Determination of the Dissociation Constants for Ca\(^{2+}\) and Calmodulin from the Plasma Membrane Ca\(^{2+}\) Pump by a Lipid Probe That Senses Membrane Domain Changes*

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The purpose of this work was to obtain information about conformational changes of the plasma membrane Ca\(^{2+}\)-pump (PMCA) in the membrane region upon interaction with Ca\(^{2+}\), calmodulin (CaM) and acidic phospholipids. To this end, we have quantified labeling of PMCA with the photoactivatable phosphatidylcholine analog [\(^{125}\)I]TID-PC/16, measuring the shift of conformation E\(_2\) to the auto-inhibited conformation E\(_1\)I and to the activated E\(_1\)A state, titrating the effect of Ca\(^{2+}\) under different conditions. Using a similar approach, we also determined the CaM-PMCA dissociation constant. The results indicate that the PMCA possesses a high affinity site for Ca\(^{2+}\) regardless of the presence or absence of activators. Modulation of pump activity is exerted through the C-terminal domain, which induces an apparent auto-inhibited conformation for Ca\(^{2+}\) transport but does not modify the affinity for Ca\(^{2+}\) at the transmembrane domain. The C-terminal domain is affected by CaM and CaM-like treatments driving the auto-inhibited conformation E\(_1\)I to the activated E\(_1\)A conformation and thus modulating the transport of Ca\(^{2+}\). This is reflected in the different apparent constants for Ca\(^{2+}\) in the absence of CaM (calculated by Ca\(^{2+}\)-ATPase activity) that sharply contrast with the lack of variation of the affinity for the Ca\(^{2+}\) site at equilibrium. This is the first time that equilibrium constants for the dissociation of Ca\(^{2+}\) and CaM ligands from PMCA complexes are measured through the change of transmembrane conformations of the pump. The data further suggest that the transmembrane domain of the PMCA undergoes major rearrangements resulting in altered lipid accessibility upon Ca\(^{2+}\) binding and activation.

Detailed structural information about the plasma membrane calcium pump (PMCA)\(^2\) is currently lacking. This pump is an integral part of the Ca\(^{2+}\) signaling mechanism (1) and is thus a crucial component of cell function. It is highly regulated by calmodulin (CaM), which activates this protein by binding to an auto-inhibitory region (2) and changes the conformation of the pump from an inhibited state to an activated one (2, 3).

Information about the structure and assembly of the transmembrane domain of an integral membrane protein can be obtained from an analysis of the lipid-protein interactions. In this work, we have used a hydrophobic photolabeling method to study the noncovalent interactions between the membrane domain of the PMCA and surrounding phospholipids under different experimental conditions that lead to known conformations. It has been previously demonstrated that both the conformation and the activity of the pump protein are preserved in either solubilized or reconstituted purified preparations compared with the native pump located in the erythrocyte (4).

In recent work, we assessed changes in the overall exposure of PMCA to surrounding lipids by quantifying the extent of protein labeling by the photoactivatable phosphatidylcholine analog [\(^{125}\)I]TID-PC/16 under different conditions (5). This showed that labeling of PMCA incubated with Ca\(^{2+}\) and calmodulin decreases by 25% and incubation with Ca\(^{2+}\) alone increases labeling by more than 50% compared with control labeling of the PMCA in the absence of Ca\(^{2+}\) and CaM. These results suggest that the PMCA assumes two different E\(_1\) conformations: one that is auto-inhibited and in which the membrane domain is in contact with a higher amount of lipids (incubating with Ca\(^{2+}\) alone, E\(_1\)I) and one in which the enzyme is fully active (incubating with Ca\(^{2+}\)-calmodulin, E\(_1\)A) and exhibits a more compact transmembrane arrangement with lesser exposure to lipids. These data provide the first evidence that there is an auto-inhibited conformation in P-type ATPases involving both the cytoplasmic regions and the transmembrane segments.

Activation of PMCA is caused by the binding of CaM to the C-terminal tail of the pump, leading to dissociation of the auto-inhibitory domain from its close proximity to the active site. This removes the self-inhibition of the enzyme and stimulates the PMCA-mediated Ca\(^{2+}\) transport severalfold (6). Previously, the binding of CaM to PMCA was measured indirectly by determining the PMCA activity (7). Recently, Liyanage et al. (8) reported a fluorescence polarization method to measure the binding of CaM modified with Oregon Green 488. This was an efficient way of measuring CaM stimulation of PMCA activity, but it did not provide information about the region of the pump domain that interacts with CaM.
Ligand Affinity of PMCA Measured by a Membrane Domain Probe

