Deletions in the Acidic Lipid-binding Region of the Plasma Membrane Ca\(^{2+}\) Pump

A MUTANT WITH HIGH AFFINITY FOR Ca\(^{2+}\) RESEMBLING THE ACIDIC LIPID-ACTIVATED ENZYME\(^*\)

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The C-terminal segment of the loop between transmembrane helices 2 and 3 (A\(_L\) region) of the plasma membrane Ca\(^{2+}\) pump (PMCA) is not conserved in other P-ATPases. Part of this region, just upstream from the third transmembrane domain, has been associated with activation of the PMCA by acidic lipids. cDNAs coding for mutants of the Ca\(^{2+}\) pump isoform h4xb with deletions in the A\(_L\) region were constructed, and the proteins were successfully expressed in either COS or Chinese hamster ovary cells. Mutants with deletions in the segment 296–349 had full Ca\(^{2+}\) transport activity, but deletions involving the segment of amino acids 350–396 were inactive suggesting that these residues are required for a functional PMCA. In the absence of calmodulin the \(V_{\text{max}}\) of mutant d296–349 was similar to that of the recombinant wild type pump, but its \(K_{\text{m}}\) for Ca\(^{2+}\) was about 5-fold lower. The addition of calmodulin increased the \(V_{\text{max}}\) and the apparent Ca\(^{2+}\) affinity of both the wild type and d296–349 enzymes indicating that the activating effects of calmodulin were not affected by the deletion. At low concentrations of Ca\(^{2+}\) and in the presence of saturating amounts of calmodulin, the addition of phosphatidic acid increased about 2-fold the activity of the recombinant wild type pump. In contrast, under these conditions phosphatidic acid did not significantly change the activity of mutant d296–349. Taken together these results suggest that (a) deletion of residues 296–349 recreates a form of PMCA similar to that resulting from the binding of acidic lipids at the A\(_L\) region; (b) the A\(_L\) region acts as an acidic lipid-binding inhibitory domain capable of adjusting the Ca\(^{2+}\) affinity of the PMCA to the lipid composition of the membrane; and (c) the function of the A\(_L\) region is independent of the autoinhibition by the C-terminal calmodulin-binding region.

The plasma membrane Ca\(^{2+}\) pump (PMCA)\(^3\) extrudes Ca\(^{2+}\) from the cytosol to the extracellular space playing an important role in the maintenance of the resting level of intracellular Ca\(^{2+}\) and in the control of the Ca\(^{2+}\) transients. The overall topology of the PMCA is similar to that of other P-type ion motive ATPases with about 10 transmembrane segments (M1–M10) and most of the protein exposed to the cytosol (1). Four genes for the PMCA have been identified in humans, and each of these genes produces additional isoforms by alternative splicing of primary transcripts (2).

The molecular mass of the PMCA (~135 kDa) is slightly higher than that of other P-ATPases, and this is mainly due to the extended C-terminal region (C region) and also to the insertion of a highly charged segment of about 60 amino acids in the loop between M2 and M3. In this report we call the latter the A\(_L\) region.

The C region includes a calmodulin-binding autoinhibitory domain, and the binding of calmodulin enhances both the Ca\(^{2+}\) sensitivity and the turnover of the pump. Alternative splicing of mRNA modifies region C leading to isoforms with different responsiveness toward calmodulin activation (3). The removal by limited proteolysis or deletion mutagenesis of the C-terminal 120 amino acids including the calmodulin-binding autoinhibitory domain suffices for a calmodulin-like activation (4–7).

It has long been known that the PMCA is also activated by acidic lipids (5, 6, 8–11), and this effect has been in part accounted for by the finding that these lipids interact with the calmodulin-binding site (12, 13). However, the fact that acidic lipids are capable of enhancing the Ca\(^{2+}\) sensitivity of the PMCA to a greater extent than calmodulin suggested the existence of an independent acidic lipid-responsive region (5, 6). Studies of controlled proteolysis with trypsin of the PMCA from human erythrocytes (mostly isoform h4xb) have shown that the activating effects of acidic lipids is lost concomitantly with the appearance of a 76-kDa peptide lacking the N-terminal portion of the molecule and the C-terminal regulatory region (5, 6). Because the proteolytic fragment of 76 kDa and its precursor, a lipid-responsive peptide of 81 kDa, have identical C termini, it was inferred that the portion of the A\(_L\) region cleaved during lipid activation (14). In addition, it was later shown that synthetic peptides made with the sequence of segment 339–360 specifically bind acidic lipids (3, 13). As with the C region, alternative splicing at the so-called site “A” can generate different versions of the A\(_L\) region. These changes were proposed to affect the sensitivity of the PMCA to acidic lipids (15, 16). However, no functional consequences of the changes in this region have been reported.

