Functional consequences of relocating the C-terminal calmodulin-binding autoinhibitory domains of the plasma membrane Ca^{2+} pump near the N-terminus

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A mutant of the plasma membrane Ca^{2+} pump (PMCA) called (nCI)hPMCA4b(ct120), in which the C-terminal regulatory segment including the calmodulin-binding autoinhibitory domains C and I had been relocated near the N-terminus, has been expressed in COS-1 cells. The measurements of Ca^{2+} transport in microsomal preparations showed that the rearranged enzyme was functional. The activity of the (nCI)hPMCA4b(ct120) mutant was compared with those of the wild-type hPMCA4b and the fully active calmodulin-insensitive mutant hPMCA4b(ct120). In the absence of calmodulin the activity of (nCI)hPMCA4b-(ct120) was higher than that of hPMCA4b but only 45 % of that of hPMCA4b(ct120). Mutant (nCI)hPMCA4b(ct120) exhibited an apparent affinity for Ca^{2+} similar to that of hPMCA4b, typical of the inhibited state of the enzyme. Calmodulin at concentrations that fully activated hPMCA4b increased the activity of (nCI)hPMCA4b(ct120) to 68 % of that of hPMCA4b-(ct120). The lower maximal activity of (nCI)hPMCA4b(ct120) was not due to a lower affinity for calmodulin because the concentration of calmodulin required for half-maximal activation of (nCI)hPMCA4b(ct120) was equal to that of the wild-type hPMCA4b. These results indicate that (1) the disturbance of the N-terminal region of the PMCA by the insertion of the C-terminal segment decreased the ability of the pump to transport Ca^{2+}, and (2) the calmodulin-binding autoinhibitory domain was still able to access its acceptor site from the N-terminal end of the molecule. However, although the calmodulin-binding and inhibitory functions of the C-domain were fully preserved, the I domain at its new position seemed less effective at inhibiting the pump.

INTRODUCTION

The plasma membrane Ca^{2+} pump (PMCA), a calmodulin-regulated P-type ATPase, has a key role in the control of intracellular Ca^{2+} [1]. Like the other P-type pumps, PMCA contains about ten transmembrane segments, and most of the polypeptide chain, including both terminal ends, is exposed to the cytosol. The central portion of the molecule contains the catalytic domain, which is homologous with those of the other members of the family. In contrast, the N- and C-terminal segments are distinctive of the PMCA. Very little is known about the functional importance of the N-terminal segment of the PMCA. We have recently found that a PMCA mutant lacking residues 18–75 is unable to transport Ca^{2+}, indicating that the N-terminal segment is essential for the expression of an active enzyme [2]. However, it is known that the C-terminal segment of the PMCA contains a calmodulin-binding autoinhibitory domain which in the absence of calmodulin binds close to the catalytic site, keeping the enzyme in a low-activity state. Recent studies [3–5] with C-terminally truncated mutants of the human PMCA isoform 4b, hPMCA4b (which has also been called hPMCA4CI [1]), have indicated that the autoinhibitory region consists of two domains, called C and I. Domain, C, which also functions as a calmodulin-binding site, consists of a segment of 28 residues and is responsible for approx. 50 % of the inhibition. Synthetic peptides resembling the C domain bind calmodulin as tightly as does hPMCA4b and also interact with the catalytic site of the enzyme after the natural autoinhibitory domain has been removed [6]. Full inhibition requires domain I, which includes approx. 45 residues downstream. The binding of calmodulin to its site or the proteolytic removal of a 15 kDa fragment from the C-terminus activates the pump, increasing the maximum velocity and apparent Ca^{2+} affinity. Alternatively the maximum velocity can be increased by the phosphorylation of residues from the I domain with protein kinase C [5]. Other regulated enzymes have been shown to possess autoinhibitory regions found at either the N-terminal or the C-terminal side of the catalytic site [7]. This raised the question of whether it is mandatory for domains C and I to be at the C-terminal end of the PMCA to perform their regulatory function.

