Mechanism of Reversal of Phospholamban Inhibition of the Cardiac Ca$^{2+}$-ATPase by Protein Kinase A and by Anti-phospholamban Monoclonal Antibody 2D12*

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Our model of phospholamban (PLB) regulation of the cardiac Ca$^{2+}$-ATPase in sarcoplasmic reticulum (SERCA2a) states that PLB binds to the Ca$^{2+}$-free, E2 conformation of SERCA2a and blocks it from transitioning from E2 to E1, the Ca$^{2+}$-bound state. PLB and Ca$^{2+}$ binding to SERCA2a are mutually exclusive, and PLB inhibition of SERCA2a is manifested as a decreased apparent affinity of SERCA2a for Ca$^{2+}$. Here we extend this model to explain the reversal of SERCA2a inhibition that occurs after phosphorylation of PLB at Ser16 by protein kinase A (PKA) and after binding of the anti-PLB monoclonal antibody 2D12, which recognizes residues 7–13 of PLB. Site-specific cysteine variants of PLB were co-expressed with SERCA2a, and the effects of PKA phosphorylation and 2D12 on Ca$^{2+}$-ATPase activity and cross-linking to SERCA2a were monitored. In Ca$^{2+}$-ATPase assays, PKA phosphorylation and 2D12 partially and completely reversed SERCA2a inhibition by decreasing $K_{c_a}$ values for enzyme activation, respectively. In cross-linking assays, cross-linking of PKA-phosphorylated PLB to SERCA2a was inhibited at only two of eight sites when conducted in the absence of Ca$^{2+}$ favoring E2. However, at a subsaturating Ca$^{2+}$ concentration supporting some E1, cross-linking of phosphorylated PLB to SERCA2a was attenuated at all eight sites. $K_{c_a}$ values for cross-linking inhibition were decreased nearly 2-fold at all sites by PLB phosphorylation, demonstrating that phosphorylated PLB binds more weakly to SERCA2a than dephosphorylated PLB. In parallel assays, 2D12 blocked PLB cross-linking to SERCA2a at all eight sites regardless of Ca$^{2+}$ concentration. Our results demonstrate that 2D12 restores maximal Ca$^{2+}$-ATPase activity by physically disrupting the binding interaction between PLB and SERCA2a. Phosphorylation of PLB by PKA weakens the binding interaction between PLB and SERCA2a (yielding more PLB-free SERCA2a molecules at intermediate Ca$^{2+}$ concentrations), only partially restoring Ca$^{2+}$ affinity and Ca$^{2+}$-ATPase activity.

$\beta$-Adrenergic stimulation of mammalian myocardium increases both the strength of contraction and the rate of relax-

2 The abbreviations used are: PLB, phospholamban; SR, sarcoplasmic reticulum; SERCA2a, isoform of Ca$^{2+}$-ATPase in cardiac SR; SERCA1a, isoform of Ca$^{2+}$-ATPase in fast twitch skeletal muscle; MOPS, 3-(N-morpholino)propanesulfonic acid; E1, high Ca$^{2+}$ affinity conformation of Ca$^{2+}$-ATPase; E2, low Ca$^{2+}$ affinity conformation of Ca$^{2+}$-ATPase; $K_{c_a}$, Ca$^{2+}$ concentration required for half-maximal effect; Me$_3$SO, dimethyl sulfoxide; WT, wild type; PKA, protein kinase A; BMH, 1,6-bismaleimidohexane; bBBr, dibromobimane; KMUS, N-(6-maleimidouracil)-succinimidyl succinimide ester; EMCS, N-(6-maleimidocaproyl)-succinimidyl succinimide ester; DTT, dithiothreitol.
This idea is strongly supported by recent cross-linking results in which the binding of PLB to SERCA2a at several sites distributed throughout both molecules was detected (14–17). Based on these cross-linking results, we proposed the following model (Scheme 1) to explain the inhibitory action of PLB on SERCA2a.

\[
\text{PLB} \quad \text{Ca}^{2+} \\
E_2 \cdot \text{PLB} \xleftrightarrow{} E_2 \xleftrightarrow{} E_1 \xleftrightarrow{} E_1 \cdot \text{Ca}^{2+}
\]

