The plasma membrane calcium ATPase (PMCA) actively transports Ca$^{2+}$ from the cytosol to the extra cellular space. The C-terminal segment of the PMCA functions as an inhibitory domain by interacting with the catalytic core. Ca$^{2+}$-calmodulin binds to the C-terminal segment and stops inhibition. Here we showed that residue Asp$^{170}$, in the putative “A” domain of human PMCA isoform 4xb, plays a critical role in autoinhibition. In the absence of calmodulin a PMCA containing a site-specific mutation of D170N had 80% of the maximum activity of the calmodulin-activated PMCA and a similar high affinity for Ca$^{2+}$. The mutation did not change the activation of the PMCA by ATP. Deletion of the C-terminal segment further downstream of the calmodulin-binding site led to an additional increase in the maximal activity of the mutant, which suggests that the mutation did not affect the inhibition because of this portion of the C-terminal segment. The calmodulin-activated PMCA was more sensitive to vanadate inhibition than the autoinhibited enzyme. In contrast, inhibition of the D170N mutant required higher concentrations of vanadate and was not affected by calmodulin. Despite its higher basal activity, the mutant had an apparent affinity for calmodulin similar to that of the wild type enzyme, and its rate of proteolysis at the C-terminal segment was still calmodulin-dependent. Altogether these results suggest that activation by mutation D170N does not involve the displacement of the calmodulin-binding autoinhibited domain from the catalytic core and may arise directly from changes in the accessibility to the calcium-binding residues of the pump.

PMCA1 and PMCA4 are the most widespread isoforms, whereas PMCA2 and PMCA3 are rather cell-specific. The PMCAs belong to the subtype 2B of P-type ATPases that are characterized by their autoregulation by internal sequences. At resting concentrations of Ca$^{2+}$, autoinhibition keeps the activity of the PMCA low. When the concentration of Ca$^{2+}$ in the cytosol increases, Ca$^{2+}$-calmodulin binds to the C-terminal portion of the molecule, switching the PMCA to an activated state of higher maximum activity and affinity for Ca$^{2+}$. The removal by partial proteolysis or deletion mutagenesis of 120 C-terminal residues of human (h)PMCA4xb, including the calmodulin-binding site, results in a constitutively active form insensitive to calmodulin (3). More recently, it has been shown that a construct named ct92, truncated after the calmodulin-binding site, displays an apparent affinity for Ca$^{2+}$ very similar to that of the full-length hPMCA4xb, but its basal activity is 2–3 times higher (4). This fact indicates that the calmodulin-binding sequence suffices to keep the enzyme in a state of low affinity for Ca$^{2+}$, whereas additional determinants downstream from the calmodulin-binding sites are required for full inhibition.

In agreement with the mechanism of autoinhibited protein kinases and phosphatases (5, 6), the inhibition of the PMCA seems to involve the interaction between the catalytic region of the molecule and the inhibitory sequence (see Fig. 1A). Indeed, it has been shown that a peptide of 28 amino acids (C28) with the sequence of the calmodulin-binding autoinhibitory site binds to segments Ile$^{206}$-Val$^{217}$ and Cys$^{253}$-Thr$^{254}$ in the minor and large cytoplasmic loops, respectively (7, 8). The homologous regions in SERCA are part of domains “A” and “N” (9). The ACA2 from Arabidopsis thaliana is also a calmodulin-regulated Ca$^{2+}$ pump containing an autoinhibitory region, which, at variance with the PMCA, is located at the N terminus of the molecule. Recently Curran et al. (10) found that mutations of residues in the stalk region of ACA2 disrupt autoinhibition. In particular the residue Asp$^{210}$ located in the stalk segment 2 was extremely sensitive, because substitutions by Asn, Glu, or Lys resulted in calmodulin-independent activated pumps. In parallel with ACA2, the stalk portion of the PMCA may also be important for autoinhibition. Indeed, homology modeling of the PMCA using the crystal structures of SERCA1 suggests that residue Asp$^{170}$ (homologous to Asp$^{210}$ in ACA2) is in a conformationally sensitive portion of M2 (see Fig. 1B).

