Mutation of Conserved Residues in Transmembrane Domains 4, 6, and 8 Causes Loss of Ca^{2+} Transport by the Plasma Membrane Ca^{2+} Pump†

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ABSTRACT: Mutants of the plasma membrane Ca^{2+} pump (PMCA), in which amino acids in transmembrane domains (TM) 4, 6, and 8 had been replaced, have been expressed in COS-7 cells. They were analyzed functionally by measuring the uptake of Ca^{2+} in microsomal preparations and by following the formation of the phosphorylated intermediate from ATP and from phosphate. The mutated residues corresponded to amino acids whose mutation in the sarcoplasmic reticulum pump (SERCA) caused loss of Ca^{2+} transport by the pump protein: however, only four of the six SERCA residues were conserved in the PMCA pump. Mutation of Glu423 (TM4), Asn879 or Asp883 (TM6), or Gln971(TM8) suppressed Ca^{2+} transport by the pump and its ability to form the phosphorylated intermediate starting from ATP. By contrast, the ability of these mutants to form the intermediate starting from phosphate was not impaired. In two mutants (Glu423 and Asp883) it was even enhanced. Two conserved Pro residues of TM4 were also mutated, leading to the loss of the ability of the pump to form the Ca^{2+}- and ATP-dependent phosphorylated intermediate. Unexpectedly, two of the mutations (Asn879 and Gln971) led to the mistargeting of the mutated proteins, i.e., to their retention in the endoplasmic reticulum.

The plasma membrane Ca^{2+} ATPase (Carafoli, 1994) belongs to the family of P-type ion pumps (Pedersen & Carafoli, 1987), which contains also the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase. The members of this family of pumps form a phosphorylated enzyme intermediate (an aspartyl-phosphate) from phosphate or ATP, a condition necessary for ion translocation. Cloning work has revealed that the different members of this family share regions of high homology. The high degree of conservation of some of these regions, e.g., the amino acids surrounding the aspartic acid that forms the phosphorylated intermediate (Verma et al., 1988), has been be used to identify new members of this protein family.

Despite the sequence homology and the very similar function, the two Ca^{2+} pumps [that of the plasma membrane (PMCA)[1] and that of the sarcoplasmic/endoplasmic reticulum (SERCA)] exhibit a number of structural and functional differences. For example, the PMCA pump is directly regulated by calmodulin, whereas no direct interaction has been described for the SERCA pump (Carafoli, 1994). The SERCA pump transports 2 Ca^{2+}/ATP hydrolyzed, the PMCA pump only one (Niggli et al., 1981; Hao et al., 1994). Finally, the two pumps are targeted to two different cellular compartments.

The expression of the SERCA pump in COS-7 cells has allowed the use of site-directed mutagenesis to study the function of amino acids located in the various domains of the pump (MacLennan; 1990; Andersen & Vilsen, 1995). It has thus been possible to identify six amino acids as prime candidates in the formation of the transprotein Ca^{2+} translocation pathway (Clarke et al., 1989a). Mutations of these amino acids, which are located in transmembrane domains 4, 5, 6, and 8, led to the loss of the ability of the SERCA pump to transport Ca^{2+} across the membrane. The mutants, however, were still partially active (Clarke et al., 1989a,b). Later work has revealed that these amino acids can be divided in two groups: those that form the Ca^{2+} binding site facing the cytosol and those that form the lumenal site (Andersen & Vilsen, 1995).

A comparison of the primary sequences of the P-type pump has revealed that the amino acids involved in the Ca^{2+} translocation in the SERCA pump are conserved in other pumps of the family: for example, five of the six residues of the SERCA (Clarke et al., 1989a) are present in the Na^+/K^+- ATPase and four are present in the PMCA pump. In the latter pump these amino acids are Glu423, Asn879, Asp883, and Gln971. The latter would correspond to Glu908 of the SERCA pump, but since its mutation to a Gln failed to affect the properties of the SERCA pump (Clarke et al., 1990), it may be assumed to be homologous to Gln971 of the PMCA pump. Other highly conserved amino acids in transmembrane domain 4 of the SERCA pump (corresponding to Pro422 and Pro426 of the PMCA4 pump) were found to be critical and were proposed to be necessary for the proper folding of the mature SERCA protein (Vilsen et al., 1989).

To establish whether the amino acids mentioned above play similar roles in the PMCA and SERCA pumps, mutants of the former were prepared, expressed in COS-7 cells, and analyzed functionally. The results have shown that the

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[1] Abbreviations: DMEM, Dulbecco's modified minimal eagle's medium; FCS, fetal calf serum; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMCA, plasma membrane Ca^{2+} ATPase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; Tris, tris(hydroxymethyl)aminomethane.

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mutation of Pro422, Glu423, Pro426, Asn879, Asp883, or Gln971 indeed resulted in the loss of the ability of the PMCA pump to transport Ca^{2+}. At variance with what was observed with the SERCA pump (Clarke et al., 1990), the Gln971Glu mutation also abolished the Ca^{2+} translocation by the PMCA pump. Unexpectedly, the substitution of Gln971 with a Glu or of Asn879 with an Asp caused the mistargeting of the mutants in COS-7 cells, i.e., their retention in the endoplasmic reticulum.

