The Plasma Membrane Calcium Pump Displays Memory of Past Calcium Spikes

DIFFERENCES BETWEEN ISOFORMS 2b AND 4b*

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To understand how the plasma membrane Ca\(^{2+}\) pump (PMCA) behaves under changing Ca\(^{2+}\) concentrations, it is necessary to obtain information about the Ca\(^{2+}\) dependence of the rate constants for calmodulin activation (k\(_{\text{act}}\)) and for inactivation by calmodulin removal (k\(_{\text{inact}}\)). Here we studied these constants for isoforms 2b and 4b. We measured the ATPase activity of these isoforms expressed in Sf9 cells. For both PMCA4b and 2b, k\(_{\text{act}}\) increased with Ca\(^{2+}\) along a sigmoidal curve. At all Ca\(^{2+}\) concentrations, 2b showed a faster reaction with calmodulin than 4b but a slower off rate. On the basis of the measured rate constants, we simulated mathematically the behavior of these pumps upon repetitive changes in Ca\(^{2+}\) concentration and also tested these simulations experimentally; PMCA was activated by 500 nM Ca\(^{2+}\) and then exposed to 50 nM Ca\(^{2+}\) for 10 to 150 s, and then Ca\(^{2+}\) was increased again to 500 nM. During the second exposure to 500 nM Ca\(^{2+}\), the activity reached steady state faster than during the first exposure at 500 nM Ca\(^{2+}\). This memory effect is longer for PMCA2b than for 4b. In a separate experiment, a calmodulin-binding peptide from myosin light chain kinase, which has no direct interaction with the pump, was added during the second exposure to 500 nM Ca\(^{2+}\). The peptide inhibited the activity of PMCA2b when the exposure to 50 nM Ca\(^{2+}\) was 150 s but had little or no effect when this exposure was only 15 s. This suggests that the memory effect is due to calmodulin remaining bound to the enzyme during the period at low Ca\(^{2+}\). The memory effect observed in PMCA2b and 4b will allow cells expressing either of them to remove Ca\(^{2+}\) more quickly in subsequent spikes after an initial activating spike.

There are only two mechanisms that actively extrude Ca\(^{2+}\) from the cytosol to the extracellular space: the Na\(^{+}/Ca\(^{2+}\) exchanger and the plasma membrane Ca\(^{2+}\) pump (PMCA). From comparison of the sequence of PMCA with the x-ray structure of the homologous sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (1), it is accepted that its basic structure contains 10 transmembrane domains and two large cytosolic loops. The larger cytosolic loop contains the sites for ATP and formation of the phosphorylated intermediate. In addition, after the last transmembrane domain, PMCAc have a cytoplasmic C-terminal region that includes the calmodulin-binding domain. Although there is strong evidence supporting the formation of oligomers of PMCA when this protein has been solubilized with detergents and purified to a high concentration (2), there is no evidence that these oligomers form when the pump is in the plasma membrane.

There are four genes encoding PMCA; the resulting isoforms are accordingly named PMCA 1, 2, 3, and 4. The diversity of PMCA isoforms is greatly enhanced by the existence of two alternative splicing sites, denoted A and C. No functional differences have yet been found for the alternative splices at site A. Splicing site C is located in the middle of the calmodulin-binding domain in the C-terminal region of the molecule. Alternative splicing at this site generates two or more versions of the C-terminal end of PMCA (named a, b, and c) that result in very different calmodulin-binding domains (3).

The response of different isoforms of the plasma membrane Ca\(^{2+}\) pump to Ca\(^{2+}\) and the modification of the response of the pump by binding of calmodulin has been extensively studied by our laboratory (4, 5) and others (6). Most of the studies of this interaction were done in the steady-state condition. However, in living cells, cytosolic Ca\(^{2+}\) varies. Ca\(^{2+}\) spikes, waves, and oscillations have been thoroughly described and are the most ubiquitous signaling systems in cell biology. Our goal is to study how different isoforms of PMCA behave in these conditions.

Early work by Scharff and Foder (7) has shown that calmodulin activation of the red cell Ca\(^{2+}\) pump, which consists mainly of PMCA4b (8), was slow and that the rate of this activation was dependent on the Ca\(^{2+}\) concentration. In a recent paper, we found that different isoforms have different rates for calmodulin activation (9). Because the rate of response to calmodulin also affects the rate of response to increases in Ca\(^{2+}\) concentration (10), the different isoforms differ in their rate of response to Ca\(^{2+}\).

