Deletions in the A_L region of the h4xb plasma membrane Ca^{2+} pump
High apparent affinity for Ca^{2+} of a deletion mutant resembling
the alternative spliced form h4zb

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Abstract Mutants of the plasma membrane Ca^{2+} pump (human isoform 4xb) with deletions in the linker between domain A and transmembrane segment M3 (A_L region) were constructed and expressed in Chinese hamster ovary cells. The total or partial removal of the amino acid segment 300–349 did not change the maximal Ca^{2+} transport activity, but mutants with deletions involving residues 300–338 exhibited a higher apparent affinity for Ca^{2+} than the wild type h4xb enzyme. Deletion of the putative acidic lipid interacting sequence (residues 339–349) had no observable functional consequences. The removal of either residues 300–314 or 313–338 resulted in a similar increase in the apparent Ca^{2+} affinity of the pump although the increase was somewhat lower than that obtained by the deletion 300–349 suggesting that both deletions affected the same structural determinant. The results show that alterations in the region of the alternative splicing site A change the sensitivity to Ca^{2+} of the human isoform 4 of the PMCA.

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Keywords: Plasma membrane Ca^{2+} pump (PMCA); Ca^{2+} apparent affinity; Alternative splicing site A; Directed mutagenesis; Heterologous expression

1. Introduction

The Ca^{2+} ATPases (PMCA) from plasma membrane are primary systems for the specific extrusion of Ca^{2+} from eukaryotic cells, and they are responsible for the fine-tuning of intracellular Ca^{2+} concentration [1]. They belong to the family of P-type ion transport ATPases, which are characterized by the formation of an aspartyl phosphate intermediate as part of their reaction cycle. Humans contain four genes coding for PMCA and a diversity of isoforms generated by the alternative splicing of their transcripts at two sites (sites “A” and “C”) [2]. The molecular mass of the PMCAs is about 135 kDa and the overall topology is predicted to be similar to that of other P-type ion ATPases. Following the domain organization described for the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) [3], the PMCA protein would contain three cytosolic domains, nucleotide-binding (N), phosphorylation (P) and actuator (A), a transmembrane region of 10 segments (M1–M10) and minor extracellular loops. The long C-terminal segment (C region) of the PMCA following M10 functions as an autoinhibitory domain and includes a calmodulin-binding site. The binding of calmodulin enhances both the Ca^{2+} sensitivity and the turnover of the pump. Alternative splicing at the so-called alternative splice “C” modifies calmodulin-binding autoinhibitory region leading to isoforms with different responsiveness toward calmodulin activation [4].

The A domain is formed by the N-terminal segment of the protein and the intracellular loop located between M2 and M3. It has been known for a long time that this region formerly identified as transduction or phosphatase domain [5,6] is involved in the conformational changes occurring during the transport cycle. Recent studies have shown that the segment linking the M3 to the A domain of SERCA is essential for the extensive rotation of domain A and its correct positioning in the active configuration of the catalytic site [7,8]. In the PMCA the A-M3 linker is longer (residues 294–348 in h4xb) and includes a segment not conserved in other P-ATPases that we have called region A_L [9]. This region of the PMCA has been associated with the increase in the apparent affinity of the enzyme for Ca^{2+} caused by long-chain polyunsaturated fatty acids and acidic phospholipids, in particular phosphoinositides [10–13]. The involvement of the A_L in the regulation of the PMCA by acidic lipids arose from studies of the lipid activation of mixtures of PMCA fragments obtained by limited proteolysis of the PMCA from human erythrocytes (mostly isoform h4xb) [14–16]. The cleavage by trypsin at residues 314 and 358, and the presumed elimination of the corresponding peptide of 44 amino acids was shown to raise the apparent Ca^{2+} affinity of the PMCA to a level similar to that attained with acidic lipids. Later it was shown that synthetic peptides made with the sequence of the segment 339–360 bind acidic lipids, reinforcing the idea of the A_L–acidic lipid interaction [17,18]. Furthermore, since the alternative splicing at site
A changes the sequence of the A1 region it was speculated that alternative splicing may influence the phospholipid regulation in the different PMCA isoforms [2,19,20].

