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Biochimica et Biophysica Acta 1419 (1999) 71–77

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Substitution of the N-terminal segment of the plasma membrane Ca pump isoform 4 by that of isoform 1 results in a fully functional chimeric enzyme

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Received 1 March 1999; accepted 24 March 1999

Abstract

The N-terminal segment of the plasma membrane Ca^{2+} pump (PMCA) is one of the most variable regions among the four isoforms of the enzyme and its functional importance is unknown. In the present work, the N-terminal segment of the highly active C-terminally truncated h4 mutant, h4(ct120) was modified either by substituting residues 18–43 by residues 43–75 or by replacing residues 1–75 by the homologous region from isoform h1 (residues 1–79). Immunoblot analysis of microsomal membranes from transfected COS-1 cells showed that the two N-terminally mutated proteins were correctly expressed at a level similar to that of h4(ct120). Measurements of the Ca^{2+} uptake by microsomal vesicles from transfected COS-1 cells indicated that mutant (18–43→43–75)h4(ct120) had only negligible Ca^{2+} transport activity while the chimeric (n1–79)h1h4(ct120) enzyme was fully capable of functioning as a calcium pump.

Like h4(ct120), the chimeric mutant was not stimulated further by calmodulin, and was inhibited to a similar degree by the C28R2 peptide corresponding to the calmodulin binding autoinhibitory region of the pump. Moreover, the apparent affinity for Ca^{2+} and the ATP dependence of the chimeric enzyme were similar to those of the h4(ct120) pump suggesting that the variability of sequence between the N-terminal segment of PMCA isoforms h1 and h4 involves amino acid substitutions that do not substantially change the behavior of the h4 enzyme.

Altogether, these results demonstrate that for activity the h4 Ca pump requires a specific amino acid sequence at its N-terminus, and the essential elements for a fully active enzyme can be provided by the N-terminal segment of isoform h1 despite the variability.

Keywords: PMCA; Ca^{2+} pump; Ca^{2+} ATPase; Chimeric enzyme; Calcium ion transport

1. Introduction

The plasma membrane Ca^{2+} pumps (PMCAs) are P-type ion-transporting ATPases involved in the maintenance of the intracellular Ca^{2+} homeostasis by actively extruding Ca^{2+} to the extracellular space. The PMCAs consist of a single polypeptide of approximately 138 kDa containing 10 transmembrane segments (M1–M10). At least four PMCAs (isoforms 1–4) exist in humans, and alternative splicing of RNA generates additional variants[1] with differences in the C-terminal region and in the region between M2 and M3. The PMCAs exhibit a high degree of conservation (about 85–90% of overall similarity)

