Single Amino Acid Mutations in Transmembrane Domain 5 Confer to the Plasma Membrane Ca\(^{2+}\) Pump Properties Typical of the 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 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 Ca\(^{2+}\) by the sarcoplasmic reticulum 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\(^{3+}\) sensitivity and decay of the phosphorylated intermediate, higher cooperativity of 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.

Ca\(^{2+}\) transporting ATPases (Ca\(^{2+}\) pumps) (1) remove Ca\(^{2+}\) from the cytosol maintaining the low intracellular concentration necessary to its second messenger functions. The plasma membrane pump (PMCA)1 shares structural (32% identity at the primary sequence level) and mechanistic properties with its intracellular counterpart (the sarco(endo)plasmic reticulum Ca\(^{2+}\) ATPase or SERCA). As all P-type pumps, both ATPases form a high energy enzyme intermediate (phosphoenzyme) (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 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 Ca\(^{2+}\), but mutation of this residue did not prevent Ca\(^{2+}\) occlusion, indicating that this residue may not have a major role in Ca\(^{2+}\) coordination (13). Based on the available results, it is now agreed that the SERCA pump contains two high affinity Ca\(^{2+}\) binding sites (sites I and II), responsible for the transport of 2 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 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 its retention in the endoplasmic reticulum (ER) (18).

In the PMCA pump, Glu-771 and Thr-799 in the domain defined as 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 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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1 The abbreviations used are: PMCA, plasma membrane Ca\(^{2+}\) pump; TM, transmembrane domain; SERCA, sarcoplasmic reticulum Ca\(^{2+}\) pump; PAGE, polyacrylamide gel electrophoresis; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; E\(_{\text{P}}\), E\(_{\text{P}}\), 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 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 matured 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 StrI (1580)-KpnI (3637) fragments of the hPMCA4I cDNA (for the numbering, refer to Ref. 25) were subcloned in pUC18 vector (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'-ggctacaatctcacaaccagtc-3'; Ala54Asp: 5'-ggctacaatctcacaaccagtc-3'; Glu975Asp: 5'-ggagggtgattggatgaacggc-3'; Glu975Glu: 5'-ggagggtgattggatgaaccgc-3'; Glu975Ala: 5'-ggagggtgattggatgaacgct-3'.

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 μg/ml gentamicin in a 6% CO2 atmosphere at 37 °C in a fully humidified incubator. The DNA transfections were carried out by using the transfection reagent N-[1-[2-diisoeleyloxy)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 × 104 cells/cm2.

Spodoptera frugiperda (Sf9) cells were grown in TMN-FH (Sigma, Division of Fluka, Buchs, Switzerland) supplemented with 10% fetal calf serum and 100 mg/ml gentamicin at 29 °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 transfer vector with a bacmid containing the genomic sequence of the AcNPV virus. The cDNA fragments were introduced in the pFastBac transfer vector with a bacmid containing the genomic sequence of the AcNPV virus. The cDNA fragments were introduced in the pFastBac transfer vector with a bacmid containing the genomic sequence of the AcNPV virus. The cDNA fragments were introduced in the pFastBac transfer vector with a bacmid containing the genomic sequence of the AcNPV virus. The cDNA fragments were introduced in the pFastBac transfer vector with a bacmid containing the genomic sequence of the AcNPV virus.

Construction and Expression of the Mutant PMCA4—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 μg of membrane proteins were resuspended in 50 μl of 20 mM MOPS-KOH, pH 6.8, 100 mM KCl, in the presence of 100 μM CaCl2, and in the presence or the absence of 100 μM LaCl3. The reaction, carried out on ice for 30 s, was started by the addition of 0.3 μM 32P-ATP (300 Ci/mmol) and stopped by the addition of 6% trichloroacetic acid, 1 mM phosphate. The samples were incubated at 15 °C for 10 min, 300 g × 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 acetic 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 μg of crude membrane proteins from Sf9 cells expressing wild type or mutated proteins, phosphorylated for 30 s in the presence of the components described above. To accumulate the ADP-insensitive F,P intermediate, the phosphorylation was performed in the presence of 0.3 μM 32P-ATP (300 Ci/mmol), 100 mM MES/Tris, pH 8.35, 100 mM CaCl2 for 30 s at 0 °C. KC1 was omitted from the reaction mixture, since it would favor F,P hydrolysis. A portion of the phosphorylated sample was acid-changed (7% trichloroacetic acid, 10 mM phosphate) directly after the 30-s phosphorylation reaction; another portion was treated with 1 mM EGTA after 30-s phosphorylation and was acid-changed 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 F,P 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,
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 [35S]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 overexpression 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). A similar 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

| Table I Conserved polar amino acids present in the TM 4, 5, 6, and 8 of P-type pumps |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| TM4 | TM5 | TM6 | TM6 | TM8 | TM8 |
| SERCA | E309 | E771 | N796 | T799 | D800 | E908 | A913 |
| PMCA | E423 | A854 | N879 | M882 | D883 | Q971 | E975 |
| 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 |