In this paper, we have analyzed how Ca\(^{2+}\) affects the rate of calmodulin activation and the rate of inactivation by calmodulin dissociation of isoforms 2b and 4b. We chose to compare these isoforms for the following reasons: 1) 2b has a higher affinity for calmodulin than 4b and a higher basal activity in the absence of the activator (11). 2) The higher calmodulin affinity of 2b is a consequence of both a faster rate of activation by calmodulin and a slower rate of inactivation by calmodulin dissociation (12). Interestingly, the two isoforms both have

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The abbreviations used are: PMCA, plasma membrane Ca\(^{2+}\) pump; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino-ethane sulfonic acid.
essentially the same splice at sites C and A. Previously we demonstrated that the different kinetics of PMCA2b and 4b are related to differences in the central cytoplasmic loop rather than in the calmodulin-binding domain region itself (12). These differences in the kinetics of calmodulin activation translate into a faster activation by Ca\(^{2+}\) of PMCA2b (10). 4) These isoforms show very different patterns of distribution: 2b is expressed in particular locations like certain regions of the brain (13), but 4b is widespread (14, 15). In the second part of this paper, we propose a model for the response of different PMCA isoforms to Ca\(^{2+}\) concentration, and we test the model experimentally. The results show that these PMCA isoforms change their behavior in response to increases in Ca\(^{2+}\) concentration depending on whether they were exposed to Ca\(^{2+}\) previously.

MATERIALS AND METHODS

Construction of Plasmid—pVL1392-h2b and pSG5-h4b were gifts from Ernesto Carafoli (University of Padova, Padova, Italy). Using BamHI and KpnI sites, h4b was excised from pSG5 and inserted into expression vector pVL-1392. Standard transformation and DNA purification techniques were followed.

Preparation and Amplification of Recombinant Baculovirus—Recombinant baculovirus was prepared using Pharmingen BaculoGold transfection kit. More than 95% of the plaques were PMCA-positive with this system. Briefly, Sf9 (Spodoptera frugiperda) cells in Grace medium without fetal bovine serum were co-transfected with 2 μg of transfer plasmid DNA and 5.0 μl of BaculoGold DNA following the manufacturer’s protocol. Plaques expressing the recombinant protein were isolated by plaque assay and screened for the pump by Western blot. The selected early viral stock was amplified using a multiplicity of infection of 0.1–0.2 following standard procedures, and the titer of the amplified stock was determined (16). The viral stock was kept at 4 °C in the dark.

Expression of PMCA in Sf9 Cells—The expression for protein production was carried out by infecting Sf9 cells in suspension in complete Grace medium with the recombinant virus at a multiplicity of infection of 1. After 48 h of incubation at 27 °C, the cells were harvested. A 500-ml culture gave 800 × 10⁶ cells. The cells were washed with phosphate-buffered saline buffer containing 1 mM EDTA and protease inhibitors, quick frozen, and then kept at −80 °C until microsome processing.

Microsomal Preparation—Crude microsomal membranes from Sf9 cells were prepared essentially as described for COS cells with some minor modifications (17). After washing the cells with phosphate-buffered saline containing increased amounts of protease inhibitors, 15 μg/ml aprotinin, 4 μg/ml leupeptin, and 4 μg/ml leupeptin, the cell pellets were immediately frozen in aliquots of 200 × 10⁶ cells until processing. The volumes of buffers used for homogenizing the relatively large cell pellet were also increased two or three times to maintain the concentrations required to promote formation of inside out vesicles. Per 200 × 10⁶ cells, 6 ml of hypotonic and 6 ml of homogenization buffers were used. Aliquots of the microsomes were stored at −80 °C.

Gel Electrophoresis—SDS gel electrophoresis was performed as described (18). After electrophoresis, the gels were stained with Coomassie Brilliant Blue.

Measurements of ATPase Activity—The ATPase activity was measured by continuously monitoring the absorbance at 360 nm, following the procedure outlined before (9). The medium contained 120 mM KCl, 30 mM Tris-triethanolamine (pH 7.2), 5 mM MgCl₂, 200 μM EGTA, 2.5 mM ATP, 0.2 mM 2-aminomethyc-p-methyl ethanolamine, 1 unit/ml urease, 0.5 μM AMP, and 1 mM diethiothreitol, 0.5 mM ouabain, 4 μg/ml oligomycin, 200 mM thapsigargin, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 117 mM calmodulin, and enough CaCl₂ to obtain the concentration of free Ca\(^{2+}\) indicated in the figure. The calmodulin concentration was chosen to be in the physiological range, to provide maximal activation of the enzyme, and to give a good time resolution for the observed phenomena.