We employed the photoactivatable phosphatidylcholine analog 1-palmitoyl-2-[9-125I]iodo-4′-(trifluoromethyldiazirinyl)-benzoylxyxycarbonyl-2-nonaoyl-sn-glycero-3-phosphocholine [125I]TID-PC/16 that has been previously used to analyze lipid-protein interfaces (9–11). This reagent partitions in the phospholipidic milieu and upon photolysis reacts indiscriminately with its molecular environment. It is thus possible to directly analyze the interaction between the hydrophobic membrane-spanning domain of a membrane protein and lipids belonging to its immediate environment (12–14). Applying this technique on the PMCA, we were able to measure equilibrium constants for the dissociation of ligands from PMCA complexes and to draw structural conclusions about the regulation of the transport of Ca²⁺ in the presence of different modulators.

EXPERIMENTAL PROCEDURES

Reagents—All chemicals used in this work were of analytical grade. Calmodulin was obtained from Calbiochem. Recently drawn human blood for the isolation of PMCA was obtained from the Hematology Section of the Hospital de Clínicas General San Martín and from Fundación Fundosal (Argentina). Blood donation in Argentina is voluntary, and therefore the donor must provide informed consent for the donation of blood and the subsequent legitimate use of the blood by the transfusion service.

Purification of PMCA from Human Erythrocytes—PMCA was isolated from CaM-depleted erythrocyte membranes by the CaM-affinity chromatography procedure (4) and stored in the CaM-affinity matrix obtained with Ca₂⁺(1 mM EGTA). The incorporation of [125I]TID-PC/16 was carried out by adding 0.1 ml of HNO₃, 0.9 ml of HClO₄, and enough CaCl₂ to give the desired final free [Ca²⁺]. The reaction was started by the addition of ATP (final concentration 2 mM). Release of P₁ was estimated according to the procedure of Fiske and Subbarow (16).

Labeling Procedure—A dried film of the photoactivatable reagent was suspended in DMPC/C₁₂E₁₀ mixed micelles (80 and 120 μM, respectively) containing 10 μg/ml of the membrane protein, 120 mM KCl, 30 mM MOPS-K (pH 7.4), 3.75 mM MgCl₂, 1 mM EGTA, and enough CaCl₂ to give the desired final free [Ca²⁺]. The samples were incubated for 20 min at 37 °C before being irradiated for 15 min with light from a filtered UV source (λ ≈ 360 nm).

Radioactivity and Protein Determination—Electrophoresis was performed according to the Tris-Tricine SDS-PAGE method (17). Polypeptides were stained with Coomassie Blue R, the isolated bands were excised from the gel, and the incorporation of radioactivity was directly measured on a γ-counter. The amount of protein was quantified by eluting each stained band as previously described (18), including bovine serum albumin in each gel as a standard for protein quantification. Specific incorporation was calculated as the ratio between measured radioactivity and amount of protein determined for each band.

Proteolysis of PMCA—Proteolysis of PMCA was performed for 5 min in the presence of 25 mM Tris-HCl, pH 7.4 at 37 °C, 2 mM EGTA, and 0.22 μg/ml of TLCK-treated chymotrypsin in water. The reaction was stopped by a 10-fold excess of ovomucoid trypsin inhibitor solution at 4 °C.

Data Analysis—All measurements were performed in triplicate to quintuplicate unless specified otherwise in the figures.

RESULTS

Incorporation of [125I]TID-PC/16 as a Conformation Marker—We first wished to determine the extent of [125I]TID-PC/16 labeling of the PMCA in its major known conformational states. The E₂ state is attained by incubating the PMCA in the absence of Ca²⁺ (1 mM EGTA). The incorporation of [125I]TID-PC/16 in this condition was considered as the control and was set as 100% (Fig. 1). The other two conformers are E₁I, which is obtained by incubating the enzyme in the presence of Ca²⁺ and binds the maximum concentration of [125I]TID-PC/16 (180%) at optimal concentration of Ca²⁺, Fig. 1) and E₁A, a conformation attainable in the presence of Ca²⁺ and CaM or with a CaM-like effector such as phosphatidic acid, oleic acid or after removal of the C terminus of PMCA. This conformation binds the least amount of [125I]TID-PC/16 (~75% of the control for E₁A obtained with Ca²⁺ and CaM) (Fig. 1). These data are as
reported earlier (5) and illustrate the sensitivity of the incorporation of labeled [125I]TID-PC/16 on the different PMCA conformations.