\text{SCHEME 1}

Binding of PLB and Ca\(^{2+}\) to SERCA2a is mutually exclusive. PLB binds to the \(E_2\) conformation of SERCA2a, the low Ca\(^{2+}\) affinity state, whereas Ca\(^{2+}\) binds to the \(E_1\) conformation of SERCA2a, the high Ca\(^{2+}\) affinity state. Consistent with this, cross-linking of PLB to SERCA2a at all previously established sites at both cytoplasmic and transmembrane residues of PLB is completely abolished by micromolar Ca\(^{2+}\) stabilizing \(E_1\). We further proposed, based on cross-linking data, that PLB binds specifically to the \(E_2\)-ATP conformation of the enzyme, physically blocking its transition from \(E_2\) to \(E_1\) (14–17). Finally, we postulated that for the kinetic cycle to proceed, PLB must fully dissociate from SERCA2a to allow \(E_1\) formation, the high Ca\(^{2+}\) affinity conformation that actively hydrolyzes ATP. Therefore, when the enzyme is actively transporting Ca\(^{2+}\), this competition between PLB and Ca\(^{2+}\) for binding to \(E_2\) and \(E_1\), respectively, is manifested as a decrease in apparent affinity of SERCA2a for Ca\(^{2+}\), as suggested in earlier kinetic experiments (18). Cross-linking results from two other groups also suggested that \(E_1\)-Ca\(^{2+}\) is incapable of binding PLB (19, 20), which is in contrast to recent claims of others that PLB remains tightly associated with SERCA2a throughout the entire reaction cycle (21, 22).

As noted above, the molecular mechanism by which phosphorylation of PLB or anti-PLB monoclonal antibodies reverse PLB inhibition of SERCA2a remains unclear. In recent studies in which the physical interactions between the two molecules were examined directly, phosphorylation of PLB by PKA has been reported to either increase its dissociation from SERCA2a (14, 19, 20) or to have no effect (21, 23–25). We observed that both phosphorylation of PLB by PKA and the anti-PLB antibody, 2D12 (which recognizes residues 7–13 of PLB), reduce the ability of PLB to dissociate from SERCA2a (14, 19, 20) and that the protein is completely abolished by micromolar Ca\(^{2+}\) stabilizing \(E_1\). This idea is strongly supported by recent cross-linking results in which the binding of PLB to SERCA2a at several sites distributed throughout both molecules was detected (14–17). Based on these cross-linking results, we proposed the following model (Scheme 1) to explain the inhibitory action of PLB on SERCA2a.

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In this study, we examined the effects of PKA phosphorylation of PLB and 2D12 on the cross-linking of PLB to SERCA2a at both cytoplasmic and transmembrane residues, and we correlated these results with Ca\(^{2+}\) activation of ATPase activity. The results are entirely consistent with our model of mutually exclusive binding of Ca\(^{2+}\) and PLB to SERCA2a (Scheme 1). We conclude that phosphorylation of PLB by PKA weakens its binding interaction with SERCA2a, giving a partial stimulation of Ca\(^{2+}\)-ATPase activity at low ionized Ca\(^{2+}\) concentration, whereas 2D12 virtually completely dissociates PLB from SERCA2a, giving the maximal increase in Ca\(^{2+}\)-ATPase activity possible.

**EXPERIMENTAL PROCEDURES**

**Materials**—The cross-linking agents BMH, EMCS, and KMUS were purchased from Pierce. bBBr was from Molecular Probes. Catalytic subunit of PKA was from Sigma. \([\gamma-^{32P}]ATP\) was from PerkinElmer Life Sciences.

**Mutagenesis and Baculovirus Production**—Mutation of canine SERCA2a and PLB cDNAs was conducted as described recently (14–17). All Cys-scanning point mutants of PLB were made on the Cys-less PLB background, which is a fully functional, canine PLB with Cys residues 36, 41, and 46 changed to Ala (14–17). V89C-SERCA2a and T317C-SERCA2a were made directly in the transfection vector pVL1393 using the QuikChange™ XL-Gold system (Stratagene) (16–17). All mutated cDNAs were confirmed by DNA sequencing of the plasmid vectors. Baculoviruses encoding mutated proteins were generated as described previously with BaculoGold™ (Pharmingen) linearized baculovirus DNA (14–17).