In this study we examined the effects of repositioning the C-terminal regulatory region of the PMCA near the other end of the molecule. In addition, to investigate the functional effects of the disruption of the N-terminal region we reinserted the C-terminal region approximately in the middle of the N-terminal segment. Using the cDNA of the fully activated calmodulin-insensitive mutant hPMCA4b(ct120), which lacks the C-terminal 120 residues [3], we constructed a new mutant called (nCI)-hPMCA4b(ct120) by inserting a segment of 129 residues from the C-terminal region of hPMCA4b at position 43 of hPMCA4b-(ct120) (Figure 1). We expressed the mutant in COS-1 cells and characterized its ability to transport Ca^{2+} and also its response to calmodulin.

EXPERIMENTAL

Materials

Reagents were purchased from the following companies: enzymes used in DNA manipulations, New England Biolabs; ^{45}Ca, DuPont–NEN; Immobilon transfer membranes, Millipore; Immunochemicals, Vector Labs.; reagents for cell culture, calmodulin and other chemicals, Sigma.

Abbreviation used: PMCA, plasma membrane Ca^{2+} pump.

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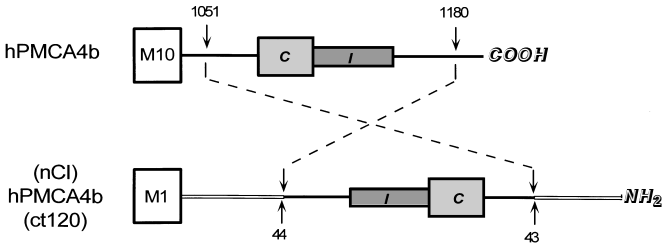

Figure 1 Schematic representation of the autoinhibitory domains of wild-type hPMCA4b and the (nCI)hPMCA4b(ct120) mutant

The C-terminal fragment of the hPMCA4b, which extends after the transmembrane segment (M10), and the N-terminal fragment of (nCI)hPMCA4b(ct120), which precedes the first transmembrane segment (M1), are shown aligned by their position with respect to the membrane. The (nCI)hPMCA4b(ct120) mutant was constructed by reinserting residues 1051–1180 of hPMCA4b at position 43 of hPMCA4b(ct120). Abbreviations: COOH, C-terminus; NH_2, N-terminus; C, calmodulin-binding autoinhibitory domain C; I, autoinhibitory domain I. The numbers refer to the amino acid sequence of hPMCA4b. The lengths of the segments are shown proportional to the number of amino acid residues that they contain.

Construction of the (nCI)hPMCA4b(ct120) mutant and expression in COS-1 cells

The construction of the cDNA of mutant hPMCA4b(ct120) was as described previously [3]. By using PCR for site-specific mutagenesis a new unique restriction site for *Mlu*I was created in the hPMCA4b(ct120) cDNA. This resulted in the alteration of Gln-43 to Arg. A cDNA fragment coding for the C-terminal segment of hPMCA4b (Thr-1051 to Asn-1180) was synthesized by PCR and inserted at the *Mlu*I site of hPMCA4b(ct120). The final construct was checked for insertion in the correct orientation by double-strand sequencing. The wild-type and mutant cDNA species were cloned into the pMM2 vector [8] for expression in COS-1 cells [9]. The transfection was performed by the DEAE/dextran/Chloroquine method [10] and cells were harvested after 48 h. The microsomal fraction was isolated as described previously [3]. Protein concentration was estimated by means of the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.), with BSA as a standard.