Titration of the E2 to E1 Conformer Shift with Ca2+—To determine the [Ca2+] dependence of the conformational shift from the E2 to the E1 state, we performed a titration experiment (Fig. 2A) in which we measured the specific incorporation of [125I]TID-PC/16 to PMCA when the enzyme was incubated in the presence of increasing concentrations of Ca2+. This experiment, we titrate the concentration of [125I]TID-PC/16 that binds to PMCA as it shifts from the E2 to the E1 conformer, i.e. as [125I]TID-PC/16 incorporation increases from 100% to near 180%. As illustrated in Fig. 2A, [125I]TID-PC/16 incorporation increases hyperbolically with low concentrations of Ca2+ (up to ~40 μM) and then decreases to a level that cannot be easily evaluated with the available information. However, the data are well described by Equation 1.

$$[PC_B] = \frac{PC_{max}[Ca^{2+}]}{K_{Ca^{2+}} + [Ca^{2+}]}$$  
(Eq. 1)

![Graph](image1.png)

**Figure 1.** Relative specific incorporation of [125I]TID-PC/16 to PMCA under different conditions. Incorporation in the presence of 1 mM EGTA is taken as 100% control (E, left bar). Middle, 15 μM Ca2+ (E1). Right, 5 μM Ca2+ and 200 nM calmodulin (E A). A similar result for E A was obtained with phosphatidic acid, oleic acid, or removing the PMCA C-terminal domain with chymotrypsin. Data are the mean ± S.E. of 6–9 independent experiments.

This empirically derived equation aims to characterize the effect of Ca2+ on the amount of [125I]TID-PC/16 bound to PMCA ([PC_B]). PC_{max} is the maximal value of [PC_B] attainable if [Ca2+] << K_{Ca^{2+}} (the constant describing the half-maximal concentration of Ca2+ for inhibition of [125I]TID-PC/16 binding to PMCA). The term [Ca2+]K_{Ca^{2+}} was included to account for the inhibitory effect by excess Ca2+ (see Fig. 2) and thus to allow a better estimation of K_{Ca^{2+}} (the concentration of Ca2+ for half-maximal binding of [125I]TID-PC/16 to PMCA) and of the maximal increase of [PC_B], i.e. PC_{max} – PC_{op} (where PC_{op} is the amount of [125I]TID-PC/16 bound in the absence of Ca2+). Fitting the experimental data points to this equation yielded PC_{op} = 99.1 ± 4.8%, PC_{max} = 176.7 ± 4.3%, K_{Ca^{2+}} = 0.52 ± 0.14 μM, and K_{i} = 1212 ± 319 μM.

The inset in Fig. 2A shows the specific incorporation of [125I]TID-PC/16 to PMCA at very low concentrations of Ca2+ to illustrate the rapid increase in binding of [125I]TID-PC/16 at submicromolar [Ca2+]. It is also worth pointing out that after reaching a maximum near 40–50 μM Ca2+, [125I]TID-PC/16 binding decreases slowly with [Ca2+] in a similar way as is observed when measuring the Ca2+-ATPase activity (Fig. 2B). It was reported earlier that excess [Ca2+] in SERCA also induced a conformation different from that attained at lower concentrations of Ca2+ (19).

Fig. 2B shows the Ca2+-ATPase activity as a function of Ca2+. The data were quantitatively evaluated using Equation 2,

$$V = \frac{[Ca^{2+}]V_{max}}{[Ca^{2+}] + K_{Ca^{2+}}} + \frac{[Ca^{2+}]^{2}}{K_{i}}$$  
(Eq. 2)

where V_{max} is the maximum velocity, K_{Ca^{2+}} is the apparent dissociation constant for Ca2+ (Ca2+ concentration at half-maximal ATPase activity), and K_{i} is the concentration of Ca2+ for half-maximal inhibition of the enzyme at high [Ca2+]. Equation 2 is an empirical equation, which describes the effect of Ca2+ on Ca2+-ATPase activity and takes into account the inhibitory effect by excess Ca2+. It is analogous to Equation 1, which describes the effect of Ca2+ on the incorporation of [125I]TID-PC/16 into PMCA. It can be seen from Fig. 2B that the Ca2+-ATPase activity increases hyperbolically with low concentrations of Ca2+ (up to about 50 μM) and then decreases at higher [Ca2+] reflecting excess substrate inhibition. Applying Equation 2 to the data yielded V_{max} = 13.4 ± 1.1 μmol Pi/mg/min, K_{Ca^{2+}} = 11.0 ± 1.7 μM, and K_{i} = 248 ± 65 μM.

The most interesting finding resulting from a comparison of the Ca2+ dependence of [125I]TID-PC/16 incorporation (Fig. 2A) and ATPase activity (Fig. 2B) is that the K_{0.5} for the binding of [125I]TID-PC/16 is much lower (~0.5 μM) than the K_{Ca^{2+}} for Ca2+ activation.