Here we report on the effects of alterations of Asp$^{170}$ of the PMCA. Similar to that described previously in ACA2, we found that the mutation D170N results in a deregulated calmodulin-insensitive PMCA. Hence, this residue seems equally critical for autoinhibition in both ion pumps. Nevertheless, we found that the D170N PMCA does not fully resemble the calmodulin-
activated enzyme, particularly because in the mutant, activation took place without fully disengaging the C-terminal domain from the catalytic core. These results indicate that the molecular mechanism involved in the activation of the PMCA by D170N is only partially common to that elicited by calmodulin, and the results suggest a direct role of Asp$^{70}$ in regulating the access to the calcium-binding residues of the pump.

**MATERIALS AND METHODS**

**Chemicals**—Polyoxyethylene 10 lauryl ether (C$_{12}$E$_{10}$), 1,2-o-phosphatidylcholine type X-E (P-5394) from dried egg yolk, phosphodiesterase-3', 5'-cyclic nucleotide activator (calmodulin) from bovine brain, calmodulin agarose, calcimycin (A23187), chymotrypsin, ATP (disodium salt, vanadium-free), sodium dodecyl sulfate, yeast synthetic drop-out media supplement without leucine, yeast nitrogen base without amino acids, dextrose, enzymes and cofactors for the synthesis of ATP$^{32}$P, and all other chemicals were obtained from Sigma. Tryptone and yeast extract were from Difco. Carrier-free [P$^{32}$]PO$_4$ was provided by PerkinElmer Life Sciences.

**Site-directed Mutagenesis**—Portions of hPMCA4xb were amplified by a two-step PCR process to obtain the desired mutation in a clonable fragment using the manufacturer's instructions. The sequence of the oligonucleotide primers were as follows (New England BioLabs Inc.): F: 5'-CTCGAGGTCGACCATGGCGCATCACCATCACA-TCACAAACCCTAGCGGCTTCTGCAGCCACTCCCTGTGGAAGGTGCTGGT-3'; B: (CTTCTGCTCAATTATAGAAAGCCTA); 5'DN, (GCTTCTGAATATTGGGCAAAGAGAAG); and 829, (AAGGATGATTCCAGTGGAGT). The products HIS-3DN and 5'DN-829 were isolated from the reaction mixture in a 1:1 agarose gel and extracted using DNA QIAEX II (Qiagen). The isolated products of the first PCR were used in a subsequent PCR step with primers HIS and 829. The amplified fragment was subcloned in the corresponding position of hPMCA4xb after digestion with SalI and PflM1. A similar strategy was used to obtain the mutation in the coding sequence of calmodulin. The mutated PMCA sequences were confirmed by DNA sequencing.

**Protein Staining**—SDS electrophoresis and immunoblotting were carried out as described previously (18). Proteins were electrohoresed on a 7.5% acrylamide gel according to Laemmli (19) and revealed by Coomassie Blue staining or subsequently electrotransferred onto Millipore Immobilon P membranes. Non-specific binding was blocked by incubating the membranes overnight at 4 °C in a solution of 160 mM NaCl, 0.05% Tween 20, and 1% nonfat dry milk. The membranes were incubated for 1 h with 50 μl of antibody (20, 21) from ascitic fluid (dilution 1:1000). For staining, biotinylated anti-mouse immunoglobulin G and avidin-horseradish peroxidase conjugate were used. The image was acquired using a Foto/Analyst documentation system from Fotodyne, and the intensity of the bands was quantified with the Gel-Pro Image Analyzer software.

**Proteolytic Digestion of PMCA**—Limited proteolysis of the membranes from yeast transformed with the appropriate construct was carried out at 37 °C essentially as described in Pedanay et al. (22). 10 μg of membranes isolated from yeast was preincubated for 5 min at 37 °C in 100 μl of medium containing 100 mM KCl, 25 mM HEPES-K, 1 mM MgCl$_2$, 100 mM EGTA-K, 200 μM CaCl$_2$ (100 μM free Ca$^{2+}$ when added), 5 mM dithiothreitol, 0.05% Triton X-100, and 485 nM calmodulin (when added). The proteolysis was initiated by the addition of ice-cold trichloroacetic acid (6% final concentration). The precipitate was supplemented with 100 μg of bovine serum albumin, washed twice with distilled water, and then dissolved in electrophoresis sample buffer.

