Ca\(^{2+}\) Binding to Site I of the Cardiac Ca\(^{2+}\) Pump Is Sufficient to Dissociate Phospholamban*

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Phospholamban (PLB) inhibits the activity of SERCA2a, the Ca\(^{2+}\)-ATPase in cardiac sarcoplasmic reticulum, by decreasing the apparent affinity of the enzyme for Ca\(^{2+}\). Recent cross-linking studies have suggested that PLB binding and Ca\(^{2+}\) binding to SERCA2a are mutually exclusive. PLB binds to the E2 conformation of the Ca\(^{2+}\)-ATPase, preventing formation of E1, the conformation that binds two Ca\(^{2+}\) (at sites I and II) with high affinity and is required for ATP hydrolysis. Here we determined whether Ca\(^{2+}\) binding to site I, site II, or both sites is sufficient to dissociate PLB from the Ca\(^{2+}\) pump. Seven SERCA2a mutants with amino acid substitutions at Ca\(^{2+}\)-binding site I (E770Q, T798A, and E907Q), site II (E309Q and N795A), or both sites (D799N and E309Q/E770Q) were made, and the effects of Ca\(^{2+}\) on N30C-PLB cross-linking to Lys\(^{328}\) of SERCA2a were measured. In agreement with earlier reports with the skeletal muscle Ca\(^{2+}\)-ATPase, none of the SERCA2a mutants (except E907Q) hydrolyzed ATP in the presence of Ca\(^{2+}\); however, all were phosphorylatable by P\(_{i}\) to form E2P. Ca\(^{2+}\) inhibition of E2P formation was observed only in SERCA2a mutants retaining site I. In cross-linking assays, strong cross-linking between N30C-PLB and each Ca\(^{2+}\)-ATPase mutant was observed in the absence of Ca\(^{2+}\). Importantly, however, micromolar Ca\(^{2+}\) inhibited PLB cross-linking only to mutants retaining a functional Ca\(^{2+}\)-binding site I. The dynamic equilibrium between Ca\(^{2+}\) pumps and N30C-PLB was retained by all mutants, demonstrating normal regulation of cross-linking by ATP, thapsigargin, and anti-PLB antibody. From these results we conclude that site I is the key Ca\(^{2+}\)-binding site regulating the physical association between PLB and SERCA2a.

PLB\(^{2}\) is a 52-amino acid, homopentameric protein that regulates the activity of the Ca\(^{2+}\)-ATPase in cardiac sarcoplasmic reticulum (SERCA2a isoform) (1). D Dephosphorylated PLB inhibits SERCA2a by decreasing its apparent affinity for Ca\(^{2+}\) ions. Phosphorylation of PLB at Ser\(^{16}\) by cAMP-dependent protein kinase A or at Thr\(^{17}\) by calmodulin kinase II reverses Ca\(^{2+}\)-ATPase inhibition, allowing greater Ca\(^{2+}\) transport at low ionized Ca\(^{2+}\) concentrations (1), with large effects on cardiac contractile kinetics (2). In the membrane there is a dynamic equilibrium between PLB pentamers, PLB monomers, and PLB/SERCA2a heterodimers, the monomer being the active form responsible for enzyme inhibition (3, 4). Because of its prominent effect on cardiac muscle physiology, the molecular mechanism by which PLB lowers the apparent Ca\(^{2+}\) affinity of SERCA2a continues to be an important area of investigation (5).

