Expression, Purification, and Characterization of Isoform 1 of the Plasma Membrane Ca\(^{2+}\) Pump

FOCUS ON CALPAIN SENSITIVITY*

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The plasma membrane Ca\(^{2+}\) ATPase isoform 1 (PMCA1) is ubiquitously distributed in tissues and cells, but only scarce information is available on its properties. The isoform was overexpressed in Sf9 cells, purified on calmodulin columns, and characterized functionally. The level of expression was very low, but sufficient amounts of the protein could be isolated for biochemical characterization. The affinity of PMCA1 for calmodulin was similar to that of PMCA4, the other ubiquitous PMCA isoform. The affinity of PMCA1 for ATP, evaluated by the formation of the phosphorylated intermediate, was higher than that of the PMCA4 pump. The recombinant PMCA1 pump was a much better substrate for the cAMP-dependent protein kinase than the PMCA2 and PMCA4 isoforms. Pulse and chase experiments on Sf9 cells overexpressing the PMCA pumps showed that PMCA1 was much less stable than the PMCA4 and PMCA2 isoforms, i.e. PMCA1 had a much higher sensitivity to degradation by calpain. The effect of calpain was not the result of a general higher susceptibility of the PMCA1 to proteolytic degradation, because the pattern of degradation by trypsin was the same in the three isoforms.

The plasma membrane Ca\(^{2+}\) ATPase (PMCA)\(^1\) pumps Ca\(^{2+}\) out of the cell, reducing its concentration in the cytosol to the level compatible with the messenger function (1). In excitable tissues, e.g. heart, it does so in concert with the Na\(^+/\)Ca\(^{2+}\) exchanger (2). The pump has been detected in all mammalian cells studied so far (1, 3), although differences in the level of its expression have been observed (4). cDNA cloning has identified four independent PMCA transcripts in human and rat tissues, and the corresponding human genes have been located on four different chromosomes (3, 5–7). The primary transcripts are expressed in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms. The primers are spliced in a complex way: two major sites of splicing, termed sites C and A, in principle produce about 30 different isoforms (1). The cloning work has indicated that certain transcripts of the PMCA isoforms and of their splicing products have striking tissue-specific expression (8–14). For example, although PMCA1 and PMCA4 have been detected in all tissues examined, PMCA2 and PMCA3 have only been found in significant amounts in the brain and a few other tissues. These results on the transcripts have been confirmed at the protein level with isoform-specific antibodies (13). Quantitative analysis has shown that, in general, the human PMCA1 product is more abundant than the PMCA4, both at the mRNA (4) and the protein level (13).

The overexpression of the PMCA4 and the PMCA2 proteins has permitted the study of their biochemical properties (15–19). A number of methods (transient transfection, stable cell lines, vaccinia virus as a vector) have been used (20–22), significantly advancing knowledge on the differential properties of the isoforms. Strikingly, however, very little information is available on PMCA1, which is the most common isoform. Only one successful attempt to express this isoform (a truncated variant C) has so far been reported (23). Difficulties in expressing this isoform have been repeatedly reported (23, 24).

The PMCA pump is a substrate of intracellular proteases. Initial observations had indicated that it was a substrate for the Ca\(^{2+}\)-dependent protease calpain (CANP, EC 3.4.22.17) (25), and more recent work has shown that effector caspases (e.g. caspases 1 and 3) also cleave PMCA pump isoforms (22, 26). Both activation and inactivation of PMCA2 and PMCA4 by caspases have been reported (22, 26). Calpain attacks calmodulin binding enzymes (27), removing portions of the calmodulin binding sequence and leading to their “irreversible” activation. The protease contains regions with strong homology to calmodulin (domains IV and VI; Refs. 28, 29), which may be important in directing it to its target sequences. Support for this concept was provided by the binding of calpain to the calmodulin binding domain of the erythrocyte pump (27). The proteolysis of the pump by calpain removes most of the C-terminal portion protruding into the cytosol, leaving behind a constitutively active fragment of about 124 kDa. The process has been studied on erythrocyte membranes or on the pump purified from them, i.e. on a mixture of the pumps isoforms present in erythrocytes, PMCA1, and PMCA4 (13). Because PMCA4 represents at least 80% of the total erythrocyte pump, it was not surprising that most of the peptides isolated after calpain digestion should have derived from this isoform (30). Nevertheless, the failure to detect high molecular mass peptides deriving from PMCA1 indicated that the latter isoform had different calpain sensitivity.

