Sf9 cells were separated on 4–12% SDS gel and stained with Coomassie Blue. The full-length (gel mass, ~142 kDa) and the 105-kDa expression products are not seen under these conditions in noninfected cell membranes. b, 200 ng of purified, expressed full-length PMCA (lane 2), of the 105-kDa product (lane 3) and of the purified erythrocyte ATPase (lane 1) were electrophoresed and visualized by silver staining. The double band detected at about 50-kDa was a buffer artefact, since it was also present in lanes containing no protein. c, a gel run as in b was used for electrophoretic transfer. The proteins were detected by a polyclonal antibody directed against the erythrocyte ATPase as described under “Experimental Procedures.”

**Fig. 5. Isolation and gel analysis of the expressed hPMCA proteins.** a, 20 μg of total membrane proteins of noninfected (lane 1), AcPMCA-infected (lane 2), and AcPMCA105-infected (lane 3) Sf9 cells were separated on 4–12% SDS gel and stained with Coomassie Blue. The full-length (gel mass, ~142 kDa) and the 105-kDa expression products are not seen under these conditions in noninfected cell membranes. b, 200 ng of purified, expressed full-length PMCA (lane 2), of the 105-kDa product (lane 3) and of the purified erythrocyte ATPase (lane 1) were electrophoresed and visualized by silver staining. The double band detected at about 50-kDa was a buffer artefact, since it was also present in lanes containing no protein. c, a gel run as in b was used for electrophoretic transfer. The proteins were detected by a polyclonal antibody directed against the erythrocyte ATPase as described under “Experimental Procedures.”

The yield of purified PMCA in the purification experiments on the infected Sf9 cells was estimated to be about 1.1% of the total membrane protein of the cells (93 μg of purified pump protein from 8.3 mg of membrane protein in the experiment shown in Fig. 5). Lane 1 shows the control purified erythrocyte Ca\textsuperscript{2+}-ATPase, which often shows, in SDS gels, degradation products of 125 and 90 kDa. As already observed in the COS cell system (see above), also in the baculovirus system the expressed PMCA apparently run with lower mobility than the erythrocyte enzyme. The immunoreactivity of the isolated expressed proteins with a polyclonal antibody against the erythrocyte pump is shown in Fig. 5c. It is important to mention at this point that the expression time was critical for the successful isolation of the recombinant pump protein. Long expression times (see “Experimental Procedures”) resulted in higher losses of the hPMCA protein into the nuclear pellet and in the inefficient solubilization of the protein from the crude membrane fraction, even if larger amounts of Coomassie Blue-stainable, immunoreactive protein were clearly present in the membranes. This is consistent with the previously mentioned observation that an area containing sample buffer was required at late stages of infection to force the protein to enter the SDS gel. This indicates that the hPMCA pump formed stable aggregates as its concentration in the cell increased as a result of the prolonged expression times.

ATPase Activity of the Expressed Proteins—To establish whether the pump expressed by Sf9 cells was active, Ca\textsuperscript{2+}-ATPase activity measurements and the formation of the phosphorylated intermediate from ATP were studied. Table I summarizes the ATPase results (see “Experimental Procedures”). Infection of Sf9 cells with AcPMCA resulted in the increase of the membrane-associated ATPase activity in the presence of Ca\textsuperscript{2+}, but in the absence of CaM, from 27 to 71 nmol of ATP hydrolyzed per min and mg protein: this activity level included contributions from other ATPases different from the Ca\textsuperscript{2+}-ATPase of the plasma membrane, chief among them the Ca\textsuperscript{2+}-ATPase of endoplasmic reticulum. The activity increased from 34 nmol in control membranes to 216 nmol in those from infected cells if 5 μg/ml CaM were included in the medium: the stimulation factor by CaM was thus 3.06 (as compared to 1.25 in the membranes from noninfected cells). Evidently, the specific expression of large amounts of the PMCA pump decreased the relative contribution of the non-CaM-stimulated ATPase(s) to the overall measured activity level. By contrast, expression of the truncated AcPMCA105 pump caused no significant changes in the basal and in the CaM-stimulated ATPase activity. The CaM-stimulated activity of the purified expressed hPMCA was 1,810 nmol × min\textsuperscript{-1}.
The ATPase activity was measured as described under "Experimental Procedures." The values are expressed as nmoles ATP hydrolyzed × mg⁻¹ × min⁻¹. The samples were either freshly prepared total cell membranes or CaM affinity-purified PMCA and PMCA105 proteins expressed by the infected cells. The activity of the membrane fractions declined by 20–27% upon freezing. The experiment was carried out on two different preparations, whose results were very similar. The table shows one of them.