Simulations—Mathematical procedures used to simulate the behavior of PMCA2b and 4b under changing Ca\(^{2+}\) concentrations are described in the “Appendix.”

RESULTS

Characteristics of PMCA Overexpressed in Sf9 Cells—In Fig. 1 we show an SDS gel of membrane preparations of Sf9 cells in which PMCA2b and PMCA4b were overexpressed. The gels were stained by Coomassie Blue. In both cases, a prominent band of approximately 135 kDa is evident, demonstrating the high level of expression obtained in this system. Confirmation that these bands belonged to PMCA was obtained by Western blots, using a 5F10 antibody (not shown).

Fig. 2 shows the dependence of the steady-state ATPase activity of PMCA2b and 4b on Ca\(^{2+}\) concentration in the absence and in the presence of 117 mM calmodulin. Activation of PMCA2b by calmodulin was about 2-fold, whereas activation of 4b was nearly 5-fold. These results are very similar to those obtained with the pump expressed in COS cells, confirming the preservation of both structural and functional properties during expression in Sf9 cells. The advantage of using the baculovirus expression system in Sf9 cells is that high levels of expression are obtained (almost 100 times higher than obtained with COS-cells), allowing better kinetic measurements of PMCA activities.

Dependence of k_{act} and k_{inact} on Ca\(^{2+}\)—Fig. 3 compares the dependence of Ca\(^{2+}\) on k_{act} (the observed time constant for activation by calmodulin) for PMCA2b and 4b. These data were obtained by measuring the time course of the ATPase activity upon addition of 117 mM calmodulin at the Ca\(^{2+}\) concentrations indicated in the figure. At all Ca\(^{2+}\) concentrations, PMCA2b was activated faster than 4b. Also, 2b showed higher apparent affinity for Ca\(^{2+}\) for the increase in k_{act} than 4b. Presumably, the higher affinity for Ca\(^{2+}\) of 2b for this effect reflects the higher affinity of this isoform for the calcium-calmodulin complex, whereas the high cooperativity is a consequence of the titration of the four Ca\(^{2+}\)-binding sites in calmodulin. A Hill equation gave maximum values of k_{act} of 5.04 × 10⁵ ± 0.43 × 10⁴ s⁻¹ M⁻¹ for 2b and 3.2 × 10⁴ ± 0.08 × 10³ s⁻¹ M⁻¹ for 4b. The K_{C} for this effect was 0.64 ± 0.03 μM Ca\(^{2+}\) for 2b and 1.19 ± 0.04 μM for 4b. In both cases the curve was cooperative, with a n of 4.6 for 2b and 3.0 for 4b. As the figure shows, if the value of n for both isoforms was held equal to 4, the fit is negligibly different, so that a value of 4 for n was assumed throughout the rest of this study.

Fig. 4 shows the dependence of k_{inact} (the observed rate constant for inactivation by calmodulin removal) on Ca\(^{2+}\) concentration for PMCA2b and 4b. In this and all other experi-
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PMCA2b and at slightly higher Ca\(^{2+}\) longer at higher Ca\(^{2+}\) ATPase. It is worth noting that Ca\(^{2+}\) binding of Ca\(^{2+}\) added 10 nM myosin light chain kinase peptide RRKWQKT-PUMP39799—

ments involving calmodulin removal, the removal was done by adding 10 \(\mu\)M myosin light chain kinase peptide RRKWQKT-GHAVRAIGRLSS (19). This method was used to measure inactivation constants in our previous study (9). \(k_{\text{inact}}\) decreased with increasing Ca\(^{2+}\) concentration along a curve that denoted positive cooperativity. The value of the parameters for a Hill plot are given in the legend of the figure. The value of \(k_{\text{inact}}\) for 2b was smaller than for 4b at all Ca\(^{2+}\) concentrations. However the \(K_i\) values for the change in \(k_{\text{inact}}\) were similar for both isoforms. This \(K_i\) was very low, which is probably due to tight binding of Ca\(^{2+}\) by calmodulin when calmodulin is bound to the ATPase. It is worth noting that Ca\(^{2+}\) has a profound effect on the dissociation of calmodulin as judged by this experimental approach. At 50 nM Ca\(^{2+}\), the half-time for inactivation after removal of calmodulin from the solution was around 20 s for PMCA4b and 50 s for 2b. But this time becomes substantially longer at higher Ca\(^{2+}\) concentration, being nearly 25 min for 2b and 12 min for 4b at 2 \(\mu\)M Ca\(^{2+}\).