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Fig. 1. Transient expression of the mutated PMCA4CI pumps in COS-7, Sf9, and HeLa cells. A and B, 30 μg 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; Δ, 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 Gln-975. C, left panel, 30 μg 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 β-galactosidase-expressing virus were used (6). The same mutants described for A and B were analyzed. Right panel, 30 μg 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 μg 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 → Gln or the Ala → Glu mutants after labeling with [35S]Met (200,000 cpm) were separated by SDS-PAGE, and exposed for autorigraphy. The asterisk indicates the bands corresponding to the recombinant 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.
antibody A52 was used for the SERCA pump (for details, see Ref. 23). Antibody 5F10 was used for the PMCA mutants, while the monoclonal staining pattern similar to that of the SERCA pump. The monoclonal identical to that of the wild type PMCA4CI. The other mutants had a pressing the Glu
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Panel A, images for the wild type (WT) and the A854E mutant (A>E) of the PMCA4CI pump were obtained with a 25 x objective for all six PMCACI mutants studied. Cells expressing the Glu -> Gln and Glu -> 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).

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. 3, A, 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)), 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);
in the presence of 0.3 mM CaCl₂ and 100 μM LaCl₃. The radioactive phosphorylated intermediates (E₈P*, E₉P) can be detected by autoradiograms. The addition of excess ADP leads to the decay of E₉P to E₈P + ATP (reaction 1) running backwards. The addition of EGTA to preformed phosphorylase intermediate prevents the formation of new intermediate. The decay of E₉P to E₈P and E₉P (data not shown) is dependent on the amount of preformed intermediate (reaction 2). The addition of excess ATP forces the decay of the phosphorylated intermediate through E₈P and E₉P (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 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 La³⁺ 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 phosphonate species formed during the catalytic cycle of calcium ATPases (E₉P, 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.
is shown in the inset of Fig. 6C. The decay of the intermediate was accelerated by ATP, reaching near completion within 60 s in both the wild type and the mutated pumps. However, at 30 s differences in the extent of the dephosphorylation reaction between the ADP-insensitive mutants was shown to be slowest.

**Formation of the State 2 Phosphoenzyme Intermediate**

(E2P) 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 E1P-E2P interconversion or of the E2P decomposition. To identify the affected step of the reaction cycle, the conversion of E1P to E2P was studied (Fig. 6D). The phosphorylation step was performed at pH 8.35 in the absence of K+, i.e., under conditions that favor the accumulation of E1P in the SERCA pump (38), and so do so in the PMCA pump as well, as shown in Fig. 6D. The E2P accumulation was revealed by the increased stability of the intermediate after the addition of ADP (only the E1P intermediate is ADP-sensitive (Ref. 14)) (Fig. 6D, lanes 2 and 3, wt). While under standard conditions (K+, pH 6.8), the addition of ADP caused the complete disappearance of the intermediate of the wild type pump (see Fig. 6A), 30–40% of it was still present at the end of the experiments shown in Fig. 6D (lane 3, wt), i.e. an amount similar to the amount of phosphorylated species observed at 0 s. 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 µg 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 µM [γ-32P]ATP (300 Ci/mmol) and 100 µM CaCl2 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 sodium 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. D, decay of the phosphoenzyme intermediate in the presence of excess non-radioactive ATP. 30 µg 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, E2P accumulation by the wild type pump and the A854E and A854Q mutants. Phosphorylation was carried out by incubating 30 µg of membrane proteins obtained from Sf9 cells expressing the recombinant proteins in the presence of 0.3 µM [γ-32P]ATP (300 Ci/mmol), 100 mM TES/Tris, pH 8.35, 100 µM CaCl2, and 100 µM LaCl3 at 0°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 E2P 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-quinched 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-quinched 10 s after the addition of ADP. The phosphoenzyme remaining after the 10-s incubation with ADP represents the ADP-insensitive E2P intermediate.
The reaction buffer contained 20 mM HEPES, pH 7.2, 100 mM KCl, 0.5 mM EGTA, and enough CaCl₂ to yield the free calcium concentration (Fig. 6). The apparent $K_m$ was similar (0.25 and 0.22 μM 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 Ca²⁺ 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 phosphorylated in the presence of Ca²⁺, free calcium concentration (%), and ADP (Table II). The ATPase activity was measured as described under “Materials and Methods.” 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 μg/ml) (Fig. 7B). The apparent $K_m$ was similar (0.25 and 0.22 μM 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 Ca²⁺ 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.

**TABLE II**

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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.
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 \( E_P \) (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 ATP only slightly reduced the calcium binding in the presence of EGTA or of ATP in the mutated pumps, an effect that was not observed in the mutated SERCA (14). The finding that the SERCA E771A mutant was inactive (12) is consistent with the idea that the calcium 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.

REFERENCES

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

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