

# Single Amino Acid Mutations in Transmembrane Domain 5 Confer to the Plasma Membrane $\text{Ca}^{2+}$ Pump Properties Typical of the $\text{Ca}^{2+}$ Pump of Endo(sarco)plasmic Reticulum\*

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Conserved residues in some of the transmembrane domains are proposed to mediate ion translocation by P-type pumps. The plasma membrane  $\text{Ca}^{2+}$  pump (PMCA) lacks 2 of these residues in transmembrane domains (TM) 5 and 8. In particular, a glutamic acid (Glu-771) residue in TM5, which is proposed to be involved in the binding and transport of  $\text{Ca}^{2+}$  by the sarcoplasmic reticulum  $\text{Ca}^{2+}$  pump (SERCA), is replaced by an alanine (Ala-854) in the PMCA pump. Ala-854 has been mutated to Glu, Asp, or Gln; Glu-975 in TM8, which is an Ala in the SERCA pump, has been mutated to Gln, Asp, or Ala. The mutants have been expressed in three cell systems, with or without the help of viruses. When expressed in large amounts in Sf9 cells, the mutated pumps were isolated and analyzed in the purified state. Two of the three TM8 mutants were correctly delivered to the plasma membrane and were active. All the TM5 mutants were retained in the endoplasmic reticulum; two of them (A854Q and A854E) retained activity. Their properties (La<sup>3+</sup> sensitivity and decay of the phosphorylated intermediate, higher cooperativity of  $\text{Ca}^{2+}$  binding with a Hill's coefficient approaching 2) differed from those of the expressed wild type PMCA pump, and resembled those of the SERCA pump.

$\text{Ca}^{2+}$  transporting ATPases ( $\text{Ca}^{2+}$  pumps) (1) remove  $\text{Ca}^{2+}$  from the cytosol maintaining the low intracellular concentration necessary to its second messenger functions. The plasma membrane pump (PMCA)<sup>1</sup> shares structural (32% identity at the primary sequence level) and mechanistic properties with its intracellular counterpart (the sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$  ATPase or SERCA). As all P-type pumps, both ATPases form a high energy enzyme intermediate (phosphoenzyme) from ATP during the reaction cycle and are organized in the

membrane with 10 putative transmembrane domains (TM) (2, 3). The second, and largest, cytosolic loop of both pumps contains the sites of phosphoenzyme formation and ATP binding (4), while regulatory regions like the calmodulin-binding domain are located at the C terminus of the PMCA enzyme (5, 6). Importantly, the inhibitor lanthanum acts differently on the two pumps; it stabilizes the phosphorylated intermediate of the PMCA pump, markedly increasing its steady state concentration (7). This effect, which is likely to reflect the inhibition of the conversion of the PMCA enzyme from the  $E_1P$  to the  $E_2P$  form (8), is not observed in the SERCA pump.

The SERCA pump transports 2 calcium ions for each ATP hydrolyzed, while the PMCA pump only transports 1 (9–11). Extensive mutagenesis of the SERCA pump has led to the identification of high affinity  $\text{Ca}^{2+}$  binding sites, mostly formed by acidic residues conserved in P-type pumps, within transmembrane domains 4, 5, and 6 (12). A glutamic acid in TM8 (Glu-908) was also suggested to be involved in the high affinity binding of  $\text{Ca}^{2+}$ , but mutation of this residue did not prevent  $\text{Ca}^{2+}$  occlusion, indicating that this residue may not have a major role in  $\text{Ca}^{2+}$  coordination (13). Based on the available results, it is now agreed that the SERCA pump contains two high affinity  $\text{Ca}^{2+}$  binding sites (sites I and II), responsible for the transport of 2  $\text{Ca}^{2+}$  ions per catalytic cycle (14–16). Site II comprises Glu-309 (TM4) and Asn-796 (TM6), site I Glu-771 (TM5), and Thr-799 (TM6). Glu-908 (TM8) contributes, at most, partially to site I, while Asp-800 (TM6) bridges the two sites (14).

Conserved acidic residues in the transmembrane domains may also form the transprotein calcium “channel” in the PMCA pump. However, a sequence comparison with the SERCA and the other P-type pumps reveals no counterpart for SERCA residue Glu-771 in TM5 in the PMCA protein (Table I); an Ala is present instead in the corresponding position (854 of TM5). Mutagenesis work (a total of about 20 PMCA mutants have now been analyzed (Refs. 17 and 18)) has yielded results compatible with the involvement of 2 of the 4 conserved residues (Glu-423 and Asp-883 in TM4 and TM6 of the PMCA4CI; Table I) in the translocation of calcium. The mutation of the other two (Asn-879 and Glu-971) resulted instead in the inactivation of the pump and in its retention in the endoplasmic reticulum (ER) (18).

In the PMCA pump, Glu-771 and Thr-799 in the domain defined as  $\text{Ca}^{2+}$  binding site I of the SERCA enzyme (19, 20) are replaced by an alanine (Ala-854 in TM5) and a methionine (Met-882 in TM6), respectively; this would indicate that only site II is conserved in the PMCA pump. The absence of any charged residues in TM 5 of the PMCA protein is worth stressing in view of the proposed essential role of Glu-771 in the transport of  $\text{Ca}^{2+}$  across the SERCA pump. By contrast, a glutamic acid (Glu-975) in TM 8 is only found in the PMCA

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<sup>1</sup> The abbreviations used are: PMCA, plasma membrane  $\text{Ca}^{2+}$  pump; TM, transmembrane domain; SERCA, sarcoplasmic reticulum  $\text{Ca}^{2+}$  pump; PAGE, polyacrylamide gel electrophoresis; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid;  $E_1P$ ,  $E_2P$ , phosphorylated intermediates; ER, endoplasmic reticulum; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammoniummethylsulfate.

pump. Ala-854 and Glu-975 were thus mutated. The effects of their mutations on the cellular targeting and catalytic cycle of the expressed protein were studied in COS and HeLa cells. High amounts of mutated recombinant PMCA pump were also expressed in Sf9 cells with the help of the baculovirus (21–23); the pump was isolated from them and tested in the purified state. The results have shown that the replacement of Ala-854 in TM5 with a glutamic acid or with a glutamine conferred to the mutated PMCA pump a number of properties similar to those of the SERCA enzyme.

#### MATERIALS AND METHODS

**Site-directed Mutagenesis and Construction of the Expression Vectors**—Mutagenesis was performed according to Deng and Nickoloff (24) using the U.S.E. (Unique Site Elimination) mutagenesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The *StuI* (1590)-*KpnI* (3637) fragments of the hPMCA4I cDNA (for the numbering, refer to Ref. 25) were subcloned in pUCBM20 (Roche Diagnostics Ltd., Rotkreuz, Switzerland). The mutated cDNA fragments were cloned back in pSG5-hPMCA4I digested with *SacI*-*KpnI*. The mutations were checked by DNA sequencing before and after cloning back in pSG5 hPMCA4I. The following oligonucleotides were used: Ala54Glu: 5'-ggctacaatcactccaccacattgac; Ala854Gln: 5'-ggctacaatcactgcccacattgac; Ala854Asp: 5'-ggctacaatcactgcccacattgac; Glu975Ala: 5'-cgggagtgattgattgaagaagctgc; Glu975Gln: 5'-cgggagtgattgattgaagaagctgc; Glu975Asp: 5'-cgggagtgattgattgaagaagctgc.

**Cell Culture and Transfection**—COS-7 cells were cultured in high glucose Dulbecco's modified Eagle's medium (Life Technologies, Inc., Basel, Switzerland), 5% fetal calf serum, and 50  $\mu$ g/ml gentamicin in a 6%  $CO_2$  atmosphere at 37 °C in a fully humidified incubator. The DNA transfections were carried out by using the transfection reagent N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethylsulfate (DOTAP; Roche Diagnostics Ltd.) according to the supplier's protocol. The cells were plated on 10-cm Petri dishes or alternatively for immunocytochemical experiments, on coverslips in six-well dishes at a density of about  $2 \times 10^4$  cells/cm<sup>2</sup>.