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PII: S0005-2736(99)00050-4

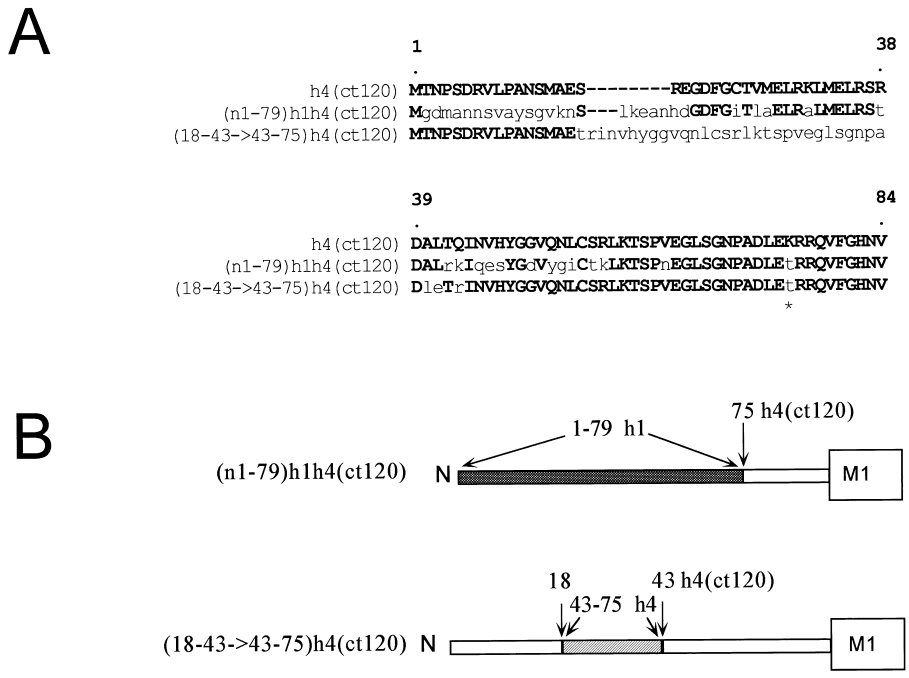

Fig. 1. A: Alignment of the N-terminal amino acid sequence of the h4(ct120) Ca^{2+} pump (wild-type h4 amino terminal segment) with that of mutants (n1–79)h1h4(ct120) and (18–43→43–75)h4(ct120). The residues of the mutants which agree with those of the wild-type h4 are indicated with bold capital letters. The numbers are those corresponding to the sequence of the h4 PMCA. The junction between the h1 and h4 sequences at position 75 is marked with an asterisk. B: Schematic representation of the N-terminal region of the mutants.

particularly in those regions which are also common to other P-type ATPases while the variation appears concentrated at the sites where the splicing options occur and at the extreme N-terminus. Recent studies have shown that the C-terminal variation has important consequences for the regulatory properties of the enzyme [1–5]. In contrast, the function of the N-terminal segment of the PMCA and the possible consequences of its variability between different PMCA isoforms are unknown.

We have shown previously that deletion of residues 19–75 produces an inactive hPMCA4 [6]. Hence, despite of its variability, the N-terminal segment is important for a functional enzyme. Moreover, it has been shown that a chimeric hPMCA4 containing the SERCA N-terminal segment up to the first transmembrane domain is unable to transport Ca^{2+} indicating that this portion of the SERCA cannot function as a surrogate of the N-terminal segment of the PMCA [7].

Some N-terminal variability between PMCA isoforms involves conservative substitutions which are preserved among the same isoform from different species. In contrast, other changes seem isoform specific because they only occur in different isoforms [8,9]. The N-terminal segment of the human isoform h4 is five amino acids shorter than that of h1, and only 40 of the 75 first residues are identical in both isoforms (Fig. 1A).

In this study we explored the consequences of two alterations in the N-terminal segment of the h4 enzyme (see Fig. 1A and B) using the highly active C-terminally truncated mutant h4(ct120) as the form of the enzyme containing the wild-type h4 amino terminus [10]. The substitution of residues 18–43 of the N-terminal segment of the h4 enzyme by residues 43–75

[1] The plasma membrane Ca^{2+} pump is referred to by a name such as 'hPMCA4' for the isoform of the plasma membrane Ca^{2+} pump derived from the fourth human gene, where the first letter indicates the species, and the number at the end indicates the gene of origin. Since this study involves only the plasma membrane Ca^{2+} pump, PMCA is frequently omitted. Other abbreviations: SERCA, sarcoplasmic/endoplasmic reticulum Ca pump; h4(ct120), mutant hPMCA4 lacking the C-terminal 120 amino acids.

resulted in an enzyme that, like our previous deletion mutant [6], was essentially unable to transport Ca^{2+}. However, by replacing residues 1–74 of h4(ct120) with residues 1–79 from isoform h1, we succeeded in creating a fully active chimeric Ca^{2+} pump we called (n1–79)h1h4(ct120). In all the conditions tested, the activity of the chimeric enzyme was similar to that of the h4(ct120) indicating that the substitution of the N-terminal segment of h4 by that of isoform h1 does not substantially change the behavior of the h4 enzyme. Altogether our results indicate that a functional PMCA has specific requirements at its N-terminus which, despite the variability of sequence, can be meet by the N-terminal segment of a different isoform.