SERCA pumps two Ca\(^{2+}\) ions into the SR (ER) lumen per ATP molecule hydrolyzed through a multistep kinetic cycle (see Fig. 1) (6–8). Recent advances in protein crystallization (6–8) coupled with earlier mutational analyses (9–13) have clearly delineated the two high affinity Ca\(^{2+}\)-binding sites, I and II, of SERCA1a, the well studied skeletal muscle isoform of SERCA. The two Ca\(^{2+}\)-binding sites of E1, the high Ca\(^{2+}\) affinity conformation, are located side by side near the cytoplasmic membrane surface and are formed by several residues located within transmembrane spans M4, M5, M6, and M8 of SERCA1a (see Fig. 2, parentheses). Ca\(^{2+}\) binding to the high affinity binding sites of E1 appears to be sequential (14), occurring first at site I (E1-Ca\(^{2+}\)) followed by a conformational change leading to cooperative binding at site II (E1-Ca\(^{2+}\)). Site I is formed by side chain oxygens contributed by Glu\(^{771}\) (M5), Thr\(^{799}\) (M6), and Glu\(^{600}\) (M6), with less contribution from Glu\(^{908}\) (M8) of SERCA1a. Side chain oxygens from Glu\(^{109}\) (M4), Asn\(^{796}\) (M6), and Glu\(^{400}\) (M6) along with several main chain carbonyls contribute to site II (8). Binding of both Ca\(^{2+}\) ions is essential for initiation of the catalytic cycle, allowing for phosphorylation of Asp\(^{351}\) by ATP to form E1P at low micromolar Ca\(^{2+}\) concentration. Subsequent translocation of Ca\(^{2+}\) across the SR/ER membrane occurs when the enzyme converts from E1P to E2P, the low Ca\(^{2+}\) affinity conformation (see Fig. 1).

Ca\(^{2+}\)-binding sites I and II have been selectively disrupted by point mutations targeting the amino acid residues above (9–13, 15, 16). Specifically, the site I substitutions (E771Q and T779Q) totally abolished Ca\(^{2+}\) binding to the skeletal muscle Ca\(^{2+}\) pump, whereas site II substitutions (E309Q and N796A) disrupted Ca\(^{2+}\) binding at site II selectively (10–12), resulting in 50% Ca\(^{2+}\) binding (13, 15, 16). Mutation of Asp\(^{351}\), which contributes to both sites (11), also abolished Ca\(^{2+}\) binding (13). From these results it was concluded that Ca\(^{2+}\) binding at site I is required to initiate cooperative binding at site II (8, 16). Neither mutants lacking site I nor site II could hydrolyze ATP and...
transport Ca\(^{2+}\) at low ionized Ca\(^{2+}\) concentration (9–13). However, all mutants were readily phosphorylated by P\(_i\) in the absence of Ca\(^{2+}\) by the technique of “back door” phosphorylation (10, 11). When back door phosphorylation of E2 by P\(_i\) was measured, it was found that retention of site I was sufficient to maintain Ca\(^{2+}\) inhibition of E2P formation (10, 11, 13), consistent with binding of the first Ca\(^{2+}\) being sufficient to deplete the E2 form reacting with P\(_i\) (12) (Fig. 1). Although the amino acid residues forming the Ca\(^{2+}\)-binding sites of SERCA1a have been extensively studied, residues contributing to the Ca\(^{2+}\)-binding sites of SERCA2a, the cardiac muscle isoform, have not been directly investigated. Nonetheless, the amino acid sequences around M4, M5, M6, and M8 of SERCA1a and SERCA2a are highly conserved (3, 17), and it is likely that the same amino acids (depicted in Fig. 2) contribute comparably to formation of the Ca\(^{2+}\)-binding sites in SERCA1a and SERCA2a.

An earlier study by Cantilina et al. (18) suggested that PLB lowers the apparent Ca\(^{2+}\) affinity of SERCA2a by affecting the kinetics of enzyme activation by bound Ca\(^{2+}\) rather than by changing the actual Ca\(^{2+}\) binding affinity of the ATPase. In this model PLB was proposed to impede the slow isomeric transition after binding of the first Ca\(^{2+}\), which triggers cooperative binding of the second Ca\(^{2+}\) and catalytic activation (Fig. 1, single asterisk) (18). Inherently to this model of PLB inhibition is simultaneous binding of Ca\(^{2+}\) and PLB to the Ca\(^{2+}\)-ATPase. However, in more recent studies in which physical interactions between PLB and SERCA2a were assessed directly by chemical cross-linking, it was found that PLB binding and Ca\(^{2+}\) binding to the cardiac Ca\(^{2+}\) pump are mutually exclusive (19–20). Based on these cross-linking results, it was proposed that PLB acts by binding to E2 and preventing the conformational transition to E1 (19–20) (Fig. 1, double asterisk). Consistent with this, Ca\(^{2+}\) inhibited PLB cross-linking to SERCA2a at both cytoplasmic and transmembrane sites distributed throughout both proteins over the same concentration range as Ca\(^{2+}\) activation of Ca\(^{2+}\)-ATPase activity, strongly suggesting that PLB dissociation from E2 occurs before Ca\(^{2+}\) binding to E1 (21–23). Thus, cross-linking results suggest that competitive binding of PLB and Ca\(^{2+}\) to the Ca\(^{2+}\)-ATPase underlies the apparent decrease in Ca\(^{2+}\) affinity of SERCA2a that occurs during active ATP hydrolysis and Ca\(^{2+}\) transport (19–23).