**Protein Expression**—Sf21 insect cells were co-infected with baculoviruses encoding PLB and SERCA2a proteins as described previously (5, 26). Microsomes were harvested 60 h after co-infection and stored frozen in small aliquots at approximately 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. PLB was expressed at a 4:1 molar ratio to SERCA2a (14–17).

**Phosphorylation of PLB by PKA**—Prior to experiments, the catalytic subunit of PKA was dialyzed overnight at 4 °C in 50 mM MOPS (pH 7.0) to eliminate DTT, phosphate, and EDTA. Control experiments revealed that the dialyzed protein remained fully functional. Phosphorylation of PLB in Sf21 membranes was performed in a master mix containing 80 units of dialyzed catalytic subunit of PKA with 110 μg of microsomal protein in 100 μl of 40 mM MOPS (pH 7.0), 3.2 mM MgCl\(_2\), 75 mM KCl, and 3 mM ATP. Phosphorylation was conducted for 10 min at 30 °C, and then 11-μg aliquots of pre-phosphorylated or control (minus catalytic subunit) membranes were diluted into concentrated Ca\(^{2+}\)/EGTA buffers to yield the final concentrations below for cross-linking experiments or for Ca\(^{2+}\)-ATPase assays. In experiments in which PLB was pre-labeled at Ser\(^{16}\) with PKA and \([\gamma-^{32P}]ATP\) (Fig. 4), 25 μM \([\gamma-^{32P}]ATP\) was substituted for 3 mM cold ATP in the phosphorylation reaction. The membranes were then cross-linked in normal buffer containing 3 mM nonradioactive ATP. Inclusion of phosphatase inhibitors did not have any effect on PLB phosphorylation,
PKA and 2D12 Effects on PLB to SERCA2a Cross-linking

which was complete; so for most experiments, phosphatase inhibitors were not added. Heat-inactivated PKA was made by heating PKA to 100 °C for 5 min.

Cross-linking—Cross-linking between Cys residues of PLB and Cys or Lys residues of SERCA2a was conducted using homobifunctional thiol (BMH and bBBr) and heterobifunctional thiol to amine (EMCS and KMUS) cross-linking reagents, respectively, as described previously (14–17). N27C and N30C of PLB were cross-linked to WT-SERCA2a at Lys2328 or Cys318 (14, 15); L31C-PLB was cross-linked to T317C-SERCA2a (16); and I48C, V49C, M50C, and L52C of PLB were cross-linked to V89C-SERCA2a (17). Reactions were conducted with 11 µg of microsomal protein pre-phosphorylated in the presence or absence of catalytic subunit of PKA as described above in 12 µl of 40 mM MOPS (pH 7.0), 3.2 mM MgCl2, 75 mM KCl, and 3 mM ATP supplemented with Ca2+/EGTA. The final concentration of EGTA was fixed at 1 mM, and ionized calcium concentrations were set by adding CaCl2 from 0 to 1 mM (5). Cross-linking reactions were conducted at room temperature with 0.1 mM final cross-linker concentrations added from concentrated stock solutions in Me2SO and terminated by adding 7.5 µl of SDS sample buffer containing 100 mM DTT (14–17). Specific cross-linking agents used and cross-linking times to achieve maximal cross-linking are indicated in the figure legends. After terminating the reactions, samples were subjected to SDS-PAGE followed by immunoblotting with anti-PLB monoclonal antibody 1F1 to detect PLB cross-linked to SERCA2a. 1F1 was raised to residues 1–10 of PLB and recognizes phosphorylated and dephosphorylated PLB equally well (14). Antibody visualization was with 125I-protein A except in experiments in which 2D12 effects on cross-linking were assessed (Fig. 5). In this case, blots were probed with 125I-and 32P-labeled bands on nitrocellulose sheets were visualized by autoradiography and then quantified with a Bio-Rad Personal FX PhosphorImager. Data analysis was done with Origin (Microcal).