The work described here was performed to characterize biochemically the human PMCA1 isoform, including its calpain sensitivity. The pump was expressed in Sf9 insect cells with the help of the baculovirus system, but a complex DNA manipula-
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EXPERIMENTAL PROCEDURES

Materials—m-CAMP was isolated from freshly collected human erythrocytes as described in Ref. 27. The TNN-FH medium was from Sigma. Antibiotics and fetal calf serum were purchased from Invitrogen. The monoclonal antibody 5F10 was obtained from Milan Analytic AG (Hoffmann-La Roche). Nitrocellulose was from Schleicher & Schuell (Dassel, Germany). Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate were from Promega (Madison, WI). All other reagents were of the highest purity grade commercially available.

Construction of the Expression Vectors—To construct a full-length cDNA of human PMCA1, two clones (t5, t8.1) were used (32). The human PMCA1 isoform was used (also known as PMCA1A). The vectors used for the constructions were the pTZ1819 (United States Biological, Swamscott, MA), the pSG5 (Stratagene, GmbH, Zurich, Switzerland), and the pVL1393 (provided by Dr. M. D. Summers, Texas A&M University, College Station, TX). DNA was purified by CsCl centrifugation (33) or by Qiagen columns (Qiagen, Chatsworth, CA). Different attempts to ligate fragments together to obtain a full-length construct resulted in a number of deletion-creating products. The use of PCR-directed mutagenesis to create suitable restriction sites or cDNA fragments was similarly unsuccessful. In a first step an XbaI, located in the 3'-end, in BamHI (nt 950, HUMMPCA, GenBank™ accession number J04027) fragment, was subcloned together with a 900-bp SalI, blunt ended, cut by BamHI fragment. This vector was used because it yielded cut vector. This vector was used because it yielded

Preparation of RNA, Northern Blotting, and Preparation of Digoxigenin Probes—2—4 × 10⁶ Sf9 cells were infected with recombinant baculovirus for 48 h. The cells were collected, washed twice in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and resuspended in 600 μl of 20 mM Tris-HCl, pH 7.4, 10 mM NAc, 3 mM MgCl₂ on ice before the addition of 100 μl of 1 M dithioerythritol (Invitrogen). Cytoplasm was purified as described in Ref. 35. Northern blotting was performed essentially as described in Ref. 36. DNA fragments were labeled with digoxigenin-dCTP (Roche Applied Science), using the protocol suggested by the manufacturer.

Preparation of Membranes—Sf9 cells were seeded at a density of 10⁶/cm² and left to attach for 1 h. They were infected at a multiplicity of infection of 5–10 for 1 h, and then the inoculum was replaced by fresh medium. After 48 h the infected cells were collected, washed twice with TBS (25 mM Tris-HCl, pH 7.5, 150 mM NaCl), and resuspended in 2 ml of 10 mM Tris-HCl, pH 7.5 (1 × 10⁶ cells/ml). After 10 min on ice, the cells were homogenized in the presence of 75 μg/ml phenylmethylsulfonyl fluoride, 0.5 mM dithioerythritol, 5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml pepstatin. The homogenate was diluted by adding an equal volume of 10 mM Tris-HCl, pH 7.5, 20% sucrose, and 300 mM KC1 and centrifuged at low speed (750 × g) for 5 min. The supernatant was centrifuged at 100,000 × g and resuspended in 20 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 500 μM MgCl₂, 50 μM CaCl₂.

In some cases the membranes were prepared by a freeze and thaw procedure: the cells were washed twice with TBS, resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM dithioerythritol, 75 μM phenylmethylsulfonyl fluoride, and 1 mM EDTA and disrupted by three cycles of freezing at –70 °C and thawing at 37 °C.