**Spodoptera frugiperda** (Sf9) cells were grown in TNM-FH (Sigma, Division of Fluka, Buchs, Switzerland) supplemented with 10% fetal calf serum and 100 mg/ml gentamicin at  $29 \pm 1$  °C. All routine procedures involving Sf9 cells were performed according to Summers and Smith (26). Recombinant baculoviruses were prepared according to a protocol provided by Life Technologies, Inc., Basel, Switzerland (27), which is based on the homologous recombination in *Escherichia coli* of a transfer vector with a bacmid containing the genomic sequence of the AcNPV virus. The cDNA fragments were introduced in the pFastBac vector utilizing the restriction sites *Bam*HI-*Kpn*I.

HeLa cells were maintained in Dulbecco's modified Eagle's medium, 5% fetal calf serum, and 50  $\mu$ g/ml gentamicin in 5%  $CO_2$ , at 37 °C. Transient expression was performed by infecting the cells ( $1 \times 10^7$  cells/cm<sup>2</sup>) with a recombinant vaccinia virus carrying a gene for the T7 polymerase (vvT7) (28) and by transfecting them with a vector (pSG5) carrying the mutated cDNA. The transfection was performed by using the transfection reagent DOTAP. 14–16 h after transfection-infection, membrane preparations were carried out.

**Immunocytochemistry**—The immunocytochemistry work was performed as described (23) using the PMCA pump antibody 5F10 (29).

**Preparation of Membranes from COS7 and HeLa Cells**—Crude membranes were prepared from COS-7 cells 48–60 h after transfection and from HeLa cells 14–16 h after transfection infection. Cells grown were harvested by scraping them in a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 10 mM EDTA, 75  $\mu$ g/ml phenylmethylsulfonyl fluoride, and 100 units/ml Trasylol. The cells were disrupted by three cycles of freeze and thaw at  $-70$  °C/37 °C, and the insoluble proteins were sedimented at  $15,000 \times g$  for 30 min (4 °C). The supernatant, which did not contain wild type or mutated PMCA proteins, was discarded and the pellet resuspended in 4 mM Tris-HCl, pH 7.5, 10% sucrose for storage at  $-70$  °C, or solubilized in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% SDS for immunoblotting or immunoprecipitation.

**Preparation of Membranes from Sf9 Cells**—Two days after infection with recombinant baculovirus, Sf9 cells were collected, washed three times in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and homogenized in 10 mM Tris-HCl, pH 7.5 (40 strokes of a Dounce homogenizer, on ice), in the presence of 75  $\mu$ g/ml phenylmethylsulfonyl fluoride, 100 units/ml Trasylol, and 1 mM dithiothreitol. After the addition of sucrose and KCl

to a final concentration of 10% and 150 mM, respectively, the nuclei were sedimented for 10 min at  $750 \times g$ . A final concentration of 10 mM EDTA was added to the post-nuclear supernatant, and the same was centrifuged at  $100,000 \times g$  for 45 min. The high speed pellet was resuspended in 4 mM Tris-HCl, pH 7.5, and 10% sucrose and stored at  $-70$  °C. Western blotting analysis showed that most of the PMCA immunoreactive material was associated with the  $100,000 \times g$  pellet. Some was recovered in the nuclear fraction of both the wild type and mutants, whereas none was observed in the post- $100,000 \times g$  supernatant.

**Purification of the PMCA  $Ca^{2+}$ -ATPase**—The  $Ca^{2+}$ -ATPases were purified from Sf9 cells infected with the recombinant baculovirus by calmodulin affinity chromatography, essentially as described by Niggli *et al.* (30).

**Measurement of  $Ca^{2+}$ -ATPase Activity**—The  $Ca^{2+}$ -ATPase activity was measured by the colorimetric method of Lanzetta *et al.* (31). The reaction buffer contained 20 mM HEPES-KOH, pH 7.2, 100 mM KCl, and 0.5 mM EGTA;  $CaCl_2$  was added to produce the free calcium concentrations indicated in the individual experiments. They were calculated with the computer program of Fabiato and Fabiato (32). The reaction was performed in a final volume of 100  $\mu$ l in the presence of 1 mM ATP. The incubation times were 30 min or 2 h at 37 °C.

**Quantification of the Phosphoenzyme Intermediate**—Quantification of the phosphoenzyme intermediate was performed by estimating the intensity of radioactive bands of scanned gels by an ImageQuant program, or, alternatively, by using a spectrophotometric quantification of silver grains eluted from autoradiograms (33).

**Phosphorylation and Dephosphorylation Kinetics of the PMCA Pumps**—The formation of the PMCA-specific phosphoenzyme intermediate from ATP was performed on membrane fractions from vaccinia virus-infected HeLa cells, from transfected COS-7 cells, and from Sf9 cells infected with recombinant baculoviruses. 25–50  $\mu$ g of membrane proteins were resuspended in 50  $\mu$ l of 20 mM MOPS-KOH, pH 6.8, 100 mM KCl, in the presence of 100  $\mu$ M  $CaCl_2$  and in the presence or the absence of 100  $\mu$ M  $LaCl_3$ . The reaction, carried out on ice for 30 s, was started by the addition of 0.3  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (300 Ci/mmol) and stopped by the addition of 6% trichloroacetic acid, 1 mM phosphate. The samples were spun down at  $15,000 \times g$  for 20 min at 4 °C. The pellet was washed once with 6% trichloroacetic acid, 1 mM phosphate, once with water, and resuspended in sample buffer prior to separation on acidic SDS-PAGE. After drying, the gels were analyzed on autoradiograms exposed to x-ray films at  $-70$  °C for 1–7 days. Kinetics studies of the dephosphorylation of the phosphoenzyme intermediate formed from ATP were performed on 30  $\mu$ g of crude membrane proteins from Sf9 cells expressing the wild type or mutated proteins, phosphorylated for 30 s in the presence of the components described above. An aliquot of the reaction medium was stopped by the addition of 7% trichloroacetic acid, 10 mM sodium phosphate. The dephosphorylation was initiated by the addition of either 1 mM EGTA, 1 mM EGTA plus 1 mM ADP, 1 mM ATP, or 1 mM ADP. The reaction was continued for 10 and 30 s (EGTA, EGTA+ADP, and ADP), or for 30 and 60 s (ATP) on ice before the addition of 7% trichloroacetic acid, 10 mM phosphate. Samples were processed and quantified as described above. To accumulate the ADP-insensitive  $E_2P$  intermediate, the phosphorylation was performed in the presence of 0.3  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (300 Ci/mmol), 100 mM TES/Tris, pH 8.35, 100 mM  $CaCl_2$  for 30 s at 0 °C. KCl was omitted from the reaction mixture, since it would favor  $E_2P$  hydrolysis. A portion of the phosphorylated sample was acid-quenched (7% trichloroacetic acid, 10 mM phosphate) directly after the 30-s phosphorylation reaction; another portion was treated with 1 mM EGTA after the 30-s phosphorylation and was acid-quenched 1 min later; the third portion was treated with 1 mM EGTA for 1 min after 30-s phosphorylation followed by a 10-s incubation with 1 mM ADP, before acid quenching. The phosphoenzyme remaining after 10 s with ADP represents the ADP-insensitive  $E_2P$  intermediate. Samples were processed as described above.