**RESULTS**

**Expression and Purification of the Wild Type PMCA and Mutants**—A novel expression system using yeasts was used for the production of recombinant PMCA. 2. S. cerevisiae strain DBY2062 cells were transformed with the pMP625 vector containing the human PMCA4xb cDNA under the control of the strong PMA1 promoter. The presence of the PMCA protein in yeast membranes was detected by SDS-PAGE and Western blot (Fig. 1C). Antibody 5P10 recognized a single band at 135 kDa indicating that the full-length polypeptide of the recombinant wild type (recWT) protein was successfully expressed. Mutants D170N and D170K were expressed at a level similar to that of the wild type protein. Membranes from yeast were solubilized with detergent C$_{12}$E$_{10}$, and the recombinant PMCA proteins were purified by calmodulin affinity chromatography. As shown in Fig. 1D, the EGTA eluate contained highly pure PMCA protein as revealed by electrophoresis and Coomassie Blue staining of the gel.

2. H. P. Adamo, L. M. Bredeston, and G. R. Corradi, manuscript in preparation.
As templates for the E1 and E2 conformations, respectively, identified with the protein data bank codes 1EUL and 1IWO were used.

The model was obtained using the SwissModel (35), an automated homology modeling server running at the Geneva GlaxoSmithKline PMCA extending from M2 up to the conserved P-ATPase motif TGES.

Asp170 is indicated ( ).

The position of the Asp170 is indicated (D170). CB, calmodulin-binding site; P, phosphorylation site. The putative autoinhibitory receptor sites in the core of the protein are indicated as empty boxes. B, model of the segment of the PMCA extending from M2 up to the conserved P-ATPase motif TGES. The coordinates of the structures of SERCA identified with the protein data bank codes 1EUL and 1IWO were used as templates for the E1 and E2 conformations, respectively. C, Western blot of yeast membranes containing the expressed recWT hPMCA4xb and mutants. 10 μg of total yeast membranes protein was applied on each lane of an SDS-7.5% polyacrylamide gel and subjected to immunoblot analysis with monoclonal antibody 5F10. Yeast transfected with the empty vector (control) or the vector containing the cDNA encoding hPMCA4xb (recWT), D170N, or D170K. pPMCA, 50 ng of PMCA purified from porcine red cells was loaded. D, gel run as in C and stained with Coomassie Blue. Yeast recWT membranes, 3.5 and 7 μg of membranes from yeast expressing hPMCA4xb, recWT; 0.6 and 1 μg of recombinant hPMCA4xb purified from yeast; D170N, 0.9 and 1.3 μg of purified D170N mutant; D170K, 1.5 and 2.3 μg of purified D170K mutant; pPMCA, 1.7 and 2.6 μg of PMCA purified from porcine red cells.

**Functional State of Mutants D170N and D170K**—The C12E10 solubilized recombinant PMCA purified from yeasts was supplemented with phosphatidylinositol to obtain a full activation (23). Under these conditions the recWT exhibited a Ca2+ ATPase activity of ~2.4 μmol P i/mg protein/min, whereas mutant D170N had a somewhat lower activity of 1.5 μmol of P i/mg of protein/min. After the reconstitution of the purified proteins in phosphatidylinositol liposomes the maximal activities of the recWT and D170N enzymes in the presence of saturating amounts of Ca2+-calmodulin were 0.72 μmol of P i/mg of protein/min and 0.43 μmol of P i/mg of protein/min, respectively. Substitution of Asp170 by Lys led to a substantial reduction in the activity, which for the soluble enzyme activated by phosphatidylinositol was 0.13 μmol of P i/mg of protein/min and thus this mutant was not studied further.