From the ratios of the fits in Figs. 3 and 4 we calculated the apparent \(K_j\) for calmodulin at various Ca\(^{2+}\) concentrations. The calculated \(K_j\) values are plotted in the inset of Fig. 4. For the calculations we used the parameters of the fittings shown in Figs. 3 and 4, assuming that \(n = 4\). The rationale for fixing the Hill coefficient to 4 is that this is the least reliable parameter of the fitting, the active binding species is Ca\(_2\)-calmodulin, and the binding of Ca\(^{2+}\) to calmodulin is highly cooperative. The results show that the \(K_j\) for calmodulin drops almost 7 orders of magnitude when Ca\(^{2+}\) is increased from 50 nM to 1000 nM. The biggest change in \(K_j\) occurs between 50 and 500 nM for both PMCA2b and at slightly higher Ca\(^{2+}\) concentrations for 4b.

"Memory" Effect—The relatively slow \(k_{\text{inact}}\) for calmodulin suggests that if there are two cytosolic Ca\(^{2+}\) spikes separated by a short interval at low Ca\(^{2+}\), when the second Ca\(^{2+}\) spike starts calmodulin would still be partially bound to the pump. Hence, PMCA would react faster to the second spike than to the first one. In this way, PMCA would have a memory of past Ca\(^{2+}\) spikes. The results in Fig. 4 predict that PMCA2b, because of its slower \(k_{\text{inact}}\), would have a longer memory than 4b. We tested this prediction by mimicking Ca\(^{2+}\) spikes. The Ca\(^{2+}\) concentration was set at 50 nM in the presence of added calmodulin, then increased to 500 nM for about 5 min, and then reduced to 50 nM by the addition of EGTA. After varying times at 50 nM Ca\(^{2+}\), the Ca\(^{2+}\) concentration was increased again to 500 nM. The ATPase activity was monitored continuously. A typical experiment is shown in Fig. 5A. In this case, the trace corresponds to PMCA2b. Increasing the Ca\(^{2+}\) concentration from 0 to 50 nM caused a small increase in activity. When the Ca\(^{2+}\) concentration was increased to 500 nM, there was a slow but very evident increase in activity. Upon decreasing Ca\(^{2+}\) concentration to 50 nM for 50 s, the activity decreased slowly; when Ca\(^{2+}\) was increased again to 500 nM the P_i production slowly increased.

Fig. 5B compares the changes in activity of 2b and 4b after decreasing the Ca\(^{2+}\) concentration from 500 to 50 nM. The activity of 4b returned immediately to its basal value, but the activity of 2b decreased more slowly. The decrease in slope for 2b could be fitted to an exponential function with an apparent \(k\) of 0.044 \(\pm\) 0.001 s\(^{-1}\).

Fig. 5C shows the change in ATPase activity of PMCA2b when the Ca\(^{2+}\) concentration is increased (for the second time) from 50 to 500 nM, after being exposed to 50 nM Ca\(^{2+}\) for 12 or 85 s. It is clear that the increase in activity for the enzyme that was exposed for 12 s is immediate, whereas for the enzyme that was exposed 85 s to 50 nM Ca\(^{2+}\) the rate showed a lag similar to that observed during the first increase in Ca\(^{2+}\). To analyze the data, we used the following function.

\[
P(t) = P_o - v_c/k_v + (v_o + v_c)k_v (v_o/k_v) \exp(-kt) \tag{Eq. 1}
\]

Where \(P_o\) is the amount of product at \(t = 0\), \(v_c\) is the activity at 50 nM Ca\(^{2+}\), \(v_o\) is the maximal increase in activity caused by the increase in Ca\(^{2+}\) to 500 nM, and \(k\) is the time constant for the change in activity. The change in the shape of the curve with different times of exposure to low Ca\(^{2+}\) could be attributed to either a change in \(k\) (meaning that calmodulin would rebind quicker after the enzyme was exposed to high Ca\(^{2+}\)) or by a change in the ratio \(v_o/(v_o + v_c)\), where \(v_o\) is the velocity at 50 nM Ca\(^{2+}\) at the end of the second exposure to low Ca\(^{2+}\). This value is higher than \(v_c\) because the enzyme remains activated after being exposed to high Ca\(^{2+}\), and it takes some time at 50 nM Ca\(^{2+}\) for the enzyme to return to its basal state. Based on the \(k_{\text{inact}}\) values from the experiment in Fig. 4 and from the results from the experiment in Fig. 6 (see below), we assume that \(k\) did not change and that the change in the shape of the curves has to be attributed to changes in the ratio \(v_o/(v_o + v_c)\). From a series of experiments like those shown in Fig. 5, we plotted the ratio \(v_o/(v_o + v_c)\) as a function of the time of exposure at 50 nM Ca\(^{2+}\) (after a 5-minm exposure to 500 nM Ca\(^{2+}\)) for both PMCA2b and 4b. The results are shown in Fig. 5D. For both isoforms, the ratio \(v_o/(v_o + v_c)\) decreased exponentially with the exposure time at 50 nM Ca\(^{2+}\). As expected, 4b had a faster time constant than 2b (0.053 versus 0.021). This means that PMCA2b has a longer memory of past Ca\(^{2+}\) spikes than 4b.

