The role of the plasma membrane Ca\(^{2+}\) pump (PMCA) is to remove excess Ca\(^{2+}\) from the cytosol to maintain low intracellular Ca\(^{2+}\) levels. Asp\(^{1080}\) lies within an acidic sequence between the C-terminal inhibitory region and the catalytic core of PMCA and is part of the caspase-3 recognition site of isoform 4b. Caspase-3 cuts immediately after this residue and activates the pump by removing the inhibitory region (Pászty, K., Verma, A. K., Padányi, R., Filoteo, A. G., Penniston, J. T., and Enyedi, Á. (2002) J. Biol. Chem. 277, 6822–6829). Asp\(^{1080}\) had not been believed to have any other role, but here we show that it also plays a critical role in the autoinhibition and calmodulin activation of PMCA4b. Site-specific mutation of Asp\(^{1080}\) to Asn, Ala, or Lys in PMCA4b resulted in a substantial increase in the basal activity in the absence of calmodulin. All Asp\(^{1080}\) mutants exhibited an increased affinity for calmodulin because of an increase in the rate of activation by calmodulin. This rate was higher when the inhibition was weaker, showing that a strong inhibitory interaction slows the activation rate. In contrast, mutating the nearby Asp\(^{1077}\) had no effect on basal activity or calmodulin activation. We propose that the conserved Asp\(^{1080}\) even though it is neither in the regulatory domain nor in the catalytic core, plays an essential role in inhibition by stabilizing the inhibited state of the enzyme.

The plasma membrane calcium pump (PMCA\(^{1}\)) is an essential element of intracellular Ca\(^{2+}\) homeostasis. Its role is to remove excess Ca\(^{2+}\) from the cell to maintain low cytosolic Ca\(^{2+}\) concentrations critical for cell survival and Ca\(^{2+}\) signaling. An increase in cellular Ca\(^{2+}\) results in an increase in substrate Ca\(^{2+}\) at the transport site of the pump, as well as an increase in activator Ca\(^{2+}\)-calmodulin complex. PMCA are encoded by four genes, and alternative splicing of the primary transcripts produces many more isoform variants (1). Two of the four PMCA gene products (PMCA1 and PMCA4) are ubiquitous, whereas the expression of PMCA2 and PMCA3 appears to be more cell- and tissue-specific.

PMCA4b has a low activity in the absence of calmodulin and thus a great stimulation by calmodulin (2). A series of experiments using peptides and C-terminally truncated mutants of hPMCA4b showed that the high affinity calmodulin binding region lay within a 28-residue sequence between Leu\(^{1086}\) and Ser\(^{1113}\) (3, 4). It has been demonstrated that the same region is responsible for most of the autoinhibition, but a downstream sequence (between Ser\(^{1113}\) and Asp\(^{1157}\)) also contributes. Removal of the whole C terminus from Leu\(^{1086}\) to the end resulted in a constitutively active enzyme; it could not be further activated by addition of calmodulin (2).

Cross-linking experiments with the calmodulin binding synthetic peptide C28 have revealed that C28 interacts with two sites within the catalytic core of PMCA; one is located downstream of the phosphoenzyme forming aspartic acid between residues 537–544, and the other is within the small cytoplasmic loop (transduction domain or activation domain) (5–7).

Although the contribution of the sequence between residues Leu\(^{1086}\) and Asp\(^{1157}\) to the regulation of the enzyme has been studied extensively, the role of the sequence between transmembrane domain 10 and Leu\(^{1086}\) is poorly understood. Because this region belongs neither to the autoinhibitory domain nor to the catalytic core of the enzyme, it may be considered as a connecting region or hinge. A highly acidic region is located just upstream of the calmodulin-binding/autoinhibitory sequence. This acidic region is rich in proline, aspartic/glutamic acid, and serine/threonine and has been suggested to play a role in calpain recognition (8); however, experiments using peptides from isoform 1 did not support this idea (9). Also, it has been suggested that this acidic region contains a high affinity Ca\(^{2+}\) binding site, but its role in the regulation of the pump has not been addressed (10). In addition, part of the upstream region (between Glu\(^{1087}\) and Arg\(^{1087}\)) was suggested to encode a “masked” signal for endoplasmic reticulum retention that was functional only when the C terminus downstream of it was removed (11).