**Dependence of Ca2+ ATPase of Mutant D170N on Ca2+ Concentration**—The activity of the recWT and D170N enzymes reconstituted in phosphatidylinositol liposomes was measured as function of Ca2+ concentration in both the presence and absence of calmodulin. In the absence of calmodulin the recWT had a low apparent affinity for Ca2+ (KCa = 3.4 μM) and a Vmax at 40% of that attained in the presence of calmodulin (Fig. 2A). The addition of calmodulin increased the Vmax and lowered the KCa to ~0.5 μM. Curves in Fig. 2B show that in the presence of calmodulin the Ca2+ dependence of D170N was identical to that of the recWT. However, in the absence of calmodulin the apparent affinity for Ca2+ of D170N remained as high as in the presence of calmodulin, and its Vmax was ~80% of that observed in the presence of calmodulin. The kinetic parameters of recWT and D170N are summarized in Table I.

**Effect of ct92 Truncation on the D170N Mutant**—The activation of mutant D170N could reflect the release of the inhibition because of the segment downstream the calmodulin-binding site. To test this idea a mutant D170Nct92 truncated after the calmodulin-binding site was constructed. The maximal specific Ca2+ ATPase activity of C12E10-solubilized D170Nct92 supplemented with phosphatidylinositol was similar to that of D170N. Fig. 3 shows that as expected, after reconstitution in phosphatidylinositol liposomes D170Nct92 had similar apparent affinities for Ca2+. However, the ct92 truncation of D170N increased the Vmax, in the absence of calmodulin, suggesting that the D170N mutation does not affect the inhibition of Vmax exerted by the portion of the C-terminal regulatory domain downstream from the calmodulin-binding site.

**Activation of recWT and D170N by ATP**—High concentrations of ATP in the range of the low affinity regulatory site ATP increase the stimulatory effect of calmodulin (24). To test whether the differences in calmodulin activation of recWT and D170N were related to a different dependence on ATP, the activity of the recWT and D170N enzymes was measured as function of ATP concentration in both the presence and absence of calmodulin. Fig. 4 shows that the D170N and recWT enzymes had a similar response to ATP either in the presence or in the absence of calmodulin. At saturating concentrations of ATP calmodulin increased the activity of recWT ~3-fold, whereas D170N was stimulated 1.4-fold.

**Effect of Vanadate on the Ca2+ ATPase of the recWT and D170N Enzymes**—Because vanadate is a transition state ana-
FIG. 2. Ca$^{2+}$ concentration dependence of the rate of ATP hydrolysis by recWT (A) and D170N (B) enzymes. The Ca$^{2+}$-ATPase activity of purified PMCA reconstituted in phosphatidylcholine liposomes was measured at 37 °C as described under "Materials and Methods." Empty symbols, no calmodulin; Filled symbols, 200 nM calmodulin. The data are the average of 3 experiments with 3 different preparations. The Ca$^{2+}$-ATPase activity of each preparation in the presence of 10 μM Ca$^{2+}$ and 200 nM calmodulin was taken as 100%. The lines represent the best fit of the data given by the Hill equation.

TABLE I

Effects of the D170N substitution on the kinetics parameters of recWT

|        | D170N | RecWT |
|--------|-------|-------|
| $V_{max}$ | 42 ± 3 | 94 ± 2 |
| $n_H$   | 1.1 ± 0.1 | 2.0 ± 0.2 |
| $K_{0.5}$ Ca$^{2+}$ (μM) | 3.4 ± 0.6 | 0.5 ± 0.1 |

and it was not affected by calmodulin.

Dependence of recWT and D170N on Calmodulin Concentration—The Ca$^{2+}$-ATPase activity of recWT and D170N enzymes was measured as a function of calmodulin concentration. The measurement was performed at 10 μM free Ca$^{2+}$ to ensure the saturation of calmodulin with Ca$^{2+}$ at all calmodulin concentrations. Under this condition the maximal activation by calmodulin was 2.5- and 1.3-fold for recWT and D170N, respectively. However, as shown in Fig. 6, both enzymes attained half-maximal activation at ~13 nM calmodulin.