To confirm the assumption that the memory effect was due to calmodulin remaining bound to the pump and not to faster rebinding, we designed an experiment using a calmodulin-binding peptide not related to PMCA. The enzyme was exposed to 500 nM Ca\(^{2+}\) for 5 min and then 50 nM Ca\(^{2+}\) for a variable time, and then the Ca\(^{2+}\) concentration was increased again to 500 nM. At this point, 10 \(\mu\)M myosin light chain kinase calmodulin-binding peptide was added to remove all calmodulin.
from the medium to prevent calmodulin rebinding. The effect (or lack of effect) of the peptide on activity will indicate whether the calmodulin that activates the enzyme is rebinding to it or remained bound to the enzyme during the period at low Ca\(^{2+}\) concentration. The results in Fig. 6 show that the myosin light chain kinase calmodulin-binding peptide had little or no effect when added to the enzyme that had been preincubated at low Ca\(^{2+}\) for 15 or 30 s. On the other hand, when the peptide was added to the enzyme that had been incubated 150 s, the activity was inhibited about 70%. This is an indication that the memory...
The ATPase activity was measured as described under Materials and Methods. The experiments show that our assumption that the ratio $v_{\text{act}} / v_{\text{inact}}$ at 500 nM Ca$^{2+}$ is warranted. We have developed a simple model that describes the experimental results in this work. The model is presented below.

\[
\begin{align*}
C + 4 \text{Ca}^{2+} & \rightleftharpoons \text{CCa} \quad \text{(Step A)} \\
P + \text{CCa} & \rightleftharpoons \text{PCCa} \quad \text{(Step B)} \\
\text{PCCa} & \rightleftharpoons \text{PC} + 4 \text{Ca}^{2+} \quad \text{(Step C)} \\
\text{P} & \rightleftharpoons \text{PC} + \text{C} \quad \text{(Step D)}
\end{align*}
\]

C is free calmodulin, CCa is the calmodulin-Ca$_4$ complex, P is free PMCA, PC is PMCA bound to calmodulin (not complexed with calcium), and PCCa is PMCA bound to the calmodulin-calcium complex. Notice that the dissociation of calmodulin (not complexed with 4 Ca$^{2+}$) from the PMCA is assumed to be irreversible, i.e., that calmodulin only binds to the pump when forming a calmodulin-Ca$_4$ complex. A second assumption is that binding and dissociation of Ca$^{2+}$ to and from calmodulin (steps A and C) is much faster than binding and dissociation of calmodulin to and from the pump (steps B and D). Therefore, steps A and C can be treated as being in equilibrium. This assumption is warranted by experimental evidence (20, 21). We did not include in the model the species of calmodulin in which there are less than 4 Ca$^{2+}$ ions bound. These may be assumed to be included in the C and PC forms of the scheme. For simplicity, the model does not take into account the potential role of these intermediate forms of the calcium-calmodulin complex, although there is evidence that they might play a role (22). The differential equations derived from the model are described in the “Appendix.” The ratio $k_3/k_{-3}$ was taken from Linse et al. (23). The rate constants $k_2$ and $k_{-2}$ were made equal to the values of $k_{\text{act}}$ and $k_{\text{inact}}$ at saturating Ca$^{2+}$ concentration, from the experiments in Figs. 3 and 4, respectively. The ratio $k_4/k_{-4}$ was taken from the value of $K_a$ in Fig. 4. The value of $k_4$ was taken from the value of $k_{\text{inact}}$ extrapolated to [Ca$^{2+}$] = 0.

The behavior predicted by the model is shown in Figs. 7–9. In Fig. 7, the model is used to describe the behavior of the pump when Ca$^{2+}$ is increased to 500 nM for the first time. The only species of pump that are present in significant amount (both for PMCA4b and 2b) are P and PCCa. PCCa increases faster for form 2b than for 4b, explaining the faster increase in activity. This is in agreement with our previous experimental results (9).