In more recent experiments, we have demonstrated that Asp\(^{1080}\), located in this hinge region five residues upstream of the calmodulin binding domain of hPMCA4b, is a target for caspase-3 cleavage. hPMCA4b is cut by caspase-3 downstream of Asp\(^{1080}\) specifically upon the early phase of apoptosis, producing a single 120-kDa fragment (12). This fragment showed the same characteristics as the constitutively active ct120, which is truncated at Glu\(^{1085}\); it was fully active in the absence of calmodulin.

In the previous paper (12) we also showed that mutation of Asp\(^{1080}\) to Ala in the Asp\(^{1077}\)-Glu-Ile-Asp\(^{1080}\) caspase recognition sequence abolished the susceptibility of hPMCA4b to
Asp1080 is Critical in PMCA Regulation

Caspar-3. In the present paper we have analyzed the functional consequences of that mutation. When we tested the caspase-3-resistant Asp1080 → Ala mutant for Ca2+-transport activity, we found that it was almost fully active in the absence of calmodulin. This was the first evidence that the hinge region referred to above may be involved in the autoinhibitory interaction. Therefore, we have studied further the role of Asp1080 in the regulation of hPMCAb. We have mutated Asp1080 to Ala, to Asn, and to Lys and found that these mutations not only activated the pump substantially but also increased its affinity for calmodulin. The increased affinity was because of an increased rate of activation by calmodulin. Our data indicate that Asp1080 plays an essential role in autoinhibition and that the rate of calmodulin activation of these mutants of hPMCAb depends on the degree of autoinhibition.

MATERIALS AND METHODS

Construction of the hPMCAb Mutants—Mutation at Asp1080 and Asp1077 was done by the double PCR method as described previously (12). Briefly, the first PCR product was made by amplification of an hPMCAb sequence using a primer containing the desired mutation and a primer including a unique restriction site (BamHI) already present in the sequence. The product of this PCR was then used as primer for a second round of PCR with the other primer including the second unique site (NsiI). The PCR products were cloned using the blunt PCR cloning kit from Invitrogen and sequenced by the Mayo Molecular Biology Core Facility. The NsiI-BamHI piece was cut out and placed in the wild type hPMCAb in pSP72. The full-length SstI-KpnI piece was then cut out of pSP72 and ligated into expression vector pMM2.

Cell Culture and Transfection—COS-7 cells were grown at 37 °C, 5% CO2 in a humidified atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml gentamicin. COS-7 cells were transfected as described before (13) using the LipofectAMINE reagent based on the protocol recommended by the manufacturer (Invitrogen). Briefly, cells were grown in 175-cm2 flasks to 70–80% confluence. Cells were incubated at 37 °C with DNA-LipofectAMINE complex (formed by incubating 5 μg of DNA and 100 μl of LipofectAMINE in 3.6 ml of serum-free OPTI-MEM medium for 15 min at 4 °C). Cells were incubated in 14.5 ml of serum-free OPTI-MEM medium. After 5 h of incubation cells were supplemented with serum; 24 h later the incubation medium was replaced with fresh tissue culture medium, and cells were harvested after an additional 24 h.