Here we addressed the role(s) of the two high affinity Ca\(^{2+}\)-binding sites of SERCA2a in regulating the physical interaction with PLB. Amino acid substitutions at site I, site II, or both sites were made, and the effects of the mutations on PLB binding to the Ca\(^{2+}\) pump were analyzed. Our results show that site I of SERCA2a is the key Ca\(^{2+}\)-binding site regulating the physical association with PLB.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Baculovirus Production**—Mutation of canine SERCA2a and PLB cDNAs was conducted as recently described (22–23). N30C-PLB was made on the Cys-less PLB background, which is fully functional, canine PLB with Cys residues 36, 41, and 46 changed to Ala. Point mutations at Ca\(^{2+}\) binding residues of SERCA2a were made directly in the transfection vector pVL1393 using the QuickChange\textsuperscript{\textregistered} XL-Gold system (Stratagene). All mutated cDNAs were confirmed by DNA sequencing of the plasmid vectors. Baculoviruses encoding mutated proteins were generated as previously described with BaculoGold\textsuperscript{\textregistered} (Pharmingen)-linearized baculovirus DNA.

**Protein Expression**—SERCA2a proteins and N30C-PLB were expressed in SF21 insect cells by baculovirus infection (19–23). Microsomes were harvested 60 h after infection and stored frozen in small aliquots at −40 °C at a protein concentration of 6–10 mg/ml in 0.25 M sucrose, 10 mM MOPS (pH 7.0). Protein assay was by the Lowry method. Expression of SERCA2a and PLB was quantified with the monoclonal antibodies 2A7-A1 and 2D12, respectively (3).

**Phosphorylation with \(^{32}\)P**—Phosphorylation of SERCA2a with \(^{32}\)P (PerkinElmer Life Sciences) to form E2P was performed as previously described (22). 11 μg of microsomal proteins expressing SERCA2a alone or co-expressing SERCA2a and N30C-PLB were added to 12 μl of buffer consisting of 40 mM MOPS (pH 7.0), 20 mM MgCl\(_2\), 25% Me\(_2\)SO, 1.0 mM EGTA, and 0.25 mm radioactive P\(_i\) to promote E2P formation. Ca\(^{2+}\) was included to yield the final concentrations indicated. After a 10-min incubation at room temperature to allow E2P formation, reactions were stopped by adding 7.5 μl of sample loading buffer, which contained 200 mM glycine (pH 2.4), 20% glycerol, 3% lithium dodecyl sulfate, 100 mM dithiothreitol, and a trace of malachite green as the tracking dye. Samples were then subjected to a gel electrophoresis in 7% polyacrylamide under acidic conditions to retain E2P and transferred to a nitrocellulose membrane. Radioactive protein bands were detected by autoradiography followed by quantification with Bio-Rad Personal FX phosphorimaging.

**Cross-linking**—Cross-linking of residue N30C of PLB to Lys\(^{328}\) of SERCA2a was conducted using the heterobifunctional thiol to amine cross-linking reagent, KMUS (Pierce), as described previously (20). Reactions were conducted with 11 μg of microsomal protein in 12 μl of 40 mM MOPS (pH 7.0), 3.2 mM MgCl\(_2\), 75 mM KCl, and 3 mM ATP supplemented with Ca\(^{2+}\) / EGTA buffer. The final concentration of EGTA was fixed at 1 mM, and ionized Ca concentrations were set by varying CaCl\(_2\) from 0 to 1 mM. Higher ionized Ca\(^{2+}\) concentrations were achieved by omitting EGTA. Cross-linking reactions were conducted at room temperature for 10 min in 0.1 mM KMUS and terminated by adding 7.5 μl of SDS sample buffer containing 100 mM dithiothreitol. Samples were then subjected to SDS-PAGE followed by immunoblotting with anti-PLB monoclonal antibody, 2D12, to detect PLB cross-linked to SERCA2a. 125I-Labeled protein A was routinely used for antibody visualization.
followed by phosphorimaging quantification. In some experiments ATP was omitted or thapsigargin or 5.5 μg of affinity purified 2D12 was included in the cross-linking buffer (see Fig. 7). In this case blots were probed with 125I-labeled 2D12 to avoid interference from 2D12 carried over in the cross-linking buffer (23). Data analysis was done with Origin (Microcal).