Fig. 8 shows the prediction of the model when Ca$^{2+}$ is dropped from 500 to 50 nM. In these circumstances, there is a transient accumulation of PC that then decays exponentially. The accumulation of PC is greater in the case of PMCA2b. If we considered that the species PCCA is the only one that displayed high ATPase activity, the simulation predicts that the activity of both PMCA4b and 2b decays very fast, which is in conflict with the experimental results (Fig. 5B). But if it is assumed that in the case of PMCA2b, PC has the same activity as PCCA, then the model prediction is very similar to the experimental results (compare Fig. 5B with Fig. 9, C and D).

In Fig. 9, the results of the simulation of a second increase in Ca$^{2+}$ from 50 to 500 nM for PMCA4b (panels A, C, and E) and for 2b (panels B, D, and F) are shown. In Fig. 9A it is shown that when Ca$^{2+}$ is raised (after only 12 s at 50 nM Ca$^{2+}$), there is an immediate increase in PCCa at the expense of PC. This is because binding of Ca$^{2+}$ to calmodulin is a fast process. This is followed by a much slower binding of Ca$_4$-calmodulin to the pump. A similar chain of events happened with isoform 2b, except more PC was converted into PCCa (Fig. 9B). After 85 s of incubation at 50 nM Ca$^{2+}$, the level of PC was decreased, so PCCA has to be generated mostly from P (Fig. 9, C and D), and the reactivation is much slower. Because the decay of PC into P is slower for PMCA2b than for 4b (Fig. 8), the differences between the 12-s incubation at 50 nM Ca$^{2+}$ and the 85-s incubation are not as dramatic for 2b as for 4b (Fig. 9, compare E and F). In summary, the simulation reproduced accurately the form of the experimental results (compare Fig. 5C and Fig. 9F) and gave rates about two times higher. This latter discrepancy was probably due to uncertainties in the total enzyme concentration.

**DISCUSSION**

The goal of this paper was to study how PMCAs react when subjected to changing cytosolic Ca$^{2+}$ concentrations. In the first part we analyzed how the rates of calmodulin activation and inactivation change with Ca$^{2+}$ concentration, and in the second part we proposed and tested a model on how these rates will affect the behavior of PMCA2b and 4b when subjected to changing Ca$^{2+}$ concentrations in the cytosol. We used baculovirus in insect Sf9 cells to overexpress the two isoforms. This system proved to be advantageous, giving a very high level of expression while preserving both the physical and functional properties of the pump.

$k_{\text{act}}$ for both 2b and 4b increased with Ca$^{2+}$ concentration along an S-shaped curve, denoting positive cooperativity. This is likely the result of the requirement of 4 Ca$^{2+}$ binding to calmodulin for activation of the enzyme. PMCA2b showed a higher apparent affinity for Ca$^{2+}$ of the increase in $k_{\text{act}}$ than 4b ($K_{act}$ was $0.64 \pm 0.03$ nM for 2b and $1.1 \pm 0.04$ nM for 4b), reflecting the higher affinity of 2b for the calcium-calmodulin complex. On the other hand, $k_{\text{inact}}$ decreased greatly with increasing Ca$^{2+}$. The $K_a$ for the decrease in $k_{\text{inact}}$ was very low in both cases: 0.11 nM for 2b and 0.14 nM for 4b. This difference in Ca$^{2+}$ dependence of association and dissociation rate constants for calmodulin was observed by Scharff and Foder (7) in the Ca$^{2+}$ pump from human erythrocytes and is the basis for the
proposed hysteretic properties of the pump. Our results also show that at all of the Ca\(^{2+}\)/H\(_{11001}\) concentrations tested, PMCA2b was activated by calmodulin faster and inactivated by calmodulin removal at a slower rate than 4b. By dividing \(k_{\text{inact}}/k_{\text{act}}\) we calculated the expected \(K_d\) for activation of PMCA2b and 4b. To calculate the dependence of \(K_d\) for calmodulin with Ca\(^{2+}\)/H\(_{11001}\), we assumed a \(n=4\). Binding of Ca\(^{2+}\)/H\(_{11001}\) to calmodulin, both in solution and when calmodulin is bound to its targets, follows a highly cooperative curve (20, 21, 24). Therefore, the assumption of \(n=4\) seems justified.

