Chimaeras reveal the role of the catalytic core in the activation of the plasma membrane Ca^{2+} pump

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Isoform 2b of the plasma membrane calcium pump differs from the ubiquitous isoform 4b in the following: (a) higher basal activity in the absence of calmodulin; (b) higher affinity for calmodulin; and (c) higher affinity for Ca^{2+} in the presence of calmodulin [Elwess, Filoteo, Enyedi and Penniston (1997) J. Biol. Chem. **272**, 17981–17986]. To investigate which parts of the molecule determine these kinetic differences, we made four chimaeric constructs in which portions of isoform 2b were grafted into isoform 4b: chimaera I contains only the C-terminal regulatory region of isoform 2b; chimaera II contains the N-terminal moiety of isoform 2b, including both cytoplasmic loops; chimaera III contains the sequence of isoform 2b starting from the N-terminus to after the end of the first (small) cytoplasmic loop; and chimaera IV contains only the second (large) cytoplasmic loop. Surprisingly, chimaera I showed low basal activity in the absence of calmodulin and low affinity for calmodulin, unlike isoform 2b. In contrast, the chimaera containing both loops showed high basal activity, and Ca^{2+} activation curves (both in the absence and in the presence of calmodulin) similar to those of isoform 2b. The rates of activation by calmodulin and of inactivation by calmodulin removal were measured, and the apparent K_d for calmodulin was calculated from the ratio between these rate constants. The order of affinity was: 2b = II > 4b = IV > III = I. From these results it is clear that the construct that most closely resembles isoform 2b is chimaera II. This shows that, in order to obtain an enzyme with properties similar to those of isoform 2b, both cytoplasmic loops are needed.

Key words: calmodulin, Ca^{2+} signal, ion transport, P-type ATPase.

INTRODUCTION

The plasma membrane calcium pump (PMCA) actively transports Ca^{2+} across the plasma membrane, and is necessary for control of the calcium concentration inside the cell. At low cytoplasmic Ca^{2+} concentrations, this pump is maintained in an inactive state by the interaction of its catalytic core with an autoinhibitory region in the C-terminus of the molecule. As discussed previously [1], activation of PMCA is accomplished by the disruption of this interaction by calmodulin. A study in which the properties of the rat PMCA2b and human PMCA4b isoforms were compared suggested that differences in the sequences of these molecules can influence this interaction. The basal activity (measured in the absence of calmodulin) of PMCA2b is much greater than that of PMCA4b, and the apparent calmodulin affinity is also about 4-fold higher [2]. Previous studies [3] using synthetic peptides representing the calmodulin-binding domains of different b isoforms have indicated that the small differences in their calmodulin-binding domains cannot be responsible for the discrepancy in their calmodulin affinity. Rather, this discrepancy comes from the sequence variations in either the catalytic core or the C-terminus downstream of the calmodulin-binding domain, or both. This indicates that further comparisons between isoforms 2b and 4b may shed light on the intramolecular interaction of the core with the C-terminus.

Predictions of the structure of PMCA, based on the amino acid sequence [4] and on structural data for the related sarcoplasmic reticulum Ca^{2+} pump [5], reveal that the cytoplasmic side of the Ca^{2+} pump contains two loops. These loops are predicted to form the site for catalysis of the hydrolysis of ATP. We therefore made several chimaeras of these two isoforms, in which we replaced portions of PMCA4b with the corresponding regions of isoform 2b (see Figure 1). The results reported here show that the regulatory C-terminal region interacts with these two loops. They also show that exchanges of the loops (included in the region Met-1–Arg-704) between isoforms 2b and 4b are important in determining the strength of its interaction with the regulatory region, while exchanges of the regulatory C-terminal regions (Ile-1100 to Val-1205) are less important.

MATERIALS AND METHODS

Materials

^{45}Ca and [γ-^{32}P]ATP were purchased from DuPont NEN. Calmodulin was obtained from Sigma. LIPOFECTAMINE™ and Optimem media were obtained from Gibco BRL Life Technologies, Inc. All other chemicals used were of reagent grade. Rat isoform 2b and human isoform 4b were used in all experiments. The sequences of the corresponding rat and human isoforms of PMCA are nearly identical.