MATERIALS AND METHODS

Site-Directed Mutagenesis and Construction of the Expression Vectors. The oligonucleotide site-directed mutagenesis was performed according to Deng and Nickoloff (1992) using the Pharmacia U. S. E. (Unique Site Elimination) mutagenesis kit (Pharmacia Biotech, Uppsala, Sweden). The *Sac*I(725)−*Sma*I(2123) and *Stu*I(1590)−*Kpn*I(3637) fragments of the hPMCA4CI cDNA [for the numbering refer to Strehler (1991)] were subcloned in pUCBM20 (Boehringer Mannheim GmbH, Zurich, Switzerland). Where possible, the mutation primers were chosen in order to insert or to abolish a restriction site. The mutated cDNA fragments were cloned back in pSG5-hPMCA4CI digested with *Sac*I−*Sma*I and *Sac*I−*Kpn*I, respectively. All the mutations were confirmed by DNA sequencing. For this purpose the complete cassette was sequenced to completion in at least one direction. The following oligonucleotides were used: Pro422Ala, 5′ gcccctctg**c**cacagccacc; Glu423Ala, 5′ggcagcccc**g**ctggcacagcc; Glu423Asp, 5′ggcagcccc**g**tctggcacagcc; Glu423Asn, 5′ggcagcccct**g**tggcacagcc; Glu423Gln, 5′ggcagccc**gtt**tggcacagcc; Pro426Ala, 5′gacagccagag**c**cagcccctc; Asn879Asp, 5′ccatgatcagat**c**aacccacaac; Asn879Ala, 5′ccatgatcaga**gc**aacccacaacatc; Asp883Asn, 5′gaagcaaaagtgt**t**catgatcag; Asp883Glu, 5′gaagcaaaagt**c**tccatgatcag; Asp883Ala, 5′gaagcaaaagtg**g**ccatgatcag; Gln971Glu, 5′cattgaagagct**c**catcagcacg; Gln971Ala, 5′cattgaagagc**gc**catcagcacgaag; Ile929Val, 5′ggataaagatgacaa**c**gagctgatag; Glu1078Gln, 5′ggtcaatct**g**atccagtccctcg; selection primer, 5′cctgataaatgcttcaata**ggcc**tgaaaaaggaagag. (The mutated nucleotides are in boldface type.)

Cell Culture and Transfection. The COS-7 cells were cultured in DMEM−high glucose, 5% FCS, and 50 μg/mL gentamicin in a 6% CO_2 atmosphere at 37 °C in a fully humidified incubator. The DNA transfections were carried out by the calcium phosphate coprecipitation method essentially as described by Chen and Okoyama (1987). The cells were plated on 10-cm Petri dishes or alternatively for immunocytochemical experiments on cover slips in 6-well dishes at a density of about 2×10^4 cells/cm^2.

Immunocytochemistry. The immunocytochemistry work was performed as described previously (Foletti et al., 1995) using the PMCA pump antibody 4N (Stauffer et al., 1995).

Preparation of Membranes from the COS-7 Cells. After transfection (48−72 h), the cells were washed twice with TBS, scraped, and recovered by centrifugation. Total membranes were prepared from the cells by a freeze and thaw procedure. Briefly, the cell pellet was resuspended (10^7 cells/mL) in 0.5 M NaCl, 50 mM Tris-HCl, pH 7.0, 1 mM DTT, 7.5 mg/mL phenylmethanesulfonyl fluoride, 100 units/mL trasylol, and 1 mM EDTA, quickly frozen in dry ice and thawed at 37 °C three times. After centrifugation, the pellet was resuspended in 10% sucrose and 10 mM Tris-HCl, pH 7.5, with a 10-s sonication and stored at −70 °C. Microsomes were prepared from the cells 60 h after the beginning of the transfection according to the method of Maruyama and MacLennan (1988) and Enyedi et al. (1993).

Ca^{2+} Uptake by Microsomes. Calcium influx into the microsomal vesicles was measured isotopically, essentially as described by Enyedi et al. (1993).

Formation of the Phosphoenzyme Intermediate from ATP and Phosphate. Membrane proteins (30 μg) in 100 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $LaCl_3$, and 20 mM MOPS-KOH, pH 6.8, were phosphorylated in the presence of 0.75 μM [γ-^{32}P]ATP (150 Ci/mmol) on ice. After 30 s on ice the reaction was stopped by the addition of 400 μL of 7% trichloroacetic acid and 15 mM NaH_2PO_4 and centrifuged. In the case of phosphorylation from phosphate, 30 μg of membrane proteins was resuspended in 5 mM $MgCl_2$, 50 mM MES-KOH, pH 6.4, and 10 mM EGTA or different $CaCl_2$ concentrations (see the legends to figures). Before the reaction was started, DMSO was added to a final concentration of 32%. The reaction was initiated by the addition of 200 μM ^{32}P-mix (5 Ci/mmol) and terminated after 10 min at room temperature by the addition of 600 μL of 7% trichloroacetic acid and 15 mM NaH_2PO_4. The precipitated proteins were washed with 7% trichloroacetic acid and 15 mM NaH_2PO_4 and with 200 μL of distilled water and separated on an acidic gel.

Other Methods. Proteins were separated according to the method described by Laemmli (1970). The sensitivity of the acyl-phosphate formed by the P-type pumps to alkaline conditions precluded the use of SDS Laemmli gels (1970) when the phosphoenzyme was to be studied. In such cases the proteins were separated on an acidic gel [7% running gel (pH 6.8) and 4% stacking gel (pH 5.6)] as described by Sarkadi et al., (1986). After electrophoresis the gel was stained, dried, and exposed to an X-ray film at −70 °C or quantified with a Phosphorimager device.