**Ca**<sup>2+</sup>-ATPase Assay—**Ca**<sup>2+</sup>-dependent ATPase activities of insect cell microsomes co-expressing PLB and SERCA2a were measured using an enzyme-coupled spectrophotometric assay (23, 24). The rate of NADH decay was measured at 340 nm in a SPECTRAmax PLUS (Molecular Devices) microplate spectrophotometer at 37 °C with 5 μg of membrane proteins in buffer containing 50 mM MOPS (pH 7.0), 3 mM MgCl<sub>2</sub>, 100 mM KCl, 5 mM NaN<sub>3</sub>, 3 μM Ca<sup>2+</sup> ionophore, A23187, 3 mM ATP, and Ca/EGTA as described above. Some membranes were pre-treated with 2D12 to reverse PLB inhibition as described previously (3). All ATPase activities reported are Ca<sup>2+</sup>-dependent.

**RESULTS**

Characterization of **Ca**<sup>2+</sup>-binding Site Mutants—The amino acid sequences surrounding the two Ca<sup>2+</sup>-binding sites (I and II) of SERCA2a and SERCA1a are identical at M4, M5, M6, and M8 (3, 17). However, the putative Ca<sup>2+</sup> binding residues occurring after Glu<sup>309</sup> in SERCA2a are numbered one less than in SERCA1a (Fig. 2) due to an amino acid deletion that occurs at residue 510 in SERCA2a (3, 17). To assess the roles of sites I and II of SERCA2a in regulating protein-binding interactions with PLB, we targeted the same residues previously characterized in SERCA1a (10–13). Specifically, we made the mutations E770Q and T798A at site I and E309Q and N795A at site II. Mutations at residue 510 in SERCA2a (3, 17) were expressed alone (open squares) or co-expressed with N30C-PLB (circles) in Sf21 cells, and membranes were isolated. Ca<sup>2+</sup>-ATPase assays were then conducted in the presence (filled circles) and absence (open circles and squares) of 2D12 as described under “Experimental Procedures.”

(Fig. 3), giving a K<sub>Ca</sub> value for ATP hydrolysis of 0.38 μM (B) compared with 0.15 μM obtained with wild-type SERCA2a (A) (Table 1). This lower apparent Ca<sup>2+</sup> affinity for E907Q has been reported previously for the skeletal muscle Ca<sup>2+</sup> pump (E908Q in SERCA1a) (13, 25). Interestingly, E907Q remained regulatable by PLB, as demonstrated by the 2-fold higher K<sub>Ca</sub> value obtained for activation of ATP hydrolysis, when this SERCA2a mutant was co-expressed with N30C-PLB (Fig. 3B) (Table 1). Moreover, the anti-PLB antibody, 2D12, which reverses PLB inhibition of SERCA2a (18), worked equally well with E907Q (B) as with wild-type SERCA2a (A).

To confirm the functionality of the Ca<sup>2+</sup>-binding sites in the remaining mutants incapable of hydrolyzing ATP, we measured Ca<sup>2+</sup> inhibition of back door phosphorylation supported by P<sub>i</sub> (E2P formation). Control experiments confirmed that the low level of endogenous SERCA in insect cells gave virtually no contribution to E2P formation (data not shown). As expected, all of the SERCA2a mutants were readily phosphorylated by P<sub>i</sub>.
**Ca\(^{2+}\) Binding Sites and PLB**