Ca2+-ATPase Assay—Ca2+-dependent ATPase activities of insect cell microsomes co-expressing PLB and SERCA2a were measured using an enzyme-coupled spectrophotometric assay (26). The rate of NADH decay was measured at 340 nm in a SPECTRamax® PLUS (Molecular Devices) microplate spectrophotometer at 37 °C with 11 µg of membrane protein in buffer containing 50 mM MOPS (pH 7.0), 3 mM MgCl2, 100 mM KCl, 5 mM Na3cit, 3 µg/ml of the Ca2+-ionophore A23187, 3 mM ATP, and Ca/EGTA as described above. Ca2+-ATPase activities were measured in the presence and absence of anti-PLB monoclonal antibody 2D12 or with prior phosphorylation of PLB at Ser16 by PKA. All ATPase activities reported are Ca2+-dependent.

RESULTS

PLB Phosphorylation and 2D12 Effects on SERCA2a Activity—
In a series of studies, we used Cys-scanning mutagenesis of PLB to identify several residues located within both the cytoplasmic and transmembrane domains of PLB that cross-link to SERCA2a (14–17). Prior to analyzing the effects of PLB phosphorylation and the anti-PLB antibody 2D12 on cross-linking at these sites, we determined whether 2D12 and phosphorylation of these mutants altered their ability to inhibit Ca2+-ATPase activity, as has been demonstrated previously for WT-SERCA2a (5). Fig. 1 shows a typical Ca2+-dose-response curve on Ca2+-ATPase activity for one of the PLB mutants tested, N30C-PLB, when it was co-expressed with the Ca2+-pump in insect cell microsomes. N30C-PLB inhibited SERCA2a by shifting the Ca2+ activation curve to the right (Fig. 1, open triangles), yielding a Kcat value of 0.35 µM, compared with 0.13 µM when SERCA2a was expressed alone (open squares). Phosphorylation of N30C-PLB at Ser16 by PKA shifted the curve to the left (Fig. 1, partially closed triangles), decreasing the Kcat value to 0.25 µM, and thus partially restoring the higher apparent Ca2+ affinity of the Ca2+-pump. In contrast, 2D12 almost completely reversed N30C-PLB inhibition of the Ca2+-ATPase (Fig. 1, closed triangles), lowering the Kcat value to 0.16 µM, which is similar to the Kcat value obtained when SERCA2a was expressed by itself. The more complete reversal of PLB inhibition of Ca2+-ATPase activity by monoclonal antibodies, compared with PKA phosphorylation of PLB, was previously demonstrated with native SR vesicles (10–12).

Similar results were obtained for all other PLB mutants tested that cross-link to SERCA2a, with Kcat values from multiple determinations listed in Table 1. For comparison, Kcat values for WT-SERCA2a co-expressed with SERCA2a are also shown, and fall within the same range. Notably, the PLB mutations N30C, V49C, and L52C produced gain of PLB function, increasing Kcat values for half-maximal SERCA2a activation more than WT-PLB, as has been reported previously (17, 27). Also consistent with previous work (5, 26), 2D12 nearly com-

![FIGURE 1. PKA and 2D12 effects on Ca2+-ATPase activity of SERCA2a co-expressed with N30C-PLB. SERCA2a was expressed by itself (squares) or with N30C-PLB (open triangles) in Sf21 cells, followed by measurement of Ca2+-ATPase activities in membranes in the presence of 5.5 µg of anti-PLB monoclonal antibody, 2D12 (+2D12), or with prior phosphorylation of PLB by PKA (+PKA). See text for Kcat values.](image-url)
PKA and 2D12 Effects on PLB to SERCA2a Cross-linking

TABLE 1
PKA and 2D12 effects on χCa values for activation of Ca2+-ATPase

| Protein expressed | Control | +PKA |
|-------------------|---------|------|
| N27C               | 0.12 ± 0.01 | 0.10 ± 0.01 |
| N30C               | 0.13 ± 0.01 | 0.12 ± 0.01 |
| N30C/C318          | 0.13 ± 0.01 | 0.12 ± 0.01 |
| V89C               | 0.14 ± 0.01 | 0.13 ± 0.01 |
| V89C/C318          | 0.14 ± 0.01 | 0.13 ± 0.01 |
| M50C               | 0.15 ± 0.01 | 0.14 ± 0.01 |
| M50C/C318          | 0.15 ± 0.01 | 0.14 ± 0.01 |
| L52C               | 0.16 ± 0.01 | 0.15 ± 0.01 |
| L52C/C318          | 0.16 ± 0.01 | 0.15 ± 0.01 |

+PKA compared to control. *p < 0.05 versus control and +PKA.