The gel was electroblotted to nitrocellulose sheets according to Towbin et al. (1979). The blots were incubated with the primary antibody (1N, 4N, or 2A; Stauffer et al., 1995) in TBST buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 0.5% Tween 20) and with the secondary anti-rabbit (mouse) Ig antibody (alkaline phosphatase conjugated), diluted 1:7500 in TBST. Each incubation was for 60 min at room temperature. The bands were visualized in 0.5 mM $MgCl_2$ and 0.1 M Tris-HCl, pH 9.5, with NBT and BCIP (Promega, Madison, WI). To quantify the expressed proteins, after the incubation with the primary antibody, the blots were tested with an anti-rabbit Ig ^{125}I-labeled F(AB′)$_2$ fragment at 0.5 μCi/10 mL (Amersham International, Buckinghamshire, England). The radioactivity was quantified using a Phosphorimager device.

RESULTS

Expression and Construction of the Mutant PMCA4. Table 1 offers a summary of the conserved amino acids in the transmembrane domains 4, 5, 6, and 8 of the PMCA, SERCA, Na^+/K^+, and H^+/K^+ pumps. As mentioned, some of these amino acids have been proposed to be involved in the translocation of Ca^{2+} by the SERCA pump (Clarke et al., 1989a). To establish whether the four amino acids of the PMCA4 pump shown in Table 1 (the PMCA4CI isoform

Table 1: Conserved Residues in the Transmembrane Domains of the SERCA, PMCA, H^+/K^+-, and Na^+/K^+-ATPases[a]

	TM4	TM4	TM4	TM5	TM6	TM6	TM6	TM8
SERCA	P308	E309	P312	E771	N796	T799	D800	E908
H^+/K^+-ATPase	P342	E343	L346	E795	E820	T823	D824	E936
Na^+/K^+-ATPase	P326	E327	L330	E779	D807	T807	D808	V924
PMCA	P422	E423	P426	A854	N879	M882	D883	Q971

[a] The sequences used in the table were those of the rabbit SERCA pump (Brandl et al., 1986), the rat gastric H^+/K^+-ATPase (Shull & Lingrel, 1986), the sheep kidney Na^+/K^+-ATPase (Shull et al., 1985), and the PMCA pump (Strehler et al., 1990).

has been used in the work to be described) were involved in the translocation of Ca^{2+}, Glu423, Asn879, Asp883, and Gln971 were each mutated to Ala. In addition, Glu423 was mutated to Gln, Asp, or Asn, Asn879 to Asp, Asp883 to Glu or Asn, and Gln971 to Glu. The two other conserved residues of transmembrane domain 4 mentioned in the introduction, Pro 422 and Pro 426, were also mutated to Ala. A third set of mutants (Ile929 to Val, Glu1078 to Gln), which were not expected to influence significantly the activity of the pump, were also prepared. These were also prepared as controls for the mutagenesis and for the back-cloning procedures. The cassettes containing the mutations were routinely sequenced to completion in at least one direction. Since the construction procedure required additional cloning steps, the expression of mutated but still fully active PMCA pumps was taken to mean that no unwanted mutations had occurred. This was important since a propensity to random mutations of DNA constructs containing parts of the PMCA4 pump had been observed in the past (Adamo et al., 1992).

The mutated PMCAs were expressed in parallel to the wild-type pump in COS cells: as shown in Figure 1A (only some of the mutants are shown there), the mutants were generally expressed at similar levels. To test whether the overexpression of the PMCA pump influenced the expression of the endogenous PMCA pump, isoform-specific antibodies were used to stain membranes containing different amounts of the overexpressed recombinant proteins: the 1N and 2A antibodies were used in these experiments (Stauffer et al., 1995). No differences were detected in the level of the endogenous PMCA1 pump (Figure 1B, independent work has shown that PMCA1 is the major isoform of COS-7 cells) even when the level of expression of the recombinant protein is high (Figure 1C, lane 4).

Ca^{2+} Uptake by COS-7 Cell Microsomes. The microsomal fraction isolated from COS-7 cells transfected with the full-length PMCA4CI cDNA catalyzed ATP-dependent Ca^{2+} transport: the uptake process was calmodulin-dependent (not shown). As reported by others, the uptake mediated by the PMCA pump was lower than that observed for the microsomal fraction of COS cells expressing the SERCA pump (Enyedi et al., 1993) but was clearly distinguishable from the endogenous PMCA pump activity (Figure 2). Figure 2 shows the time dependency of Ca^{2+} uptake by some of the Ala mutants. The results with these and all the other mutants are summarized in Table 2. The Ile929Val and the Glu1078Gln mutants were the only ones that were still able to accumulate Ca^{2+} in the microsomes of the transfected COS-7 cells: all others were inactive (Figure 2, Table 2). The result with the Gln971Glu mutant was rather unexpected since the mutation of Glu908 to Gln in the SERCA pump failed to influence the activity of the pump (Clarke et al., 1990). The observation on the mutation of the Gln971 to Glu was confirmed in three independent experiments.