Chimaera construction

PMCA isoform 4b, cloned into the *Sal*I and *Kpn*I sites of pSP72, and PMCA isoform 2bz, cloned into the *Sal*I and *Kpn*I sites of the PMM2 expression vector, served as starting points for chimaera construction. The unique restriction enzyme sites *Sca*I, *Stu*I, *Sma*I and *Bam*HI in isoform 4b provided points at which

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

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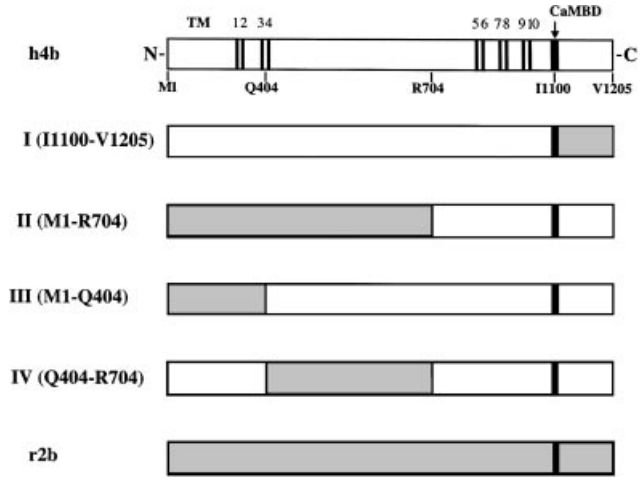

Figure 1 Chimaeras used in the present study

The 10 transmembrane (TM) regions are indicated by the thin vertical lines, which are numbered to identify them. 'CaMBD' refers to the calmodulin-binding domain (which does not pass through the membrane). Substitution of regions of isoform 2b for the corresponding regions of isoform 4b is indicated by grey shading of the region. Note that chimaera II (Met-1–Arg-704) contains both cytoplasmic loops, while chimaera III (Met-1–Gln-404) contains the small cytoplasmic domain, and chimaera IV (Gln-404–Arg-704) contains the large cytoplasmic domain. The prefixes h and r denote human and rat respectively.

Table 1 Primers used to make the chimaeras

Nucleotides that introduce mutations are shown italicized and in bold. New restriction endonuclease sites are underlined.

Primer	Sequence
A	atc ccc gag gaa gag tt
B	cca ggc gac a***gg tac c***
C	gca ***a***gc cta tgt cgg tga tgt cc
D	ggg cag gcc ***t***cc ctc ttt tt
E	ggt gct tca ggg caa gct
F	aag agg g***a***g ***g***cc ***t***gc c
G	ggt gga taa tgc cac act t

to substitute segments of isoform 4b with those of isoform 2bz. These sites correspond to amino acids Gln-404, Gly-526, Arg-704 and Ile-1100 respectively.

Chimaera I did not require any intricate manipulations. Both isoforms 4b and 2bz have a *Bam*HI site at corresponding locations. Primer A (Table 1) extends through this site. Primer B introduces a *Kpn*I site. While isoform 2bz already had a *Kpn*I site after the stop codon, it also had a substantial 3′ untranslated region. Addition of the *Kpn*I site to primer B eliminates the 3′ untranslated region that the original *Kpn*I site would have introduced into chimaera I. Construction of chimaera II was straightforward, because isoform 2bz shares the *Sma*I site at a corresponding position. Thus a simple replacement of the *Sal*I/*Sma*I fragment in 4b with the corresponding fragment from 2bz created chimaera II.

Chimaera III was more challenging, because the ampicillin resistance gene has a *Sca*I site. Therefore it was not possible to directly replace the *Sal*I/*Sca*I fragment in pSP72, even though the *Sca*I sites in isoforms 2bz and 4b occur at corresponding points. This dilemma was resolved by destroying the *Sca*I site in pSP72 containing isoform 4b. A 1340 bp *Stu*I/*Stu*I fragment from the pACYC177 vector, which contains the kanamycin resistance gene, was blunt-cloned into the *Sca*I site of pSP72. This vector is designated pSP72;Kn. The *Sal*I/*Sca*I replacement was then made directly and selected using kanamycin resistance.