2. Materials and methods

2.1. Construction of mutant DNAs

The construction of the h4(ct120) mutant was described previously [10]. The chimerical mutant (n1–79)h1h4(ct120) was assembled using the cDNA of a mutant h4(d18–75)(ct120) constructed earlier [6]. The digestion of h4(d18–75)(ct120) with *Sal*I and *Mlu*I (New England Biolabs) produced a fragment coding for h4 residues 76–1085. A cDNA coding for the amino terminal segment of the human isoform 1 was obtained by reverse transcription and PCR using total RNA from human brain with oligonucleotides 5′-GCATCGGTCGACCATGGGCGACATGGC-3′ containing a site for *Sal*I, and 5′-ACTGCTTCA-CGCGTTTCTAAATCTGCA-3′ containing a site for *Mlu*I. The amplified DNA was digested with *Sal*I and *Mlu*I and the product, coding for residues 1–79 of h1 isoform, was ligated with the fragment coding for h4 residues 76–1085 to produce the chimeric (n1–79)h1h4(ct120) DNA. The final construct was cloned into the expression vector pMM2 [11] and the sequence checked by double-strand sequencing at the INTA-CICA sequencing facility, Argentina. Because the strategy used in assembling (n1–79)h1h4(ct120) involved the use of a *Mlu*I site not originally present in either of the PMCA DNAs, the residue at position 80 of the (n1–79)h1h4(ct120) protein became threonine instead of either arginine or lysine of the corresponding h1 and h4 position respectively.

A strategy similar to that described above was used for the construction of the (18–43→43–75)-h4(ct120) DNA. A short DNA segment coding for residues 43–75 and containing unique sites for *Mlu*I was amplified by PCR, digested with *Mlu*I. The h4(d18–75)(ct120) DNA was cut with *Mlu*I and ligated with the PCR fragment coding for residues 43–75. A clone containing a double insertion of this fragment was identified by restriction analysis and DNA sequencing.

2.2. Cell culture and transfection

Reagents for cell culture were obtained from Sigma. COS-1 cells [12] were maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin, 100 μg/ml streptomycin. Two days before transfection the cells were split in 15-cm dishes. The cells were transfected by treatment with DEAE-dextran-chloroquine as described in a previous publication, using 20 μg of affinity purified DNA (Qiagen) per dish and harvested after 48 h [11].

2.3. Membrane preparation and detection of expressed Ca^{2+} pump protein

The microsomal fraction was isolated as described previously [10]. Protein concentration was estimated by means of the Bio-Rad protein assay, with bovine serum albumin as a standard.

Sodiumdodecylsulfate polyacrylamide gel electrophoresis and immunoblotting were carried out as previously described [13]. Proteins were electrophoresed on a 7.5% acrylamide gel according to Laemmli [14] and subsequently transferred to Millipore Immobilon 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% non-fat dry milk. The membranes were incubated at room temperature for 1 h with monoclonal antibodies 5F10 (dilution 1:1000) or JA9 (dilution 1:250) from ascites fluid. For staining, biotinylated anti-mouse immunoglobulin G and avidin-horseradish peroxidase conjugate (Vector Labs.) were used.

2.4. Assay of Ca^{2+} transport

Ca^{2+} uptake assays were performed as described previously [10]. The reaction mixture contained 100 mM KCl, 50 mM Tris/HCl (pH 7.3 at 37°C), 5 mM NaN_3, 400 nM thapsigargin, 20 mM sodium phosphate, 95 μM EGTA, 6 mM ATP and enough $MgCl_2$ and $CaCl_2$ (labeled with ^{45}Ca) to give the desired concentrations of free Mg^{2+} and free Ca^{2+}. The free concentrations of Ca^{2+} and Mg^{2+} were estimated using the program of Fabiato and Fabiato [15] and unless stated otherwise they were 8 μM and 1.2 mM respectively. In order to ensure a constant level of free Mg^{2+}, when the ATP concentration was varied the concentration of $MgCl_2$ was also changed.

The rate of Ca^{2+} uptake was measured by preincubating the vesicles (5 to 10 μg of protein) at 37°C for 5 min in the reaction mixture and initiating the reaction by the addition of ATP. Under these conditions the Ca^{2+} taken up by the vesicles increases linearly with time up to 15 min [10]. The reaction was terminated after 5 min by filtering the samples through a 0.45 μm filter. The ^{45}Ca taken up by the vesicles was then determined by counting in a liquid scintillation counter. Uptake activities were expressed per mg of COS-1 cell membrane protein.