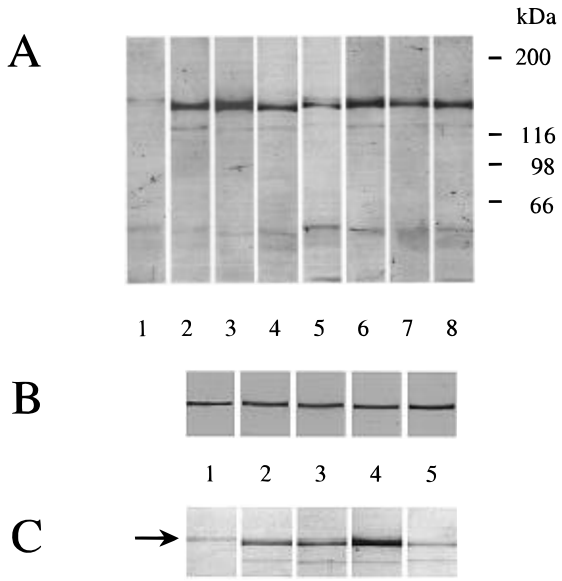

FIGURE 1: Transient expression of the mutated PMCA4CI pumps in COS-7 cells. (A) Crude membrane proteins (30 μg) obtained from transfected COS-7 cells (prepared by the freeze and thaw method) were separated by SDS−6% PAGE, transferred to nitrocellulose, and stained with the 4N antibody, which is specific for PMCA pump isoform 4 (Stauffer et al., 1995). COS-7 cells were transfected with the vector alone, lane 1; with the Pro422Ala construct, lane 2; with the Glu423Ala construct, lane 3; with the Pro426Ala construct, lane 4; with the Asn879Ala construct, lane 5; with the Asp883Asn construct, lane 6; with the Gln971Ala construct, lane 7; with the Glu1078Gln construct, lane 8. (B, C) Crude membrane proteins (30 μg) from transfected COS-7 cells were divided into two aliquots and transferred to nitrocellulose sheets. One aliquot was probed with the 1N antibody (specific for the endogenous PMCA pump isoform 1, panel B) and the other with antibody 2A, which recognizes all PMCA isoforms (panel C). Lanes 1, COS-7 cells transfected with the vector alone; lanes 2, with the wild-type PMCA4CI construct; lanes 3, with the Pro422Ala construct; lanes 4, with the Ile929Val construct; lanes 5, with the Gln971Ala construct. The arrow indicates the position of the endogenous PMCA pump.

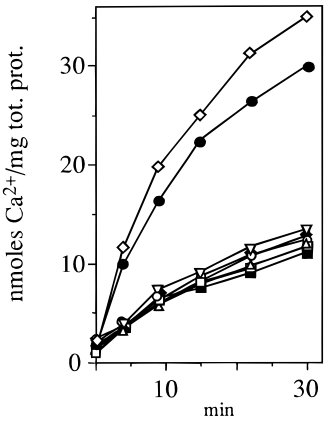

FIGURE 2: Ca^{2+} uptake by microsomes prepared from COS-7 cells overexpressing the wild-type or the mutant PMCA4CI pump. Microsomal proteins (30 μg) were resuspended in 1 mL of uptake buffer. The reaction was started by the addition of ATP. At the times indicated a 150-μL aliquot was withdrawn and rapidly filtered. The figure shows a typical experiment (representative of three independent experiments). Untransfected COS-7 cells (□), wild-type PMCA4CI (●), Ile929Val construct (◇), Pro422Ala construct (▽), Glu423Ala construct (△), Asn879Ala construct (◆), Asp883Ala construct (○), Gln971Ala construct (■).

Table 2: Summary of the Ca^{2+} Uptake by Microsomes of COS-7 Cells Transfected with the PMCA4 Pump Mutants

mutant	location	Ca^{2+} uptake[a,b]
P422A	TM5	0
E423A	TM5	0
E423D	TM5	0
E423N	TM5	8 ± 8[c]
E423Q	TM5	0
P426A	TM5	10 ± 8[c]
N879A	TM6	0
N879D	TM6	0
D883A	TM6	0
D883E	TM6	0
D883N	TM6	5 ± 5[c]
I929V	TM7	160 ± 35
Q971A	TM8	0
O971E	TM8	0
E1078Q	C-terminus	65 ± 20

[a] The Ca^{2+} uptake was measured 10 min after the addition of ATP. It is expressed as the percent of the activity (± standard deviation) obtained with the wild-type pump calculated according to the following equation: $(A_{mut} - A_{cos})/(A_{pmca} - A_{cos}) \times 100$, where A_{mut} corresponds to the uptake measured for the PMCA4 mutant, A_{cos} to the uptake of mock-transfected COS cells, and A_{pmca} to wild-type pump activity. [b] The 0 value means that in these experiments the standard deviation was within 5% of the value obtained for nontransfected cells. [c] Statistically not significant.