Chimaera IV was the most challenging, because the kanamycin resistance gene has a *Sma*I site. Therefore neither the native nor the modified pSP72 vector could be used for a direct replacement of the *Sca*I/*Sma*I fragment. Therefore this chimaera was constructed from the Gln-404–Gly-526 and Gly-526–Arg-704 chimaeras (not shown). These constructs took advantage of the unique *Stu*I site in isoform 4b. However, isoform 2bz does not share a *Stu*I site at the corresponding point; it has a *Stu*I site 140 nucleotides upstream from the corresponding position in isoform 4b. A megaprimer PCR approach was used to construct the Gln-404–Gly-526 chimaera. Primers C and D were used with isoform 2bz as template to create the megaprimer. This primer destroyed the native *Stu*I site in isoform 2bz and added a *Stu*I site at a position corresponding to that in isoform 4b. Primer E and the megaprimer were used with isoform 2bz as template to create a product with the *Sca*I/*Stu*I fragment needed for this chimaera. This product was cloned into the pCR-Blunt II vector, and the replacement of the *Sca*I/*Stu*I fragment in the kanamycin-resistant isoform 4b was carried out using standard molecular biology techniques. This construct was then transferred to the native pSP72 vector in preparation for making chimaera IV. Primer F introduces a *Stu*I site in isoform 2bz at the same position as primer C. Primers F and G with isoform 2bz as template created a *Stu*I/*Sma*I fragment. Creation of the Gly-526–Arg-704 chimaera only required replacing the *Stu*I/*Sma*I segment of isoform 4b with this fragment. Also, with this fragment, creation of chimaera IV was a simple matter of replacing the *Stu*I/*Sma*I fragment of the Gln-404–Gly-526 chimaera cloned into native pSP72. It should be noted that the mutations introduced by primers D and F are not silent in isoform 2b. Therefore Gly-526 was left instead of the corresponding Ala from isoform 2b. Hence chimaera IV contains the large cytoplasmic loop of PMCA2, with an A → G substitution at position 526. This has to be taken into account when interpreting the results.

Transfection of COS-1 cells

Transfection was carried out using LIPOFECTAMINE™ (Gibco BRL Life Technologies, Inc.), based on the protocol described by the manufacturer and in Enyedi et al. [6]. Briefly, transfection was initiated when the cells were 70–80% confluent in 150 cm^2 flasks. The cells were incubated at 37 °C with the DNA–LIPOFECTAMINE™ complex (formed by incubating 8 μg of DNA and 100 μl of LIPOFECTAMINE™ in 3.6 ml of serum-free Optimem medium) in 14.5 ml of serum-free Optimem medium. After a 5 h incubation, the cells were supplemented with serum, and incubation was continued for a total of 24 h. The medium containing the DNA–LIPOFECTAMINE™ complex was then replaced with fresh tissue culture medium containing 10% (v/v) serum, and the cells were cultured for an additional 24 h.

Isolation of microsomes from COS cells

Crude microsomal membranes from COS cells were prepared as described by Enyedi et al. [7].

Ca^{2+} transport assay

Ca^{2+} uptake by microsomal vesicles was carried out in a 200 μl reaction mixture and assayed by rapid filtration through

Millipore membrane filters (0.45 μm pore size; type HA) as described previously [6,7]. The reaction mixture contained 100 mM KCl, 25 mM Tes/triethanolamine, pH 7.2, 40 mM KH_2PO_4/K_2HPO_4, pH 7.2, 200 nM thapsigargin, 5 mM NaN_3, 4 μg/ml oligomycin, 7 mM $MgCl_2$, 100 μM $CaCl_2$ (labelled with ^{45}Ca; specific radioactivity 100000–150000 c.p.m./nmol) and enough EGTA to obtain the desired free Ca^{2+} concentration. Microsomes at 10–20 μg/ml concentration were preincubated in the presence or absence of 2 μM calmodulin for 2 min at 37 °C, and Ca^{2+} uptake by the vesicles was started by the addition of 6 mM ATP. The reaction was terminated by rapid filtration of the microsomes using Millipore membrane filters.