Determination of the Protein Stability by Pulse and Chase Experiments—48 h after infection the cells were washed twice, incubated with methionine-deficient minimal Eagle’s medium (Invitrogen), and buffed to pH 6.2 with 25 mM MES-KOH. After 20 min at 27 °C, the same medium containing 200 μCi/ml [³⁵S]Met (Amersham Biosciences) was added and incubated for 3 h at 27 °C. A portion of the cells was collected (time = 0). To the remainder a 10,000 excess of cold Met in TNM-FH, 10% fetal calf serum, 50 μg/ml gentamicin was added, and the cells were transferred to 27 °C. Membranes were prepared from them by the freeze and thaw method described above, solubilized in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1% SDS and boiled for 5 min. Membrane proteins corresponding to 3 × 10⁶ cpm were diluted in 500 μl of NEM formalin (Tris-HCl, 7.5, 150 mM NaCl, 1 mM EDTA, 0.2% gelatin, 0.1% Na₂O₃, 0.2% SDS, 0.3% Nonidet P-40) and mixed with 1–2 μl of monoclonal antibody 5F10. After gentle shaking for 1–2 h at 4 °C, 20–30 μl of protein A-Sepharose CL-4B were added, and the incubation was continued for 2 h or overnight at 4 °C. The pellet was washed twice with NEM, twice with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Na₂O₃, 0.3% Nonidet P-40, twice with 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, and resuspended in SDS-PAGE loading buffer.

Activity Measurements—The ATPase activity was measured as described in Ref. 37. The assays were performed on 10–30 μg of membrane proteins or, in the case of the purified protein, with 50–200 ng of protein in 500–1000 μl of 120 mM KC1, 30 mM HEPES-KOH, pH 7.2, 1 mM MgCl₂, 0.5 mM EGTA, 0.5 mM EDTA, 1 μM thapsigargin, and 5 μM calmodulin (Calbiochem). The amount of CaCl₂ used was such that the free calcium concentration desired was calculated as described in Ref. 38. The formation of the phosphoenzyme intermediate from ATP was followed as described before (18): additional details are given in the legends for the figures. In brief, total membrane proteins or purified enzymes were diluted in 20 mM MOPS-KOH, pH 6.8, 100 mM KC1 in the presence of different concentrations of Ca²⁺—La³⁺. Incubation was started by the addition of [γ-³²P]ATP (100–300 Ci/mmol) on ice and stopped after 20 s by the addition of 6% trichloroacetic acid, 1 mM KH₂PO₄. The pelleted proteins were separated on acidic gels (39). After drying, the gels were exposed at −70 °C for 2–5 days.

SDS-PAGE, Western Blotting, and Protein Determination—Proteins were separated on SDS-polyacrylamide gels, and immunoblotting was performed as described in Refs. 40 or 39. Protein concentrations were determined as described in Ref. 37. In some cases the gels were stained with a silver impregnation method (41). The proteins were transferred to nitrocellulose or to polyvinylidene difluoride membranes (Schleicher and Schuell, New York, NY). The transferred proteins were detected by a peroxidase-conjugated anti-mouse antibody (Amersham Biosciences). Membranes were stained with Coomassie blue and scanned with a computerized densitometer (Canon, Japan). The presence of each isoform was determined by computerized video densitometry (Pharmacia, Uppsala, Sweden) and quantitated relative to the signal obtained with the PMCA1 isoform, which was used as a standard.
The immunoprecipitated proteins were fractionated by SDS-PAGE gels. The dried gels were exposed at −70°C for 3–5 days. The arrows on the left of the gels indicate the position of the pumps.

\[ \text{Calmodulin Overlay} \]

The proteins were transferred to nitrocellulose membrane sheets (42). Nonspecific binding was blocked by 1% bovine serum albumin in TBSM. The filters were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate in 10 ml of alkaline phosphatase buffer.

\[ \text{Formation of the Phosphorylated Intermediate by the Pumps} \]

The phosphorylation was started by adding 0.2 \( \mu \)l of [\( \gamma^{32P} \)]ATP (500 Ci/mmol). After 30 s on ice, the reaction was stopped with 600 \( \mu \)l of cold 7% trichloroacetic acid, 10 \% NaOH. The precipitated proteins were divided into two aliquots (20 and 5 \( \mu \)g) and separated by acidic SDS-PAGE (39). The portion of the gel containing the 20-\( \mu \)g aliquot was stained with Coomassie Brilliant Blue, dried, and exposed overnight at −70°C with an intensifying screen. The other portion of the gel was subjected to Western blot analysis using the 5F10 antibody.

\[ \text{Trypsin Digestion of Membrane Protein-expressing PMCA Isoforms} \]

100 \( \mu \)g of Sf9 cell membrane proteins were resuspended in 200 \( \mu \)l of 20 mM Hepes-KOH, pH 7.4, 130 mM NaCl, 0.05% Triton X-100, 0.5 mg/ml phosphatidylcholine, 10 mM MgCl\(_2\), 2 mM EDTA, 1 mM EGTA, 10 \( \mu \)g of reconstituted protein kinase A (Sigma) for 30–60 min at 37°C. The phosphorylated proteins were treated with 0.2 \( \mu \)l of hydroxylamine for 30 min at room temperature and separated by SDS-PAGE.