We have shown previously that the elimination by deletion mutagenesis of the A1 region of the h4xb corresponding to residues 296–349 increase the apparent affinity for Ca$^{2+}$ and reduce the activation by acidic lipids [9]. Here, we have narrowed down the region that affects the apparent Ca$^{2+}$ affinity by analyzing the Ca$^{2+}$ dependence of mutant PMCA containing specifically designed shorter deletions. We found that deletion of the segment 339–349 including the putative lipid-binding site is not responsible of activation. In contrast the deletion of segments 300–338, 313–338 or 300–314 increased the apparent affinity for Ca$^{2+}$. Since the later deletion resembles the naturally occurring splice variant h4zb the results suggest that modifications in isoform 4 of the PMCA occurring as consequence of splicing site A affect the Ca$^{2+}$ sensitivity of the pump.

2. Results

2.1. Expression and activity of Ca$^{2+}$ pumps lacking residues from the segment 300–349

Initially the segment 300–349 whose removal was shown to activate the h4xb [9] was divided in two by making deletions 300–338 and 339–349 (Fig. 1). The last deletion eliminates most of the sequence corresponding to the peptides that have been shown to bind acidic-lipids [17,18]. Then the segment 300–338 was again divided in two by making deletions 300–314 and 313–338. These deletions were made taking in account that the tryptic cleavage leading to the proteolytic fragment of 90 kDa occurs after Lys315 [16]. In addition by eliminating residues 300–314 the sequence of the h4xb became similar to that of the alternative splicing variant h4zb [30].

Immunoblots of microsomes from transfected Chinese hamster ovary (CHO) cells showed that the mutant proteins were expressed and had the expected migration (Fig. 2). The deletions did not produce significant changes in the amount of expressed PMCA protein. The functional state of the mutants was evaluated by measuring their Ca$^{2+}$ uptake activity at a saturating concentration of Ca$^{2+}$ of 10 μM. Under these conditions all the mutants exhibited a high activity similar to that of the wild type enzyme indicating that the deletions did not alter the ability of the PMCA to transport Ca$^{2+}$ (Table 1).

2.2. Apparent affinity for Ca$^{2+}$

The apparent Ca$^{2+}$ affinity of the mutant PMCA was examined by measuring the dependence of Ca$^{2+}$ uptake on free Ca$^{2+}$ concentration. As shown in Fig. 3 the activity of the wild type enzyme gradually increased with the concentration of Ca$^{2+}$, reaching half maximal activity at about 1.6 μM Ca$^{2+}$. In
contrast the activity of mutant d300–349 at low Ca $^{2+}$ concentrations was higher than the wild type and displayed a higher apparent affinity for Ca$^{2+}$ ($K_{0.5} = 0.5 \mu M$). The mutant d339–349 had a Ca$^{2+}$ dependency similar to the wild type. However, mutant d300–338 exhibited an increase in the apparent affinity for Ca$^{2+}$ comparable to that of mutant d300–349. Fig. 4 shows that also both mutants d300–314 and d313–338 had an apparent affinity for Ca$^{2+}$ higher than the wild type enzyme although the magnitude of the increment was somewhat lower than that produced by the removal of the sequence 300–338.

2.3. Effect of deleting residues 300–314 on the apparent Ca$^{2+}$ affinity of the ct120 enzyme

The removal of the last 120 residues of the h4xb (ct120 mutant) eliminates the calmodulin-binding autoinhibitory domain leading to a higher $V_{max}$ and apparent affinity for Ca$^{2+}$. The effect of the simultaneous removal of the C-terminal 120 amino acids and residues 300–314 was investigated. As shown in Fig. 5, the activity of the ct120 enzyme rapidly increased with

Table 1

<table>
<thead>
<tr>
<th>Ca$^{2+}$ pump</th>
<th>Rate of Ca$^{2+}$ uptake (nmol Ca$^{2+}$/mg membrane protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>h4bx</td>
<td>9.2 ± 1.2 (17)</td>
</tr>
<tr>
<td>d(300–349)</td>
<td>9.0 ± 0.8 (17)</td>
</tr>
<tr>
<td>d(300–338)</td>
<td>10.2 ± 0.5 (17)</td>
</tr>
<tr>
<td>d(339–349)</td>
<td>9.8 ± 0.8 (9)</td>
</tr>
<tr>
<td>d(313–338)</td>
<td>11.2 ± 0.9 (13)</td>
</tr>
<tr>
<td>d(300–314)</td>
<td>9.5 ± 0.5 (15)</td>
</tr>
</tbody>
</table>

The rate of Ca$^{2+}$ uptake (±S.D.) from CHO cell microsomes was estimated at 10 $\mu M$ Ca$^{2+}$ as described under Section 4 for 5 min at 37 °C. The number of determinations is shown in parentheses.
increasing concentrations of Ca\textsuperscript{2+} (K\textsubscript{0.5} = 0.36 \textmu M). However deletion of residues 300–314 in mutant d(300–314)(ct120) increased even further the apparent affinity for Ca\textsuperscript{2+} of the ct120 enzyme (K\textsubscript{0.5} = 0.24 \textmu M).