Accessibility of the C-terminal Region to Degradation by Chymotrypsin—It has been shown recently that the interaction between the C-terminal autoinhibitory domain and the catalytic core of hPMCA4xb affects the sensibility of the C-terminal segment to proteolysis (22). Results in Fig. 7 show the effect of chymotrypsin on the recWT and D170N pumps. In agreement with Ref. 22, the degradation of the full-length recWT at the C-terminal segment producing a 124.5-kDa fragment proceeded slowly in the absence of Ca$^{2+}$, slightly faster in the presence of Ca$^{2+}$, and much faster in the presence of Ca$^{2+}$-calmodulin. Similarly, the degradation of D170N was minimally affected by Ca$^{2+}$ but was significantly enhanced by Ca$^{2+}$-calmodulin. However, even in the presence of calmodulin, the D170N mutant was more resistant to proteolysis than the recWT enzyme.

DISCUSSION

Disruption of PMCA Autoinhibition by the Mutation of Asp$^{170}$ to Asn—Recent mutagenesis studies (26, 27) have lead to the identification of several residues from the C-terminal regulatory segment of the PMCA that are critical for calmodulin binding and autoinhibition. In contrast, the role of the catalytic core in autoinhibition is much less known. Ba-Thein et al. (28) reported that activation of the PMCA4 requires the substitution of both its small and large cytoplasmic loops by those of the naturally activated PMCA2. The results presented here show that mutation of residue Asp$^{170}$ to Asn suffices for the activation of PMCA4 and thus they provide the first evidence of the importance of a single residue from the core of the PMCA molecule in autoinhibition. Curran et al. (10) used complementation of the yeast strain K616 to select for random
Role of Asp<sup>170</sup> in PMCA Autoinhibition

**Materials and Methods**

The Ca<sup>2+</sup>-ATPase activity of recombinant recWT (A) and D170N (B) enzymes as a function of ATP concentration. The reaction medium contained 10 μM free Ca<sup>2+</sup> and enough MgCl₂ to give 1.25 mM free Mg<sup>2+</sup> in the absence (empty symbols) or in the presence of 200 nM calmodulin (filled symbols). The Ca<sup>2+</sup>-ATPase activity of each preparation in the presence of 3000 μM ATP and 200 nM calmodulin was taken as 100%. The data were fitted to a simple hyperbola with the following parameters:

- Maximum activity in the absence of calmodulin was subtracted from each data point, and the value at 200 nM calmodulin was taken as 100%. The lines represent the best fit of the data given by the Hill equation. The apparent Kᵦ (nM) for calmodulin was 13 ± 1 for recWT and 12 ± 2 for D170N.

**Fig. 5. Sensitivity to vanadate.** The Ca<sup>2+</sup>-ATPase activity of the recWT (circles) and D170N (squares) enzymes was measured in a medium containing 20 mM HEPES-K, pH 7.00 at 37 °C, 100 mM KCl, 0.5 mM EGTA, 2 μM A23187, 10 μM free Ca<sup>2+</sup>, 4 mM MgCl₂, and the indicated concentrations of vanadate, in the absence (empty symbols) or in the presence (filled symbols) of 200 nM calmodulin. The activity of each preparation in the absence of vanadate was taken as 100%. The curves were fitted by the equation

\[ v = V_{\text{max}} \frac{K_i}{K_i + [\text{vanadate}]} \]

where \( V_{\text{max}} \) is the maximum velocity in the absence of vanadate and \( K_i \) is the inhibition constant. The best fitted values of \( K_i (\mu M) \) were for recWT, without calmodulin 11.2 ± 0.6 and with calmodulin 2.6 ± 0.1, and for D170N, without calmodulin 20.6 ± 1.5 and with calmodulin 21.9 ± 1.7.

**Fig. 6. Calmodulin dependence of the rate of ATP hydrolysis by the recWT and D170N.** ATP hydrolysis was measured as described under "Materials and Methods" at a constant free Ca<sup>2+</sup> of 10 μM. Calmodulin was varied from 0 to 200 nM. The data points are the average of 2 experiments with different purified preparations. The activity in the absence of calmodulin was subtracted from each data point, and the value at 200 nM calmodulin was taken as 100%. The lines represent the best fit of the data given by the Hill equation. The apparent Kᵦ (nM) for calmodulin was 13 ± 1 for recWT and 12 ± 2 for D170N.