In this study, we have investigated the effects of deletions in mutant h4xb Ca\(^{2+}\) pump lacking the segment of amino acids n1 to n2, where n1 and n2 indicate the first and the last deleted amino acid, respectively; CHO, Chinese hamster ovary.
the segment 296–356 of the human PMCA4b. This segment encompasses the variable region between M2 and M3 (Alg region) close to the site of tryptic cleavage associated with the acidic lipid activation (Fig. 1). The mutant proteins were successfully expressed either transiently in COS-1 cells or in a stable form in CHO cells. Contrary to our expectations, the recombinant PMCAs with deletions involving residues 350–356 were completely inactive suggesting that, in addition to its proposed regulatory role, this segment is also necessary for the biogenesis of a functional enzyme. In contrast, deletion of residues 296–349 resulted in a fully active pump with a high affinity for Ca\(^{2+}\) characteristic of the activated form of the PMCA that would result from binding of acidic lipids at the lipid-binding site of region Alg.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents were purchased from the following companies: enzymes used in DNA manipulations, New England Biolabs; oligonucleotide primers, DNAgency, Malvern, PA; columns for DNA purification, Qiagen; [\(^{45}\)Ca and \([\beta\)\(^{32}\)P\]ATP, PerkinElmer Life Sciences; Immobilon transfer membranes and nitrocellulose filters, Millipore; immunobiochemicals, Vector Laboratories and Amersham Biosciences; and reagents for cell culture, thapsigargin, phosphatidic acid, and other chemicals, Sigma. The expression vector pED was a generous gift of Dr. Randall J. Kaufman, Genetics Institute, Boston.

**Construction of the cDNAs**—The construction of the cDNAs coding for the wild type h4xb and the C-terminally truncated mutant c120 were described previously (7, 17). They were cloned into the eukaryotic expression vector pED (18). To obtain a cDNA coding for a mutant lacking residues 300–356, two fragments were synthesized by PCR using the h4xb cDNA as template and the primer pair of primers A1–5EGE–2096. Primer sequences are as follows: A1, 5'-ctgcgttgccatgcggacacact-3'; 3EGE, 5'-gaacgagcaagctgctctttctctctctctc-3'; 5EGE, 5'-gagaaaaaaagagctgctctttctctctctc-3'; and 2096, 5'-cagctggcttcttccttc-3'.

Primer A1 has a SalI site incorporated at its 5' position, whereas primer 5EGE-3EGE contains a restriction site for nuclease MluI. The PCR products were digested either with SalI and MluI or MluI and BspEI (internal site naturally occurring in the wild type h4xb DNA at position 1911) to produce cohesive fragments coding for residues 1–299 and 357–719, respectively, and cloned into the corresponding position of pED-h4xb or pED-c120. Mutant d296–349 was obtained using oligonucleotides A1 and 857 5'-ctcctggtcagccgctctctc-3'. A similar strategy was used to obtain the other mutants. The nucleotide sequence of the mutant cDNAs extending between the SalI and BspEI sites was verified by dideoxy chain termination sequencing.

**Protein Expression and Isolation of Cellular Membranes**—COS-1 or CHO(dhfr) cells were lipofected using either the ESCORT transfection reagent (Sigma) or polyfect (Qiagen) according to the manufacturer's protocol. COS-1 cells were harvested 48 h after transfection for the preparation of microsomes. Stable CHO cell lines expressing the recombinant wild type h4xb was described previously (19). To express in a stable form the d300–356 and d296–349 proteins, the transfected CHO cells were split into dishes of 15 cm in diameter 48 h post-transfection and cultured in a selective Eagle's minimum essential medium without antibiotics and 10% of dialyzed fetal calf serum. After 3 weeks about 3–5 of the resulting colonies were cloned and expanded, and the expression of the pump was investigated by immunoblotting. The crude microsomal membrane fractions were prepared by the procedure of Enyedi et al. (7). Protein concentration was estimated by means of the Bio-Rad protein assay, with bovine serum albumin as a standard.