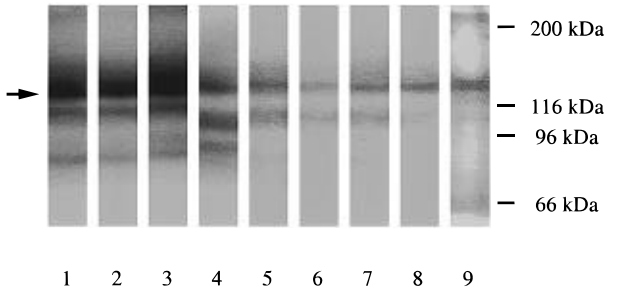

FIGURE 3: Formation of the phosphorylated intermediate starting from ATP. Microsomal proteins (30 μg) were phosphorylated in the presence of 500 μM $CaCl_2$, 500 μM $LaCl_3$, and 0.3 μM ATP (300 Ci/mmol) as described in the Materials and Methods section. The phosphorylated proteins were separated on acidic gels. The gel was stained with Coomassie Brilliant Blue, dried, and exposed to a Fuji RX film at −70 °C for 2 days. Lane 1, wild-type PMCA4CI; lane 2, Glu1078Gln mutant; lane 3, Ile929Val mutant; lane 4, microsomal proteins of untransfected COS-7 cells; lane 5, Glu423Ala mutant; lane 6, Asn879Ala mutant; lane 7, Asp883Ala mutant; lane 8, Gln971Ala mutant; lane 9, Pro426Ala mutant. The arrow indicates the position of the PMCA-specific phosphoenzyme intermediate. For the band in lanes 1−3 a (2.3 ± 0.24)-fold (PMCA4CI), a (2 ± 0.2)-fold (Glu1078Gln mutant), and a (2.8 ± 1.2)-fold (Ile929Val mutant) increase of the 135-kDa phosphorylated band as compared to untransfected cells or to cells transfected with the remaining mutants (lanes 4−9) was observed. Values are the average of at least three independent experiments ± the standard deviation.

Unfortunately, the high background activity made it impossible to establish whether some of the inactive mutants still had a residual activity, i.e., lower than 10% that of the wild-type pump.

Formation of the Phosphorylated Intermediate by the PMCA Mutants. A number of phosphorylated bands were present in membrane preparations of COS-7 cells, but the overexpression of PMCA4 clearly resulted in a 3−5-fold increase of the phosphorylated band at 135 kDa [Figure 3, compare lane 1 with lane 4; see also Heim et al. (1992)]. High concentrations of La^{3+} were routinely used in the assay system, since this ion specifically augmented the steady-state level of the phosphoenzyme intermediate of the PMCA pump. As was the case for the experiments on Ca^{2+} uptake, only the Ile929Val and the Glu1078Gln mutants were able to form a phosphoenzyme intermediate from ATP: a strong radioactive band at 135 kDa could indeed be seen in the lanes corresponding to membranes containing these two mutants (Figure 3, lanes 2 and 3). This band comigrated with that of the wild-type PMCA4 protein (Figure 3, lane 1). In agreement with the experiments presented in Figure 2 and Table 2, none of the other mutants showed increased intensity of the phosphorylated band at 135 kDa with respect to untransfected cells (Figure 3; only part of the mutants have been shown). The small differences in the level of the phosphorylated intermediate (Figure 3, lanes 4−9) were not significant and did not reflect the activity of the mutants. Similar results were obtained using membranes from cells expressing different amounts of the mutant pumps: none of the mutated pumps (apart from those in the Ile929Val and the Glu1078Gln mutants) were able to form the phosphorylated intermediate from ATP. At variance with what observed in the SERCA pump, the Pro mutant (Pro426Ala; Figure 3, lane 9) was also unable to form the phosphorylated intermediate: the Pro mutation of the SERCA pump was unable to transport Ca^{2+} but was still able to form a phosphoenzyme intermediate from ATP (Vilsen et al., 1989). The higher level of intermediate formed by the Ile929Val compared to that of the PMCA4CI wild-type pump and to that of the Glu1078Gln mutant was consistent with the observation that this mutant reproducibly had a higher Ca^{2+} uptake rate than the wild-type protein (Table 2 and Figure 2).

The PMCA pump can also form the phosphorylated intermediate starting from phosphate, provided that Ca^{2+} is absent and DMSO is present in the reaction mixture (Chiesi et al., 1984). Unfortunately La^{3+} cannot stabilize the intermediate formed from phosphate, i.e., only low levels of the intermediate can be detected when starting from phosphate (Figure 4). In the case of the wild-type PMCA4 protein a 2-fold increase of the radioactivity associated with the band at 135 kDa could be observed upon incubation with ^{32}P (Figure 4, compare lanes 2 and 1). The radioactive band was sensitive to hydroxylamine (not shown). The 2-fold increase, albeit low, was nevertheless reproducibly observed in four independent experiments (not shown). A more accurate evaluation of the amount of intermediate formed by the overexpressed proteins and by the endogenous pump was not possible given the high background, which was due to the high amount of radioactive phosphate that had to be used in these experiments. Membranes of COS-7 cells transfected with the Glu423Ala, Asp883Ala, and Gln971Ala mutants formed the phosphorylated intermediate from phosphate (Figure 4). In the case of the Asn879Ala mutant a (1.4 ± 0.2)-fold increase with respect to untransfected cells (determined with a Phosphorimager; Figure 4, lane 4) was obtained, indicating that this mutant was likely to be partially active. Consistent with this, the weak signal for the Asn879Ala was only observed in membrane preparations containing high amounts of the expressed mutant. Surprisingly, however, the level of intermediate formed by mutants Glu423Ala and Asp883Asn was clearly higher as compared to the wild-type pump: that of the Glu423Ala mutant was up to 5 times higher (Figure 4, lanes 3 and 5). The same