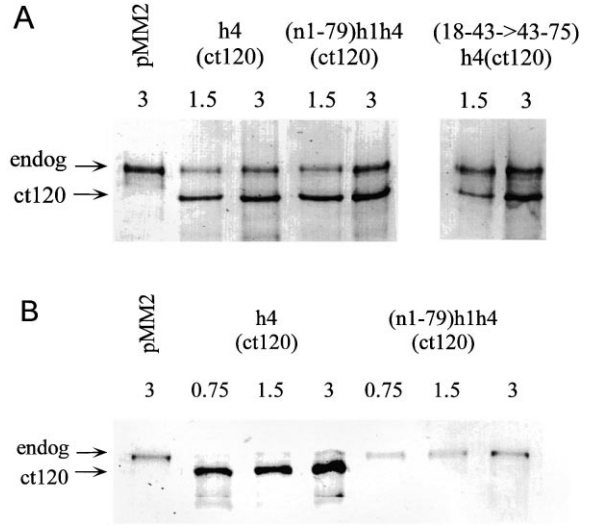

Fig. 2. Immunoblotting of microsomal proteins from COS-1 cells. The microsomal fraction of COS-1 cells transfected with the DNAs indicated in the figure was electrophoresed on a 7.5% sodiumdodecylsulfate polyacrylamide gel and immunoblotted with antibody 5F10 (panel A) or antibody JA9 (panel B). The number on top of each lane indicates the number of micrograms of microsomal protein from transfected COS cells loaded.

3. Results

COS-1 cells were transfected with pMM2 plasmid either empty or containing the DNA coding for mutants (18–43→43–75)h4(ct120), (n1–79)h1h4(ct120) or h4(ct120), and the expression of the proteins was investigated using monoclonal antibodies 5F10 and JA9 [16]. Antibody 5F10 recognizes all the isoforms of the PMCA by reacting with an epitope which in the h4 is located between residues 719–738. Fig. 2A shows that in membranes from cells transfected with the empty pMM2, 5F10 reacted with only one major band corresponding to the endogenous PMCA from COS cells. On the other hand, in microsomes from cells transfected with the mutant DNAs, 5F10 revealed an additional band with the expected migration of the C-terminally truncated mutants. Thus, the mutants containing the mutated N-terminal segment were correctly expressed and as judged by the intensity of the bands, their expression level was similar to that of h4(ct120). Because antibody JA9 reacts with an epitope located between residues 51–75 of h4, and does not recognize isoform h1, the (n1–79)h1h4(ct120) protein is not expected to be detected by JA9. Fig. 2B shows that a weak band corresponding to the endogenous PMCA was revealed by JA9 in microsomes from transfected COS-1 cells, in agreement with previous observations indicating that most of the COS-1 cells PMCA correspond to isoform 1b [16,17]. Consistently with the expression of a chimeric protein, no additional bands were revealed by JA9 in microsomes of COS-1 cells transfected with the (n1–79)h1h4(ct120) DNA.

The functional state of the (18–43→43–75)-h4(ct120) and (n1–79)h1h4(ct120) enzymes was assessed by measuring the ATP-dependent Ca^{2+} uptake by microsomes of transfected COS-1 cells. Fig. 3A shows the rate of Ca^{2+} uptake in reaction media containing 8 μM Ca^{2+} and either in the absence or in the presence of saturating amounts of calmodulin. Microsomes containing the (18–43→43–75)h4-(ct120) protein exhibited a low rate of Ca^{2+} uptake which, independently of the presence or absence of