![Graph](image2.png)

**Figure 2.** [Ca2+] dependence of incorporation of [125I]TID-PC/16 to PMCA and of PMCA activity. A, purified PMCA devoid of CaM was incubated in the presence of different amounts of Ca2+, and after 3 min [125I]TID-PC/16 was added as described under “Experimental Procedures.” The inset shows the incorporation of TID-PC at low concentration of Ca2+. B, Ca2+-dependent ATPase activity as a function of [Ca2+]. The inset shows the data at low concentrations of Ca2+.
of the PMCA (11 μM). This strongly suggests that the intrinsic Ca\(^2+\) affinity of the PMCA in equilibrium is very different from its apparent Ca\(^2+\) affinity for activation by Ca\(^2+\).

**Titration of the Ca\(^2+\) Dependence of the E\(_2\) to E\(_{1A}\) Conformer Shift in the Presence of Calmodulin and Calmodulin-like Treatments**—We next wished to compare the Ca\(^2+\) dependence of the E\(_2\) to E\(_{1A}\) shift with that of the shift from E\(_2\) to the activated E\(_{1A}\) conformation of the PMCA. To this end, we performed a series of titrations of \(^{125}\)I-TID-PC/16 incorporation in the presence of various activators of the pump. We chose the well-known “prototypical” PMCA activator CaM, the lipid activators phosphatidic acid (PA) and oleic acid (OA), as well as limited proteolytic treatment using TLCK-chymotrypsin. The latter treatment results in a C-terminally truncated and fully active pump lacking its auto-inhibitory tail (20). These activators thus reflect different mechanisms and pathways of PMCA activation.

**Activation in the Presence of CaM**—Fig. 3A shows the incorporation of \(^{125}\)I-TID-PC/16 as a function of concentration of Ca\(^2+\) in the presence of 1 μM CaM. Initially, incorporation of \(^{125}\)I-TID-PC/16 decreases rapidly with Ca\(^2+\) and then slowly decreases to a constant value. The experimental data are well described by Equation 3,

\[
[PC_B] = [PC_{min}] + \frac{([PC_0] - [PC_{min}])}{1 + \left(\frac{[Ca^{2+}]}{K_{Ca^{2+}}}\right)^n}
\]  
(Eq. 3)

where [PC\(_B\)] is the \(^{125}\)I-TID-PC/16 bound to PMCA at a given concentration of ionic calcium; [PC\(_{min}\)] is the final minimal concentration (%) of \(^{125}\)I-TID-PC/16 bound to PMCA (at non-limiting concentration of Ca\(^2+\)), [PC\(_0\)] is the initial concentration (%) of PC bound to PMCA (at zero [Ca\(^2+\)]), “n” is the Hill coefficient, and \(K_{Ca^{2+}}\) is the concentration of Ca\(^2+\) for half-maximal binding to PMCA. This empirical equation describes the binding of \(^{125}\)I-TID-PC/16 to PMCA as a function of Ca\(^2+\). It can be seen from Fig. 3A that the amount of \(^{125}\)I-TID-PC/16 bound to PMCA decreases in a sigmoidal fashion with Ca\(^2+\)
from 100% (PC$_0$, in the absence of Ca$^{2+}$) to a constant minimal value (PC$_{min}$ at non-limiting concentration of Ca$^{2+}$), which indicates that the PMCA conformation in this latter condition is more compact than that in the absence of Ca$^{2+}$. Evaluating the data with Equation 3 yielded the following experimental parameters: [PC$_{min}$] = 76.7 ± 0.5%, [PC$_0$] = 100.2 ± 0.5%, n = 1.7 ± 0.2, and $K_{Ca^{2+}} = 0.46 ± 0.04$ μM.

The inset in Fig. 3A shows the CaM-stimulated Ca$^{2+}$-ATPase activity as a function of concentration of Ca$^{2+}$. The experimental points were adjusted to a simple hyperbolic function with a $V_{max}$ of 12.8 ± 0.2 μmol Pi mg$^{-1}$ min$^{-1}$, and $K_{Ca^{2+}} = 0.75 ± 0.07$ μM.

In contrast to the large difference between the equilibrium Ca$^{2+}$ affinity and the steady state (apparent) Ca$^{2+}$ affinity for ATPase activation by Ca$^{2+}$ alone (E$_2$-E$_1$I shift), the Ca$^{2+}$ affinity for CaM stimulation of [125]TID-PC/16 incorporation during the E$_2$-E$_1$A conformational shift is much closer to the true equilibrium Ca$^{2+}$ affinity of the pump.