#### RESULTS

##### Construction and Expression of the Mutant PMCA4

The experiments were particularly aimed at understanding the role of Ala-854 and Glu-975, which are only found in the PMCA pump (Table I). Glu-975 was mutated to alanine, aspartic acid, or glutamine, while Ala-854 was mutated to glutamic acid, aspartic acid, or glutamine. The cassettes containing the mutations were routinely sequenced to completion in at least one direction. The mutated PMCA4s were expressed in parallel with the wild type pump in three different cells types (COS,



TABLE I  
Conserved polar amino acids present in the TM 4, 5, 6, and 8  
of P-type pumps

The sequences used in the table were those of the rabbit SERCA pump (43), the rat gastric  $H^+/K^+$ -ATPase (44), the sheep kidney  $Na^+/K^+$ -ATPase (45), and the PMCA4 pump (46). SMA1, SERCA of *Schistosoma mansoni* (47); PMA1,  $H^+$ -ATPase of *Saccharomyces cerevisiae* (48); ECA1, calcium ATPase of *Arabidopsis thaliana* (49). Single-letter amino acid codes are used.

|                    | TM4  | TM5         | TM6  | TM6  | TM6  | TM8  | TM8         |
|--------------------|------|-------------|------|------|------|------|-------------|
| SERCA              | E309 | E771        | N796 | T799 | D800 | E908 | A913        |
| PMCA               | E423 | <b>A854</b> | N879 | M882 | D883 | Q971 | <b>E975</b> |
| SMA1               | E307 | E766        | N795 | T798 | D799 | E908 | A859        |
| ECA1               | E316 | E800        | N825 | T828 | D829 | E906 | S910        |
| $H^+/K^+$ -ATPase  | E343 | E795        | E820 | T823 | D824 | E936 |             |
| $Na^+/K^+$ -ATPase | E327 | E779        | D807 | T807 | D808 | V924 |             |
| PMA1               | E291 | E721        | N74  | N750 | S751 | Q840 |             |

HeLa, and Sf9) using three different expression systems. In all three cell types, the mutants and the wild type pump were expressed at about the same levels, indicating that the mutations had not increased the propensity of the pump to become proteolyzed. In COS-7 cells two different PMCA-specific antibodies were used: the monoclonal 5F10, which recognizes all PMCA isoforms (29), and the polyclonal 94.2, which is specific for isoform 4 (34) (Figs. 1, A and B). In cells transfected with the wild type hPMCA4CI DNA, both antibodies recognized a protein migrating with an apparent molecular mass of 135 kDa, which is the size of the PMCA pump (Fig. 1, A and B). In agreement with previous reports (18, 23), hardly any cross-reaction of the 94.2 antibody was observed with the endogenous pump of COS-7 cells; these cells express both isoforms 1 and 4, but the latter only in minimal amounts (35). At variance with 94.2, antibody 5F10 reacted instead with a band of about 135 kDa (corresponding to PMCA isoform 1) in control cells (Fig. 1B) and with a second band in cells overexpressing the 4CI pump. Fig. 1B also shows that the overexpression of the PMCA4 pump failed to influence the expression of the endogenous PMCA protein. Using the viral expression system, the recombinant proteins were consistently expressed at much higher levels in Sf9 and HeLa cells than in COS cells. In Sf9 cell membranes (Fig. 1C, right panel), the recombinant protein was recognizable in Coomassie Brilliant Blue-stained gels, and in HeLa cells after [ $^{35}$ S]Met labeling experiments (Fig. 1D, right panel).

In crude membranes of Sf9 and HeLa cells, a band migrating with an apparent molecular mass of 135 kDa was recognized by both the 94.2 and 5F10 antibodies. In some cases (for example in Fig. 1D), a signal above that at 135 kDa was also detected in cells expressing the wild type pump and the E mutants, but not in those expressing the A mutants.

#### Membrane Targeting of the Expressed Mutated PMCA4 Pump

The transfection efficiency in the experiments used for the immunofluorescence detection of wild type and mutated PMCA4CI was 10–20% (Fig. 2A). A selection of the immunofluorescence images is shown in Fig. 2, at both low (A) and high magnification (B). COS-7 cells overexpressing the wild type hPMCA4CI pump (Fig. 2A, WT) had a staining pattern typical of proteins targeted to the plasma membrane (23, 36), *i.e.* diffuse fluorescence throughout the cell with well defined staining of the cell border. As expected, the staining pattern of cells overexpressing pumps retained in the endoplasmic reticulum was different; the fluorescent signal had the appearance of a fine reticular network, and the rim of the cell was not visible. Strong staining in the perinuclear region, due to the overex-

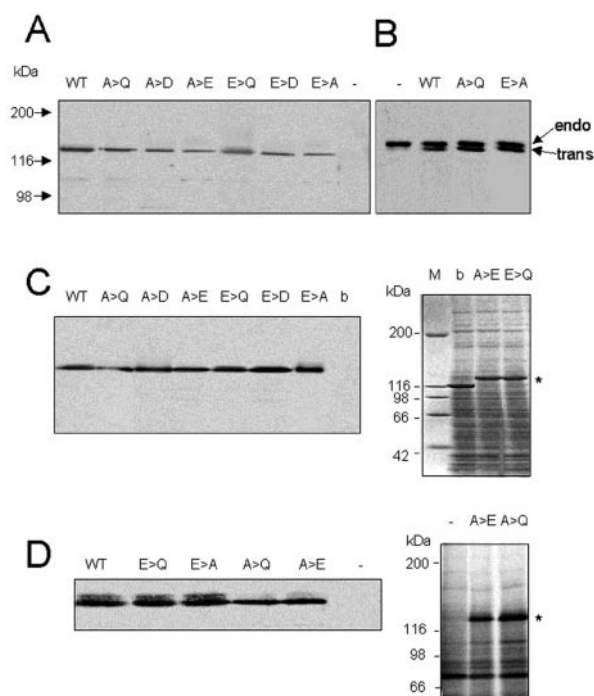
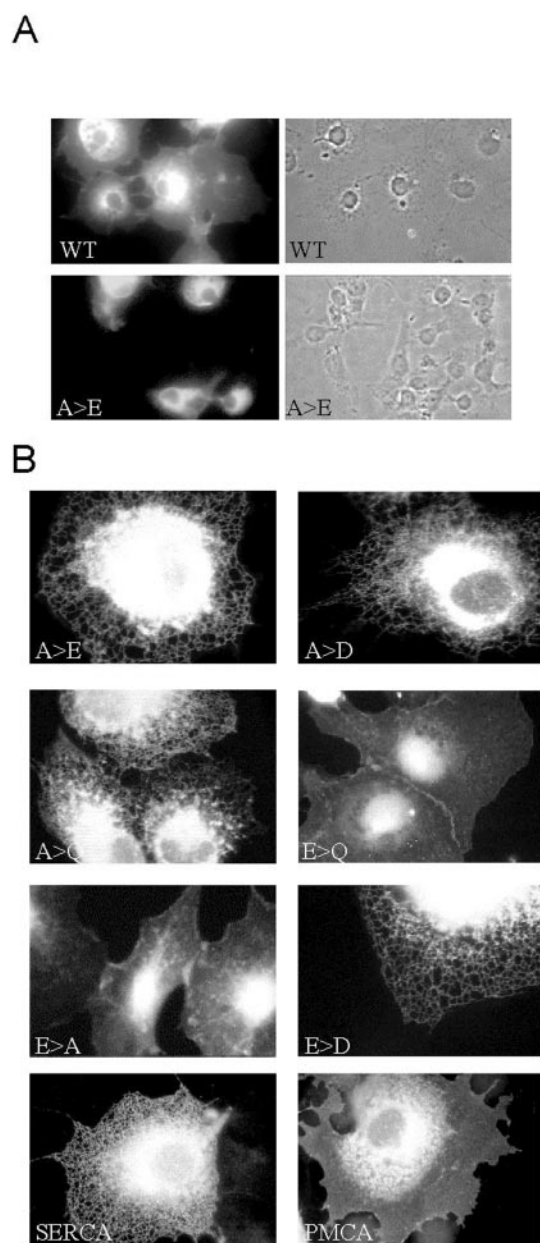