3. Discussion

Earlier studies of limited trypsinolysis of the PMCA from human erythrocytes showed the progressive appearance of fragments of 90, 85, 81 and 76 kDa. The last conversion from a fragment of 81 to 76 kDa by the cleavage after arginine 358 was shown to activate the enzyme by increasing the apparent Ca\textsuperscript{2+} affinity to a level similar to that produced by acidic lipids [14–16]. In line with these results we have previously shown that a site directed deletion of the region A\textsubscript{L} of h4xb (residues 296–349) increased the apparent Ca\textsuperscript{2+} affinity of the pump [9]. Here we found that a similar activation can be obtained by deletion of residues 300–338 while a partial effect was observed by deletion of either residues 300–314 or 313–338. These results suggest that a higher Ca\textsuperscript{2+} apparent affinity is due to the disruption of a domain extending between residues 300–338.

Because of the particular location in the PMCA of the A\textsubscript{L} region, as a hinge between the domain A and the transmembrane segment M3, the observed changes in the Ca\textsuperscript{2+} sensitivity may reflect the modulation by the A\textsubscript{L} region of the communication between the Ca\textsuperscript{2+} site in the membrane and the movements of domain A required for ion pumping. Indeed both segments linking the A domain with the rest of the protein seems to be important for regulation since mutation of Asp 170 by Asn in the stalk region of M2 also activates the h4xb [31].

In contrast with the higher apparent Ca\textsuperscript{2+} affinity observed upon deletion of residues 300–338, the deletion of the sequence corresponding to the proposed lipid-binding site had no effect on the apparent affinity for Ca\textsuperscript{2+} of the enzyme. Therefore it would seem that the proposed interaction of acidic lipids with the segment 339–360 does not by itself promote the activation of the PMCA. Nevertheless the interaction of acidic lipids with the segment 339–360 does not by itself promote the activation of the PMCA. Nevertheless the interaction of acidic lipids with the segment 339–360 may trigger a conformational change involving the segment 300–338 with the consequent rise of the apparent Ca\textsuperscript{2+} affinity.

The sequence of the A\textsubscript{L} region of the PMCA is changed by alternative splicing in the so-called splicing site A. Although the functional consequences of these variations are not known, recently it has been shown that the splice variant w of isofrom 2 is delivered to the apical membrane of polarized MDCK epithelial cells in contrast with the PMCA2x and PMCA2z forms that were confined to the basolateral membrane [32]. In the case of isofrom 4 no specific function has so far been assigned to the spliced-in sequence at site A. The amino acid sequence of mutant d300–314 is similar to the splice variant h4xb with the only differences being that mutant d300–314 lacks residue Lys300 and has residues Ala313–Lys314 substituted by Thr–Arg. We found that deletion of residues 300–314 increased the apparent affinity for Ca\textsuperscript{2+} of the h4xb and this effect persisted after the removal of the C-terminal 120 residues, showing that it did not result from a change in the intramolecular interaction between the catalytic site and the C-terminal autoinhibitory domain. Thus, the results obtained with the d300–314 mutant suggest that in isofrom 4 the splicing site A variants x and z differ in their apparent affinity for Ca\textsuperscript{2+}.

4. Experimental procedures

4.1. Materials

4\textsuperscript{5}Ca\textsuperscript{2+} was purchased from Perkin–Elmer Life Sciences Reagents. The reagents used in DNA manipulations were obtained from New England Biolabs and Quiagen; oligonucleotide primers were purchased from DNAGen, Malvern, PA; Immobion transfer membranes and nitrocellulose filters were from Millipore. Other reagents were purchased from Sigma. The expression vector pED was a generous gift of Dr. Randall J. Kaufman, Genetics Institute, Boston.