**Activation in the Presence of Phosphatidic Acid**—Fig. 3B shows the incorporation of [125]TID-PC/16 as a function of concentration of Ca$^{2+}$ in the presence of 58 μM PA. Incorporation of [125]TID-PC/16 decreases with [Ca$^{2+}$] in a sigmoidal manner and then reaches a constant minimal value. Applying Equation 3 yielded [PC$_{min}$] = 91.2 ± 0.4%, [PC$_0$] = 99.8 ± 0.5, n = 3.7 ± 0.9, and $K_{Ca^{2+}} = 0.67 ± 0.05$ μM.

Whereas the values for most parameters are similar for the PA- and CaM-stimulated E$_2$-E$_1$A transition, the value of $n$ describing the sigmoidicity of the Ca$^{2+}$ dependence is much higher for PA than for any of the other treatments. Equation 3 is a Hill equation that shows in the denominator term the exponent $n$ for the Ca$^{2+}$ concentration, where $n$ can be higher than one. In fact the value for $n$ is near 2 for the effect of Ca$^{2+}$ on [125]TID-PC/16 binding in the presence of CaM (as well as of OA and for the enzyme that lacks the C-terminal region; see below), indicating that more than one molecule of Ca$^{2+}$ is needed for the E$_2$ to E$_1$A shift. However, in the presence of PA, $n$ is near 4 for the effect of Ca$^{2+}$ on [125]TID-PC/16 binding. This may be caused by a more complex behavior between PA and Ca$^{2+}$: (i) PA could be at a non-optimal concentration and (ii) PA could associate with free Ca$^{2+}$. However, when we measured the concentration of Ca$^{2+}$ with a Ca$^{2+}$-sensitive electrode in the absence and in the presence of 60 μM of PA, we found the value for free [Ca$^{2+}$] to be similar. We also determined the [125]TID-PC/16 binding to PMCA as a function of PA, and the results indicate that the concentration used in the experiments of this work is sufficient to obtain the maximal effect, i.e. to reach PC$_{min}$ (data not shown).

Measuring the PA-stimulated Ca$^{2+}$-ATPase activity as a function of concentration of Ca$^{2+}$ (inset in Fig. 3B) yielded a $V_{max}$ of 11.3 ± 0.2 μmol Pi mg$^{-1}$ min$^{-1}$, and $K_{Ca^{2+}} = 0.83 ± 0.07$ μM. These values are comparable to those determined for the activation of the PMCA by CaM and indicate that the apparent Ca$^{2+}$ affinity for activation by either mechanism approaches the equilibrium Ca$^{2+}$ affinity of the PMCA.

**Activation in the Presence of Oleic Acid**—The incorporation of [125]TID-PC/16 as a function of concentration of Ca$^{2+}$ in the presence of 10 μM OA is shown in Fig. 3C. As in the case of PA, incorporation of [125]TID-PC/16 decreases with Ca$^{2+}$ and then reaches a constant value. Using Equation 3 to evaluate the experimental points resulted in [PC$_{min}$] = 83.5 ± 3.6%, [PC$_0$] = 100.3 ± 2.3%, n = 1.8 ± 1.1, and $K_{Ca^{2+}} = 0.75 ± 0.28$ μM.

The inset in Fig. 3C shows the Ca$^{2+}$-ATPase activity in the presence of 10 μM OA as a function of Ca$^{2+}$. The experimental points were adjusted to a simple hyperbolic function yielding $V_{max}$ of 11.1 ± 0.3 μmol Pi mg$^{-1}$ min$^{-1}$, and $K_{Ca^{2+}} = 0.96 ± 0.14$ μM.

**Removal of the PMCA C-Terminal Domain by Proteolysis with TLCK-chymotrypsin**—As mentioned above, proteolysis of PMCA by chymotrypsin yields a pump that is fully active, lacks the C-terminal region, and hence does not bind CaM. We used TLCK-chymotrypsin to avoid digestion of the N-terminal domain, which remains intact based on the reaction of the truncated PMCA with the specific antibody JA9 (9). Fig. 3D shows the incorporation of [125]TID-PC/16 to chymotrypsin-treated PMCA as a function of [Ca$^{2+}$]. Equation 3 was again fitted to the experimental points, yielding [PC$_{min}$] = 83.6 ± 0.6%, [PC$_0$] = 100.2 ± 1.2%, n = 2.3 ± 0.5, and $K_{Ca^{2+}} = 0.79 ± 0.10$ μM.

The Ca$^{2+}$-ATPase activity as a function of [Ca$^{2+}$] of the C-terminally truncated pump is shown in the inset of Fig. 3D. Adjusting the experimental points to a simple hyperbolic function gave a $V_{max}$ of 13.9 ± 0.4 μmol Pi mg$^{-1}$ min$^{-1}$, and $K_{Ca^{2+}} = 0.53 ± 0.07$ μM.