Isolation of Microsomes from COS-7 Cells—Crude microsomal membranes from COS-7 cells were prepared as described (2) with the following modifications. Cells were washed with ice-cold phosphate-buffered saline solution, pH 7.4, then harvested in the same medium containing 0.1 mM phenylmethylsulfonyl fluoride, 6 μg/ml aprotinin, 2.2 μg/ml leupeptin, and 1 μM EDTA. After centrifugation, cells were resuspended in an ice-cold hypotonic solution containing 10 mM Tris-HCl, pH 7.4, 1 mM MgCl2, 0.5 mM EDTA, 2 μg/ml aprotinin, and 4 mM dithiothreitol. After lysis, homogenization, and centrifugation, the final pellet was resuspended in a solution of 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 0.15 M KCl, 2 mM dithiothreitol, and 20 μM CaCl2, and the suspension was stored in liquid N2.

Ca2+ Transport Assay—Ca2+ uptake by microsomal membrane vesicles was carried out as described previously (14, 15) in a reaction mixture containing 25 mM TES-triethanolamine, pH 7.2, 100 mM KCl, 7 mM MgCl2, 100 μM CaCl2 (labeled with 45Ca), 40 mM KH2PO4/K2HPO4, pH 7.2, 200 mM thapsigargin, 4 μg/ml oligomycin, and enough EGTA to obtain the desired Ca2+ concentration. Calmodulin was added as indicated in the figure legends. The free Ca2+ was calculated as described previously (16, 17). Micromoles of 20 μg/ml concentration were added, and Ca2+ uptake was initiated by the addition of 5 mM ATP. The reaction was terminated by rapid filtration of the microsomes using Millipore filters (0.45-μm pore size). Data were analyzed with GraphPad Prism (GraphPad Software Inc.).

Solubilization of PMCA for ATPase Assays—COS cell membrane preparations were solubilized and reconstituted as described (18). Briefly, COS cell membrane preparations containing 100–200 μg of protein were pelleted by centrifugation in a microfuge tube and resuspended in 80 μl of solubilization buffer (60 mM TES-triethanolamine, pH 7.2, 240 mM KCl, 10 mM MgCl2, 400 μM EGTA, 10 mM NaF, 2 mM dithiothreitol, 1 mM ouabain, 8 μg/ml oligomycin, 400 mM thapsigargin, 4 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5% Triton X-100) at 4 °C. After 4 min, 320 μl of dilution buffer (same as solubilization buffer except 0.5 mg/ml phosphatidy chololine replaced Triton X-100) was added and then 200 μl of Bio-Beads SM-2 were added to remove the Triton X-100. The tube was placed on a gel rocker for 1 h at 4 °C. The bulk of the Bio-Beads were removed by centrifugation, and the residual beads were removed by filtration with a 0.45-μm spin filter.

Pre-steady State Rate of Activation by Calmodulin—The assay medium contained 30 mM TES-triethanolamine, pH 7.2, 120 mM KCl, 5 mM MgCl2, 2.5 mM ATP, 5 mM NaF, 1 mM dithiothreitol, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 200 mM EGTA, enough CaCl2 to obtain 0.5 μM free Ca2+, ±235 nM calmodulin, 0.2 mM 2-amino-6-mercaptop-7-methyl purine ribonucleoside (MESG), and 1 units/ml of the coupled enzyme purine nucleoside phosphorylase. Phosphorolysis of MESG was monitored by an increase in absorbance at 360 nm at 37 °C on a Beckman DU 70 spectrophotometer. Data were fitted to the following equation: Yt = Y0 - v0t/k + v0Mt + (v0/k)exp(-kt), where Y0 is the absorbance at 360 nm at time zero, v0 is the steady state slope in the absence of calmodulin, and (v0 + vM) is the steady state slope in the presence of 235 nM calmodulin (18).

Inactivation by Calmodulin Removal—Experiments to measure the rate of inactivation upon calmodulin dissociation were conducted in the same assay medium as for calmodulin activation. The enzyme was allowed to reach steady state at 0.5 μM free Ca2+ and 235 nM calmodulin and then 10 μM calmodulin binding peptide RRKWQKTH-GHAIRAGLRSS from chicken smooth muscle myosin light chain kinase (19) was added to the reaction. Data were fitted to the following equation: Y(t) = Y0 - v0t + (v0/k1) + (v1/k2)exp(-kt).