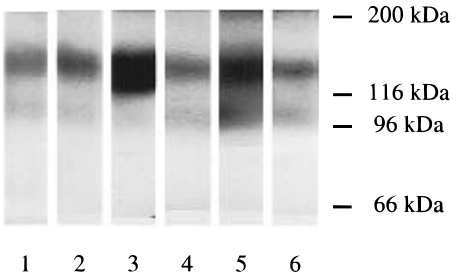

FIGURE 4: Formation of the phosphoenzyme intermediate from P_i. Proteins (crude membranes prepared by the freeze and thaw method, 50 μg) were phosphorylated in the presence of 200 μM P_i (5 Ci/mmol), 32% DMSO, and 10 mM EGTA as described in the Materials and Methods section. The labeled proteins were prepared for autoradiography as described in the legend for Figure 3. Lane 1, membrane proteins from untransfected COS-7 cells; lane 2, membrane proteins from COS-7 cells overexpressing the wild-type PMCA4CI; lane 3, Glu423Ala mutant; lane 4, Asn879Ala mutant; lane 5, Asp883Asn mutant; lane 6, Gln971Ala mutant. The difference in the intensity of the radioactive 135−140-kDa band of the endogenous pump (lane 1) with respect to the bands of the PMCA4 and the PMCA4 pump mutants (other lanes) was observed in several experiments, in which high amounts of the mutant pumps were expressed. The calculated means (*x*-fold increase as compared to the untransfected cells ± standard deviation) were the following: PMCA4CI, 1.6 ± 0.3; Glu423Ala mutant, 4.9 ± 1.3; Asn879Ala mutant, 1.4 ± 0.2; Asp883Asn mutant, 2.5 ± 0.35; Gln971Ala mutant,1.3 ± 0.1.

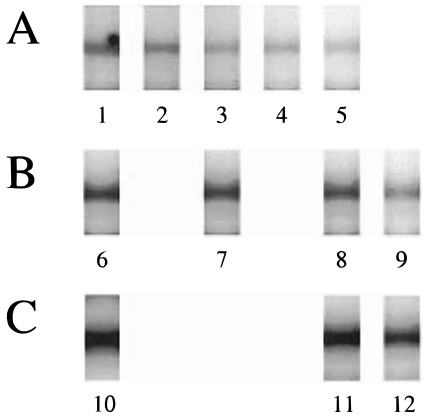

FIGURE 5: Ca^{2+} dependence of the formation of the phosphorylated intermediate starting from phosphate. Proteins (50 μg) of transfected COS-7 membranes were phosphorylated as described in the legend for Figure 4 in the presence of different Ca^{2+} concentrations. (A) Membrane proteins of COS-7 cells overexpressing the wild-type PMCA4CI pump were phosphorylated in the presence of, lane 1, 10 mM EGTA; lane 2, 30 μM $CaCl_2$; lane 3, 100 μM $CaCl_2$; lane 4, 300 μM $CaCl_2$; or lane 5, 1000 μM $CaCl_2$. (B) Membrane proteins of COS-7 cells overexpressing the Asp883Asn PMCA4CI mutant were phosphorylated in the presence of, lane 6, 10 mM EGTA; lane 7, 250 μM $CaCl_2$; lane 8, 1000 μM $CaCl_2$; or lane 9, 10 000 μM $CaCl_2$. (C) Membrane proteins of COS-7 cells overexpressing the Glu423Ala PMCA4CI mutant were phosphorylated from phosphate in the presence of, lane 10, 10 mM EGTA; lane 11, 1000 μM $CaCl_2$; or lane 12, 10 000 μM $CaCl_2$.

result was obtained with the Glu423Gln, Glu423Asp, and Glu423Asn or the Asp883Ala and Asp883Glu mutants, although the strongest effect was normally observed with the corresponding Ala mutants.

The formation of the intermediate from phosphate by the endogenous PMCA pump of the COS-7 cells (not shown) and the overexpressed PMCA4CI pump was strongly inhibited by Ca^{2+} (Figure 5A). Similar experiments with the Asp883Asn (Figure 5B) and Glu423Ala mutants (Figure 5C) indicate that the mutation of these two amino acids, in addition to stabilizing the pump intermediate, also caused a marked loss of its sensitivity to Ca^{2+}: a 50−60% decrease of the intensity of the intermediate could only be observed at a Ca^{2+} concentration of 10 mM for the first of the two mutants. In the case of the second mutant, even at 10 mM Ca^{2+} the inhibition was minor (Figure 5B,C). The very low level of the phosphoenzyme intermediate formed from phosphate in the case of the Asn879 and Gln971 mutants prevented the determination of Ca^{2+} dependency.

Targeting of the Mutated PMCA4. The expressed PMCA4 pump is located in the plasma membrane of COS cells (Zvaritch et al., 1995; Foletti et al., 1995). The proper targeting of the pump can also be considered a test of its structural integrity and proper folding. The cellular location of the PMCA Glu423Ala, Asn879Ala, Asp883Ala, Gln971Ala, and Gln971Glu mutants was studied after transfection and staining of the permeabilized COS cells with the 4N antibody (Stauffer et al., 1995) (Figure 6). A cell transfected with the wild-type PMCA4CI pump has been included in the figure (panel A) as a control. The Glu423Ala and Asp883Ala mutants showed the typical pattern for plasma membrane localization (Figure 6, panels B and D) whereas the Asn879Ala, Gln971Ala, and Gln971Glu mutants yielded a staining indistinguishable from that of the proteins retained in the endoplasmic reticulum [Figure 6, panels C, E, and F; for an example of typical reticular staining of the endoplasmic reticulum, see Foletti et al. (1995)]. The cellular staining shown in Figure 6 corresponds to that seen in at least 95% of the transfected cells. The two mutants that induced stabilization of the intermediate formed from phosphate (Glu423Ala and Asp883Ala; see Figures 4 and 5) were correctly delivered to the plasma membrane, whereas the others (Asn879Ala, Gln971Ala, and Gln971Glu) were retained in the endoplasmic reticulum.