**Comparison of Apparent Ca$^{2+}$ Affinities for PMCA Measured in Equilibrium and in the Steady State**—Table 1 summarizes the results of the experiments described above and compares the Ca$^{2+}$ affinities obtained through binding of [125]TID-PC/16 and through the determination of Ca$^{2+}$-ATPase activity in similar experimental conditions. From an inspection of this table we can draw the following conclusions: 1) Treatments

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**TABLE 1**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Conformation</th>
<th>Ca$^{2+}$-ATPase activity $K_{Ca^{2+}}$ and $V_{max}$</th>
<th>Specific incorporation of [125]TID-PC/16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$</td>
<td>E$_1$I</td>
<td>11.0 ± 1.7 μmol Pi mg$^{-1}$ min$^{-1}$</td>
<td>[PC$<em>0$]-[PC$</em>{max}$] or [PC$<em>0$]-[PC$</em>{min}$]</td>
</tr>
<tr>
<td>Ca$^{2+}$ + CaM</td>
<td>E$_1$A</td>
<td>0.75 ± 0.07 12.8 ± 0.2 μmol Pi mg$^{-1}$ min$^{-1}$</td>
<td>0.52 ± 0.14 77 ± 4%</td>
</tr>
<tr>
<td>Ca$^{2+}$ + PA</td>
<td>E$_1$A</td>
<td>0.83 ± 0.07 11.3 ± 0.2 μmol Pi mg$^{-1}$ min$^{-1}$</td>
<td>0.46 ± 0.04 24 ± 1</td>
</tr>
<tr>
<td>Ca$^{2+}$ + OA</td>
<td>E$_1$A</td>
<td>0.96 ± 0.14 11.1 ± 0.3 μmol Pi mg$^{-1}$ min$^{-1}$</td>
<td>0.67 ± 0.05 9 ± 1</td>
</tr>
<tr>
<td>Ca$^{2+}$ + Chym</td>
<td>E$_1$A</td>
<td>0.53 ± 0.07 13.9 ± 0.4 μmol Pi mg$^{-1}$ min$^{-1}$</td>
<td>0.75 ± 0.28 17 ± 1</td>
</tr>
<tr>
<td>OA</td>
<td>E$_1$I</td>
<td>0.53 ± 0.07 13.9 ± 0.4 μmol Pi mg$^{-1}$ min$^{-1}$</td>
<td>0.79 ± 0.10 17 ± 1</td>
</tr>
</tbody>
</table>

* [125]TID-PC/16 bound to PMCA increases or decreases with [Ca$^{2+}$] from 100% (PC$_0$) to a new maximum or minimum value (PC$_{max}$ or PC$_{min}$) bound at non-limiting concentration of Ca$^{2+}$.

* The negative value reflects the fact that for Ca$^{2+}$ alone, PC$_{max}$ is higher than PC$_0$.

As mentioned above, proteolysis of PMCA by chymotrypsin yields a pump that is fully active, lacks the C-terminal region, and hence does not bind CaM. We used TLCK-chymotrypsin to avoid digestion of the N-terminal domain, which remains intact based on the reaction of the truncated PMCA with the specific antibody JA9 (9). Fig. 3D shows the incorporation of [125]TID-PC/16 to chymotrypsin-treated PMCA as a function of [Ca$^{2+}$]. Equation 3 was again fitted to the experimental points, yielding [PC$_{min}$] = 83.6 ± 0.6%, [PC$_0$] = 100.2 ± 1.2%, n = 2.3 ± 0.5, and $K_{Ca^{2+}} = 0.79 ± 0.10$ μM.

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that alter the equilibrium between E₁ and E₂ are recognized by a change in the incorporation of the probe. 2) Ca²⁺ affinities correlate well between the two methods employed when CaM or CaM-like treatments modified the PMCA. However, 3) The affinity for Ca²⁺ alone is similar to that in the presence of CaM or CaM-like treatments only when this parameter is evaluated according to [¹²⁵I]TID-PC/16 incorporation on PMCA undergoing the shift between E₂ to E₁A or E₂ to E₁I. Taken together, these results indicate that the affinity for Ca²⁺ of the PMCA is not regulated at the Ca²⁺ site in the membrane but rather through the auto-inhibitory cytoplasmic domain.