RESULTS

We have shown previously (12) that mutation of the aspartate residues of the 1077DEID1082 caspase recognition sequence made hPMCA4b partially (Asp1077 → Ala) or completely (Asp1080 → Ala, and Asp1077 → Ala/Asp1080 → Ala) resistant to caspase cleavage. In this paper we have made additional mutants (Asp1080 → Asn and Asp1080 → Lys) and studied further the effect of mutations in the 1077DEID1082 sequence on the activity of the pump. The mutants made are shown in Fig. IA. Fig. LB shows that the mutants have the expected size and are expressed equally well in COS cells. ATP-dependent Ca2+ uptake by COS cell membrane vesicles was measured at a saturating Ca2+ concentration in the presence and absence of calmodulin. Fig. 2 shows that all Asp1080 mutants had higher basal activity in the absence of calmodulin than the wild type. The Asp1080 → Ala and Asp1080 → Lys mutants were almost fully active in the absence of calmodulin. Although the Asp1080 → Asn mutant showed considerably lower basal activity than the alanine or lysine mutants, it still had about 2–3 times higher basal activity than the wild type enzyme. In contrast, the basal activity of the Asp1077 → Ala mutant did not differ substantially from the wild type and was between 20 and 30% of that of the maximum. The basal activity of the wild type hPMCA4b is typically between 15 and 20% of the maximal activity in the presence of calmodulin. Although surprising, these data pointed out that the sequence between transmembrane domain 10 and the calmodulin binding/autoinhibitory sequence can play an important role in autoinhibition and that Asp1080 must be one of the key residues of that region that are involved in stabilizing the inhibited conformation of the enzyme in the resting state (i.e. in the absence of calmodulin).

The curves in Fig. 3 show the activity of the mutants as a function of Ca2+ concentration. The activities were measured in both the presence and absence of calmodulin. In the presence of calmodulin the curves of all constructs were nearly identical as shown in Fig. 3B and indicated by the parameters shown in Table 1. This suggests that mutation of Asp1080 increased specifically the basal activity of the pump with no effect on the characteristics of the full active form of the enzyme. In the
Absence of calmodulin the Asp₁₀₈₀ mutants all had substantially higher maximal velocity and Ca²⁺ affinity than the wild type (see Fig. 3A and Table I). At saturating Ca²⁺ concentrations calmodulin increased the activity of the Asp₁₀₈₀→Ala and Asp₁₀₈₀→Lys mutants only by a factor of 1.2–1.3 whereas the Asp₁₀₈₀→Asn mutant retained more autoinhibition. This latter mutant was stimulated by calmodulin about 2.5 times at saturating Ca²⁺ concentrations. However, this is still much less than the 5–7-fold stimulation observed in the wild type. In all cases the affinity for Ca²⁺ was further increased by calmodulin.

Then we tested the effect of mutation at Asp₁₀₈₀ on the calmodulin affinity. We measured the Ca²⁺ transport activity of the constructs as a function of calmodulin concentration at a relatively low (0.3 μM) Ca²⁺ concentration (Fig. 4). This low Ca²⁺ concentration was chosen, because under these conditions the stimulation with calmodulin was greater than at saturating Ca²⁺ concentrations. To reach steady state or near steady state conditions, the membranes were preincubated with calmodulin for 5–10 min at 37 °C. Fig. 4 shows that each Asp₁₀₈₀ mutant had higher affinity for calmodulin than the wild type. In contrast mutation at Asp₁₀₇₇ did not affect calmodulin affinity. The K₅₅₉ for calmodulin of the Asp₁₀₈₀→Ala, Asp₁₀₈₀→Lys, and Asp₁₀₈₀→Asn mutants were 14 ± 2, 17 ± 3, and 29 ± 6 nM, respectively, compared with 65 ± 11 nM for the wild type hPMCA4b. It is apparent from Table I, that the increase in calmodulin affinity was greater for the mutants Asp₁₀₈₀→Ala and Asp₁₀₈₀→Lys, which had higher basal activity than for the mutant Asp₁₀₈₀→Asn, which had lower basal activity.