**Detection of the Ca\(^{2+}\) Pump Protein**—SDS-PAGE and immunoblotting were carried out as described previously (20). Proteins were electrophoresed on a 7.5% acrylamide gel according to Laemmli (21) and subsequently transferred to Millipore Immobilon membranes. The membranes were incubated at 37 °C for 1 h with 5\(^{10}\) monocolonal antibody (22, 23) from ascites fluid (dilution, 1:2000). For staining, biotinylated anti-mouse immunoglobulin G and avidin-streptavidin peroxidase conjugate were used.

**Ca\(^{2+}\) Transport Assay**—Ca\(^{2+}\) uptake assays were performed as described previously (19). The reaction mixture contained 100 mM KCl, 55 mM Tris-HCl (pH 7.3 at 37 °C), 5 mM NaCl, 0.1 nM thapsigargin, 4 μg/ml oligomycin, 20 mM sodium phosphate, 1.5 mM ATP, 95 μg/ml EGTA, 2.5 mM MgCl\(_2\) and CaCl\(_2\) (labeled with 45Ca) to give the desired concentration (3 Ca\(^{2+}\)). Three concentrations of free Ca\(^{2+}\) were used during the run using the program of Fabiato and Fabiato (24). Vesicles (10 μg of protein) were preincubated at 37 °C for 5 min, and the reaction was initiated by the addition of ATP. The reaction was finished after 5 min by filtering the samples through a 0.45-μm filter. The 45Ca uptake was determined by counting in a scintillation counter. Uptake activities were expressed per mg of membrane protein. For each data point the activity of the recombinant PMCA was estimated by subtracting the activity of the endogenous PMCA (Ve) from the overall transport activity. The Ve was obtained as Ve = (Ve + Ic) \times (Vc + Ie), where Ve is the rate of Ca\(^{2+}\) uptake by microsomal vesicles made from cells transfected with vector without insert; Ic is the intensity of the endogenous PMCA band in microsomes containing endogenous and recombinant enzymes as determined by immunoblotting, and Ic is the intensity of the endogenous PMCA band in microsomes made from cells transfected with vector without insert. The values of Ve were less than 20% of the total Ca\(^{2+}\) uptake.

To measure the effect of PA on the activity of the recombinant PMCA, PA was dissolved in methanol:chloroform (2:1) at a concentration of 17 mg/ml. Immediately before use, the methanol:chloroform was dried off, and the lipid was resuspended in 5 mM Tris-HCl (pH 7.3 at 37 °C), 10 mM KCl. The suspension was refrigerated for 5–10 min, then an aliquot was added to the reaction media. The microsomes were preincubated at 37 °C with PA for 5 min in the reaction medium before initiating calcium uptake by the addition of ATP.

**Detection of the Phosphorylated Intermediate**—The phosphorylation reaction was carried out at 4 °C in a medium containing 30 μg of microsomal protein, 160 mM KCl, 25 mM Tris-HCl (pH 7.0 at 4 °C), 4 μM thapsigargin, 0.2 mM CaCl\(_2\), and 0.06 mM LaCl\(_3\) in a reaction volume of 200 μl.
slow migrating band seen in each lane corresponds to the endogenous
with antibody 5F10 as described under
Experimental Procedures. The slower migrating band seen in each lane corresponds to the endogenous PMCA from COS cells.

0.25 ml La3+ was added because it is known to stabilize the phosphoenzyme of the plasma membrane Ca2+ pump (25). The reaction was initiated by the addition of 1 μM [γ-32P]ATP and terminated after 1 min with 15 μl of a solution containing 100% trichloroacetic acid. The precipitated proteins were dissolved in sample buffer and separated by SDS-PAGE in a 7% acrylamide gel according to Sarkadi et al. (4). After drying the gel, autoradiographs were produced with 24–72 h of exposure at −70 °C using X-OMat x-ray films.

Tryptic Digestion of Microsomes—Microsomes from CHO cells were suspended in a medium containing 0.05 mM EGTA, and the reaction was initiated by the rapid addition of 5 μl of trypsin 0.1 μg/μl (ratio of microsomal protein:trypsin of 20:1). After incubation on ice for 30 s or 1, 10, or 25 min the reaction was stopped with 4 μl of aprotinin 1.2 mg/ml. Controls (0 s) were done without trypsin.