**Titration of the E,I to E,A Conformer Shift with Calmodulin—** Activation of the PMCA is due to the binding of CaM, which dissociates the auto-inhibitory domain, removes the self-inhibition by the enzyme and thus stimulates PMCA transport severalfold (6). One way to measure the binding of CaM to the PMCA has been to follow pump activity as a function of the CaM concentration (7). We measured the binding of CaM to PMCA by quantifying the amount of bound [¹²⁵I]TID-PC/16 to PMCA as a function of [CaM] in the presence of a saturating concentration of Ca²⁺. Fig. 4 shows the CaM-dependent incorporation of [¹²⁵I]TID-PC/16 in the presence of 100 μM Ca²⁺. Equation 3 was fitted to the experimental points, but now the variable was the concentration of CaM instead of the concentration of Ca²⁺. Accordingly, \( K_{Ca^{2+}} \) of Equation 3 is replaced by \( K_{D(CaM)} \) and \( [PC]_0 \) reflects the initial amount of [¹²⁵I]TID-PC/16 bound in the absence of CaM rather than in the absence of Ca²⁺. This resulted in the following values: \( [PC]_{min} = 71.9 \pm 1.8\% \), \( [PC]_0 = 154.8 \pm 2.6\% \), n = 1.9 ± 0.2, and \( K_{D(CaM)} = 9.6 \pm 0.8 \) nM.

The **inset** shows the Ca²⁺-ATPase activity as a function of [CaM]. The experimental points were fitted to a hyperbolic function plus a term \( V_o \) that indicates the ATPase activity in the absence of CaM, yielding \( V_o = 4.9 \pm 0.5 \) μmol Pi mg⁻¹ min⁻¹, \( V_{max} = 12.5 \pm 0.6 \) μmol Pi mg⁻¹ min⁻¹, and \( K_{D(CaM)} = 7.2 \pm 1.4 \) nM. These results show that the values for the dissociation constant of CaM calculated from the change in [¹²⁵I]TID-PC/16 incorporation and from enzyme activation are very similar, validating the use of [¹²⁵I]TID-PC/16 incorporation to follow the conformational shift from E₁I to E₁A.

**DISCUSSION**

[¹²⁵I]TID-PC/16 has previously been used to identify and characterize regions within membrane proteins that interact with lipids (11, 14). Its physicochemical behavior in terms of mobility in thin layer chromatography is indistinguishable from PC, and its interaction with the transmembrane region of integral membrane proteins also appears to be identical to that of PC (12, 13). PC is generally chosen as the reference lipid against which relative lipid association constants of integral membrane proteins are compared, because these proteins show no selectivity for this phospholipid (21). This fact simplifies the interpretation of our results, as it allows a direct correlation between level of reagent incorporation and amount of protein surface exposed to surrounding lipids.

By using [¹²⁵I]TID-PC/16 as a sensitive probe of the hydrophobic protein environment, we were able to study the transmembrane region of the PMCA in different conformations. Quantification of the amount of labeling by [¹²⁵I]TID-PC/16 allowed us to calculate the apparent affinity of the PMCA for Ca²⁺ and CaM as the pump shifts between different conformers in equilibrium with surrounding lipids.

Activation of the PMCA by CaM is commonly explained by the binding of CaM to the CaM binding domain in the C-terminal tail of the pump, followed by release of the inhibitory interactions from the cytosolic core. It should be noted that this hypothesis for the mechanism of auto-inhibition and activation does not predict changes in the transmembrane region. However, in a previous report, we demonstrated that the auto-inhibited conformation is distinct in its membrane domain, and that the conformational changes induced by auto-inhibition do expose additional hydrophobic surfaces of the protein to phospholipids. The auto-inhibited conformation was also obtained by adding the CaM-binding peptide C28 to an E₁CaCaM state of the PMCA (corresponding to the activated pump), showing the reversibility of this conformational transition (5). On the basis of these observations we postulated that the PMCA possesses two different E₁-Ca²⁺ conformations: one that is auto-inhibited and is in contact with a higher amount of lipids (incubating with Ca²⁺ alone, E₁I) and one in which the enzyme is fully active (incubating with Ca²⁺-calmodulin, E₁A) and that exhibits a more compact transmembrane arrangement with a smaller surface area exposed to lipids.

According to kinetic enzyme activity measurements the activated conformer E₁A can be obtained in the presence of CaM, acidic phospholipids, unsaturated fatty acids, protein oligomerization, or after removing the C-terminal tail by controlled proteolysis, e.g. with chymotrypsin (2, 4, 6, 22). In this report, we used several of these activating modes to compare the apparent affinity of the PMCA for Ca²⁺ as determined in two entirely different ways: (a) by evaluating the \( K_{0.5(Ca^{2+})} \) for activation of the Ca²⁺-ATPase activity, i.e. in steady-state conditions of the enzyme, and (b) by measuring the equilibrium constants that drive the conformer E₂ to E₁A as reflected in a change in
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[125I]TID-PC/16 incorporation. The results clearly show that the PMCA possesses a high-affinity site for Ca2+ ($K_D \sim 0.5 \mu M$) regardless of the presence or absence of activators. Therefore, modulation of the pump activity is exerted through the C-terminal domain, which induces an auto-inhibited conformation but does not modify the affinity for Ca2+ at the site where it binds for transport into the transmembrane domain. This finding is analogous to an earlier study on the cardiac muscle SERCA (SERCA2a), which showed that the equilibrium Ca2+ binding affinity of the pump was unaffected by the inhibitory protein phospholamban (23). Similar to CaM activation of the PMCA, phospholamban phosphorylation stimulates SERCA activity by releasing the inhibitory interaction, which is reflected in an increase in the apparent Ca2+ affinity of the pump.