FIG. 1. Transient expression of the mutated PMCA4CI pumps in COS-7, Sf9, and HeLa cells. A and B, 30  $\mu$ g of crude membrane proteins from transfected COS-7 cells, prepared by the freeze and thaw method, were separated by SDS-PAGE, transferred to nitrocellulose filters, and stained with antibodies specific for the PMCA pump. The polyclonal antibody 94.2 (which only recognizes PMCA4CI (Ref. 34)) was used for panel A and the monoclonal antibody 5F10 (which recognizes all isoforms of the pump (Ref. 29)) for panel B. In panel B the positions of the endogenous (*endo*) and the transfected (*trans*) pump are given on the right. The recombinant proteins used were: WT, wild type PMCA4CI pump; -, cells transfected with empty vector; A>Q, A>D, and A>E, mutations inserted in TM5 at position Ala-854; E>Q, E>D, and E>A, mutations inserted in TM8 at position Glu-975. C, left panel, 30  $\mu$ g of crude membrane proteins from Sf9 cells infected with the recombinant baculovirus encoding the wild type or the mutated PMCA4 were separated by SDS-PAGE, transferred to a nitrocellulose filter, and incubated with the 5F10 antibody. As controls, cells infected with a  $\beta$ -galactosidase-expressing virus were used (b). The same mutants described for A and B were analyzed. Right panel, 30  $\mu$ g of some of the samples used for the Western blots of the left panel were stained with Coomassie Brilliant Blue. An asterisk indicates the positions of the recombinant pumps. Protein markers were run in the M lane. D, left panel, 30  $\mu$ g of crude membrane proteins from HeLa cells infected with vaccinia virus were separated by SDS-PAGE, transferred to nitrocellulose filters, and incubated with antibody 94.2. The cells were infected with T7vv and transfected with an empty vector (-) or with vectors carrying the DNA for the wild type PMCA4CI (*wt*), the E975Q (E>Q) mutant, the E975A (E>A) mutant, the A854Q mutant (A>Q), or the A854E mutant (A>E). Right panel, crude membranes from cells infected with T7vv and transfected with an empty vector (-), or with vectors carrying the DNA for the Ala  $\rightarrow$  Gln or the Ala  $\rightarrow$  Glu mutants after labeling with [ $^{35}$ S]Met (200,000 cpm) were separated by SDS-PAGE, and exposed for autoradiography. The asterisk indicates the bands corresponding to the recombinant pumps.

pression of the recombinant proteins, was generally also seen. The staining pattern of the A854E (A>E), A854D (A>D), A854Q (A>Q) mutants but also of the E975D (E>D) mutant was typical of protein retained in the endoplasmic reticulum (see the SERCA pump offered as a control in the bottom left panel). An analysis on more than 1500 positive cells from four separate transfection experiments on the A854E (A>E), A854D (A>D), A854Q (A>Q), and E975D (E>D) mutants showed that about 90% of the positive cells had a staining pattern corresponding to that of the cells expressing the SERCA pump. By contrast, cells transfected with the other two TM8 mutants (E975Q (E>Q) and E975A (E>A)) showed a staining pattern suggesting the delivery of the recombinant proteins to the

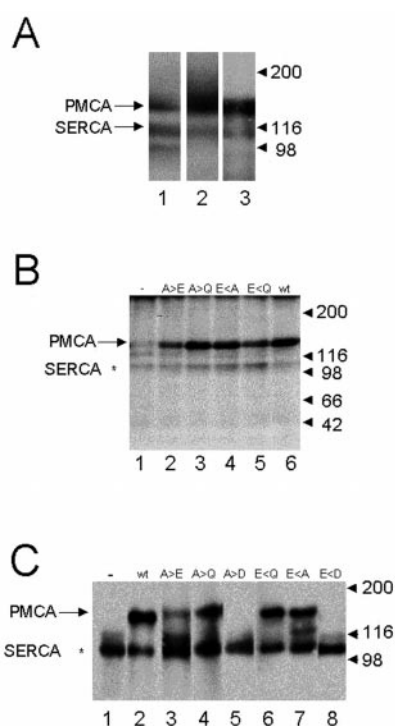


**FIG. 2. Subcellular targeting of the wild type and mutated PMCA pump.** Indirect immunofluorescence microscopy of COS-7 cells expressing different recombinant pumps. *Panel A*, images for the wild type (WT) and the A854E mutant (A>E) of the PMCA4CI pump were obtained with a 25 $\times$  objective; fluorescence images are on the left, phase contrast images on the right. *Panel B*, fluorescence images obtained with a 100 $\times$  objective for all six PMCA4CI mutants studied. Cells expressing the Glu  $\rightarrow$  Gln and Glu  $\rightarrow$  Ala mutants had a staining pattern identical to that of the wild type PMCA4CI. The other mutants had a staining pattern similar to that of the SERCA pump. The monoclonal antibody 5F10 was used for the PMCA mutants, while the monoclonal antibody A52 was used for the SERCA pump (for details, see Ref. 23).

plasma membrane, *i.e.* identical to that of the wild type PMCA4 pump (lower panel on the right of Fig. 2B).

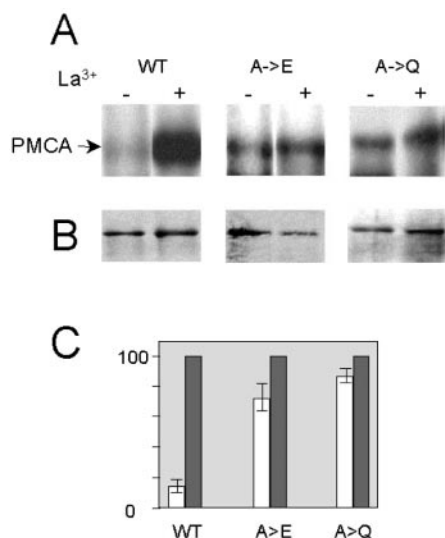
#### Formation of the Phosphorylated Intermediate by the PMCA Mutants

The initial experiments were carried out on COS7 cells, where the phosphoenzyme intermediate of the recombinant proteins was visible but difficult to quantify (Fig. 3A, lanes 2 and 3). The intermediate was instead easily quantifiable in HeLa and Sf9 cells, which showed a strong radioactive band at 135 kDa (Fig. 3, B (lanes 2–6) and C (lanes 2–4, 6, and 7)),



**FIG. 3. Formation of the phosphorylated intermediate from ATP.** *Panel A*, 40–50  $\mu$ g of membrane proteins from control COS-7 cells (lane 1) or cells expressing the wild type PMCA4CI pump (lane 2) and the A854E mutant (lane 3) were phosphorylated under conditions promoting the formation of the phosphorylated intermediate from ATP, in the presence of high calcium and lanthanum (see “Materials and Methods”). The figure shows the autoradiography of acidic gels after drying. The positions of the phosphoenzyme intermediate of the endogenous SERCA pump and the endogenous plus transfected PMCA4CI pumps are indicated. The increase of the PMCA phosphoenzyme intermediate band in lanes 2 and 3 was reproducible, although its amount was dependent on the efficiency of the transfection. The results of Western blotting on the membranes of these cells were identical to those presented in Fig. 1 (A and B) and confirmed the expression of the recombinant pumps. *Panel B* and *C*, 30  $\mu$ g of membrane proteins from expressing HeLa (B) or Sf9 (C) cells were assayed in the presence of 0.3  $\mu$ M ATP ( $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  300 Ci/mmol), 100  $\mu$ M  $\text{LaCl}_3$ , and 100  $\mu$ M  $\text{CaCl}_2$  as described under “Materials and Methods.” The proteins were separated by SDS-PAGE under acidic conditions and analyzed by autoradiography. *B*, the cells were infected with T7v $\nu$  and transfected with an empty vector (lane 1), or with a vector carrying the DNA for the A854E mutant (lane 2), the A854Q mutant (lane 3), the E975A mutant (lane 4), the E975Q mutant (lane 5), and the PMCA4CI wild type protein (lane 6). *C*, Sf9 cells infected with a control virus expressing  $\beta$ -galactosidase (lane 1), wild type PMCA4CI pump (lane 2), and the A854E (lane 3), A854Q (lane 4), A854E (lane 5), E975Q (lane 6), E975A (lane 7), and E975D (lane 8) mutants. The location of the PMCA-specific phosphoenzyme is indicated by an arrow, and that of the endogenous SERCA pump by an asterisk.