Detection of expressed Ca^{2+} pump protein

SDS/PAGE and immunoblotting were performed as described previously [11]. Proteins were electrophoresed on a 7.5% (w/v) polyacrylamide gel by the method of Laemmli [12] and subsequently transferred to Millipore Immobilon membranes. Non-specific binding was blocked by incubating the membranes overnight at 4 °C in 160 mM NaCl/0.05% (v/v) Tween-20/1% (w/v) non-fat dried milk. The membranes were incubated at 37 °C for 1 h with 5F10 monoclonal antibody [13] from ascitic fluid (dilution 1:1000). For staining, biotinylated anti-(mouse IgG) and avidin conjugated with horseradish peroxidase were used. To estimate the expression of the (nCI)hPMCA4b(ct120) protein compared with that of the wild-type hPMCA4b, different amounts of membranes containing either the mutant protein or hPMCA4b were loaded in parallel wells of the same gel. The immunoblots were scanned digitally and the intensities of the bands were quantified with the program SIGMA SCAN (Jandel Scientific). The level of expressed ATPase in each microsome preparation was referred to that of hPMCA4b.

Assay of Ca^{2+} transport

Ca^{2+} uptake assays were performed as described previously [3] in the presence of thapsigargin to inhibit the activity of the endogenous endoplasmic reticulum Ca^{2+} pump. 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, 8 mM $MgCl_2$ and $CaCl_2$ to give the concentration of free Ca^{2+} indicated in each experiment. The free Ca^{2+} concentration was calculated by using the program of Fabiato and Fabiato [14]. Vesicles (5–10 μg of protein) were preincubated at 37 °C for 5 min in the reaction mixture and the reaction was initiated by the addition of 6 mM ATP. The reaction was terminated after 5 min by filtering the samples through a 0.45 μm pore-size filter. The ^{45}Ca taken up by the vesicles was then determined by being counted in a liquid-scintillation counter. The Ca^{2+} uptake was linear with time for at least 15 min. Uptake activities were expressed per mg of COS-1 cell membrane protein. The activity of the endogenous Ca^{2+} pump from COS-1 cells accounted for 10–20% of the total Ca^{2+} uptake and was subtracted from each data point. The observed rates of Ca^{2+} transport were corrected in accordance with the level of expressed ATPase as judged by immunoreactivity in each microsomal preparation, as described above.

RESULTS AND DISCUSSION

The expression of (nCI)hPMCA4b(ct120) was investigated in microsomes isolated from transfected COS-1 cells by immunoblotting with 5F10 monoclonal antibody, which reacts in the region of residues 719–738 in the central portion of the molecule [13]. Figure 2 shows that the (nCI)hPMCA4b(ct120) protein was successfully expressed and migrated with the expected apparent size, which was close to that of the full-length hPMCA4b. The intensity of the (nCI)hPMCA4b(ct120) band indicates that the mutant was expressed at a level similar to that attained for hPMCA4b and hPMCA4b(ct120).

The results in Table 1 show that (nCI)hPMCA4b(ct120) was a functional Ca^{2+} pump. In the absence of calmodulin the Ca^{2+} transport activity of (nCI)hPMCA4b(ct120) was higher than that of the wild-type hPMCA4b, although substantially lower than that of the fully activated hPMCA4b(ct120). The addition of 240 nM calmodulin to the reaction mixture increased the activity of the (nCI)hPMCA4b(ct120) mutant to approx. 68% of that of hPMCA4b(ct120), indicating that the enzyme was sensitive to calmodulin.

The characteristics of the (nCI)hPMCA4b(ct120) mutant were analysed further by comparing the Ca^{2+} dependence of its

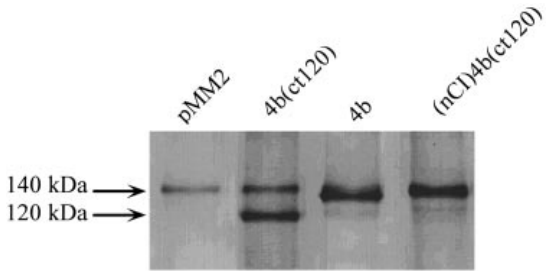

Figure 2 Immunoblot of microsomes from COS-1 cells transfected with the empty vector pMM2 or with pMM2 containing the cDNA encoding hPMCA4b-(ct120), hPMCA4b or (nCI)hPMCA4b(ct120)

Membrane protein (3 μg) was applied on each lane of an SDS/7.5% polyacrylamide gel and subjected to immunoblot analysis with monoclonal antibody 5F10.