An increase in steady state calmodulin affinity could be because of an increase in the rate of activation by calmodulin (the product of conformational opening of PMCA and calmodulin association) or a decrease in the rate of inactivation by calmodulin removal (the product of calmodulin dissociation and conformational closing of PMCA). We have shown previously that activation of the wild type hPMCA4b by 235 nM calmodulin in the presence of 500 nM free Ca²⁺ has a half-time of about 40 to 60 s and that the rate of inactivation is much slower, with a half-time of about 20 min (18). To measure the rate of activation, wild type, Asp₁₀₈₀→Ala or Asp₁₀₈₀→Asn pumps were allowed to reach steady state ATPase activity in the absence of calmodulin at 50 nM Ca²⁺ and then activation was initiated by the addition of 235 nM calmodulin. These Ca²⁺ and calmodulin concentrations provide for a rate of activation that is slow enough to monitor by a conventional spectrophotometer yet yield a sufficient change in steady state activity. Fig. 5 shows a typical experiment with the Asp₁₀₈₀→Ala mutant. The acti-
Asp<sup>1080</sup> Is Critical in PMCA Regulation

Calmodulin of both mutants fully accounted for the 2–4 times higher affinity for calmodulin observed in the steady state experiments we assumed that the mutation did not affect substantially the rate of inactivation by calmodulin removal. To test this assumption, the rate of inactivation was measured for one of the mutants. Inactivation measurements were performed by incubating wild type or mutant hPMCA4b with 500 nM free calcium and 235 nM calmodulin and allowing the reaction to reach a constant ATPase activity. Then a vast excess of calmodulin binding peptide from myosin light chain kinase was added to the reaction mixture (18). The myosin light chain kinase peptide has no direct effect on PMCA but will sequester any calmodulin that dissociates from PMCA. The inactivation rate constant (k<sub>inact</sub>) was obtained by a fit to the equation for inactivation. Inactivation experiments were done only with the Asp<sup>1080</sup>→Asn mutant, because its larger activation with calmodulin allowed an accurate determination of the inactivation rate constant. As expected, mutation at Asp<sup>1080</sup> did not affect k<sub>act</sub>. The rate constants of the mutant and the wild type were nearly identical within the range of the variability of the assay system (Table II). Based on the k<sub>act</sub> and k<sub>inact</sub> constants, we calculated the K<sub>s</sub> for the mutants and the wild type. When compared with the wild type, the K<sub>s</sub> for the mutants were

![Graph of Ca<sup>2+</sup> transport](image)

**Fig. 3.** Dependence of Ca<sup>2+</sup> transport of the Asp<sup>1080</sup>→Ala (DA), Asp<sup>1080</sup>→Lys (DK), and Asp<sup>1080</sup>→Asn (DN) mutants and wild type pump (wt) on Ca<sup>2+</sup> concentration. Ca<sup>2+</sup> transport was assayed at variable free Ca<sup>2+</sup> concentrations in the absence (A) or presence of 235 nM calmodulin (B). The activities are expressed relative to the maximum activity measured at saturating Ca<sup>2+</sup> in the presence of 235 nM calmodulin. Data points represent the means ± S.E. of two or three independent determinations. Data were fitted to the Hill equation as given by the software GraphPad Prism (GraphPad Software Inc.) The kinetic parameters given by this fit are shown in Table I. The filled squares and the filled circles are the wild type data points in the absence or presence of calmodulin, respectively. The other symbols are the same for both panel A and B; the downward pointing filled triangles are the Asp<sup>1080</sup>→Asn mutant (DN), the open circles are the Asp<sup>1080</sup>→Ala mutant (DA), and the filled diamonds are the Asp<sup>1080</sup>→Lys (DK) mutant.