Determination of k_{on} and k_{off} for activation by calmodulin

These constants were measured in experiments in which activation by calmodulin of Ca^{2+}-ATPase activity, and its inactivation by calmodulin removal, were measured. This is described in detail in [8]. The kinetic method used to measure ATPase activity was also described in that paper [8]. Ca^{2+}-ATPase activity was measured in medium containing 30 mM Tes/triethanolamine (pH 7.2), 120 mM KCl, 5 mM $MgCl_2$, 200 μM EGTA, 0.2 mM 2-amino-6-mercapto-7-methylpurine, 1 unit/ml purine nucleoside phosphorylase, 5 mM NaN_3, 1 mM dithiothreitol, 0.5 mM ouabain, 4 μg/ml oligomycin, 200 nM thapsigargin, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin and enough $CaCl_2$ to obtain 0.4 μM free Ca^{2+}.

RESULTS

Out of 28 residues, 26 are identical between the calmodulin-binding domains of PMCA isoforms 2b and 4b. Despite the great similarity in their calmodulin-binding domains, the apparent calmodulin affinity of isoform 2b is approx. 4 times higher than that of isoform 4b [2].

To test whether the differences between isoforms 2b and 4b are due to their C-termini, we made a chimaera (I) which has the C-terminal regulatory region of isoform 2b replacing that of isoform 4b. Figure 2 shows that the apparent calmodulin affinity of chimaera I was even lower than that of isoform 4b, indicating

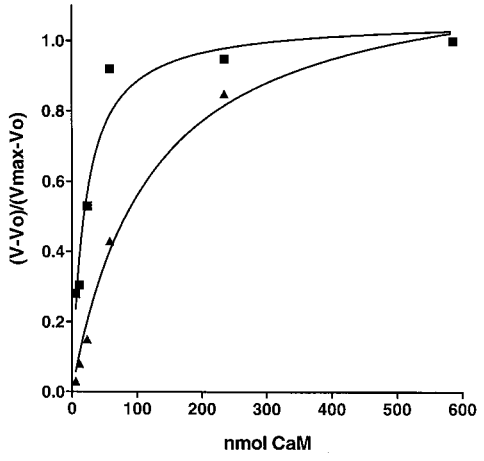

Figure 2 Effects of calmodulin on calcium uptake by PMCA4b and chimaera I

Calcium transport was measured in COS cell microsomes expressing isoform 4b (■) or chimaera I (▲), as described in the Materials and methods section. V_0 is the residual activity in the absence of calmodulin, Vmax is the maximal activity, and V is the observed activity at a given calmodulin concentration.

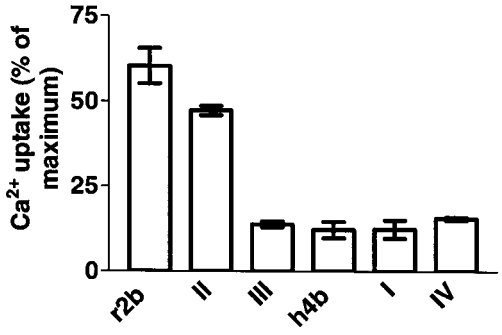

Figure 3 Ca^{2+} uptake by PMCA isoforms 4b and 2b and by the various chimaeras in the absence of calmodulin

The conditions used were as described in the legend of Figure 2. The prefixes h and r denote human and rat respectively.

that differences in the sequence of the C-terminus (including the calmodulin-binding domain) cannot be responsible for the high calmodulin affinity observed for isoform 2b.

We then made several more chimaeras in which various portions of isoform 4b were replaced by the corresponding regions of isoform 2b. Figure 1 shows the chimaeras used in the study, along with the exact portion of the isoform 4b molecule that was replaced by portions of isoform 2b. Chimaera II contains a large portion of the N-terminal region of isoform 2b, including both of the cytoplasmic loops. Previous cross-linking studies suggested that both of these loops are involved in the interaction with the regulatory region of isoform 4b [9]. Chimaera III contains the sequence of isoform 2b starting from the N-terminus to after the end of the first (small) cytoplasmic loop; chimaera IV contains only the second (large) cytoplasmic loop.