Table 1 Ca^{2+} transport activities of the hPMCA4b(ct120), (nCI)hPMCA4b-(ct120) and hPMCA4b Ca^{2+} pumps

The Ca^{2+} transport activities were estimated from the initial rate of Ca^{2+} uptake by microsomal vesicles after 5 min at 37 °C in the presence of 8 μM free Ca^{2+} with or without 240 nM calmodulin. The activities were corrected depending on the level of expression in each microsomal preparation and are given as percentage of the activity of the hPMCA4b(ct120), which varied in different transfections from 3 to 8 nmol/min per mg of protein. The Ca^{2+} uptake from pMM2-transfected cells (endogenous Ca^{2+} pump) was substrated from each data point. The values shown are means ± S.D. for three experiments.

	Ca^{2+} transport	
Type of Ca^{2+} pump	Without calmodulin	With calmodulin
hPMCA4b(ct120)	99 ± 7	100 ± 4
(nCI)hPMCA4b(ct120)	45 ± 6	68 ± 8
hPMCA4b	32 ± 9	100 ± 7

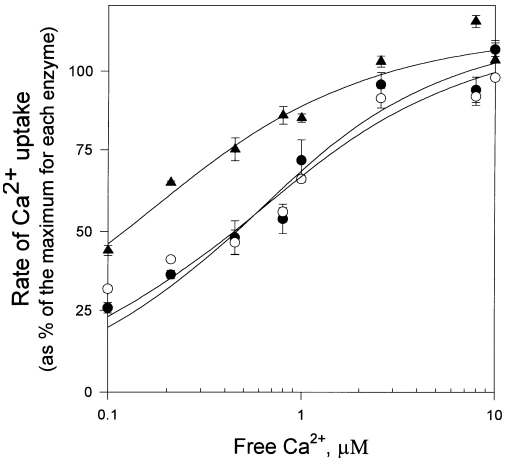

Figure 3 Ca^{2+} concentration dependence of the rate of Ca^{2+} uptake by microsomes of cells transfected with hPMCA4b(ct120) (▲), (nCI)hPMCA4b-(ct120) (○) and hPMCA4b (●)

The rate of Ca^{2+} uptake was measured at different Ca^{2+} concentrations in the absence of calmodulin. The activities are expressed as a percentage of the maximum Ca^{2+} uptake rate of each sample. The lines are the best fit to the data with the Hill equation.

transport activity with those of the hPMCA4b and hPMCA4b-(ct120) enzymes in the absence of calmodulin. The activities shown in Figure 3 were expressed as a percentage of the activity of each enzyme at saturating concentrations of Ca^{2+}. The (nCI)hPMCA4b(ct120) mutant displayed an apparent Ca^{2+} affinity ($K_{1/2} = 0.55\ \mu$M) lower than that of hPMCA4b(ct120) ($K_{1/2} = 0.18\ \mu$M) and similar to that observed for the full-length hPMCA4b ($K_{1/2} = 0.60\ \mu$M). This result indicates that the N-terminal autoinhibitory region of (nCI)hPMCA4b(ct120) was fully effective in decreasing the apparent Ca^{2+} affinity of the enzyme to a value similar to that of the hPMCA4b.