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\[ \text{Phosphorylation of PMCA by the cAMP-dependent Kinase} \]

Prior to the phosphorylation the membranes were treated with alkaline phosphatase (3 units/\( \mu \)g protein) (Roche Applied Science) for 10 min at 37°C. After addition of DNase (20 \( \mu \)g/ml) and of RNase (10 \( \mu \)g/ml) on ice, the different PMCA isoforms were purified on calmodulin-Sepharose. The proteins were phosphorylated in a final volume of 400 \( \mu \)l of 20 mM Hepes-NaOH, pH 7.4, 130 mM NaCl, 0.05% Triton X-100, 0.5 mg/ml phosphatidylcholine, 10 mM MgCl\(_2\), 2 mM EDTA, 1 mM EGTA, 10 \( \mu \)g of [\( \gamma^{32P} \)]ATP (3000 Ci/mmol), 1–5 \( \mu \)g of reconstituted protein kinase A (Sigma) for 30–60 min at 37°C. The phosphorylated proteins were treated with 0.2 \( \mu \)l of hydroxylamine for 30 min at room temperature and separated by SDS-PAGE.

\[ \text{Calpain Digestion of the Ca\textsuperscript{2+}-ATPases} \]

After the phosphorylation the membrane fractions were incubated with endogenous calpain at 35°C for 30 min. After 30 min in 50 \( \mu \)l of 20 mM MOPS-KOH, pH 6.8, 100 mM KCl, 100 \( \mu \)M Ca\textsuperscript{2+}, and 100 \( \mu \)M LaCl\(_3\). The phosphorylation was started by adding 0.2 \( \mu \)l of [\( \gamma^{32P} \)]ATP (500 Ci/mmol). After taking the zero time point, 40 \( \mu \)l of cold 7% trichloroacetic acid, 10 \% NaOH was added and the precipitated proteins were divided into two aliquots (20 and 5 \( \mu \)g) and separated by acidic SDS-PAGE (39). The portion of the gel containing the 20-\( \mu \)g aliquot was stained with Coomassie Brilliant Blue, dried, and exposed overnight at −70°C with an intensifying screen. The other portion of the gel was subjected to Western blot analysis using the 5F10 antibody.

\[ \text{Expression, stability, and purification of the human PMCA1 pump expressed in Sf9 cells} \]

A, membrane proteins prepared from Sf9 cells (lane 1, 10 \( \mu \)g) or from Sf9 cells infected with PMCA1 (lane 2, 10 \( \mu \)g), PMCA2 (lane 3, 5 \( \mu \)g), and PMCA4 (lane 4, 5 \( \mu \)g) recombinant baculoviruses were transferred to nitrocellulose sheets and stained with the 2A polyclonal antibody (13). Similar results were obtained with the 5F10 antibody (56). Quantitative analysis of the bands indicated that the amount of expressed PMCA1 protein was 5–10-fold lower than that of PMCA4 and PMCA2 isoforms. Densitometric quantification of the Western blotting in Fig. 3C yielded the following results, expressed in arbitrary units/\( \mu \)g membrane proteins: PMCA1 = 1.4, PMCA2 = 13, PMCA4 = 10.6. B, the PMCA1 protein was purified from Sf9 cells infected with the recombinant viruses by affinity chromatography on calmodulin-Sepharose. 50–100 ng of PMCA1 protein were separated by SDS-PAGE and visualized by silver staining (lane 1). The identity of the protein was confirmed by Western blotting (not shown). Protein markers are given in lane 2. Samples of purified PMCA4 and PMCA2 pumps yielded similar results (not shown). C, Western blotting of 20 \( \mu \)g of membrane proteins prepared from Sf9 cells (lane 1) or from Sf9 cells infected with seven different PMCA1 baculoviruses (lane 2). Lane 3 shows the identity of the protein was confirmed by Western blotting (not shown). Protein markers are given in lane 2. D, Northern blot of 20 \( \mu \)g of total RNA isolated for Sf9 infected with PMCA4 or PMCA1 recombinant baculoviruses. The bands were hybridized with digitoxigenin-labeled probes for PMCA4 (4) or PMCA1 (1). E, stability of the PMCA1 and PMCA4 proteins expressed in the Sf9 cells. Sf9 cells were infected with PMCA4 and PMCA1 recombinant baculoviruses, labeled for 3 h with [\( ^{35} \)S]Met (56). Quantitative analysis of the bands indicated that the amount of expressed PMCA1 protein was 5–10-fold lower than that of the PMCA4 and PMCA2 isoforms. Densitometric quantification of the Western blotting in Fig. 3C yielded the following results, expressed in arbitrary units/\( \mu \)g membrane proteins: PMCA1 = 1.4, PMCA2 = 13, PMCA4 = 10.6.
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RESULTS