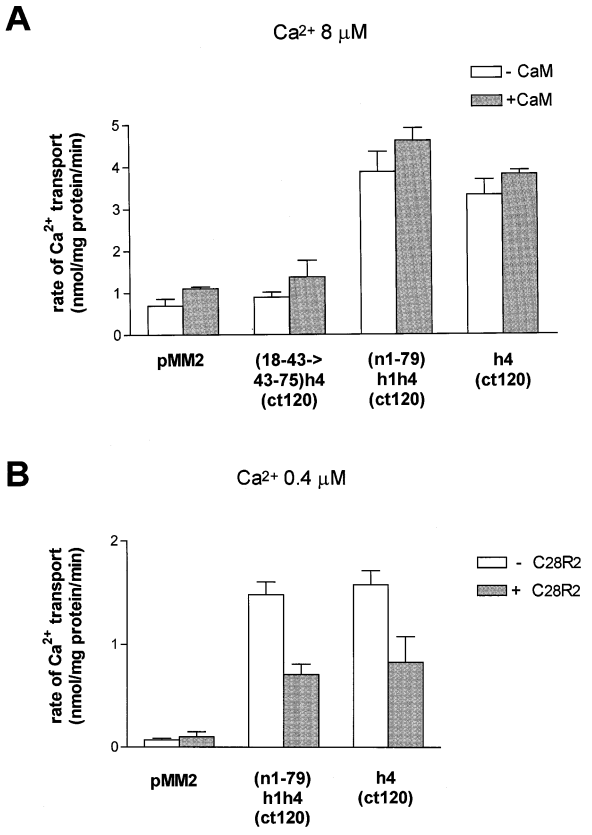

Fig. 3. Ca^{2+} transport activity of microsomes obtained from COS-1 cells transfected with the empty vector pMM2 or vector containing the cDNA of mutant plasma membrane Ca^{2+} pumps. The rate of ATP-dependent thapsigargin insensitive Ca^{2+} uptake by microsomal vesicles isolated from transfected COS-1 cells was measured at 37°C for 5 min as described in Section 2. A: The reaction media contained 8 μM and either 0 or 240 nM calmodulin. B: The reaction media contained 0.4 μM Ca^{2+} and either 0 or 16 μM of the peptide C28R2. The activities shown are the average ± standard error of the mean from two to ten experiments conducted in duplicate using membranes from different transfections. The difference between the average activities of h4(ct120) and (n1–79)h1h4(ct120) was not significant according to the Student's *t*-test.

calmodulin, was similar to that of microsomes from cells transfected with the empty pMM2 indicating that the ability of the (18–43 → 43–75)h4(ct120) mutant to transport Ca^{2+} was severely impaired. On the other hand, the rate of Ca^{2+} uptake from microsomes expressing the mutant (n1–79)h1h4(c120) was about five times higher than that from pMM2 microsomes and equal or slightly higher than that of h4(ct120). As expected, the addition of calmodulin to the reaction media increased the rate of Ca^{2+} uptake. However the increase of Ca^{2+} uptake from microsomes containing the expressed ct120 enzymes could be accounted for by the activation of the endogenous PMCA from COS cells, indicating that their activity was not significantly affected. Thus, the (n1–79)h1h4(ct120) enzyme, like the h4(ct120), had maximal activity even in the absence of calmodulin.

The C-terminal region of the PMCAs contains an autoinhibitory domain that in the absence of calmodulin binds near the catalytic site and keeps them in a state of low activity. To investigate whether differences between the N-termini of isoforms h4 and h1 affected the response toward the inhibitory domain, the inhibition of h4(ct120) and (n1–79)h1h4(ct120) by the synthetic peptide C28R2 corresponding to the calmodulin binding autoinhibitory region of the r2b isoform [18] was compared. Because the C28R2 is more effective at low Ca^{2+} concentrations [18], the free Ca^{2+} in the uptake media was lowered to 0.4 μM to make the effect of C28R2 more apparent. Fig. 3B shows that the addition of 16 μM of C28R2 decreased in a similar degree the activities of h4(ct120) and (n1–79)h1h4-(ct120).