**TABLE 1**

| Mutant | Wild type | E309Q | N795A | E770Q | T798A | E907Q | D799N | E309Q/E770Q |
|--------|-----------|-------|-------|-------|-------|-------|-------|------------|
| \(K_i\) values (µM) for Ca\(^{2+}\) activation of Ca\(^{2+}\)-ATPase activity | 0.15 ± 0.01 | 0.30 ± 0.04 | ND | ND | ND | 0.38 ± 0.01 | 0.70 ± 0.06 | ND |
| SERCA2a | 0.15 ± 0.01 | 0.30 ± 0.04 | ND | ND | ND | 0.38 ± 0.01 | 0.70 ± 0.06 | ND |
| SERCA2a + N30C-PLB | 2.06 ± 0.26 | 2.25 ± 0.24 | 2.5 ± 0.75 | 1.93 ± 0.26 | >10,000 | 26.7 ± 12.4 | 26.5 ± 9.3 | 12.4 ± 6.6 |
| \(K_i\) values (µM) for Ca\(^{2+}\) inhibition of E2P formation | 0.01 | 0.30 | 0.01 | 10,000 | 0.04 | 2.06 | 0.26 | 12.4 |
| SERCA2a | 0.01 | 0.30 | 0.01 | 10,000 | 0.04 | 2.06 | 0.26 | 12.4 |
| SERCA2a + N30C-PLB | 0.01 | 0.30 | 0.01 | 10,000 | 0.04 | 2.06 | 0.26 | 12.4 |
| Cross-linking | 0.15 | 0.30 | ND | ND | ND | 60.6 | 60.6 | 60.6 |
| SERCA2a | 0.15 | 0.30 | ND | ND | ND | 60.6 | 60.6 | 60.6 |
| SERCA2a + N30C-PLB | 0.15 | 0.30 | ND | ND | ND | 60.6 | 60.6 | 60.6 |

**FIGURE 4.** Ca\(^{2+}\) inhibition of E2P formation. Wild-type SERCA2a (WT) and SERCA2a mutants were expressed alone (A) or co-expressed with N30C-PLB (B) in S121 cells. Mutations made at site I, II, or both sites are indicated in the left margin. For SERCA2a, residues contributing to site I are highlighted in red, to site II in blue, and to both sites in green. Membranes were incubated with \(^{32}\)P, at the indicated Ca\(^{2+}\) concentrations (top), and autoradiographs of the phosphorylated Ca\(^{2+}\) pumps are displayed in the upper panels. The lower graphs plot Ca\(^{2+}\) inhibition of E2P formation on a percentage basis (n = 3–5 for each mutant).
in regulating the physical interaction between PLB and the Ca\(^{2+}\) pump. In this case cross-linking was measured in buffer similar to that used for measurement of Ca\(^{2+}\)-ATPase activity, which contains 3 mM MgATP and promotes optimal PLB binding (3, 19–23). First, as a control, cross-linking of N30C-PLB to SERCA2a with mutations at both Ca\(^{2+}\)-binding sites (E309Q/E770Q) was measured; cross-linking of PLB to this double mutant should be unresponsive to Ca\(^{2+}\). This result is confirmed in Fig. 5. For wild-type SERCA2a, cross-linking of N30C of PLB to Lys\(^{328}\) of SERCA2a (with KMUS) was inhibited by micromolar Ca\(^{2+}\) (Fig. 5A, PLB/SER), yielding a \(K_I\) value of 0.46 \(\mu M\), consistent with previous reports (20, 22). Cross-linking of PLB to E309Q/E770Q was equally strong but, importantly, virtually completely resistant to Ca\(^{2+}\) at concentrations as high as 10 \(\mu M\) (Fig. 5B). The results of Fig. 5, therefore, demonstrate that Ca\(^{2+}\) binding to the high affinity site(s) of SERCA2a regulates PLB binding to the Ca\(^{2+}\) pump.

Fig. 5 also points out the highly specific nature of the cross-linking reaction. Of all the proteins present in insect cell membranes, PLB cross-linked only to the expressed cardiac Ca\(^{2+}\)-pump with or without functional Ca\(^{2+}\)-binding sites. It should also be noted that uncross-linked PLB is visible on the autoradiogram as a population of monomers (PLB\(_1\)) and dimers (PLB\(_2\)), the pentameric form of N30C-PLB being unstable at the concentration of SDS (5%) used to solubilize membranes (19). In the remaining figures, only PLB cross-linked to SERCA2a is displayed (PLB/SERCA2a heterodimers).