The calmodulin dependence of the activities of (nCI)-hPMCA4b(ct120) and hPMCA4b was studied in more detail in the experiment shown in Figure 4. Although the magnitudes of the response to calmodulin differed, the concentrations of calmodulin required for half-maximal activation were identical,

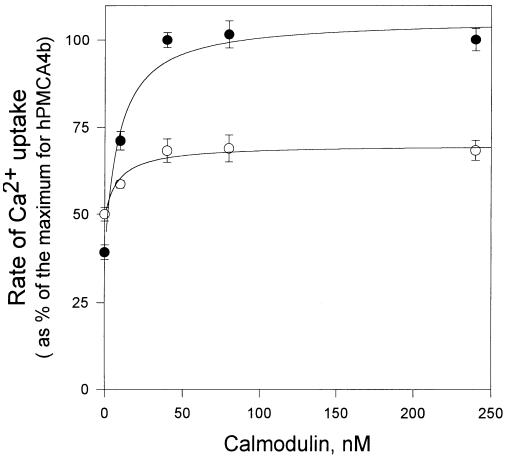

Figure 4 Calmodulin dependence of the rate of Ca^{2+} uptake by the (nCI)hPMCA4b(ct120) (○) and wild-type hPMCA4b (●) enzymes

The microsomal vesicles were preincubated at 37 °C in a medium containing 8 μM Ca^{2+} and the appropriate concentration of calmodulin, and Ca^{2+} uptake was initiated by the addition of ATP. The rates of Ca^{2+} uptake are expressed as a percentage of that of hPMCA4b measured in the presence of 3 μM calmodulin, which was 8 ± 1 nmol/min per mg of protein. The lines represent the best fit to the data given by the following equation $V = V_0 + V_c/[1 + (K_c/[CaM])]$, where CaM is calmodulin, K_c is an apparent dissociation constant for calmodulin, V_0 is the activity in the absence of calmodulin and V_c is the maximum value of the calmodulin-dependent activity. The means ± S.D. for each parameter were, for hPMCA4b, $V_0 = 38 \pm 6$, $K_c = 8 \pm 3$ nM, $V_c = 68 \pm 7$ and, for (nCI)hPMCA4b(ct120), $V_0 = 49 \pm 2$, $K_c = 8 \pm 5$ nM and $V_c = 21 \pm 3$.

suggesting that both enzymes bound calmodulin equally tightly. This result indicates that the N-terminal localization of the C domain did not impair its calmodulin-binding function and therefore the observed lower maximal activity of (nCI)-hPMCA4b(ct120) in the presence of 240 nM calmodulin (Table 1) could not be due to a decrease in the affinity for calmodulin. Therefore the decrease in the ability of the (nCI)hPMCA4b-(ct120) enzyme to transport Ca^{2+} should be related to the disruption of the N-terminal segment by the insertion at position 43. This result is consistent with our recent findings showing that the N-terminal segment including residues 18–75 is a functionally important region of the pump [2].

The kinetic properties of (nCI)hPMCA4b(ct120) are reminiscent of those recently reported for a mutant, termed hPMCA4b(ct92), lacking the C-terminal 92 residues including the I domain [4], in the sense that both mutants have a low apparent affinity for Ca^{2+}, are activated by calmodulin with high affinity but remain partly activated even in the absence of calmodulin. It therefore seems that in (nCI)hPMCA4b(ct120) the function of the C domain is preserved, whereas that of the I domain is not. It is possible that the proper functioning of the I domain requires its location to be closer than the C domain to the extreme of the molecule.

The results presented here show that the insertion of the C-terminal regulatory region of hPMCA4b near the N-terminus of hPMCA4b(ct120) partly restored its calmodulin regulation. These results suggest that a calmodulin-sensitive Ca^{2+} pump might originate from the fusion of a calmodulin-binding auto-inhibitory domain at either side of the catalytic site of the enzyme. Consistent with this idea, a cDNA sequence of a Ca^{2+} pump from plant vacuolar membranes with a putative regulatory domain naturally found at its N-terminus has been reported [15].

This work was supported in part by a grant from Fundación Antorchas (Argentina).

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Received 11 July 1997/6 October 1997; accepted 15 December 1997