4.2. Constructions of the cDNAs

The constructions of the cDNAs coding for the recombinant wild type h4xb and the C-terminally truncated mutant ct120 were described previously [21]. To obtain a cDNA coding for a mutant lacking residues 300–314, one fragment was synthesized by PCR using the h4xb cDNA as template and the pair of primers 5′ 937–2096. Primers sequences are as follows: 5′ 937, 5′-aacaataggcaagcagaagggtgccg-3′; and 2096, 5′-caatggcggcgttcagtgctg-3′. Primer 5′ 937 includes a restriction site for nuclease MluI. The PCR product was digested with MluI and BspEI (internal site naturally occurring in the wild type h4xb DNA at position 1911) to produce a cohesive fragment coding for residues 314–719, and cloned into the corresponding position of the cDNA of the previously obtained mutant d(300–356) [9]. A similar strategy was used to obtain mutants d(300–338) and d(300–349) using oligonucleotides 2096–1009 and 2096–1042, respectively. Primer sequences are as follows: 1009, 5′-aggaataaggctagggactgaagggc-3′; A1, 5′-cggcgggtgcgctgctgg-3′; and 5′-aaaaagacgtagttgctgcc-3′. Mutant d(313–338) was constructed by digestion of the previously obtained cDNA of mutant d(313–356) [9] with nuclease Sall and MluI to obtain the fragment coding for sequence 1–313. This sequence was cloned into cDNA of mutant d(300–349) [9] previously digested with the same restriction nuclease to eliminate the sequence coding for residues 1–300. In order to obtain mutant d(339–349) a cDNA of the previously obtained mutant d(339–356) was digested with nucleases Sall and MluI to obtain the sequence coding for amino acids 1–338. This fragment was cloned into cDNA of mutant d(300–349) [9] previously digested with the same restriction nuclease to eliminate the sequence coding for residues 1–300.

The nucleotide sequence of the mutants cDNA extending between the Sall and BspEI sites was verified by dyeoxy chain termination sequencing. For expression in eukaryotic cell the DNAs were cloned into the expression vector pED [22].

4.3. Protein expression and isolation of cellular membranes

CHO(dhfr-) cells were lipofected using polyelect (Quiagen) according to the manufacturer’s protocol. The selection of stable CHO cell lines expressing the wild type recombinant h4xb and mutants was described previously [23]. The crude microsomal membranes fractions were prepared according to [24]. Protein concentration was estimated by means of Bio-Rad protein assay, with bovine serum albumin as a standard.

4.4. Detection of the Ca\textsuperscript{2+} pump protein

Proteins were electrophoresed on a 7.5% acrylamide gel according to Laemmli [25] and subsequently transferred to Millipore Immobilon membranes [26]. The blots were incubated at room temperature for 1 h with 5F10 monoclonal antibody [27,28] from ascites fluid (dilution, 1:2000). For staining, biotinylated anti-mouse immunoglobulin G and avidin–streptavidin peroxidase conjugate were used.

4.5. Ca\textsuperscript{2+} transport assay

The Ca\textsuperscript{2+} uptake by microsomal vesicles was measured as described previously [23]. The reaction mixture contained 100 mM KCl, 50 mM Tris–HCl (pH 7.3 at 37 °C), 5 mM Na\textsubscript{2}SO\textsubscript{4}, 0.1 mM thapsigargin, 4 \mu g/ml oligomycin, 20 mM sodium phosphate, 1.5 mM ATP, 95 \mu M EGTA, 2.5 mM MgCl\textsubscript{2} and CaCl\textsubscript{2} (labeled with 45Ca) to give the desired concentration of free Ca\textsuperscript{2+}. The free concentrations of Ca\textsuperscript{2+} were calculated using the program of Fabiato and Fabiato [29]. Vesicles (10 \mu g 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-\mu m filter. The 45Ca taken up by the vesicles was determined by counting in a scintillation counter. Uptake activities were expressed per mg of membrane protein. The
activity of the recombinant PMCA was estimated by subtracting the activity of the endogenous PMCA from CHO cells transfected with the empty plasmid pED as described previously [9]. For each microsomal preparation the maximal rate of Ca\(^{2+}\) uptake measured at a saturating concentration of 10 \(\mu\text{M}\) was taken as 100%. The Ca\(^{2+}\) dependence of the rate of Ca\(^{2+}\) uptake was analyzed using the Hill equation. The lines in the figures show the best fit to the data and the parameters with standard errors are indicated.

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**References**