Characterization of the PMCA1 Pump Overexpressed in SF9 Cells—Blots of membrane proteins obtained from SF9 cells infected with recombinant baculoviruses for PMCA1, and with recombinant viruses for PMCA4 (15) and PMCA2 (18) used for comparison, were exposed to polyclonal antibody 2A (13), which recognizes all three isoforms. A reacting band of the expected size (135–140 kDa) was observed for the three proteins (Fig. 1A). Quantitative analysis showed that the expression level of PMCA1 was about 10 times lower than that of the other two isoforms. Despite the low level of expression, the PMCA1 protein could still be purified to homogeneity using a calmodulin-affinity trypticum column (Fig. 1B).

To establish whether the low level of expression of the PMCA1 pump was a property of the selected recombinant virus, the expression by seven independent isolated viruses was analyzed. All seven viruses directed the expression of a protein of about 140 kDa (Fig. 1C, lanes 2–8) that was recognized by the PMCA-specific antibody and that was absent in non-infected SF9 cells (Fig. 1C, lane 1). All seven viruses expressed similar amounts of PMCA1 protein, and additional clones produced essentially the same results (not shown). Protein bands of lower molecular mass, which are likely to be degradation products of the pump, were also detected in the blots of all clones (Fig. 1, C and E). From some of the clones, viral DNA was prepared and analyzed by Southern blotting, yielding the expected band pattern (not shown).

To test whether the stability of the mRNA was responsible for the different expression level of the two isoforms, RNA was prepared from SF9 cells infected with recombinant PMCA1 and PMCA4 baculoviruses and characterized by Northern blotting using specific cDNA probes (Fig. 1D). Signals of similar intensity were obtained for the mRNAs of the two pumps, showing that the differences in the level of expression were not explained by a transcription difference. The stability of the expressed proteins was therefore tested in pulse and chase experiments (Fig. 1E). 18 h after labeling the SF9 cells with<sup>35</sup>S]Met and chasing them with cold Met, only trace amounts of PMCA1 (less than 5–10% of the original amount) were detected (Fig. 1E, lane 3), whereas a significantly higher amount of PMCA4 was still present. Thus, the lower level of expression of PMCA1 was apparently due to the peculiarly low stability of the protein in SF9 cells.

Proteolysis of the Expressed Pump Isoforms—To explore the reasons for the low stability of the PMCA1 pump, membranes of SF9 cells expressing pump isoforms PMCA1, PMCA2, and PMCA4 were incubated with trypsin or with calpain (Fig. 2). The degradation pattern and the kinetics of the digestion of the three isoforms were different: i.e., the PMCA1 protein was digested much faster (Fig. 2, A and B) and failed to generate significant amounts of long-lasting products larger than 100 kDa, even when the reaction was stopped after only 2 min (not shown). By contrast, in agreement with previous results on the purified pump of erythrocytes (30), calpain digestion of the overexpressed PMCA4 protein (Fig. 2A) produced a relatively stable major polypeptide of 124 kDa. Calpain digestion of the PMCA2 protein resulted in the appearance of two polypeptides of 126–128 kDa (Fig. 2A) and of additional fragments of lower molecular mass (Fig. 2A). Addition of calmodu-

µg of membrane proteins from SF9 cells expressing the PMCA1 pump (lanes 1–3) and 1–2 µg of membrane proteins from SF9 cells expressing the PMCA2 (lanes 4–6) or PMCA4 pumps (lanes 7–9) were incubated with trypsin (protein/trypsin weight ratio, 50:1) for 0.1 min (lanes 1, 4, and 7), 1 min (lanes 2, 5, and 8), and 10 min (lanes 3, 6, and 9). The samples were processed as described above for the digestion with calpain.