**PLB Cross-linking to SERCA2a with Mutations at Site I or II**—We next used the site-selective mutants of SERCA2a to identify which Ca\(^{2+}\)-binding site of SERCA2a regulates PLB binding. As shown in Fig. 6A, PLB cross-linked strongly to all SERCA2a mutants in the Ca\(^{2+}\)-free condition (first lane), suggesting that PLB does not interact critically with any side chains forming the Ca\(^{2+}\)-binding sites. However, the effect of Ca\(^{2+}\) on PLB cross-linking was different among the SERCA2a mutants analyzed. In particular, PLB cross-linking to mutants at site II (E309Q and N795A) was inhibited by micromolar Ca\(^{2+}\) like wild-type SERCA2a. The \(K_I\) values for Ca\(^{2+}\) inhibition of N30C-PLB cross-linking to SERCA2a were 1.20 \(\pm\) 0.24 \(\mu M\) for E309Q and 0.41 \(\pm\) 0.10 \(\mu M\) for N795A, compared with 0.46 \(\pm\) 0.04 for wild-type SERCA2a (Table 1). It should be noted that because E309Q and N795A are catalytically inactive and do not hydrolyze ATP or translocate Ca\(^{2+}\), cross-linking to these mutants was measured under equilibrium conditions with respect to Ca\(^{2+}\) binding. The results obtained with E309Q and N795A, therefore, strongly suggest that Ca\(^{2+}\) occupancy of site I is sufficient to dissociate PLB from SERCA2a.

In contrast, PLB cross-linking to the E770Q and T798A mutants at site I was highly resistant to Ca\(^{2+}\). The \(K_I\) values for inhibition of N30C-PLB cross-linking to E770Q and T798A were 645 \(\pm\) 121 and 697 \(\pm\) 69 \(\mu M\), respectively, at least 600-fold greater than that obtained with wild-type SERCA2a or the E309Q or E770Q mutants at site II (Fig. 6B, Table 1) (dissociation of PLB from E770Q and T798A at very high Ca\(^{2+}\) concentrations can be explained by residual, very weak binding of Ca\(^{2+}\) at site I under these conditions, as reported earlier with the corresponding mutants in SERCA1a (10, 11)). For the E907Q mutant with reduced Ca\(^{2+}\)-binding affinity at site I, a 3.6-fold higher Ca\(^{2+}\) concentration (\(K_I = 1.65 \pm 0.26\)) was required to inhibit PLB cross-linking compared with wild-type SERCA2a (\(K_I = 0.46 \pm 0.04\)), which correlates well with the higher Ca\(^{2+}\) concentration required to stimulate Ca\(^{2+}\)-ATPase activity of E907Q (Fig. 3). Finally, cross-linking of PLB to the mutant, D799N, affecting both sites was nearly totally resistant to Ca\(^{2+}\), giving results comparable with that obtained with the double mutant E309Q/E770Q (Fig. 4, bottom two panels).

**Other Effectors**—To ensure that the SERCA2a mutations specifically affected the Ca\(^{2+}\)-binding sites, we tested the effects of other allosteric regulators (ATP and thapsigargin) on PLB cross-linking. Also, to further verify the specificity of the protein binding interactions between PLB and SERCA2a, we measured the effect of the anti-PLB antibody, 2D12, on cross-linking (Fig. 7). We have previously shown that ATP stimulates PLB cross-linking to SERCA2a by binding to the modulatory ATP site forming E2-ATP, whereas the specific inhibitor thapsigargin prevents cross-linking by stabilizing the dead-end complex,
TG. 2D12 inhibits cross-linking by scavenging free PLB (19–23). Consistent with these earlier reports, we found that the addition of ATP gave a 2–4-fold increase in cross-linking intensity for all the SERCA2a mutants tested (second lane), whereas thapsigargin and 2D12 strongly inhibited cross-linking (fourth and fifth lanes). Consistent with the results of Fig. 6, 100 μM Ca²⁺ completely inhibited PLB cross-linking to wild-type SERCA2a, E309Q, N795A, and E9907Q but had no effect on PLB cross-linking to the other SERCA2a mutants (lane 3). Thus, only the sites of Ca²⁺ binding were substantially affected in any of the mutants tested.