![Graph of Ca<sup>2+</sup> transport](image)

**Fig. 4.** Dependence of Ca<sup>2+</sup> transport of the Asp<sup>1080</sup>→Ala (DA), Asp<sup>1080</sup>→Lys (DK), Asp<sup>1080</sup>→Asn (DN), and Asp<sup>1077</sup>→Ala (D1077A) mutants on calmodulin concentration as compared with the wild type pump (wt). Ca<sup>2+</sup> transport was assayed at non-saturating 0.3 μM Ca<sup>2+</sup> concentration. Data points are expressed as f = (V - V<sub>0</sub>)/(V<sub>max</sub> - V<sub>0</sub>), where V<sub>0</sub> is the activity in the absence of calmodulin, V<sub>max</sub> is the activity in the presence of saturating calmodulin, and v is the activity at each given calmodulin concentration. The data were fitted with a one-site binding equation using GraphPad Prism. The parameters given by the fit are shown in Table I. The filled squares are the wild type data points, the downward pointing filled triangles are the Asp<sup>1080</sup>→Asn mutant, the filled diamonds are the Asp<sup>1080</sup>→Ala mutant, the open triangles are the Asp<sup>1077</sup>→Ala mutant, and the open circles are the Asp<sup>1080</sup>→Lys mutant.

### Table I

**Kinetic characteristics of the Asp-1080 and Asp-1077 mutants as judged by steady state Ca<sup>2+</sup> transport assays**

Ca<sup>2+</sup> transport assays for the mutants were done as described in the legends of Figs. 3 and 4. Maximal Ca<sup>2+</sup> transport activities were determined for each mutant at saturating Ca<sup>2+</sup> and calmodulin concentrations. Data of three independent determinations were fitted using the Hill equation as given in GraphPad Prism (GraphPad Software Inc.).

|            | K<sub>1/2</sub> for Ca<sup>2+</sup> (μM) | V<sub>max</sub> (% of maximum) | K<sub>1/2</sub> for Calmodulin (nm) |
|------------|----------------------------------------|-------------------------------|-----------------------------------|
| Wild type  | 1.49 ± 0.25                           | 0.25 ± 0.08                   | 22 ± 6                           |
| D<sup>1077</sup>A | 0.57 ± 0.11                           | 0.17 ± 0.05                   | 32 ± 6                           |
| DN         | 0.62 ± 0.12                           | 0.18 ± 0.04                   | 43 ± 3                           |
| DA         | 0.52 ± 0.15                           | 0.13 ± 0.05                   | 79 ± 8                           |
| DK         | 0.58 ± 0.13                           | 0.15 ± 0.03                   | 75 ± 6                           |

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Asp\textsuperscript{1080} Is Critical in PMCA Regulation

2–3-fold times lower, in good correlation with the steady state assays.

**DISCUSSION**

In this study we analyzed the role of a conserved aspartic acid residue (Asp\textsuperscript{1080}) between transmembrane domain 10 and the calmodulin binding/autoinhibitory domain of PMCA isoforms. The pump is one of the ubiquitously expressed isoforms of PMCA, and the effect of the mutation was tested by kinetic analysis. Because Asp\textsuperscript{1080} is highly conserved among PMCA, our data should provide a more general information of how PMCA are regulated by calmodulin.