confirming the high levels of expression of active recombinant pumps. Only a weak radioactive band corresponding to the endogenous PMCA4CI pump was seen in HeLa cells infected with the empty vector (Fig. 3A, lane 1). The intensity of the phosphoenzyme intermediate of the E975A and A854Q mutants (Fig. 3B, lanes 3, 4, and 6) was similar to that of the wild type pump, while slightly weaker bands were seen for the A854E and E975Q mutants (Fig. 3B, lanes 2 and 5). The intensity of the phosphoenzyme intermediate of the A854E mutant was also reproducibly lower in the experiments on Sf9 cells (Fig. 3C, lane 3), while that of the other active mutants was similar to that of the wild type pump. The difference was not due to the lower expression level of the A854E mutant, since Western blotting showed that the amounts of expressed protein were similar. The E975D and A854D mutants failed to form the phosphoenzyme intermediate (Fig. 3C, lanes 5 and 8);



**FIG. 4. Effect of lanthanum on the phosphoenzyme intermediate formed by the wild type pump and the A854E and the A854Q mutants.** A and B, 30  $\mu g$  of membrane proteins from Sf9 cells overexpressing the wild type pump or two mutants TM5 (A854E, A854Q) were incubated under conditions promoting the formation of the phosphorylated intermediate from ATP (see "Materials and Methods"). The reaction was performed in the presence of 0.3  $\mu M$  [ $\gamma$ - $^{32}P$ ]ATP (300 Ci/mmol), 100  $\mu M$   $CaCl_2$ , with (+) or without (-) 100  $\mu M$   $LaCl_3$ . The arrows indicate the position of the PMCA4CI radioactive band. Panel A shows the autoradiogram, panel B the Coomassie Brilliant Blue-stained portion of the gels containing the PMCA band (compare also Fig. 1C). C, the effect of lanthanum is represented graphically for the wild type pump (WT) and the A854E (A>E) and A854Q (A>Q) mutants. The data are the average of four to six experiments on independent membrane preparations and show the amount of the phosphoenzyme formed in the presence of 100  $\mu M$   $CaCl_2$  (open bars) or 100  $\mu M$   $CaCl_2$  and 100  $\mu M$   $LaCl_3$  (closed bars). The amount of phosphoenzyme intermediate formed in the presence of 100  $\mu M$   $CaCl_2$  and 100  $\mu M$   $LaCl_3$  was taken as 100%.

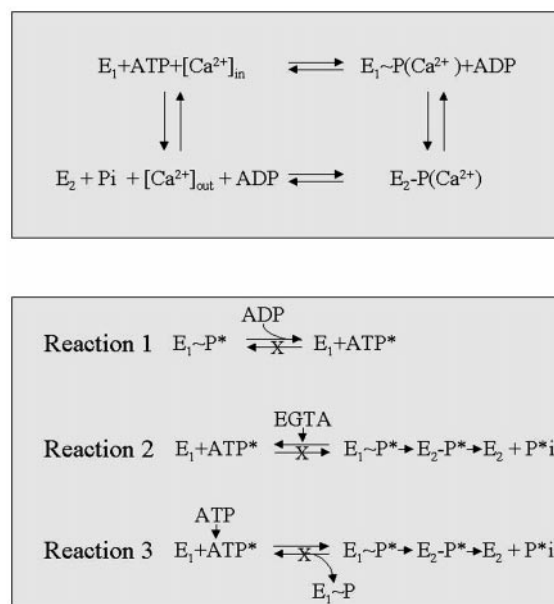
exposure of their autoradiographs for up to 1 week failed to reveal any radioactive bands.

#### Effect of Lanthanum on the Phosphoenzyme Intermediate of the Wild Type Pump and of the A854Q and the A854E Mutants

Since the A854E and A854Q mutants were active even if retained in the endoplasmic reticulum (see above), they were of particular interest. Their  $La^{3+}$  sensitivity was explored first in overexpressing Sf9 cells. Surprisingly, the phosphoenzyme intermediate of both mutants was only marginally affected by the inhibitor (Fig. 4A), which induced instead its expected large increase in the wild type pump (Fig. 4A, wt). The difference was not due to different expression levels, since similar amounts of pumps were revealed by the Coomassie Brilliant Blue staining (Fig. 4B). The change in lanthanum sensitivity was observed in four independent experiments (Fig. 4C) and was observed also when the lanthanum concentration was increased or lowered (data not shown).

#### Kinetic Properties of the Phosphoenzyme Intermediate Formed by the Wild Type Pump and the A854Q and A854E Mutants

**ADP-dependent Decay of the Phosphorylated Intermediate**—The first phosphoenzyme species formed during the catalytic cycle of calcium ATPases ( $E_1P$ , Fig. 5) is ADP-sensitive, i.e. it efficiently donates the phosphoryl group back to ADP to form ATP (Fig. 5, reaction 1) (37). Fig. 6A shows a comparison of the ADP sensitivity of the wild type and mutated pumps (A854E and A854Q) expressed in Sf9 cells. In all cases the phosphoenzyme disappeared rapidly upon addition of ADP.

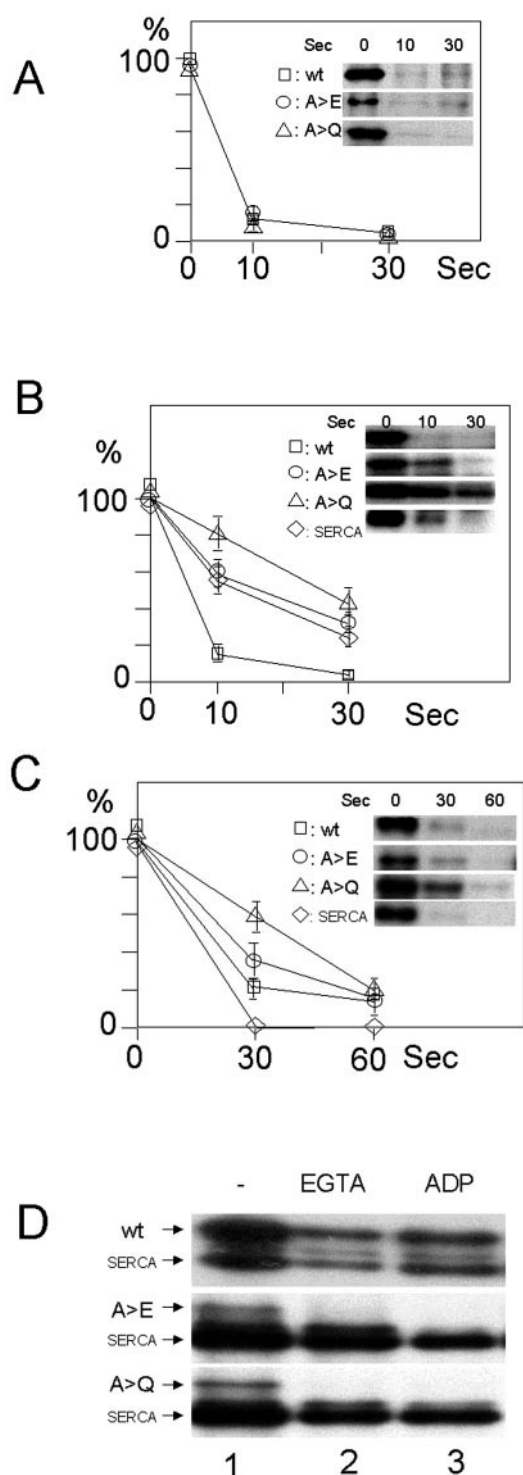


**FIG. 5. A simplified scheme of the catalytic cycle of the PMCA pump.** Upper portion, the major reaction steps are summarized. ~ indicates the high energy and - the low energy phosphoenzyme intermediate. Lower portion, the kinetics of the decay of the intermediates ( $E_1P$  and  $E_2P$ ) can be modified by the addition of ADP, EGTA and an excess of cold ATP. Reactions 1, 2, and 3 show how the radioactive phosphorylated intermediate ( $E_1P^*$ ,  $E_2P^*$ ) disappears when ADP, EGTA, and ATP, respectively, are added to the preformed intermediate. The radioactive phosphorylated intermediates ( $E_1P^*$ ,  $E_2P^*$ ) can be detected by autoradiograms. The addition of excess ADP leads to the decay of  $E_1P$  to  $E_1 + ATP$  (reaction 1) running backwards. The addition of EGTA to preformed phosphoenzyme intermediate prevents the formation of new intermediate. The decay of  $EP$  ( $E_1P$  and  $E_2P$ ) is dependent on the amount of preformed intermediate (reaction 2). The addition of excess ATP forces the decay of the phosphorylated intermediate through  $E_1P$  and  $E_2P$  (reaction 3). Newly formed intermediate after the addition of cold ATP will not be detected in autoradiograms. Alkali conditions (pH 8.35, absence of alkali metal ions) are used to enrich the  $E_2P$  intermediate.  $E_2P$  is not sensitive to ADP.