The maximal values of \(k_{\text{inact}}\) extrapolated to 0 Ca\(^{2+}\)/H\(_{11001}\) (0.016 s\(^{-1}\) for 2b and 0.033 s\(^{-1}\) for 4b) correspond to half-times of 43 and 21 s, respectively, for inactivation by calmodulin removal. We have shown previously that slow \(k_{\text{act}}\) and \(k_{\text{inact}}\) for calmodulin result in slow response to changes in Ca\(^{2+}\)/H\(_{11001}\) concentration (10). From the \(k_{\text{inact}}\) values we can predict what happens when two Ca\(^{2+}\)/H\(_{11001}\) spikes are separated by a period of a few seconds at low Ca\(^{2+}\)/H\(_{11001}\) (as happens during Ca\(^{2+}\)/H\(_{11001}\) oscillations); when the second spike starts, some calmodulin would still be bound to the pump, and the pump will increase its activity faster in response to the high Ca\(^{2+}\)/H\(_{11001}\) concentration. The experiments in Figs. 5 and 6 confirm this prediction. Furthermore, our results show that, as predicted, PMCA2b exhibits memory of the previous Ca\(^{2+}\)/H\(_{11001}\) spike for a longer time than 4b. The rate constant for this memory effect was 0.021 s\(^{-1}\) for 2b and 0.052 s\(^{-1}\) for 4b, which is close to the values for the rate constant for inactivation by calmodulin removal at low Ca\(^{2+}\)/H\(_{11001}\). This suggests, as predicted above, that the memory effect is a consequence of slow calmodulin dissociation from the enzyme.

In this paper we present a simple model that accounts for the experimental results. The model is based on the rapid dissociation of Ca\(^{2+}\)/H\(_{11001}\) from calmodulin but slow dissociation of calmodulin from the pump.

The relative rates of activation and inactivation of the pump by calmodulin not only have consequences upon increases in Ca\(^{2+}\)/H\(_{11001}\) concentration but also upon sudden decreases in Ca\(^{2+}\)/H\(_{11001}\).
Fig. 5B showed that when the Ca²⁺ concentration is reduced from 500 to 50 nM, PMCA4b immediately returned to basal levels of activity. On the other hand, PMCA2b decreased more slowly to its basal activity. The model also was able to reproduce this behavior if it was assumed that calmodulin, bound to the pump but already devoid of Ca²⁺, is still able to activate PMCA2b but not 4b. There are examples where apo-calmodulin binds to enzymes and serves as a subunit for sensing Ca²⁺ (25, 26), but we know of no examples where the bound apo-calmodulin activates the enzyme. Although the data gave rise to this interesting conclusion, the logic used might be misleading, because in the model we are only considering Ca₄-calmodulin and apo-calmodulin, ignoring intermediate species of calcium-calmodulin. Nonetheless, this gives an intriguing hint of notable differences in the action of calmodulin on PMCA, suggesting that further investigations might yield additional novel results. Such unusual characteristics of the PMCA calmodulin-binding domain may be due to its primary structure. As was previously discussed, the PMCA calmodulin-binding domain is related to IQ motifs, except that one of its IQs is reversed to Q1 (4).

The differences between isoforms are correlated with the localization of these isoforms in different cell types. Isoform 4b is primarily located in cells derived from hematopoietic stem cells (erythrocytes and other blood cells), whereas 2b is located in more quickly responding cells such as neurons and cardiac cells. In the latter case, it is easy to understand why a somewhat longer term memory is needed, because of its effect upon long term potentiation and inhibition. In the case of 4b, memory effects are probably less important because the cells involved are less subject to repeated trains of Ca²⁺ spikes.

Earlier work by Tepikin and Petersen (27) pointed out the importance of Ca²⁺ extrusion through the plasma membrane during Ca²⁺ spikes and oscillations. Moreover, a recent study by Brini et al. (28) showed that overexpression of PMCA was more efficient than overexpression of sarco/endoplasmic reticulum Ca²⁺-ATPase in returning cytoplasmic Ca²⁺ to basal levels in Chinese hamster ovary cells. In recent years, there have been several reports indicating that delayed activation of PMCA contributes to the shape of intracellular Ca²⁺ signals (29, 30). Snitsarev and Taylor (31) found that there is an overshoot of cytosolic Ca²⁺ when capacitative Ca²⁺ entry is activated in endothelial cells. This overshoot seems to be mediated by delayed activation of PMCA. In agreement with the results that we show here, the delay in the PMCA response shortens when cytoplasmic Ca²⁺ was previously kept at high levels for longer times. All of these lines of evidence emphasize the physiological relevance of the results presented here.