In order to assess the properties of the chimaeras, we began by measuring their basal activities. We already knew that isoform 2b has a much higher basal activity than isoform 4b, indicating a weaker intramolecular interaction between the C-terminal regulatory region and the catalytic core. Figure 3 shows the activities of each of the chimaeras in the absence of calmodulin. It is clear that only chimaera II (containing both cytoplasmic loops) had a basal activity approaching that of isoform 2b, while the other chimaeras were indistinguishable from isoform 4b. This shows the necessity of the presence of both cytoplasmic loops of isoform 2b to obtain higher basal activity. Our results suggest that both cytoplasmic loops are involved in determining the strength of the intramolecular interaction.

Reinforcing this point, Figure 4 shows the dependence of the activity on Ca^{2+} concentration for each of the chimaeras. Each activity is shown in the presence and in the absence of calmodulin. The chimaeras naturally divide into two groups, those that have a low basal activity like isoform 4b (chimaeras I, III and IV) and that which has a high basal activity like isoform 2b (chimaera II). The data in Figure 4 demonstrate that the results of the experiment in Figure 3 are valid at all Ca^{2+} concentrations observed here.

Another crucial parameter that differentiates isoform 2b from isoform 4b is the affinity for calmodulin. In Figure 5 the on and off rates for activation by calmodulin of isoforms 2b and 4b and the chimaeras are shown. The on-rate constants (Figure 5, top panel) can be classified in the following order: 2b = II > 4b = IV > III = I. The off-rate constants (Figure 5, middle panel) were in the following order: III > 4b ~ IV > 2b = I = II. The k_{off}/k_{on} ratio is the K_d for calmodulin for each of the constructs.

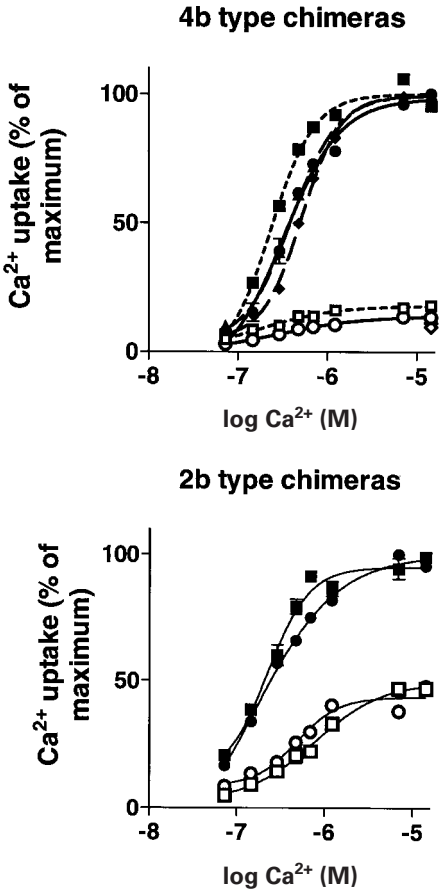

Figure 4 Ca^{2+}-dependence of activities of full-length PMCA isoforms 4b and 2b, and of the chimaeras containing each of the cytoplasmic loops

The filled symbols show the activity in the absence of calmodulin, and the open symbols that in the presence of calmodulin. Measurements in the absence of calmodulin were made with chimaeras I and IV only at the lowest and highest Ca^{2+} concentrations. All measurements on other constructs covered a full set of Ca^{2+} concentrations. The conditions used were as described in the legend of Figure 2. Upper panel: ●, isoform 4b + calmodulin; ◆, chimaera I + calmodulin; ■, III + calmodulin; ▲, IV + calmodulin; ○, 4b minus calmodulin; ◇, I minus calmodulin; □, III minus calmodulin; X, IV minus calmodulin (this symbol is obscured by the open circle symbol in the lower right-hand corner of the Figure). Lower panel: ●, 2b + calmodulin; ■, II + calmodulin; ○, 2b minus calmodulin; □, II minus calmodulin.