DISCUSSION

The primary aim of this study was to elucidate the roles of the two Ca²⁺-binding sites of SERCA2a in regulating the physical association with PLB. To accomplish this it was necessary to confirm that mutation of the six residues comprising the two putative Ca²⁺-binding sites of SERCA2a gave similar catalytic effects as those previously reported with SERCA1a (10–13). Indeed, we showed that binding of both Ca²⁺ ions was required for Ca²⁺ activation of ATP hydrolysis, whereas binding of the first Ca²⁺ at site I was sufficient to prevent phosphorylation by Pi. Thus, the same amino acid residues of SERCA2a appear to contribute comparably to formation of the two Ca²⁺-binding sites as in SERCA1a, although some differences in the effects of the specific mutation E309Q were observed (see below). We then went on to show that the same mutations at Ca²⁺-binding sites I and II produced qualitatively identical effects on PLB cross-linking as on Ca²⁺ inhibition of E2P formation. Most importantly, inhibition of PLB binding to SERCA2a by micromolar Ca²⁺ was completely lost in Ca²⁺ pump mutants lacking the ability to bind Ca²⁺ altogether (E770Q and T798A at site I or D799N at both sites, for example) but maintained in Ca²⁺ pump mutants capable of binding Ca²⁺ at the first site only (E309Q and N795A mutants at site II). Therefore, we conclude that Ca²⁺ binding at site I is primarily...
responsible for regulating the physical association of the Ca$^{2+}$ pump with PLB.

**Role of Ca$^{2+}$ Binding Site I**—Binding of the two Ca$^{2+}$ ions to SERCA is sequential (14). Ca$^{2+}$ binding at site I occurs first, leading to a conformational change enabling cooperative binding of the second Ca$^{2+}$ at site II (6, 8). Importantly, as originally proposed by Cantilina et al. (18) and further supported here, PLB appears to specifically interfere with Ca$^{2+}$ handling at site I. However, whereas Cantilina et al. (18) suggested that PLB lowers the apparent Ca$^{2+}$ affinity of the enzyme through the kinetic effect of slowing the isomeric transition that occurs after binding of the first Ca$^{2+}$ (Fig. 1, *single asterisk*), we now propose that PLB actually competes physically for the binding of Ca$^{2+}$ at site I. In the study of Cantilina et al. (18), no direct effect of PLB on Ca$^{2+}$ binding by SERCA2a was found in a variety of systems at equilibrium; therefore, it was concluded that PLB did not lower the Ca$^{2+}$ affinity of the enzyme directly. However, these earlier observations were dependent upon the ability of the anti-PLB antibody, 2D12, to reverse the effects of PLB on the Ca$^{2+}$-ATPase in cardiac SR vesicles, and direct interactions between PLB and SERCA2a could not be measured. On the other hand, more recent cross-linking results (19–23) and the results now shown with the Ca$^{2+}$-binding site mutants strongly suggest that PLB binding to E2 directly competes for Ca$^{2+}$ binding at site I and point to a simple mechanism to explain how PLB decreases the apparent Ca$^{2+}$ affinity of the enzyme by hindering the transition from E2 to E1 (Fig. 1, *double asterisk*). For example, under our cross-linking conditions, Ca$^{2+}$ pump mutants that can only bind the first Ca$^{2+}$ (E309Q and N795A) and, therefore, cannot hydrolyze ATP and cycle kinetically, will reach an equilibrium between two conformations; that is, E1 with bound Ca$^{2+}$ (E1-Ca) and E2 with bound PLB (E2-PLB), the latter being detectable by chemical cross-linking. Under these conditions, for each Ca$^{2+}$ concentration tested the amount of PLB cross-linking to the Ca$^{2+}$-ATPase reflects the equilibrium population of E2-PLB, whereas the degree of inhibition of cross-linking by Ca$^{2+}$ reflects the equilibrium population of SERCA2a molecules with site I occupied (E1-Ca). Thus, by mass action, PLB binding to E2 decreases equilibrium levels of E1 and the amount of Ca$^{2+}$ bound at site I, thereby decreasing the equilibrium constant for Ca$^{2+}$ binding directly.

The conclusion that PLB directly decreases the Ca$^{2+}$ binding affinity of SERCA2a is totally consistent with three-dimensional crystal structures of the enzyme recently reported (6, 8). When SERCA2a is in the E2 state, PLB is projected to fit into a groove formed between M2, M4, and M9 (22, 26). With PLB bound, the Ca$^{2+}$ pump is locked into this conformation, and formation of the two high affinity Ca$^{2+}$-binding sites cannot occur (26). Therefore, dissociation of PLB is required to allow the conformational transition to E1, enabling the binding of the first Ca$^{2+}$ (26).