### Table I

**ATPase activity of membranes from PMCA-infected Sf9 cells and of the isolated PMCA protein**

| Membranes from | Erythrocytes | Noninfected Sf9 | AcPMCA-infected Sf9 | AcPMCA105-infected Sf9 |
|----------------|--------------|-----------------|----------------------|------------------------|
| Ca²⁺ (control) | 5.0          | 27.2            | 70.7                 | 29.2                   |
| Ca²⁺/CaM       | 20.0         | 34.0            | 216.2                | 31.0                   |
| Stimulation by CaM | 4.00       | 1.25            | 3.06                 | 1.06                   |

| Isolated PMCA from | Erythrocytes | AcPMCA-infected Sf9 | AcPMCA105-infected Sf9 |
|-------------------|--------------|----------------------|------------------------|
| Ca²⁺ (control)    | 780.0        | 525.0                | 0                      |
| Ca²⁺/CaM          | 3770.0       | 1810.0               | 0                      |
| Stimulation by CaM | 4.83        | 3.45                 | 0                      |

Fig. 6. Ca²⁺ affinity of the expressed, purified PMCA. The activity was measured at different free Ca²⁺ concentrations in the presence (open symbols) and absence (filled symbols) of CaM by monitoring colorimetrically the release of inorganic phosphate (see the "Experimental Procedures"). The values reported are expressed in absorbance units at 660 nm.

× mg⁻¹ as compared to 525 in the absence of CaM, i.e. the stimulation factor was 3.45. The expressed 105-kDa product was completely inactive. The Ca²⁺ affinity of the isolated expressed hPMCA, estimated by following the release of inorganic phosphate from ATP (Fig. 6), yielded a $K_M$ value of about 1 and 5 μM in the presence and absence of CaM, respectively. The values were thus similar to those of the erythrocyte pump. The findings of Table I and Fig. 6 were further supported by the experiments of Fig. 7. The expressed PMCA protein was able to form the hydroxylamine-sensitive phosphorylated intermediate (Fig. 7a). However, the extremely small amounts of protein available made the visualization of the pump band in the absence of the stabilizing agent La³⁺ very difficult; as expected of the plasma membrane Ca²⁺ pump (Schatzmann and Burgin, 1978; Szasz et al., 1978), the steady state concentration of the phosphorylated intermediate was enhanced by La³⁺. At about 110 kDa, a second hydroxylamine-sensitive, but Ca²⁺-independent phosphorylation band was visible. It probably derived from small amounts of a contaminant (P-type pump) protein which apparently became strongly phosphorylated since no corresponding band was visible in silver-stained gels (see Fig. 5b).

To avoid interference by this contaminant protein with the expressed 105-kDa truncated hPMCA, the latter was subjected to a second round of CaM affinity purification with the expressed 105-kDa truncated hPMCA, whose results were very similar. The table shows one of them.