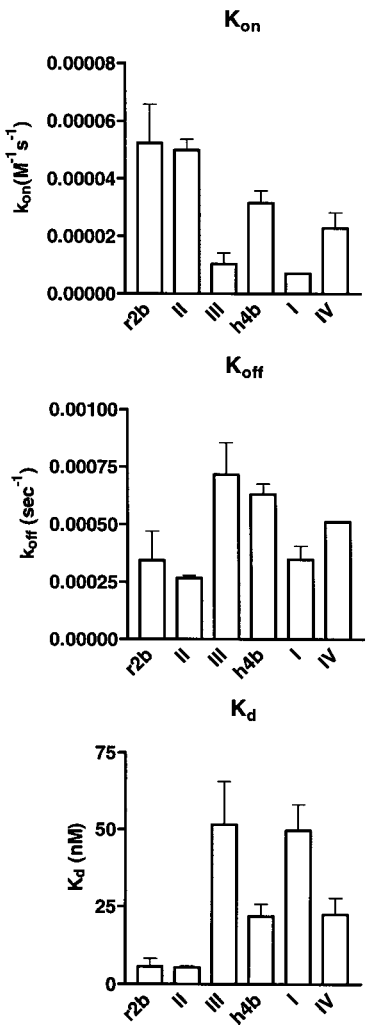

Figure 5 Analysis of the on and off rates for calmodulin binding to PMCA isoforms 4b and 2b and chimaeras I–IV

The rates of activation and inactivation, along with the overall K_d, were determined from ATPase activity assays, as described in the Materials and methods section. The prefixes h and r denote human and rat respectively.

Thus the calculated K_d values for isoform 2b and chimaera II are very low, while isoform 4b and chimaera IV have higher K_ds, and chimaeras I and III have the highest values. It is noticeable that the order of K_d values for the different chimaeras approximately reflects the distribution of basal activities, i.e. the constructs with the lowest K_d values have the highest basal activity (see Figure 3).

DISCUSSION

The goal of this work was to study the role of the C-terminus and the cytoplasmic loops in the regulation of PMCA2b and PMCA4b by calmodulin. Early studies indicated that calmodulin binding to the C-terminal region of PMCA activates the pump by preventing this region from interacting with the cytoplasmic core of the enzyme. The discovery of the existence of several isoforms of PMCA was followed by the finding that these isoforms differ in the properties of their activation by calmodulin [2,10–12]. Differences between isoforms 2b and 4b were especially striking: PMCA2b has a higher affinity for calmodulin and higher basal activity in calmodulin's absence than isoform 4b. The differences in primary structure between the two isoforms are located mainly in the C-terminal region of the pump, downstream of the calmodulin-binding domain. Furthermore, calmodulin-binding peptides having the sequence of either PMCA2b or PMCA4b bind calmodulin equally well with very high affinity. Because of this, we tried to explain the different behaviour of these isoforms on the basis of the differences between their sequences in the C-terminus, downstream of the calmodulin-binding domain. However, chimaera I (made up of the isoform 4b core and the C-terminus of isoform 2b) had the same basal activity as and even lower calmodulin affinity than isoform 4b, which is the opposite of what we expected. From these data it is evident that the regions responsible for the differences in the response to calmodulin between isoforms 4b and 2b lie somewhere else. Of all the chimaeras that we prepared, only chimaera II (containing both cytoplasmic loops of isoform 2b) had a calmodulin activation pattern like that of isoform 2b. Its basal activity, K_d for calmodulin and Ca^{2+} activation kinetics

(both with and without calmodulin) are similar to those of isoform 2b. On the other hand, chimaeras III (containing only the small cytoplasmic loop of isoform 2b) and IV (containing only the large cytoplasmic loop of isoform 2b) have a calmodulin activation pattern similar to that of isoform 4b.

Despite binding calmodulin with extremely high affinity, the calmodulin-binding domain by itself does not determine the apparent affinity for calmodulin's activation of the pump. In fact, there are two orders of magnitude difference between the apparent affinities for calmodulin activation of PMCA isoform 2b and the K_d for binding of calmodulin to C28R2, a calmodulin-binding peptide prepared based on the sequence of PMCA2b [3]. This observation reinforces the concept that activation by calmodulin is a complex process involving the interaction of several domains in the pump.

Summarizing, the present results are consistent with the idea that both cytoplasmic loops of PMCA are involved in the intramolecular interaction with the C-terminal region that is disrupted upon binding of calmodulin. Our results are in agreement with those of the Carafoli laboratory [9], who, based on chemical cross-linking experiments, proposed that the calmodulin-binding domain interacts with two regions in the pump: one in the small cytoplasmic loop and the other in the large one.

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