De Koninck and Schulman (32) showed that calmodulin-kinase II also has time-dependent kinetic properties. Because one of the localizations of calmodulin-kinase II is in post-synaptic densities of hippocampal neurons, it was proposed that the memory properties of this enzyme can relate Ca²⁺ signals to long term potentiation (32). Both the kinase and PMCA, because of their memory properties, can sense the frequency and intensity of the Ca²⁺ signal. PMCA uses this information to determine its role in shaping subsequent signals, whereas the kinase uses it to determine the degree to which it phosphorolysis proteins that are governed by the signals. The mechanism of the memory effect in calmodulin-kinase II seems to be different than in the case of PMCA. In calmodulin-kinase II, the rate of calmodulin dissociation only decreases after autophosphorylation of the enzyme, and it is apparently mediated by oligomeric interaction. In the case of PMCA2b and 4b, even at Ca²⁺ concentrations as low as 50 nM the rate of calmodulin dissociation is relatively slow (although it is much slower at higher Ca²⁺ concentrations), so there is no need to propose additional events, such as oligomeric interaction, to explain the memory effect. In summary, this paper describes...
for the first time the ability of PMCA isoforms to react differently to increases in Ca\(^{2+}\) concentration depending on the previous history of cytosolic Ca\(^{2+}\).

**APPENDIX**

The proposed reaction model yields the following rate equations (based on the law of mass action) for PCCA and free PMCA concentrations.

\[
dt[PCCA] = k_4[P][CCa] + k_2[PC][Ca^{2+}]^4 - (k_2 + k_0)[PCCA] \quad (\text{Eq. A1})
\]
\[
dt[P] = k_2[PC][PCCa] - k_3[P][CCa] \quad (\text{Eq. A2})
\]

The concentration of PMCA bound to calmodulin, [PC], can be expressed in terms of [PCCA] and [P] by using the following mass conservation equation.

\[
[PC] = P_0 - [P] - [PCCA] \quad (\text{Eq. A3})
\]

where \(P_0\) is the initial free PMCA concentration. One can further express [CCa] in terms of [P] and [PCCA] by assuming rapid equilibrium for binding calcium ions to calmodulin, as discussed above. If the corresponding equilibrium constant is \(K = k_1/k_{-1}\), one obtains the following equation.

\[
[CCa] = K[C][Ca^{2+}]^4 \quad (\text{Eq. A4})
\]

Combining this equation with the mass conservation equation for calmodulin, one obtains the following.

\[
[C] = C_0 - [PC] - [CCa] - [PCCA] \quad (\text{Eq. A5})
\]

With Equation A3 one obtains the following.

\[
[CCa] = \frac{K[C_0 + [P] - P_0][Ca^{2+}]^4}{1 + K[Ca^{2+}]^4} \quad (\text{Eq. A6})
\]

Finally, by substituting Equations A3 and A6 into Equations A1 and A2, we obtain the following system of two differential equations.

\[
dt[PCCA] = -(k_2 + k_0 + k_{-2})[Ca^{2+}]^4[PC][PCCA] + k_2[Ca^{2+}]^4[P_0 - [P]] + k_0 Beta(C_0 - P_0)[P] + [P]^4 \quad (\text{Eq. A7})
\]
\[
dt[P] = (k_2 - k_0)[PCCA] + k_0 P_0 - [P] - k_3 Beta(C_0 - P_0)[P] + [P]^4 \quad (\text{Eq. A8})
\]

with

\[
\beta = (1 + 1/(K[Ca^{2+}]^4))^{-1} \quad (\text{Eq. A9})
\]

The initial conditions are \([P](0) = P_0, [PCCA](0) = 0\). The experimental conditions were such that free calcium concentration \([Ca^{2+}]^c\) can be considered constant. We have solved the above equations numerically by using the MATLAB software platform (33) and the following rate constants: \(K = 1.58 	imes 10^{-4} \mu M^{-4}, k_2 = 5 	imes 10^{-5} s^{-1} \mu M^{-1}, k_{-2} = 5 	imes 10^{-4} s^{-1}, k_3 = 1 s^{-1}, k_4 = 10^4 s^{-1} \mu M^{-4}, k_0 = 0.014 s^{-1}\).
The Plasma Membrane Calcium Pump Displays Memory of Past Calcium Spikes:
DIFFERENCES BETWEEN ISOFORMS 2b AND 4b
Ariel J. Caride, Alan R. Penheiter, Adelaida G. Filoteo, Zeljko Bajzer, Ágnes Enyedi and John T. Penniston

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