**Dynamic Equilibrium between PLB and SERCA2a**—In a series of studies we recently showed that micromolar Ca$^{2+}$ prevents PLB cross-linking to SERCA2a at multiple sites distributed throughout both molecules at both cytoplasmic and transmembrane domains, suggesting that PLB completely dissociates from SERCA2a when the enzyme binds Ca$^{2+}$ (19–23). The dynamic nature and conformational specificity of the PLB to SERCA2a binding interaction was further evidenced with use of the effectors ATP, thapsigargin, and 2D12. Cross-linking was substantially augmented by ATP (E2-ATP) (19) but drastically attenuated by thapsigargin (E2-TG) (20) or by binding of 2D12 to PLB (18, 23). Here we demonstrated that regulation of this dynamic equilibrium by the effectors ATP, thapsigargin, and 2D12 is maintained even in Ca$^{2+}$-pumps altogether devoid of Ca$^{2+}$ binding. For example, although Ca$^{2+}$ at concentrations as high as 100 μM had no effect on PLB cross-linking to the mutants E770Q, T798A, and D799N (Fig. 7), PLB cross-linking to these mutants was nonetheless strongly enhanced by ATP and completely inhibited by thapsigargin and 2D12, like wild-type SERCA2a. Thus, PLB binding to SERCA2a is a dynamic process, PLB interacting most avidly with the Ca$^{2+}$ pump in the E2-ATP conformation but not at all when the enzyme is in the E2P (22), E2-TG (20), E1-Ca$_{2+}$ (19), or E1-Ca (shown here) states.

**Differences between SERCA2a and SERCA1a**—The data of Table 1 demonstrate that the apparent Ca$^{2+}$ affinity at site I is reduced substantially by the E309Q mutation at site II but unaffected by the N795A mutation, also at site II. This was true whether Ca$^{2+}$ affinities were assessed by Ca$^{2+}$ inhibition of E2P formation or by monitoring Ca$^{2+}$ inhibition of PLB cross-linking. These results with E309Q are apparently opposite to those recently obtained with SERCA1a, in which Ca$^{2+}$ binding affinities of the skeletal muscle Ca$^{2+}$ pump were estimated by the technique of net charge transfer (16). In this latter study it was found that the E309Q mutation actually increased Ca$^{2+}$ binding affinity at site I (but see Refs. 11–13, where these effects were not noted). At present, it is unclear whether these opposite effects of the E309Q mutation reflect real differences in interactions between the two Ca$^{2+}$-binding sites in SERCA1a versus SERCA2a or are only apparent and more a consequence of the different techniques used to estimate Ca$^{2+}$ binding.

**N30C-PLB as a Reporter Molecule**—In the present study we used N30C-PLB cross-linking to Lys$^{328}$ of the Ca$^{2+}$-ATPase to measure the Ca$^{2+}$ binding affinities of various SERCA2a mutants. Differences in relative Ca$^{2+}$ binding affinities of the seven SERCA2a mutants tested were very similar whether determined by PLB cross-linking (Fig. 6) or by monitoring Ca$^{2+}$ inhibition of E2P formation (Fig. 4). However, considerably lower Ca$^{2+}$ concentrations inhibited PLB cross-linking compared with E2P formation (Table 1), which may be explained by the different experimental conditions. Specifically, cross-linking of N30C-PLB to SERCA2a could be carried out in standard physiological buffer used for measurement of Ca$^{2+}$-ATPase activity, whereas measurement of E2P formation required high concentrations of Me$_3$SO and omission of ATP, which reduces the Ca$^{2+}$ affinity of SERCA considerably (12, 13). Thus, PLB cross-linking is a more accurate means of measuring high affinity Ca$^{2+}$ binding to wild-type and mutant Ca$^{2+}$ pumps under conditions used traditionally for measurement of Ca$^{2+}$-ATPase activity and Ca$^{2+}$ transport. Because of its high sensitivity and specificity, PLB cross-linking is also useful for distinguishing different conformational states of the Ca$^{2+}$-ATPase and its mutants, which are not easily detected by other methods, for example, conformational states stabilized by allosteric regulators like ATP and thapsigargin.
Acknowledgments—We thank Glen Schmeisser and Marcelle Stucky for technical assistance.

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Ca\textsuperscript{2+} Binding Sites and PLB