**Decay of the Phosphoenzyme Intermediate in the Presence of EGTA**—The decay of the two phosphoenzyme species  $E_1P$  and  $E_2P$  was examined next. The pump expressed in Sf9 cells was phosphorylated with ATP in the presence of 100  $\mu M$   $Ca^{2+}$  and in the absence of lanthanum. The decay of the phosphorylated intermediate was initiated by the addition of EGTA (Fig. 6B) or EGTA and ADP (data not shown). Since no new phosphoenzyme intermediate can be formed under these conditions (Fig. 5, reaction 2), its decay could be monitored. The latter could either occur by conversion of  $E_1P$  to  $E_2P$  and subsequent hydrolysis of  $E_2P$  or, in principle, by phosphorylation of ADP by  $E_1P$  with the formation of ATP (Fig. 5). The intermediates formed by the A854E and the A854Q mutants were much more stable following the addition of EGTA (Fig. 6B) than those of the wild type pump, but decayed as rapidly as that of the latter following the addition of EGTA + ADP (data not shown), (the phosphoenzyme of the A854Q mutant was slightly more stable than that of the A854E mutant). The inclusion of lanthanum during the phosphorylation step failed to affect the decay of the intermediate of the wild type and mutated pumps following the addition of EGTA (data not shown).

**ATP-dependent Decay of the Phosphoenzyme Intermediate Formed from [ $\gamma$ - $^{32}P$ ]ATP**—The kinetics of the phosphoenzyme decay was studied next by chase experiments in the presence of excess non-radioactive ATP (1 mM). After ATP addition, any newly formed phosphoenzyme would be non-radioactive, thus permitting to follow the disappearance of the preformed radioactive phosphoenzyme (Fig. 5, reaction 3). A typical experiment





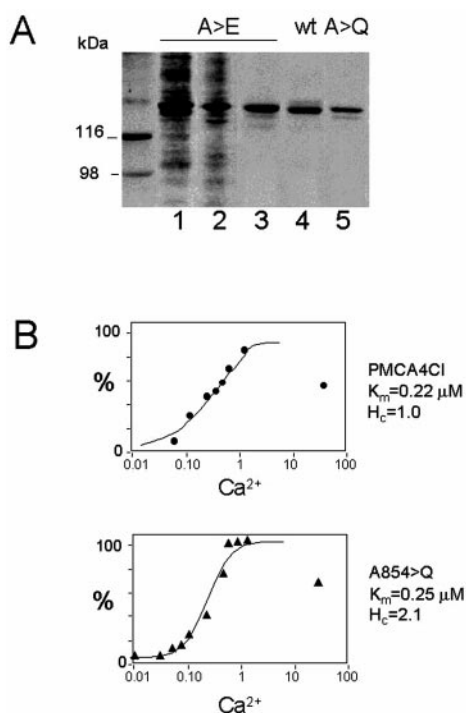
**FIG. 6. Sensitivity of the phosphoenzyme intermediate formed in the presence of ATP to ADP, EGTA, ATP, and alkali.** *A*, decay of the phosphoenzyme intermediate formed from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  in the presence of ADP. 30  $\mu\text{g}$  of membrane proteins from Sf9 cells expressing the PMCA4CI pump (*wt*), the A854E (*A>E*), and the A854Q (*A>Q*) mutants were incubated in the presence of 0.3  $\mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (300 Ci/mmol), 100  $\mu\text{M}$   $\text{CaCl}_2$ , and 100  $\mu\text{M}$   $\text{LaCl}_3$ . After 30 s, the reaction was stopped by 7% trichloroacetic acid, 10 mM sodium phosphate; otherwise, 1 mM ADP was added and the reaction was continued for 10 or 30 s on ice before the addition of trichloroacetic acid-phosphate. Samples containing equivalent amounts of expressed pump were separated by acidic SDS-PAGE and analyzed by autoradiography. The data points in the curves are the average of four independent experiments. The amounts of the phosphorylated proteins are expressed as the percentage of the amount of phosphorylated species observed at 0 s. The *inset* shows the autoradiogram of a typical experiment and the *symbols* used for the curve. Quantification was performed using the ImageQuant V1.1 pro-

gram or the spectrophotometric analysis of silver grains eluted from the autoradiograms (see "Materials and Methods"). *B*, decay of the phosphoenzyme intermediate formed from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  in the presence of EGTA. 30  $\mu\text{g}$  of crude membrane proteins from Sf9 cells expressing the PMCA4CI pump (*wt*) or the (A854E (*A>E*) and A854Q (*A>Q*)) mutants were incubated in the presence of 0.3  $\mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (300 Ci/mmol) and 100  $\mu\text{M}$   $\text{CaCl}_2$  and were phosphorylated for 30 s. Dephosphorylation was initiated by the addition of 1 mM EGTA. The reaction was stopped by the addition of 7% trichloroacetic acid and 10 mM phosphate. The data summarized in the graph are the average of four independent experiments and include measurements of the stability of the phosphoenzyme intermediate of the endogenous SERCA pump (*SERCA*). The percentage indicates the amount of the phosphoenzyme intermediate remaining after the treatment as compared with the amount before the addition of EGTA. Quantification was performed as described in the legend to *panel B*. The *inset* shows the autoradiogram of a typical experiment and the *symbols* used for the curves. *C*, decay of the phosphoenzyme intermediate in the presence of excess non-radioactive ATP. 30  $\mu\text{g}$  of crude membrane proteins from Sf9 cells expressing the PMCA4CI pump or the A854E (*A>E*) and A854Q (*A>Q*) mutants were phosphorylated for 30 s (see *panel B*). Dephosphorylation was initiated by the addition of 1 mM ATP and the reaction was stopped by the addition of 7% trichloroacetic acid, 10 mM phosphate. The result of a typical experiment is shown in the *inset* of the figure, which also shows the *symbols* used for the curves. The data in the graph are the average of three to four independent experiments. Measurements of the stability of the phosphoenzyme intermediate of the endogenous SERCA pump (*SERCA*) are included. The percentage of phosphoenzyme was calculated as described in the legend to *panel B*. *D*,  $E_2\text{P}$  accumulation by the wild type pump and the A854E and A854Q mutants. Phosphorylation was carried out by incubating 30  $\mu\text{g}$  of membrane proteins obtained from Sf9 cells expressing the recombinant proteins in the presence of 0.3  $\mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (300 Ci/mmol), 100 mM TES/Tris, pH 8.35, 100  $\mu\text{M}$   $\text{CaCl}_2$  for 30 s at 0  $^\circ\text{C}$ . No KCl was present in the reaction mixture (see "Materials and Methods"). The wild type (*WT*) PMCA and the endogenous SERCA pumps (indicated by the *arrows*) formed high amounts of ADP-insensitive  $E_2\text{P}$  intermediate. The intermediates of the mutants are indicated. In *lanes 1*, the phosphorylated samples were stopped after 30 s of incubation; in *lanes 2*, 1 mM EGTA was added after 30 s and the samples were acid-quenched 1 min later; in *lanes 3*, 1 mM EGTA was added after 30 s, followed by 1 mM ADP 1 min later. The samples were acid-quenched 10 s after the addition of ADP. The phosphoenzyme remaining after the 10-s incubation with ADP represents the ADP-insensitive  $E_2\text{P}$  intermediate.