The effects of the N-terminal variability between

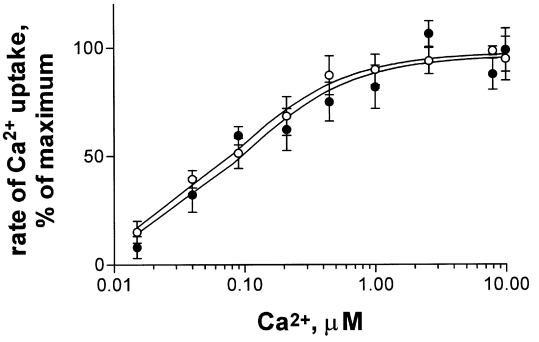

Fig. 4. Ca^{2+} dependence of the rate of Ca^{2+} uptake by mutant Ca^{2+} pumps. Open circles, (n1–79)h1h4(ct120); closed circles, h4(ct120). The rate of Ca^{2+} was measured at 37°C in a media containing 6 mM ATP, 1.2 mM free Mg^{2+} and no added calmodulin. The rate of Ca^{2+} uptake by the endogenous Ca^{2+} pump (from COS-1 cells transfected with the empty pMM2) was subtracted from each data point. The rate of Ca^{2+} uptake of each enzyme at 10 μM Ca^{2+} was taken as 100% activity. The points shown are the averages of four experiments performed in duplicate using different microsomal preparations. The error bars are the standard errors of the mean. The lines represent the best fit to the data given by the Hill equation. The $K_{1/2}$ ± the standard deviation were 0.14 ± 0.02 μM and 0.17 ± 0.05 μM for h4(ct120) and (n1–79)h1h4(ct120) respectively.

isoforms h1 and h4 on the dependence of the activity of the pump with the concentrations of Ca^{2+} was investigated. Fig. 4 shows that Ca^{2+} dependence of (n1–79)h1h4(ct120) and h4(ct120) was similar, and both enzymes reached half-maximal activity at about 0.17 μM Ca^{2+}. The ATP dependence of (n1–79)-h1h4(ct120) and h4(ct120) at physiologically relevant concentrations of ATP which occupy the low affinity regulatory site was also compared. Results in Fig. 5 show that the response to ATP of the (n1–79)-h1h4(ct120) and the h4(ct120) enzymes was virtually identical.

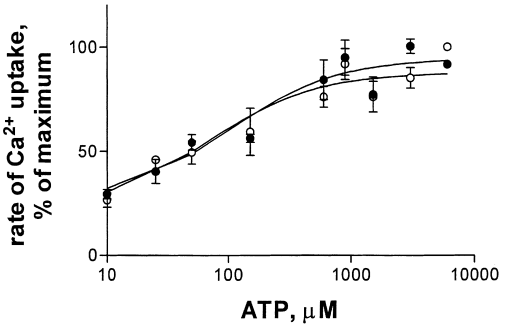

Fig. 5. ATP dependence of the rate of Ca^{2+} uptake by mutant Ca^{2+} pumps. Open circles, (n1–79)h1h4(ct120); closed circles, h4(ct120). The uptake media contained 8 μM free Ca^{2+}, 1.2 mM free Mg^{2+} and the concentrations of ATP indicated in the figure. The rate of Ca^{2+} uptake by the endogenous Ca^{2+} pump was subtracted from each data point. The rate of Ca^{2+} uptake of each microsomal preparation at 6 mM ATP was taken as 100% activity. The points shown are the averages of two or three experiments performed in duplicate using different microsomal preparations. The error bars are the standard errors of the mean. The lines represent the best fit to the data given by the following equation: $V = V_1 + [V_2 \cdot \mathrm{ATP}/(K_{m2} + \mathrm{ATP})]$ where V_1 is the maximum velocity at the high affinity site for ATP, V_2 is the maximum velocity at the low affinity regulatory site, and K_{m2} is the apparent dissociation constant for ATP from the low affinity site. The best-fitting values of the parameters ± S.D. were for h4(ct120): $V_1 = 25 \pm 10\%$, $V_2 = 70 \pm 10\%$, $K_{m2} = 102 \pm 70$ μM; and for (n1–79)h1h4(ct120): $V_1 = 22 \pm 11\%$, $V_2 = 67 \pm 11\%$, $K_{m2} = 70 \pm 40$ μM.

4. Discussion

With the exception of differences related to the C-terminal regulatory domain, the precise knowledge of the functional diversity between PMCA isoforms is lacking. The N-terminal segment of the PMCA is one of the regions of greatest diversity between PMCAs [19] and its function is unknown. This variability may indicate the involvement of the N-terminal segment in specific functional differences between PMCA isoforms. Alternatively it may be a consequence of its irrelevant influence on function.