Fig. 2. Digestion of PMCA pump isoforms by calpain and trypsin. A, digestion of PMCA1, PMCA2, and PMCA4 proteins by calpain. Membrane proteins from SF9 cells expressing the PMCA1, PMCA2, or PMCA4 pumps were incubated with calpain as described under “Experimental Procedures” in the absence (lanes 1–3) or in the presence of 580 nM calmodulin (lanes 4 and 5). The digestion was stopped at 0 min (prior to the addition of the protease, lane 1), at 15 min (lanes 2 and 4), and at 60 min (lanes 3 and 5). 15 µg (in the case of PMCA1) or 1–2 µg (in the case of PMCA2 and PMCA4) of proteins were separated on SDS-PAGE, transferred to nitrocellulose, and incubated with the 5F10 antibody. B, densitographic quantification of the results of experiments similar to those of Fig. 2A. The 100% value on the ordinate refers to the amount of pump protein present before the addition of calpain. Only the amount of the full-length protein was considered in this analysis. The values are the average of two to five independent experiments. The error bar represents the S.D. PMCA1, PMCA2, PMCA4, Δ, C, digestion of the PMCA1, PMCA2, and PMCA4 proteins by trypsin. 15

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biochemical properties of the PMCA1 pump protein expressed in Sf9 cells—The low expression level of PMCA1 made the direct measurement of the Ca2+-dependence of the ATPase activity in the membranes of Sf9 cells difficult. It was therefore decided to purify the expressed protein by calmodulin affinity chromatography on detergent extracts of Sf9 cell membranes (Fig. 1B, lane 1). The ATPase activity of the purified PMCA1 protein had the same Ca2+-dependence of the PMCA4 pump prepared under the same conditions (Fig. 3A). The two isoforms had equivalent Vmax and Keq responses to the increase of added Ca2+ in the presence of calmodulin (Fig. 3B). The calmodulin dependence of the ATPase activity of PMCA1 was compared with that of PMCA4 and was found to be essentially the same (Fig. 3B). The binding of calmodulin was also estimated in gel overlay experiments in which the affinity of PMCA1 for calmodulin appeared to be slightly higher than that of PMCA4 (Fig. 3C).

A strong radioactive band of about 140 kDa was observed in extracts of membrane proteins of Sf9 cells infected with PMCA1 baculoviruses after incubation with [γ-32P]ATP in the presence of La3+ and Ca2+ (Fig. 4A). These are conditions that promote the formation of the phosphoenzyme intermediate of the PMCA pump while inhibiting the formation of that of the sarcoplasmic/endoplasmic reticulum (SERCA) pump. No radioactive band was observed in control Sf9 cells (Fig. 4A, lane 2) or when 1 mM EDTA was included in the reaction medium (Fig. 4A, lanes 1 and 3). The radioactive band was sensitive to NH4OH, and its intensity was strongly decreased by removing La3+ from the Ca2+-containing medium, as shown for the PMCA4 (Fig. 4A, lanes 5–7). Similar results were obtained with PMCA2 (not shown, see Ref. 18). A band of about 110 kDa was also observed in the samples incubated with Ca2+ and La3+. This band was the phosphorylated intermediate of the endogenous SERCA pump, because the intensity of this band was strongly reduced when the membranes were preincubated with thapsigargin (see also Ref. 44). Another band at 96 kDa, which was present in EGTA and strongly inhibited by La3+, was likely to correspond to the o-subunit of the Na+/K+ pump.

The formation of the phosphoenzyme intermediate by PMCA1 was studied in the presence of different concentrations of ATP. The PMCA1 pump was more efficient in the formation of the intermediate at low concentrations of ATP than the PMCA4 pump (Fig. 4B). The expressed PMCA1 (Fig. 4B) had an apparent Km ATP of 0.053 μM, as compared with one of 0.34 μM estimated for PMCA4. PMCA2 and PMCA4 had been previously shown to have significantly different affinities for ATP (18). The affinity of PMCA1 was thus even higher than that of PMCA2 (the apparently Km of the latter 0.2 μM) (18).