RESULTS
Expression and Activity of Ca2+ Pumps Lacking Residues from the Segment 296–356—Our initial goal was to see how much of the region ΔA could be removed from the PMCA molecule without changing the maximum capacity of the enzyme to transport Ca2+. To detect changes in the activity without the interference of the calmodulin-binding autoinhibitory domain, the deletions were made in the fully active ct120 pump (7). Immunoblots of microsomes from transfected COS cells showed two major bands, one of about 140 kDa corresponding to the endogenous PMCA and the other of faster migration corresponding to the recombinant ct120 enzymes (Fig. 2). Despite some variation in the amount of expressed proteins due to the efficiency of the transfection, none of the deletions significantly affected the amount of recombinant PMCA.

We began making deletions at downstream residue 300 because the amino acid sequences of different PMCA isoforms started to diverge approximately at that position. In fact, residues 301–312 were absent in the alternatively spliced option h4xb of isoform 4 (26). The deletion was extended to residue 356 because previous proteolysis work suggested that the segment 315–358 was not essential for activity (5–7, 14). Table I shows the Ca2+ uptake activity of the deletion mutants measured at a saturating concentration of Ca2+ of 10 μM. Mutants lacking residues from segment 300–349 were as active as the ct120 pump. Moving the deletion upstream to residues 296–349 to remove a cluster of three consecutive lysine residues still resulted in a fully active enzyme. In contrast, shifting the deletions downstream to residue 356 led to a near total loss of the Ca2+ transport activity.

In order to characterize in more detail mutants d296–349 and d300–356, they were stably expressed as full-length Ca2+ pumps in CHO cells. The expression levels estimated by immunoblotting were 100 and 60% of the wild type for d296–349 and d300–356, respectively (not shown).

The ability of the mutants to form an acyl phosphate from ATP was investigated (Fig. 3). Membranes containing the h4xb or the ct120 enzymes showed strong bands corresponding to the phosphorylated recombinant pumps. The active mutants d300–314(ct120) and d300–349(ct120) were also capable of forming phosphoenzyme. In contrast, only a weak band similar to that of the endogenous PMCA was observed in microsomes containing mutants with deletions involving residues 350–356. Thus, in these mutants the lack of Ca2+ transport activity was accompanied by the loss of the ability to react with Ca2+ and ATP to form the phosphorylated intermediate.
Partial Proteolysis of the d300–356 and d296–349 Mutants—

The structure of the mutant proteins was investigated by examining their sensitivity to degradation by a brief exposure to trypsin (Fig. 4). The proteolytic PMCA fragments were recognized either with antibody 5F10, which reacts in the central portion of the molecule (amino acids 719–738), or antibody JA9, which reacts near the N-terminal end (amino acids 16–75). As reported before (4, 6, 20), a small amount of PMCA is more active than the recombinant wild type pump. However, as deletion of residues 296–349 activated the PMCA by enhancing its response to Ca$^{2+}$, calmodulin had a similar effect on the recombinant d296–349 and wild type enzymes increasing the $V_{max}$ at saturating concentrations of Ca$^{2+}$ and reducing the $K_{0.5}$ for Ca$^{2+}$. In addition the cooperativity for Ca$^{2+}$ activation, which was shown to be a characteristic of the calmodulin-activated pump (5), was less evident in the d296–349 enzyme.

Effect of Phosphatidic Acid on d296–349—Because the only mechanisms that have been reported to decrease the $K_{0.5}$ for Ca$^{2+}$ to values lower than those obtained with calmodulin are the removal by proteolysis of region A, and the treatment of the enzyme with acidic lipids (5, 6), in the following experiment we investigated whether the activation caused by deletion of residues 296–349 was additive to that of acidic lipids.
In apparent contradiction with these findings, we found that mutations that are no longer responsive to activation by acidic lipids. Indeed this is exactly what results from the removal of the catalytic site at position 315, it also suggests that the global structure of the protein was not grossly altered by the deletions. Furthermore, the proteolysis patterns suggest that in these mutants the cleavage at position 358 was impaired. Nevertheless, at the longest times of proteolysis a fragment of about 85 kDa detected by JA9 became more intense in mutant d300–356 than in either the recombinant wild type or mutant d296–349, a fact that may reflect a structural change associated with the lack of activity of mutant d300–356.