DISCUSSION

Site-directed mutagenesis has been extensively used to explore the role of different amino acids in the SERCA pump [for a summary see MacLennan (1990) and Andersen and Vilsen (1995)]. An essential ingredient of the work on the SERCA pump has been its successful expression in COS-7 cells (Maruyama & MacLennan, 1988). Only limited information has so far been obtained on the PMCA pump due to the difficulties in expressing it at high levels in COS-7 cells. It has nevertheless recently been possible to reproducibly measure the activity of isoform 4 of the PMCA pump expressed in these cells (Heim et al., 1992; Enyedi et al., 1993).

The work presented here has focused on six amino acids located in transmembrane domains 4, 6, and 8 of the PMCA pump. These amino acids are all highly conserved in P-type pumps (see Table 1) and have been proposed to mediate Ca^{2+} transport in the SERCA pump (Clarke et al. 1989a,b; Vilsen et al., 1989): a similar role could thus be tentatively predicted for these amino acids in the PMCA pump. Two of these six amino acids (Pro308 and Pro312 in the rabbit SERCA pump, see Table 1) have been proposed to be important for the folding of the peptide chain surrounding the high-affinity Ca^{2+}-binding domain (Vilsen et al., 1989). In the SERCA pump, their mutation to Ala resulted in reduced Ca^{2+} affinity (Pro308) and in the loss of the capacity to pump Ca^{2+} (Pro 312): the Pro312 mutant, however, could still bind Ca^{2+} with

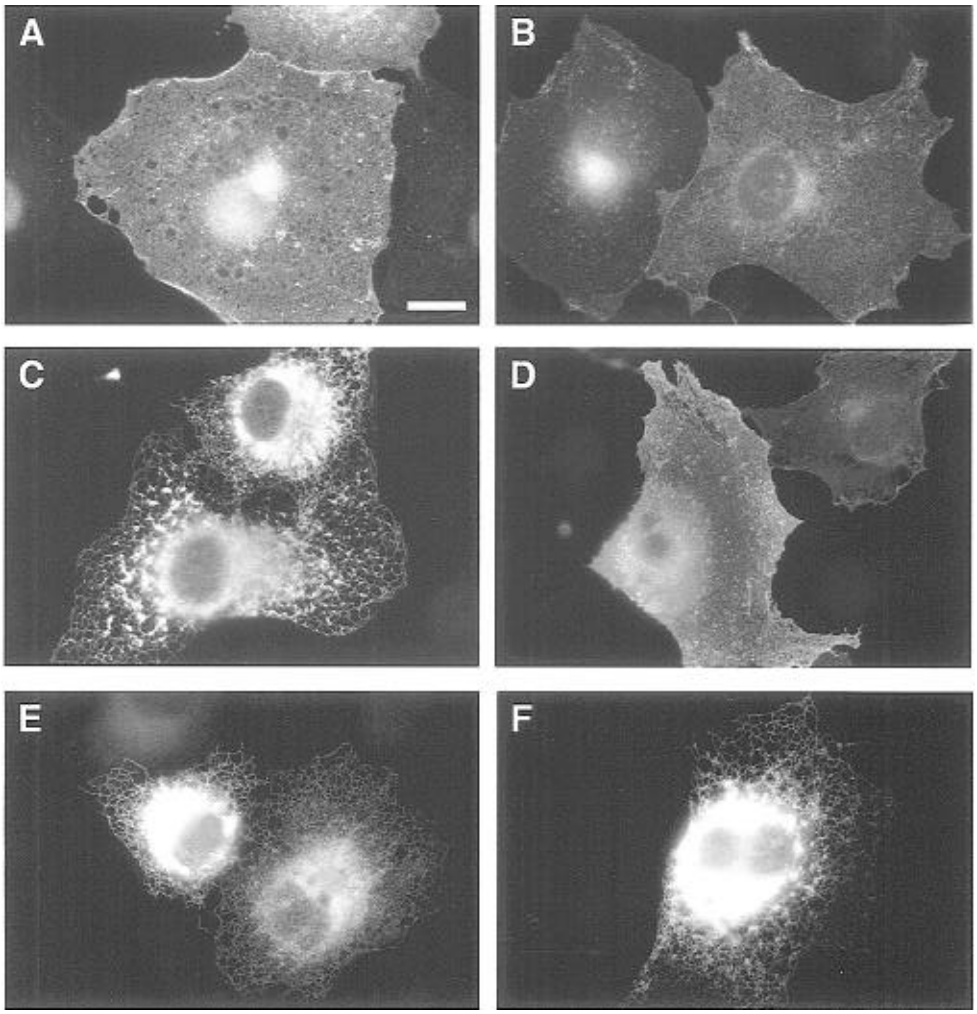

FIGURE 6: Cellular localization of the mutated PMCA4CI pump. COS-7 cells were prepared for immunocytochemistry as described in Foletti et al. (1995) 48 h after the start of the transfection. The cells were fixed and permeabilized with 0.1% Triton X-100 for 3 min. Antibody 4N was used for the staining. The cells were transfected with the wild-type PMCA4CI cDNA (panel A), and with the DNA of PMCA4CI mutants Glu423Ala (panel B), Asn879Ala (panel C), Asp883Ala (panel D), Gln971Ala (panel E), and Gln971Glu (panel F). Other technical details are found in Foletti et al. (1995).