**Electrophoretic Mobility of PMCA**—As mentioned above for the PMCA4 isoform expressed in COS cells (Fig. 2) also the pump expressed in the Sf9 insect cells (Fig. 5) showed a lower gel mobility than the pump of erythrocytes. It comigrated instead with the COS cells endogenous pump (see Fig. 2). To test whether this was a specific property of the PMCA4 isoform or an artifact of the expression system employed, other cell types were analyzed. The ATP-dependent, Ca²⁺- and La³⁺-stimulated phosphorylated intermediates of three different cell lines are shown in Fig. 8. The membranes of L-M (TK−) cells (a mouse, connective tissue-derived line), of HeLa cells (a human epithelial-like line), and of PtK1 cells (a marsupial kidney-derived line), showed two major phosphoenzymes: one at 110 kDa, which was Ca²⁺-dependent but La³⁺-insensitive (or even La³⁺-inhibited) and thus most likely belonged to the ER Ca²⁺ pump, and one at about 142 kDa,

![Fig. 6](image_url)

**Fig. 7. Phosphorylation of the expressed and purified PMCA and 105-kDa proteins.** a, 200 ng of isolated PMCA protein were phosphorylated in the presence of either 400 μM Ca²⁺ (lane 1), 400 μM Ca²⁺ plus 400 μM La³⁺ (lane 2), 4 mM EGTA (lane 3), or 400 μM Ca²⁺ plus 400 μM La³⁺ followed by hydroxylamine treatment (lane 4). Lane 5 represents the control phosphorylation of the purified erythrocyte Ca²⁺-ATPase in presence of Ca²⁺ and La³⁺. The phosphorylated samples were separated on 6% polyacrylamide gels. The dried gels were exposed for 2 days at −70 °C. Additional details are found under "Experimental Procedures." b, 200 ng of the isolated 105-kDa protein (lanes 1–4) and of the erythrocyte ATPase (lane 5) were phosphorylated and electrophoresed as in a. The 105-kDa protein was repurified on a micro-CaM column as discussed in the text using a more exhaustive washing with Ca²⁺ to eliminate the 110-kDa contaminating protein.
of L-M(TK-) membrane proteins were phosphorylated in the presence of 50 μM La3+ and 50 μM Ca2+ as described under "Experimental Procedures" and separated on acidic 5–12.5% gradient SDS-polyacrylamide gels. The dried gels were exposed for 2 days at -70 °C. Other details are found under "Experimental Procedures."

which was specifically detected in the presence of Ca2+ and La3+ (Fig. 8, lanes 1–3). The band corresponding to this phosphoenzyme migrated with slower velocity than that of the erythrocyte Ca2+ pump (Fig. 8, lane 4) but had the same mobility as the Ca2+-La3+-dependent phosphoenzyme of PMCA4 expressed in the Sf9 and COS cells (compare Figs. 2 and 5). A number of other cell lines derived from rat, hamster, and human tissues yielded essentially the same results (not shown).

**DISCUSSION**

The importance of expressing P-type ion pumps in vitro is obvious. Problems like the identification of functionally important sites, the targeting of pumps to different membrane systems, the structural reasons for the specificity of the transported ions all largely depend on the successful in vitro expression of the enzymes. The family of P-type ion-motive ATPases (Pederesen and Carafoli, 1987a; Pederesen and Carafoli, 1987b) has now grown to include enzymes that transport Na+, K+, Ca2+, Mg2+, H+, Cd2+, either isolated or in an obligatorily concerted reaction, in eucaryotic or procaryotic cells. Three of these pumps have now been expressed in vitro systems: the Ca2+ pump of sarcoplasmic/endoplasmic reticulum (Campbell et al., 1991; Maruyama and MacLennan, 1988), the H+ pump of the fungal plasma membrane (Holzer and Hammes, 1989; Portillo and Serrano, 1989), and the α and β subunits of the Na+/K+ pump (Price and Lingrel, 1988; Takeyasu et al., 1987). The expression systems have been successfully used for site-directed mutagenesis work that has permitted others to assign with a high degree of probability important sites, e.g. that are responsible for the inhibition by vanadate (Ghislain et al., 1987) and, more generally, a number of sites/residues that are apparently essential to the function of all pumps of this class, even if the reasons for their critical importance were not immediately obvious at the outset (MacLennan, 1990). Very important sites on which mutagenesis work is now beginning to shed light are those linked to the binding and transport of the ions during the catalytic cycle: contrary to earlier expectations (Brandl et al., 1986) recent work on the expressed sarcoplasmic reticulum Ca2+ pump (Clarke et al., 1989) has provided strong indications that these sites may be located within four of the ten predicted transmembrane domains of the pump.