The crystal structure of SERCA has shown that the M3 and other transmembrane helices are longer than predicted previously (28). If this is also the case for the PMCA, the N-terminal end of M3 would be at valine 351, and hence the deletion of residue 350–356 would directly affect the structure of M3. This transmembrane segment has been shown to be critical for the function of SERCA (31) and is part of the binding site for the highly specific SERCA inhibitor thapsigargin (32).

We found that at saturating concentrations of Ca2+, mutants with deletions involving amino acids from the segment 296–349 had a Ca2+ transport activity similar to that of the recombinant wild type pump. The importance of a net excess of positive charges on the cytoplasmic end of transmembrane segments for a proper membrane topology is well known (29, 30). However, because the activity of mutant d296–349 was preserved, it seems that residues 296–349 are not critical for the correct folding or insertion of the PMCA in the membrane despite the fact that the deletion of amino acids 296–349 effectively removed 15 positively charged residues from the segment preceding M3.

Although the removal of residues 296–349 did not significantly affect the $V_{\text{max}}$ of Ca2+ transport, it changed the behavior of the pump at low concentrations of Ca2+. Like acidic lipids, the deletion of residues 296–349 increased the Ca2+ affinity of the pump, and it did so more effectively than calmodulin. Moreover, when the calmodulin-binding site was saturated with calmodulin, the d296–349 enzyme was not significantly stimulated by phosphatidic acid. These results suggest that the d296–349 mutant resembles the activated form of the PMCA resulting from the binding of acidic lipids to their site in the $\Delta_3$ region of the molecule.

The N-terminal acidic lipid-responsive segment of the PMCA is bracketed by two segments near the catalytic region of the pump that were proposed to form a receptor site for the C-terminal autoinhibitory sequence (33). Because calmodulin had a similar effect on the recombinant d296–349 and wild type enzymes, it seems that the activation of the PMCA by the binding of acidic lipids to the $\Delta_3$ region does not modify the inhibitory interaction of the C-terminal segment with its receptor site.

Because acidic lipids also bind and activate via the C-terminal autoinhibitory domain (12, 13), the independence of the effects associated with the interaction of acidic lipids at a site in region $\Delta_3$ has been difficult to assess. This in part was due to the fact that it has not been possible to use partial proteolysis to remove selectively the region $\Delta_3$ of the molecule without also cleaving the C-terminal segment. In this respect, the mutant d296–349 may be useful to study in more detail the effects of acidic lipids on the enzyme not activated by disruption of the catalytic region of the pump.

According to a model proposed previously (5, 6) the Ca2+ pump may be in three distinct functional states as follows: (i) the resting state with low $V_{\text{max}}$ and low Ca2+ affinity ($K_{0.5}$ > 1 $\mu$M); (ii) the calmodulin activated state of high $V_{\text{max}}$ and intermediate affinity for Ca2+ ($K_{0.5}$ − 0.5 $\mu$M); and (iii) acidic lipid-
activated state of high $V_{\text{max}}$ and high Ca\(^{2+}\) affinity ($K_{\text{d50}} \sim 0.2 \mu M$). The results of the deletion 296–349 suggest that, through the interaction of the A\(_{L}\) region with the acidic lipids of the membrane, the PMCA could adopt a fourth state characterized by low $V_{\text{max}}$ and intermediate or high affinity for Ca\(^{2+}\).

It has been suggested that a lower rate of Ca\(^{2+}\) extrusion by the PMCA due to a decrease in the level of activating phosphoinositides facilitates the rapid transient elevation of intracellular Ca\(^{2+}\) after stimulation by agonists (34, 35). Because the affinity for acidic lipids at region A\(_{L}\) would seem higher than that of the C region (9, 12, 13), it is tempting to speculate that the d296–349 enzyme, in analogy with the d296–349 sensitive to anionic phospholipids and fatty acids, and their interaction of the A\(_{L}\) region just upstream M3 may turn the function of this transmembrane segment unusually sensitive to the lipid composition of the membrane.

The nature of the apparent increase in Ca\(^{2+}\) affinity of the PMCA mediated by acidic lipids is a major quest for future work. It is worth mentioning that recent studies (31) of the SERCA have led to the proposal that the N-terminal portion of M3 is involved in the control of the access to the Ca\(^{2+}\) transport sites. In the PMCA, the occurrence of the A\(_{L}\) region just upstream M3 may turn the function of this transmembrane segment unusually sensitive to the lipid composition of the membrane.

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