In this work we investigated the effects of two alterations in the N-terminal segment of the PMCA. In mutant (18–43 → 43–75)h4(ct120) residues 18–43 of the highly active h4(ct120) pump were substituted by residues 43–75 while in the other mutant the N-terminal segment of h4(ct120) was replaced by the homologous region of isoform h1. Immunoblot analysis of microsomal proteins from transfected COS-1 cells showed that both mutant proteins were successfully expressed. Because the substitution of the N-terminal segment did not affect the amount of expressed pump, a possible relationship between the N-terminal diversity and the expression of the h1 and h4 proteins can be excluded.

The overexpression levels of PMCA in COS-1 cells are relatively low, however, they are adequate for the activity measurements [5,10,11]. It is worth mentioning that the COS-1 cells have been shown to possess PMCA mostly of type 1b [16,17], and therefore they are likely to faithfully process heterologous PMCA. In addition the use of the highly active C-terminally truncated h4(ct120) PMCA mutant as the enzyme with wild-type N-terminus allowed us to separate more clearly the activity of the expressed enzymes from that of the endogenous PMCA which is comparatively inactive in the absence of calmodulin.

The measurements of Ca^{2+} uptake showed that the (18–43 → 43–75)h4(ct120) enzyme had very low or no activity. Because the length of the N-terminal segment of (18–43 → 43–75)h4(ct120) is similar to that of the wild-type enzyme, the observed loss of Ca^{2+} transport activity in mutant (18–43 → 43–75)h4-(ct120) suggest that the preservation of the number of amino acid residues at the N-terminal end does not suffice for a functional PMCA. This result seems to indicate that residues 18–43 are critical for the activity of the PMCA and is in agreement with our previous studies indicating the importance of the N-terminal segment [6]. However, because of the radical nature of the alteration it is possible that the observed effects are related to structural alterations of the protein extending outside the segment 18–43. On the other hand, the Ca^{2+} transport activity of the

chimeric (n1–79)h1h4(ct120) enzyme was similar to that of h4(ct120), showing that replacement of the N-terminal segment of hPMCA4 by that of h1 produced a fully functional pump. Taken together, these results show that to be active, the hPMCA4 needs a specific amino acid sequence at its N-terminus, and that this sequence can be provided by the amino terminal segment of h1.

The Ca^{2+} transport activities of the (n1–79)h1h4-(ct120) and h4(ct120) were similar either in the absence or in the presence of saturating amounts of calmodulin. Moreover, the peptide C28R2, made after the sequence of the C-terminal inhibitory domain of the r2 enzyme, reduced the activity of both pumps to a similar degree. These results support the idea that the N-terminal variation between h1 and h4 is not related to their regulation by the C-terminal autoinhibitory region.

The substitution of the N-terminal segment of h4 by that of h1 did not significantly change either the apparent affinity for Ca^{2+}, or the ATP dependency of the h4 enzyme. It has been previously noticed that a region near the N-terminus of h1 (residues 22–33) has some resemblance to the loop of Ca^{2+} binding regions of other Ca^{2+} binding proteins [8]. Some residues from this region are also conserved in the N-terminal segment of h4. However, if this sequence was actually a Ca^{2+} binding site, it is unlikely that it would be related to the high affinity Ca^{2+} transport site because, despite of the sequence variability, the Ca^{2+} dependence of the Ca^{2+} transport by h4(ct120) and (n1–79)h1h4(ct120) was similar.

The results from this work indicate that the variability of sequence at the extreme N-terminal segment of isoforms h1 and h4 has little influence on the most relevant kinetic parameters of the h4 enzyme. Although effects on functions other than those we measured cannot be discarded, our results may indicate that the observed divergence between the N-terminus of the PMCA isoforms 1 and 4 results from neutral variation, i.e. involves amino acid substitutions that do not change the behavior of the h4 enzyme sufficiently to influence selection. Alternatively, differences in the function between different isoforms may require, in addition to the N-terminal segment, other isoform specific regions, as it has been proposed for the Na,K-ATPase [20].

Acknowledgements

This work was supported by Fundación Antorchas, Agencia Nacional de Promoción Científica y Tecnológica, Consejo Nacional de Investigaciones Científicas y Tecnológicas and Universidad de Buenos Aires. We thank Dr. J.T. Penniston for kindly providing the JA9 antibody.

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