high affinity and could form the phosphoenzyme intermediate from ATP (Vilsen et al., 1989). The experiments on the PMCA4 pump presented here have shown that the Pro → Ala mutations (Pro422 and Pro426) eliminated the transport of Ca^{2+} and the ability to form the phosphoenzyme intermediate from ATP. This indicates that these two Pro residues have a more critical role in the PMCA pump than in the SERCA pump, i.e., they could act on the proper folding of the high-affinity Ca^{2+}-binding pocket.

The other set of mutations investigated had the aim to establish whether four charged or polar amino acids, also located in the transmembrane domain (Glu423, Asn879, Asp883, and Gln971) were necessary for the high-affinity binding of Ca^{2+} to the PMCA pump. Mutagenesis work on the SERCA pump had indeed led to the suggestion that the four homologous amino acids are integral components of the high-affinity Ca^{2+} binding site, which would be formed by the appropriate three-dimensional arrangement of the transmembrane domains, i.e., by bringing four of them (4, 5, 6, and 8) close to each other (Clarke et al., 1989a,b). It is noteworthy that only four of the six SERCA pump amino acids identified by Clarke et al. (1989a) are conserved in the PMCA4 pump. The remaining two (Glu771 and Thr799 in the SERCA pump, see Table 1) correspond to an Ala (Ala854) and to a Met (Met882) in the PMCA pump: mutagenesis work by Adebayo et al. (1994) has shown that Met 882 cannot be one of the ligands in the high-affinity Ca^{2+}-binding pocket. Also noteworthy is the fact that no charged residues are found in transmembrane domain 5 of the PMCA protein, whereas in all other P-type pumps this transmembrane domain contains a highly conserved Glu (Table 1). It is naturally possible that other, still unidentified residues in the PMCA pump's transmembrane domains contribute to the high-affinity binding site; further mutagenesis work, e.g., on residues of transmembrane domain 5, is certainly in order. At the present state of knowledge, however, it is tempting to relate the lower Ca^{2+}/ATP stoichiometry of the PMCA to the lower number of potential Ca^{2+}-binding amino acids.

None of the mutants of these residues showed Ca^{2+}-transporting activity upon transfection in COS-7 cells, and none of them was able to form the phosphoenzyme intermediate from ATP. The loss of pump activity in the Gln971Glu mutant was surprising, since Glu908 in the SERCA pump could be changed to a Gln without changes in pump activity (Clarke et al., 1989b). The formation of the phosphoenzyme intermediate from phosphate by these mutants showed two types of effects: in one case (Glu423 and Asp883) the level of the phosphoenzyme intermediate was increased, while in the second (Asn879, Gln971) it was

similar to that of the wild-type pump. Interestingly, when residue Glu423 or Asp883 was mutated, the formation of the phosphoenzyme from phosphate was much less sensitive to Ca^{2+} than in the wild-type pump. Only concentrations of Ca^{2+} in the vicinity of 10 mM brought about a significant decrease in the amount of phosphoenzyme intermediate. The phenotype of these mutants is thus consistent with the disruption of the high-affinity binding of Ca^{2+}, which is supported by the observed stabilization of the E2 intermediate (the intermediate formed from phosphate). This stabilization is likely to be the result of the inhibition of the decay of the intermediate via the E1 state (these mutants do not form the phosphoenzyme intermediate from ATP). The differences in the amount of intermediate in the Glu423 and Asp883 mutants probably reflect subtle differences in the perturbations of the Ca^{2+}-binding pocket. Since none of the substitutions reversed the loss of Ca^{2+} binding, steric constraints in addition to the charge effects must also play a critical role.

Proper folding is a requirement for the targeting of the PMCA to the plasma membrane [see also Foletti et al. (1995)]. The pumps carrying mutations at the amino acids Glu423 or Asp883 were correctly delivered to the plasma membrane, indicating that these mutants were properly folded. By contrast, mutations of Asn879 or Gln971 resulted in the retention of the recombinant proteins in the endoplasmic reticulum. This indicates (major) perturbations in the folding of the overexpressed protein. The possibility that the finding was due to artifacts of the preparation is very unlikely, since cells expressing different amounts of the proteins showed the same reticular staining. It will thus be difficult to establish whether these residues are directly involved in the binding to Ca^{2+}: the structural perturbations leading to the retention of the mutated proteins in the endoplasmic reticulum probably have a stronger effect on the activity of the mutants. The mistargeted mutants were able to form the phosphoenzyme intermediate from phosphate, but its low level prevented the (accurate) evaluation of its properties.

The results presented here are thus consistent with the involvement of Glu423 and Asp883 in the high-affinity site of Ca^{2+} binding, and this would be in line with the finding that these two residues are the most conserved charged amino acids in P-type pumps (see Table 1). The possibility that they are involved in the formation of a general ionic channel, rather than a specific Ca^{2+} channel, should be seriously considered, especially since site-directed mutagenesis experiments involving these amino acids in the Na^+/K^+-ATPase led to findings similar to those reported here (Andersen & Vilsen 1995).

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