#### Formation of the State 2 Phosphoenzyme Intermediate ( $E_2\text{P}$ ) in the Wild Type Pump and in the A854E and A854Q Mutants

The experiments above (Fig. 6, *B* and *C*) indicated that EGTA or ATP promoted the decay of the radioactive phosphoenzyme intermediate at a lower rate in the A854 mutants than in the wild type enzyme. This might have been due to a defect of the  $E_1\text{P}$ - $E_2\text{P}$  interconversion or of the  $E_2\text{P}$  decomposition. To identify the affected step of the reaction cycle, the conversion of  $E_1\text{P}$  to  $E_2\text{P}$  was studied (Fig. 6*D*). The phosphorylation step was performed at pH 8.35 in the absence of  $\text{K}^+$ , *i.e.* under conditions that favor the accumulation of  $E_2\text{P}$  in the SERCA pump (38), and do so in the PMCA pump as well, as shown in Fig. 6*D*. The  $E_2\text{P}$  accumulation was revealed by the increased stability of the intermediate after the addition of ADP (only the  $E_1\text{P}$  intermediate is ADP-sensitive (Ref. 14)) (Fig. 6*D*, *lanes 2* and 3, *wt*). While under standard conditions ( $\text{K}^+$ , pH 6.8), the addition of ADP caused the complete disappearance of the intermediate of the wild type pump (see Fig. 6*A*), 30–40% of it was still present at the end of the experiments shown in Fig. 6*D* (*lane 3*, *wt*), *i.e.* an amount similar to

gram or the spectrophotometric analysis of silver grains eluted from the autoradiograms (see "Materials and Methods"). *B*, decay of the phosphoenzyme intermediate formed from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  in the presence of EGTA. 30  $\mu\text{g}$  of crude membrane proteins from Sf9 cells expressing the PMCA4CI pump (*wt*) or the (A854E (*A>E*) and A854Q (*A>Q*)) mutants were incubated in the presence of 0.3  $\mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (300 Ci/mmol) and 100  $\mu\text{M}$   $\text{CaCl}_2$  and were phosphorylated for 30 s. Dephosphorylation was initiated by the addition of 1 mM EGTA. The reaction was stopped by the addition of 7% trichloroacetic acid and 10 mM phosphate. The data summarized in the graph are the average of four independent experiments and include measurements of the stability of the phosphoenzyme intermediate of the endogenous SERCA pump (*SERCA*). The percentage indicates the amount of the phosphoenzyme intermediate remaining after the treatment as compared with the amount before the addition of EGTA. Quantification was performed as described in the legend to *panel B*. The *inset* shows the autoradiogram of a typical experiment and the *symbols* used for the curves. *C*, decay of the phosphoenzyme intermediate in the presence of excess non-radioactive ATP. 30  $\mu\text{g}$  of crude membrane proteins from Sf9 cells expressing the PMCA4CI pump or the A854E (*A>E*) and A854Q (*A>Q*) mutants were phosphorylated for 30 s (see *panel B*). Dephosphorylation was initiated by the addition of 1 mM ATP and the reaction was stopped by the addition of 7% trichloroacetic acid, 10 mM phosphate. The result of a typical experiment is shown in the *inset* of the figure, which also shows the *symbols* used for the curves. The data in the graph are the average of three to four independent experiments. Measurements of the stability of the phosphoenzyme intermediate of the endogenous SERCA pump (*SERCA*) are included. The percentage of phosphoenzyme was calculated as described in the legend to *panel B*. *D*,  $E_2\text{P}$  accumulation by the wild type pump and the A854E and A854Q mutants. Phosphorylation was carried out by incubating 30  $\mu\text{g}$  of membrane proteins obtained from Sf9 cells expressing the recombinant proteins in the presence of 0.3  $\mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (300 Ci/mmol), 100 mM TES/Tris, pH 8.35, 100  $\mu\text{M}$   $\text{CaCl}_2$  for 30 s at 0  $^\circ\text{C}$ . No KCl was present in the reaction mixture (see "Materials and Methods"). The wild type (*WT*) PMCA and the endogenous SERCA pumps (indicated by the *arrows*) formed high amounts of ADP-insensitive  $E_2\text{P}$  intermediate. The intermediates of the mutants are indicated. In *lanes 1*, the phosphorylated samples were stopped after 30 s of incubation; in *lanes 2*, 1 mM EGTA was added after 30 s and the samples were acid-quenched 1 min later; in *lanes 3*, 1 mM EGTA was added after 30 s, followed by 1 mM ADP 1 min later. The samples were acid-quenched 10 s after the addition of ADP. The phosphoenzyme remaining after the 10-s incubation with ADP represents the ADP-insensitive  $E_2\text{P}$  intermediate.



**FIG. 7.  $\text{Ca}^{2+}$ -dependent ATPase activity of the purified pumps.** A, purification of the pumps. The recombinant proteins were purified from the membranes of Sf9 cells expressing the wild type or mutant pumps by affinity to calmodulin-Sepharose (21). Gels of 20  $\mu\text{g}$  of membrane protein containing the A854E mutant before (lane 1) and after the treatment with Triton X-100 (lane 2) are shown. 1–2  $\mu\text{g}$  of the purified A854E mutant (lane 3), the A854Q mutant (lane 5), and the wild type PMCA4CI (WT) (lane 4) are also indicated. Molecular mass markers are indicated in the far left lane. B,  $\text{Ca}^{2+}$ -dependent ATPase activity of the wild type pump (circles) and the A854Q mutant (triangles). The reaction buffer contained 20 mM HEPES, pH 7.2, 100 mM KCl, 0.5 mM EGTA, and enough  $\text{CaCl}_2$  to yield the free calcium concentrations indicated (see “Materials and Methods”). The reaction was performed in a total volume of 100  $\mu\text{l}$  in the presence of 1 mM ATP and of 3  $\mu\text{g}/\text{ml}$  CaM. After 2 h at 37  $^\circ\text{C}$ , aliquots were removed and the ATP hydrolysis was quantified by measuring the released  $\text{P}_i$ . The values are the average of two to three experiments on two independent purifications. The ATP hydrolysis is given as the percentage of the maximum activity observed at saturating concentrations of  $\text{Ca}^{2+}$  (1  $\mu\text{M}$ , see Table II). The experimental data were fitted using the equation  $V/V_{\text{max}}\% = \text{Ca}^{\text{HC}} / (\text{K}_m^{\text{HC}} + \text{Ca}^{\text{HC}})$ , where  $\text{K}_m$  is the free calcium concentration for 50% of maximum activity,  $\text{H}_c$  is Hill’s coefficient.  $\text{Ca}^{2+}$  on the abscissa, free calcium concentration ( $\mu\text{M}$ ). The values at 30  $\mu\text{M}$   $\text{Ca}^{2+}$  were not considered for fitting, since a decrease of activity was detected at this and higher concentrations, as had already been observed in previous work (41).

that observed after incubation with EGTA (Fig. 6D, lane 2, wt). In contrast, in addition to the very low amounts of accumulated phosphoenzyme intermediate, no  $\text{E}_2\text{P}$  was detected after 30 s in the A854E and A854Q mutants, as demonstrated by the complete dephosphorylation in the presence of ADP and EGTA (Fig. 6D, lanes 2 and 3). The phosphorylated intermediate of the endogenous SERCA pump was only partially dephosphorylated by ADP, as was the case for the wild type PMCA pump.