Ca2+-ATPase activation yields an “apparent” affinity for Ca2+, which is not the same as the actual binding affinity under equilibrium conditions. Our approach measures the real substrate affinity for Ca2+ in different conditions through the binding of [125I]TID-PC/16, which is directly proportional to the transmembrane surface of PMCA exposed to surrounding lipids. These two affinities should not be compared directly because they are determined in different states of the system. The experiments of this paper were designed to show that the true affinity of PMCA for Ca2+ is high, a fact that is masked by the steady-state condition, which shows a low affinity site for Ca2+.

Our findings raise interesting questions concerning the structural features of the transmembrane region of the PMCA in its auto-inhibited versus activated $E_1$-Ca conformation. In the SERCA pump, for which atomic-resolution structural information is available for several conformers in both the $E_1$ and $E_2$ state (24–28), calculations of the membrane-embedded accessible surface area revealed only relatively modest differences between different Ca2+-bound $E_1$-conformers (5). Major rearrangements involving the membrane-spanning region are, however, observed upon the $E_1$ to $E_2$ (or $E_2$-E1) transition of the enzyme cycle. Because of the gross architectural similarity of the PMCA to the SERCA, it is assumed that comparable structural rearrangements accompany the reaction cycle in the PMCA, including large domain movements of the A (actuator) and N (nucleotide-binding) domains with respect to each other and the P (phosphorylation) cytosolic domain. Our data based on the changes in [125I]TID-PC/16 lipid binding to the PMCA clearly suggest that a major difference exists in the membrane domain of the pump in the auto-inhibited $E_1$-Ca conformation ($E_1$I) compared with the activated $E_1$-Ca ($E_1$A) conformation where the auto-inhibitory tail is dissociated from the core of the enzyme. The most salient difference between the SERCA and PMCA pumps is the presence of the extended C-terminal tail in the PMCA that encompasses the auto-inhibitory region. How could the presence of this tail, which is thought to be cytosolic, have such a dramatic effect on the membrane portion of the PMCA? Cross-linking studies have shown that in the auto-inhibited state, the tail is in close proximity to sequences in both the A- and N-domain (29, 30) and FRET studies indicate that the N and C termini of the pump are separated by less than 50 Å in the inhibited state (3). Therefore, the definition of the characteristics of the conformers $E_1$A is purely operational and involves a series of structurally different conformers with a similar apparent high affinity for Ca2+.

Using a similar approach as for the determination of Ca2+ affinities we followed the transition from $E_1$I to $E_1$A by titrating this conformational shift with CaM. This allowed us to calculate the dissociation constant for binding of native non-labeled CaM to PMCA in equilibrium. The resulting value of $9.6 \pm 0.8 \text{ nm}$ is similar to the $K_D$ for CaM obtained in steady state conditions by evaluating the half-maximal activation of Ca2+-ATPase ($7.2 \pm 1.4 \text{ nm}$ measured in this study (4), $11.6 \pm 2.4 \text{ nm}$, reported by Penheiter et al. (31)). This indicates that the apparent affinity for CaM is indistinguishable in steady state and in equilibrium experiments. Recently, Liyanage et al. (8), using a fluorescence polarization assay to evaluate the binding of a modified CaM to the PMCA, determined a $K_D$ of 5.8 $\pm 0.5 \text{ nm}$, a value comparable to the one obtained in this work. However, it is possible that the small difference in these $K_D$ values is attributable to different sources of CaM or to the Oregon Green 488 modified CaM used in their method. Regardless, our approach allows an independent calculation of the dissociation constant of non-labeled CaM from the PMCA.

The data presented here represent the first example where equilibrium constants for the dissociation of ligands from PMCA complexes are measured through the change of transmembrane conformations of the pump. The method is sensitive, precise, and requires a very low amount of protein. This should enable its application to other membrane proteins that

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cycle through different conformations, such as all members of the P-type ATPase family. Experiments are currently underway using this methodology to evaluate the affinity constants of PMCA for ATP under different experimental conditions.

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REFERENCES