average of three to four experiments. An EC50 (calmodulin) for PMCA1 of 28 ± 8.6 nM and of 35 ± 11.7 nM for PMCA4 was determined by using a sigmoidal fitting. C, binding of calmodulin to the PMCA1 and PMCA4 pumps. The binding of biotinylated calmodulin was performed as described under "Experimental Procedures." The filters were quantified with the help of a scanner. ( ) PMCA1; ( ) PMCA4. Each value is the average of three independent experiments. The calculated EC50 values were 2.49 ± 0.41 nM for PMCA1 and 5.22 ± 0.58 nM for PMCA4.
Previous work had shown that the cAMP-dependent kinase phosphorylates the PMCA pump isolated from erythrocytes, which is a mixture of isoforms 1 and 4 (45). A consensus site for the cAMP kinase (KRKNSS) has been identified (31) in PMCA1 downstream of the calmodulin binding domain. Phosphorylation experiments using the cAMP-dependent kinase were thus performed on PMCA1, PMCA2, and PMCA4 (Fig. 4C). The results presented show that PMCA1 was phosphorylated to a much higher degree than the other isoforms, in which only very low levels of phosphorylation were obtained. Evidently, the consensus site, which is canonical in PMCA1, is less adequate in the other isoforms.

Characterization of the Proteolytic Products of the Pump Isoforms—To characterize functionally the fragments of the PMCA2 and PMCA4 isoforms generated after 30 min of cleavage by calpain, the fragments were separated by SDS-PAGE, blotted, and incubated with isoform-specific antibodies or with biotinylated calmodulin. In parallel, the formation of the phosphorylated intermediate was explored (Fig. 5). In the case of the PMCA4 protein, the fragment of a molecular mass of 124 kDa that was generated failed to react with biotinylated calmodulin and thus evidently lacked the calmodulin binding domain. The fragment was even more active in the phosphorylated intermediate assay than the original intact pump still present in the preparation (Fig. 5). This could possibly reflect previous findings that the removal of the C-terminal domain relieves the pump from autoinhibition, making it fully active (30, 46, 47) (see below). The pattern of digestion of the PMCA2 pump was more complex. Fragments of 130, 124, 95, and 42 kDa were generated; they evidently contained the N-terminal portion of the PMCA2 pump because they were recognized by the 2N polyclonal antibody (Fig. 5), which is specific for the N-terminal part of the PMCA2 protein (13). As expected, the 95- and the 42-kDa fragments were not recognized by calmodulin in the overlay experiments. However, a 45-kDa fragment that was recognized by the 5F10 monoclonal antibody but not by antibody 2N was also recognized by calmodulin (Fig. 5). Thus, it apparently contained the C-terminal portion of the pump, including the calmodulin binding domain. The large active fragments generated by the digestion of PMCA2 were active in the phosphorylated intermediate assay. It has so far proven impossible to identify conditions under which calpain could generate PMCA1 fragments active in the phosphorylation assay or which were recognized by calmodulin (not shown).

**DISCUSSION**

Cloning of the cDNA sequences has revealed numerous isoforms of the PMCAAs (3). The cDNA for the PMCA1 was the first...
to be isolated (8, 32). Peptides encompassing the C-terminal region (48, 49), the N-terminal region (13), and the central region have been expressed, but attempts to isolate or to construct full-length PMCA1 cDNA have been frustrated by DNA stability problems (23, 24). The only report describing the overexpression of an active PMCA1 isoform (23) refers to a terminally truncated variant termed PMCA1a, which corresponds to PMCA1CH in the nomenclature followed in this laboratory (1). The study did not characterize the biochemical properties of the pump nor did it compare them to those of other PMCA isoforms. Special efforts were thus made to produce a mutation-free full-length cDNA of the isoform (PMCA1C1 or PMCAb in the alternative nomenclature) and to transfer the full-length coding sequence to a recombinant baculovirus expression vector. Because other PMCA isoforms had already been expressed in Sf9 cells, the use of this system has allowed the direct comparison of the biochemical properties of the isoforms.

The lower expression level of PMCA1 as compared with that of the PMCA4 and PMCA2 isoforms (15, 18) was unexpected. The recombinant viruses for PMCA1 behaved as those isolated for the PMCA2 and PMCA4 counterparts; thus, it was obvious to look for alternative explanations. In principle, the transcription efficiency of PMCA1 in Sf9 cells could have become inherently lower, for instance, because of the interference of the 5′-untranslated sequences with the transcription mediated by the polyhedrin promoter (34): At variance with PMCA4 and PMCA2 (15, 18, 24), in the case of PMCA1 the 5′-untranslated region was not entirely deleted. However, this was unlikely to be the reason for the lower protein yield, because the levels of mRNA for the pump isoforms in the Sf9 cells were practically identical. This also ruled out the instability of the PMCA1 mRNA as the reason for the lower expression. Finally, the PMCA1 protein could have been more sensitive to proteolytic degradation or, more generally, have a higher turnover than PMCA4 and PMCA2. This was indeed found to be the case, as revealed by the pulse and chase experiments of Fig. 1E. Because PMCA1 and the PMCA pump mutants expressed in Sf9 cells behave like those expressed in other cell lines (21), this is likely to reflect a peculiar property of the PMCA1 pump and could, for instance, also explain why so much more PMCA4 than PMCA1 protein has been detected in red blood cells, at variance with other cells (13); erythrocytes do not synthesize proteins, therefore, the more stable isoform PMCA4 is preferentially enriched with respect to PMCA1.