All of the expression systems so far used have resulted in the enrichment of the P-type pumps in the membranes of the expressing cells. The whole cells, or membrane preparations derived from them, have then been the objects of study. In no case, however, have expression experiments been published in which the system used has permitted the production of amounts of pumps sufficiently large for purification attempts. As a consequence, no protein chemistry work has so far been possible on any of the expressed P-type pumps.

The work described in this contribution has shown that the human PMCA4 isoform could be successfully expressed in the two systems studied. This isoform is the only human one for which full-length DNA clones have been constructed, and was thus practically an obligate choice among the dozen or so isoforms presently known. Full-length clones of the other isoforms will presumably soon become available, and reasonable hopes can thus be entertained that their expression will rapidly follow. It must be emphasized, however, that the expression of the plasma membrane Ca2+ pump has proven to be unexpectedly difficult: the amounts of active protein expressed, as compared for example to those of the Ca2+ pump of sarcoplasmic reticulum (Maruyama and MacLennan, 1988), have been substantially smaller under all experimental conditions tested in the COS cell system. Densitometric tracings from a number of experiments of the type shown in Fig. 2 have yielded levels of expression of the active ERCA pump in the COS cell system averaging 12-fold the controls. Expression of the PMCA4b isoform in the COS cell system to levels approximately similar to those reported here have been recently detected by Penniston and co-workers3 in experiments in which the successful expression was attributed to the modification of the consensus sequence of the PMCA4b cDNA around the starting codon. Since in the present experiments the original PMCA4b cDNA sequence was used, the reason for the modest levels of active Ca2+ pump expression in the COS cell system is thus apparently not a "weak" consensus sequence around the starting codon. Still unknown intracellular regulatory mechanisms may prevent the overproduction of the pump protein in COS cells. The baculovirus system compares favorably to the COS cell system, but even in its case the activity tests (i.e. the CaM-stimulated Ca2+-dependent ATP hydrolysis) have indicated apparent expression levels less than 10-fold higher than those of noninfected Sf9 cells. However, for the reasons mentioned under "Results," these enrichment factors are most likely underestimated. This is also indicated by the finding mentioned above that no endogenous pump could be isolated from Ac-PMCA105-infected Sf9 cells. The reasons for the lower expression levels of the plasma membrane pump, as compared, for example, to those of the Ca2+ pump of sarcoplasmic reticulum, are presently being investigated: one possibility for which preliminary supporting evidence has been obtained, at least for the COS cell system, is the particular susceptibility of the expressed pump to intracellular proteases. It has been shown that the plasma membrane Ca2+ pump is peculiarly sensitive to the action of the intracellular Ca2+-dependent protease calpain (James et al., 1989), as compared for example to the Ca2+ pump of sarcoplasmic reticulum, which is completely resistant to it (Wang et al., 1989). Work on alternative expression systems is currently underway. Despite the difficulties experienced, it has been possible to isolate the pump expressed in Sf9 cells using a CaM affinity chromatography column. As mentioned in the Introduction section, this is thus the first report of a successful isolation of an expressed P-type pump: suitable scaling-up procedures can now be expected to yield amounts of the expressed protein adequate for protein chemistry work.