In the absence of Ca\textsuperscript{2+}-calmodulin, the pump is in an inhibited or “closed” conformation. In isoform hPMCA4b the closed conformation has very low activity. It has been demonstrated that the calmodulin binding domain itself is responsible for most of the inhibition (4). Experiments using synthetic peptides have revealed that in the inhibited state the calmodulin binding domain interacts with both cytoplasmic loops (5–7). This interaction makes the catalytic sites less accessible. Activation by Ca\textsuperscript{2+}-calmodulin frees the catalytic sites from the autoinhibition and increases the apparent Ca\textsuperscript{2+} affinity and maximum activity of the enzyme. Truncation of hPMCA4b at the C terminus has shown that residues downstream of the calmodulin binding domain between Ser\textsuperscript{1113} and Asp\textsuperscript{1157} also contribute to inhibition, because removal of these contacts caused partial activation of the pump (4).

Previous studies showed that mutations and truncations in the calmodulin binding domain (4, 20), downstream inhibitory region (15), and catalytic core (21) all affected the basal activity in the absence of calmodulin. Here we have shown that mutation of a residue outside of the three regions described previously also causes such a change. We have demonstrated that mutation of a single aspartate residue upstream of the conventional calmodulin binding/autoinhibitory domain of hPMCA4b increased the basal activity (i.e. the activity in the absence of calmodulin) substantially but did not affect the overall catalytic function of the enzyme. Calmodulin was still able to increase the activity of the mutants, however, to a much smaller extent at saturating Ca\textsuperscript{2+} concentrations 1.2–1.3 times in the case of the alanine and lysine mutants and about 2.5 times for the asparagine mutant as compared with a 5–7-times stimulation of the wild type. It is very unlikely that the higher basal activities of the mutants were because of a higher calmodulin content of the membrane, because 1) the microsomes were washed extensively with Ca\textsuperscript{2+} chelators, and 2) the mutation did not affect the rate of calmodulin removal. All these data suggest that these mutants have their conformational equilibrium shifted more toward the open, active conformation. The role of Asp\textsuperscript{1080} appears to be rather specific, because mutation of another aspartate (Asp\textsuperscript{1077}) in close proximity to Asp\textsuperscript{1080} did not have any effect.

The change in the basal activity was greatest when the acidic aspartate side chain was replaced by a non-polar alanine or a positively charged lysine side chain. These mutations affected the activity equally. The more conservative substitution of an amido group for the carboxyl group had less effect. These data indicate that the aspartate side chain plays an essential role in autoinhibition by stabilizing the autoinhibited closed conformation of the pump. Mutation at Asp\textsuperscript{1080} destabilized this conformation and caused activation of the protein as each mutant showed an increased basal activity and affinity for Ca\textsuperscript{2+} activation. Because no structural data are available for PMCA, the nature of the interactions remains unclear. We

![Fig. 5. Rate of ATPase activation by calmodulin.](image)

Reconstituted preparations containing h4b (upper panel) and Asp\textsuperscript{1080} → Ala (lower panel) were allowed to reach steady state in 500 nM Ca\textsuperscript{2+} and then 235 nM calmodulin was added to the reaction (time zero). Data were fitted to Equation 5 from Caride et al. (18), $Y(t) = Y_o - v_c / k_{act} + (v_o + v_c) t + (v_o / k_{act}) \exp(-k_{act} t)$, where $Y_o$ is the absorbance at 360 nm at time zero, $v_c$ is the steady state slope in the absence of calmodulin, and $(v_o + v_c)$ is the steady state slope in the presence of calmodulin. The apparent rate constant for activation ($k_{act}$) at 235 nM calmodulin was $5.82 \times 10^4 \pm 0.87 \times 10^3$ s\textsuperscript{-1}, M\textsuperscript{-1} for h4b and $1.85 \times 10^5 \pm 0.12 \times 10^5$ s\textsuperscript{-1}, M\textsuperscript{-1} for Asp\textsuperscript{1080} → Ala. These values correspond to reaction half-times of 51 and 16 s, respectively. The data shown are representative of at least three experiments, and the constants are given as means ± S.E. The dotted straight lines are lines fitted to the maximum rate achieved at long times ($v_o + v_c$) and are shown to allow a visual estimate of the curvature.