The sequence identity of the four basic isoforms of the plasma membrane Ca\(^{2+}\) ATPases averages 75\% (51). It was thus surprising to find that the three isoforms studied (PMCA1, PMCA2, and PMCA4) had such a significant difference in the susceptibility to calpain. Because the sensitivity to trypsin was similar, the effect of calpain was evidently specific. It could thus be (cautiously) suggested that calpain controls the turnover of the pump isoforms, regulating their concentration. One could mention in this context that the erythrocyte calcium pump has been shown to be a preferred substrate of calpain \textit{in vivo} (52). At variance with the results on the purified enzyme (30, 53), the degradation of the erythrocyte pump \textit{in vivo} rapidly proceeded past the stage of the 124-kDa product until the pump disappeared completely. In light of the results presented here on the two overexpressed erythrocyte isoforms, PMCA4 and PMCA1, it appears likely that their complete disappearance \textit{in vivo} was the result of the action of other (unknown) proteases attacking the (initial) products of calpain cleavage. At least in erythrocytes, then, the result of the activation of calpain would not be the increased ejection of calcium by the stimulated PMCA pump but its inhibition due to its complete degradation.

The calpain proteolytic pattern of the PMCA4 protein was similar (if not identical) to that of the isolated erythrocyte PMCA pump, \textit{i.e.} a major polypeptide of 124 kDa was generated that lacked the calmodulin binding domain (30). The digestion of the PMCA2 protein by calpain produced instead a more complex pattern. The two high molecular mass fragments (130 and 124 kDa) contained the N-terminal part of the protein and still bound calmodulin, but another calmodulin binding peptide of about 45 kDa was also generated. Evidently, calpain attacked different sites in the PMCA2 and PMCA4 isoforms. The finding was somewhat unexpected, because two of the major calpain cleavage sites in the PMCA4 protein (Arg-1089, Arg-1100; Ref. 30) are conserved in the se-

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\(5\) B. Seiz, unpublished observation.
 Isoform 1 of the PMCA Pump

receptor sequence next to the active site of the pump. Alternatively, it could be suggested that an inhibitory sequence acts downstream the calmodulin binding domain (54, 55).

Although the PMCA1 pump was expressed at a lower level than the PMCA4 and PMCA2 isoforms, it still proved possible to purify low amounts of it and to establish that its Ca$^{2+}$- and calmodulin-dependent ATPase activity did not differ from that of PMCA4. However, the overlay experiments with biotinylated calmodulin revealed a small but reproducible difference in the affinities of the two pumps for the activator. The higher affinity of PMCA1 could, in principle, be related to the stronger affinity of its autoinhibitory calmodulin binding domain for the catalytic region of the pump (46, 47). However, the difference in $K_m$ was too small (2.5 versus 5.2 nm) to result in a measurable difference when the stimulation of the ATPase activity by calmodulin was measured. Surprisingly, the PMCA1 and PMCA2 enzymes showed a much higher affinity for ATP in the experiments on the phosphoenzyme formation from ATP than PMCA4. The difference was even more pronounced for PMCA1 than for PMCA2 (18), but it would be difficult to suggest that it was significant under physiological conditions, where the ATP concentration is much higher than in the experiments reported here and in previous work (18). Nevertheless, it indicates differences in the kinetic (mechanistic) properties of the pumps. Another interesting point concerns the phosphorylation by the cAMP-dependent kinase. In agreement with earlier observations on the erythrocyte enzyme (45, 31), the work has shown that PMCA1 is a better substrate for protein kinase A than isoforms 2 and 4. It remains to be established whether the limited phosphorylation of the other isoforms is functionally significant, i.e. whether it influences the activity of the pump in vivo.

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