Mutations that constitutively activate the ACA2 pump. The mutation D219N (homologous to D170 in hPMCA4xb) was found to be present in 80% of the potentially deregulated pumps generated by the chemical mutagenesis of ACA2. Therefore, this change is very effective in activating the enzyme. In the PMCA we found that despite its activating effects, mutations D170N and D170K decreased the specific activity to 63 and 5%, respectively, suggesting a role for Asp<sup>170</sup> beyond autoinhibition.

**Fig. 4. Effect of mutation D170N on the activation by ATP.** Ca<sup>2+</sup>-ATPase activity of recombinant recWT (A) and D170N (B) enzymes as a function of ATP concentration. The reaction medium contained 10 μM free Ca<sup>2+</sup> and enough MgCl₂ to give 1.25 mM free Mg<sup>2+</sup> in the absence (empty symbols) or in the presence of 200 nM calmodulin (filled symbols). The Ca<sup>2+</sup>-ATPase activity of each preparation in the presence of 3000 μM ATP and 200 nM calmodulin was taken as 100%. The lines represent the best fit of the data given by the Hill equation. The apparent Kᵦ (nM) for calmodulin was 13 ± 1 for recWT and 12 ± 2 for D170N.
also by the accessibility of the binding site, which is dependent upon its interaction with the core of the molecule (31). In previous studies (26–28) mutants with a basal activity higher than that of the recWT enzyme were shown to also have a higher apparent affinity for calmodulin, a fact that was taken as indicative of a weaker interaction between the catalytic core and the C-terminal autoinhibitory domain. We found that mutant D170N despite having a higher basal activity and hence a small degree of calmodulin activation, did not exhibit a significant change in the apparent affinity for calmodulin. This result suggests that this mutation does not sufficiently affect the interaction between the autoinhibitory region and the catalytic core as to modify the accessibility of calmodulin to its site.

An alternative test of the strength of the interaction between the C-terminal regulatory region and the catalytic core of the PMCA has been reported recently (22). Activation of the PMCA either by calmodulin or mutations in the C-terminal segment was shown to increase the proteolytic digestion of the C-terminal region. Consistent with the idea that mutation of Asp170 to Asn did not abolish the interaction between the C terminus and the core of the PMCA, we found that the chymotryptic digestion of mutant D170N at the C-terminal segment proceeded slowly in the absence of Ca2+ and much faster after the addition of calmodulin. However the mutant appeared to be more resistant to proteolysis at the C terminus than the recWT protein, suggesting the persistence of the interaction with the core even in the presence of calmodulin.

The deregulation by mutation at residue Asp170 in PMCA and the corresponding residue in ACA2 suggests that different autoinhibited pumps share a similar mechanism for activation. However different residues may be involved in the contacts between the autoinhibitory region and the central catalytic portion of the pumps. Indeed a number of mutations that cause activation have been reported in other pumps (30, 32, 33). On the other hand, the results presented here point out that mechanisms other than a weaker interaction with the C terminus may result in a calmodulin-like activation. It is noteworthy that our modeling of the PMCA by homology with the SERCA1 pump suggests that in the E2 conformation Asp170 is part of a long and continuous M2 helix, which extends up to Cys182, whereas in the E2 conformation a portion of the M2 helix between Phe168 and Gln176 becomes unwound. It has been recently proposed (34) that in the SERCA pump the C-terminal end of the M2 helix plays a critical role in the conformational changes leading to the occlusion by closing off the access to the cytosol of the bound ion. Thus, it is conceivable that the activation of the PMCA by the mutation of Asp170 involves conformational rearrangements in the core of the enzyme that directly modify the accessibility of Ca2+ transport site.

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Role of Asp$^{170}$ in PMCA Autoinhibition

41625

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Loss of Autoinhibition of the Plasma Membrane Ca\textsuperscript{2+} Pump by Substitution of Aspartic 170 by Asparagine: ACTIVATION OF PLASMA MEMBRANE CALCIUM ATPase 4 WITHOUT DISRUPTION OF THE INTERACTION BETWEEN THE CATALYTIC CORE AND THE C-TERMINAL REGULATORY DOMAIN

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