| Table II Pre-steady state parameters of the Asp-1080 mutants |
|---------------------------------------------------------------|
| The parameters of the constructs were determined as described under "Materials and Methods" and in the legend of Fig. 5. |

|       | $k_{act}$ (s\textsuperscript{-1}, M\textsuperscript{-1}) ± S.E. | $k_{max}$ (s\textsuperscript{-1}) ± S.E. | $K_d$       |
|-------|---------------------------------------------------------------|----------------------------------------|------------|
| Wild type | $0.58 \times 10^0 \pm 0.09 \times 10^3$ | $0.84 \times 10^{-2} \pm 0.13 \times 10^{-3}$ | 14.5       |
| DN    | $1.28 \times 10^2 \pm 0.14 \times 10^2$ | $1.00 \times 10^{-7} \pm 0.19 \times 10^{-3}$ | 7.9        |
| DA    | $1.85 \times 10^0 \pm 0.12 \times 10^3$ | ND (assume 4b)\textsuperscript{*} | 4.6        |

\*The $k_{max}$ for the D1080A mutant was not determined. Instead the value obtained for the wild type pump was used for calculating the $K_d$ of that mutant.
may hypothesize that electrostatic interactions are involved; however, the greater inhibition retained in the asparagine mutant suggests that the oxygen atoms of the carboxylate group may form hydrogen bonds that are only partially perturbed in the asparagine mutant.

In addition to the change in the basal activity, mutation at Asp1080 increased the apparent affinity of the pump for calmodulin. The increase in affinity was because of an increased rate of activation by calmodulin. The rate of inactivation by calmodulin removal was not affected by the mutation. This change in calmodulin affinity was greatest for the mutants that had the highest basal activity (alanine and lysine). Thus, the increase in calmodulin affinity was proportional to the increase observed in the basal activity of the mutants.

A general property of calmodulin is that it is able to induce secondary structure in a peptide that is unstructured prior to binding (22, 23). Thus, the Asp1080 mutation, which is outside of the calmodulin binding domain, should not affect the structure of the final calmodulin-bound state of the pump. This is consistent with the observation that mutation at Asp1080 did not affect the rate of inactivation by calmodulin removal, i.e. dissociation of calmodulin from the calmodulin binding domain. Rather mutation at this residue seems to have an indirect effect on the interaction of the calmodulin binding/autoinhibitory domain with the catalytic core. We suggest that calmodulin activates the Asp1080 mutants faster because of a looser competing interaction of the calmodulin binding domain with the catalytic core of PMCA that is reflected in a higher basal activity. This appears to be a general property of PMCAs, because all mutations and truncations made so far that increased the basal activity of the pump (18, 20, 21) also increased the rate of activation by calmodulin.

It had been demonstrated that the free calmodulin binding peptide C28 representing the calmodulin binding domain of PMCA4b had a much higher affinity for calmodulin than intact hPMCA4b (3). More recent experiments showed that the rate of activation by calmodulin of the truncated mutant ct120 with bound C28 peptide was much slower than binding of calmodulin to the C28 peptide in solution. This indicates that association of the calmodulin binding domain with the cytoplasmic loops slows down the reaction with calmodulin. Moreover, the rate of activation of the ct120-C28 complex was about three times faster than the rate of activation of ct92 in which the C28 sequence is tethered to the enzyme. This is consistent with our present finding, which shows that an activating mutation in the hinge region also increases the rate of activation by calmodulin three times.

In summary, our data suggest that Asp1080 serves as a contact point in the connecting region or hinge upstream of the calmodulin binding domain that assists in orienting the calmodulin binding domain to the catalytic sites to form stable autoinhibited conformation of the enzyme. Other contact points could be within the downstream inhibitory region, because ct92 still has higher basal activity than the wild type protein. We also conclude that weaker autoinhibitory interactions result in an increased rate of activation by calmodulin.

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