#### Activity of the Purified A854E and A854Q Mutants

The high level of expression of the recombinant proteins in infected Sf9 cells permitted their purification using the calmodulin-affinity column method originally developed for the purification of the erythrocyte plasma membrane calcium-ATPase (21, 30). Total membrane proteins from Sf9 cells infected with the DNAs of the wild type PMCA4CI and the A854E and A854Q mutants were prepared and used to purify the PMCA pumps (Fig. 7A). Their ATPase activity was measured (see “Materials and Methods”) in the presence of calmodulin (CaM)

TABLE II  
Specific activity of the purified pumps

The ATPase activity was measured as described under “Materials and Methods.” Percentile specific activity was calculated by setting the value of the wild type pump to 100%. WT, wild type.

| WT   | A854E   | A854Q   |
|--|---|---|
| 1750 ( $\pm 10\%$ ) nmol<br>ATP/min $\cdot$ mg | 675 ( $\pm 10\%$ ) nmol<br>ATP/min $\cdot$ mg | 510 ( $\pm 10\%$ ) nmol<br>ATP/min $\cdot$ mg |
| 100%   | 38%   | 30%   |

or EGTA (data not shown). The specific activity of the A854E and A854Q mutants was lower than that of the wild type pump: 675 and 510 nmol/min/mg of protein ATP, respectively, were hydrolyzed, as compared with 1750 for the wild type pump (Table II). The calcium dependence of the activity of the wild type PMCA4CI and of the A854Q mutant was measured in the presence of saturating concentrations of CaM (3  $\mu\text{g}/\text{ml}$ ) (Fig. 7B). The apparent  $\text{K}_m$  was similar (0.25 and 0.22  $\mu\text{M}$  for the wild type pump and the A854Q mutant, respectively), but the activity curve of the mutant enzyme was much steeper than that of the wild type pump. By fitting Hill’s equation to the measured points (see Fig. 7B), Hill’s coefficients of approximately 2 in the case of the A854Q mutant and of approximately 1 in the case of the wild type pump were obtained. This indicated higher calcium cooperativity in the A854Q mutant. Similar experiments were attempted with the A854E mutant. Although sufficient recombinant protein could be purified to determine its specific activity (Table II), the amounts obtained were too low to reliably determine its  $\text{Ca}^{2+}$  dependence. The reason why similar amounts of the wild type and of the A854Q mutant proteins could be purified, whereas only lower amounts of the A854E mutant protein could be obtained, are not yet clear.

#### DISCUSSION

The presence of an alanine in the place of the conserved glutamic acid in TM5 is peculiar to the PMCA pump; in the other P-type pumps, the Glu residue in TM5 is necessary for ion translocation (12). In addition, TM8 of the PMCA pump contains a glutamic acid (Glu-975), for which no homologue has been found in the SERCA pump (Table I). The recombinant proteins carrying mutations at Ala-854 and Glu-975 became expressed at equivalent levels, indicating that the mutations did not affect the propensity of the pumps to become proteolyzed. However, although the E975Q and E975A mutants were correctly delivered to the plasma membrane, the E975D, A854E, A854Q, and A854D mutants were retained in the endoplasmic reticulum. Since the properties of the correctly sorted E975A and E975Q mutants were indistinguishable from those of the wild type PMCA pump, no detailed analysis was performed on them. Nor was it performed on the A984D and E975D mutants, which were retained in the endoplasmic reticulum, and were inactive (*i.e.* they lost the ability to form the phosphoenzyme intermediate from ATP or from phosphate). Perturbations in the folding of these mutants had in all likelihood occurred. As shown in previous work, the membrane sorting of the PMCA pump is evidently very sensitive to mutations, its correct delivery to the plasma membrane only occurring when the pump is at least partially active (18, 23).

It was thus surprising to find that two mutants, the A854Q and A854E, were active, *i.e.*, showed significant  $\text{Ca}^{2+}$ -CaM-dependent ATPase activity in the purified state despite their retention in the endoplasmic reticulum. These two active mutants had replaced the alanine residue in TM5 with a glutamate, *i.e.* with the residue found in the homologous position in the SERCA pump, or with a glutamine, whose sterical proper-

ties are similar to those of glutamic acid. Although previous work has shown that the first 28 N-terminally amino acids are important in the retention of the SERCA pump in the ER (23, 39), the tertiary structure of the pump evidently plays an important role in the process. The substitution of Ala-854 by Glu and Gln may structurally perturb the transmembrane domains, resulting in a conformational change that prevents the delivery of the mutant pumps to the PM, causing their retention in the ER.

The largest portion of the work described in this contribution has focused on the active, but mistargeted A854E and A854Q mutants. Their calcium affinity was essentially unaffected, and their affinity for ATP, although not studied in detail, failed to reveal significant differences with respect to the wild type pump (data not shown). The ADP-promoted dephosphorylation of  $E_1P$  (Fig. 5, reaction 1) in the A854E and A854Q mutants was also similar to that of the wild type pump (Fig. 6A). Unfortunately, times shorter than 5 s could not be reproducibly studied; thus, differences in the initial phase of the reaction could have gone undetected.

Other properties of the pump, however, were affected by the mutation; the slower decay of the phosphoenzyme intermediate in the presence of EGTA or of ATP in the mutated pumps suggests that reactions 2 and 3 in Fig. 5 were affected. The effect was more evident in the A854Q mutant, consistent with the higher amount of phosphoenzyme intermediate formed by it.

At alkaline pH (8.35) and in the absence of  $K^+$ , the wild type pump accumulated higher amounts of phosphoenzyme intermediate than under the standard, slightly acidic conditions (pH 6.6), an effect that was not observed in the mutated PMCA. The addition of EGTA and ADP only slightly reduced the amount of phosphorylated intermediate of the wild type pump, indicating that a large portion of it was in the  $E_2P$  form (the  $E_2P$  form is assumed to be ADP-insensitive). Since the intermediate of the mutants had already disappeared following the addition of EGTA, the A854E and A854Q mutants accumulated lower amounts of  $E_2P$ . Thus, the mutation of Ala-854, at least under alkaline conditions, had apparently slowed down the  $E_1P$  to  $E_2P$  conversion (Fig. 5, reaction 2).

The results on the stability of the phosphoenzyme intermediate of the protein in the membrane were consistent with those on the purified pump, *i.e.* the A854Q and A854E mutants had a lower specific ATPase activity than the wild type pump. Importantly, the phosphoenzyme intermediate of two mutants had very low lanthanum sensitivity at variance with the wild type PMCA pump and in line with the effect of  $La^{3+}$  on the SERCA pump. The step at which lanthanum influences the formation of the phosphoenzyme intermediate in the PMCA pump is still obscure, but the inhibition of the hydrolysis of the  $E_2P$  intermediate has been used to explain the 15–20-fold increase in the amount of phosphorylated enzyme at steady state induced by  $La^{3+}$  in the wild type pump (40). This may suggest the loss of a PMCA-specific lanthanum-binding site, which would be another indication that the conformation of the Ala-854 mutants is different from that of the wild type pump.

In summary, then, some of the properties of the A854Q and the A854E PMCA mutants resembled those of the SERCA pump; specifically, the two mutants were retained as active proteins in the ER (in principle, however, this could reflect the interference with the pump delivery to the plasma membrane, rather than the specific targeting to the ER SERCA site), and the formation of their phosphorylated intermediate had low sensitivity to lanthanum. Importantly, the A854Q mutant showed higher cooperativity for calcium binding (Hill's coefficient approaching 2) than the wild type PMCA pump, suggest-

ing that the mutation might have created an additional calcium-binding site. Despite the similarities, however, the properties of the Ala-854 mutants did not exactly duplicate those of the SERCA pump; in particular, the kinetics of their phosphoenzyme intermediate showed important differences. This was to be expected since other amino acids would be necessary, *e.g.* Thr-799 in TM6 and probably Glu-908 in TM8, to construct a fully functional  $Ca^{2+}$  binding site I such as that of the SERCA (14). The finding that the SERCA E771A mutant was inactive (12) is consistent with the idea that two  $Ca^{2+}$  binding sites are necessary to make the SERCA pump functional. By contrast, the creation of additional  $Ca^{2+}$ -binding capacity in a pump that only requires site II to be functional *in vivo* may be much better tolerated.

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**Single Amino Acid Mutations in Transmembrane Domain 5 Confer to the Plasma Membrane Ca<sup>2+</sup> Pump Properties Typical of the Ca<sup>2+</sup> Pump of Endo(sarco)plasmic Reticulum**

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