The expressed protein, embedded in the native membrane system in both the COS and the Sf9 cells, or purified from

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3 J. T. Penniston, personal communication.
the latter cells, has proven to be active. Actual tests of ATP-dependent Ca\(^{2+}\) transport have been carried out only occasionally, since it is generally assumed that the plasma membrane Ca\(^{2+}\) pump never becomes uncoupled (see Schatzmann (1985) for a comprehensive discussion of the matter). Ca\(^{2+}\)-dependent ATP hydrolysis (and formation of the phosphorylated intermediate) are thus adequate tests for the functional integrity of the pump. The CaM stimulation factors observed on the purified pump were in the range of those routinely measured in normal pump preparations, e.g. from erythrocytes. Although not shown under "Results" for reasons of space, gel overlay experiments with \(^{125}\)I-CaM have yielded the expected high affinity for the modulator (i.e. K\(_m\) values in the nanomolar range). In addition to being active, most of the expressed pump was apparently correctly targeted to the appropriate membrane system, i.e. the plasma membrane. The confocal microscopy experiments on the baculovirus system have shown that the expressed plasma membrane pump was concentrated in the extreme periphery of S9 cells compared to the expressed endoplasmic reticulum Ca\(^{2+}\text{-ATPase}\) which occupied the entire space between the nucleus and the plasma membrane. Although the spatial resolution of the technique did not permit the exclusive assignment of the expressed plasma membrane pump and its truncated version to the plasma membrane, it appears very probable that this was indeed the case. Thus, at least a significant fraction of the pump was apparently targeted correctly to the plasma membrane, while the expressed SERCA pump was clearly distributed throughout the perinuclear space, i.e. in the cell region where the endoplasmic reticulum is normally mostly concentrated. One unexpected result with both expression systems has been the slightly higher apparent molecular mass of the expressed protein with respect to that of the erythrocyte pump, so far considered as the reference enzyme for this multigene family of proteins. Clearly, the mass of the erythrocyte protein now appears to be the exception rather than the rule, as also indicated from the results on the apparent mass of the endogenous pump in other cell lines shown in Fig. 8. The reasons for the (slightly) lower apparent molecular mass of the erythrocyte pump are presently being investigated, the working hypothesis being some form of post-translational processing.

An important portion of the work has dealt with the expression of the N-terminally truncated 105-kDa fragment of the pump. The reasons for the importance of expressing this fragment have been discussed in the Introduction section: although the N-terminally truncated fragment had been isolated in the original trypsin proteolysis work by Zurini et al. (1984) and claimed to be active, its very low activity levels could have been due to the contamination of the fragment preparation with traces of the intact ATPase. The decision to use the entire sequence from the N terminus of the 90-kDa fragment to the C terminus of the pump, rather than to the C terminus of the 90-kDa fragment (Zvaritch et al., 1990) in the expression experiments was based on the observation that the portion of the pump downstream of the C terminus of the 90-kDa fragment was certainly removed by trypsin. Since this portion of the pump is not involved in the catalytic cycle, and is entirely extramembranous, its absence or presence most likely does not influence the basic function and membrane architecture of the pump. The results have shown that the N-terminally truncated fragment could be expressed by both systems used. They have also shown that the fragment was inactive, i.e. the activity of the purified fragment preparations observed in the early work by Zurini et al. (1984) was evidently due to traces of contaminant intact pump. This was shown for the fragment purified from S9 cells by CaM affinity chromatography, and also for the fragment embedded in the membrane environment for both expression systems. This last point is worth a comment: the bulk of the expressed truncated fragment was apparently still membrane-associated, as also shown by the confocal microscopy experiments on S9 cells. Thus, whatever the reasons for the inactivity of the fragment, which are obviously related to the absence of the portion of the pump containing the first two transmembrane domains and the so-called transducing domain, they apparently do not prevent the targeting of the truncated pump to the membrane, at least in S9 cells. Whether the insertion process leads to the correct folding of the truncated product in the membrane is, however, an open question.

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