PKA and 2D12 Effects on PLB to SERCA2a Cross-linking

Well, we did note that at the cytoplasmic domain of PLB, phosphorylation of the gain-of-function mutant, N30C-PLB, by PKA reduced cross-linking of N30C of PLB to Cys318 of SERCA2a by BMH by 50% (Fig. 2A, N30C/C318), confirming our previous

![Figure 2](image-url)
result (14). In addition, phosphorylation of the loss-of-function mutant, L31C-PLB, also decreased cross-linking of L31C of PLB to T317C of SERCA2a by BMH by 80% (Fig. 2A, L31C/T317C). Pre-phosphorylation with heat-inactivated PKA (HI PKA) had no effect on cross-linking at these sites (Fig. 2A, 4th lane).

However, at all other sites tested, including both cytoplasmic and transmembrane residues of PLB, PKA phosphorylation of PLB had no effect on cross-linking. For example, at the cytoplasmic domain, cross-linking of N27C-PLB and N30C-PLB to Lys328 of SERCA2a with EMCS and KMUS, respectively,
remained unaffected after PKA phosphorylation (Fig. 2A, N27C/K328 and N30C/K328). It was shown previously that 100% of SERCA2a molecules are cross-linked at these two sites under these conditions (15). Moreover, at the transmembrane domain of PLB, intensities of cross-linking of I48C through L52C of PLB to V89C of SERCA2a were high as noted previously (17), and virtually identical irrespective of the phosphorylation state of PLB (Fig. 2B). These results demonstrated that phosphorylated PLB still binds to the E2 state of SERCA2a at both cytoplasmic and transmembrane domains, although local conformational changes in PLB appeared to interfere with cross-linking at two sites. Fig. 2 also points out the efficient phosphorylation of PLB at Ser16 by PKA in the experiments, as indicated by the upward mobility shift in free PLB monomers, dimers, tetramers, and pentamers visible for several mutants that was induced by phosphorylation (28) (Fig. 2, 3rd lane, PLB1, PLB2, PLB4, and PLB5).

Ca$^{2+}$ Effect on Cross-linking of Phosphorylated PLB to SERCA2a—We reasoned that if PLB and Ca$^{2+}$ compete for binding to SERCA2a, then a decreased binding affinity of PKA-phosphorylated PLB for the Ca$^{2+}$ pump might be more readily demonstrated at an intermediate Ca$^{2+}$ concentration allowing some formation of E1 during the cross-linking reactions (see Scheme 1). Accordingly, we examined the Ca$^{2+}$ concentration dependence of cross-linking of phosphorylated and dephosphorylated PLB to SERCA2a, testing directly the hypothesis of mutually exclusive binding of Ca$^{2+}$ and PLB to SERCA2a.

As shown in Fig. 3, micromolar Ca$^{2+}$ totally inhibited the cross-linking of both phosphorylated and dephosphorylated PLB to SERCA2a at all PLB sites examined in Fig. 2. More importantly for the focus of this work, however, Ca$^{2+}$ inhibition curves on cross-linking of phosphorylated PLB to SERCA2a were all shifted to the left for all of the PLB mutants studied, whether Cys replacements occurred at cytoplasmic (Fig. 3A) or transmembrane residues of PLB (Fig. 3B). These results strongly suggest that phosphorylated PLB binds more weakly to SERCA2a than dephosphorylated PLB, when competing with Ca$^{2+}$ for enzyme binding. Table 2 lists the $K_{Ca}$ values for half-maximal inhibition of cross-linking of phosphorylated and dephosphorylated PLB to SERCA2a. For dephosphorylated PLB, the $K_{Ca}$ values were all in low micromolar range, in agreement with previous reports (14–17). Phosphorylation of PLB by PKA decreased these $K_{Ca}$ values by 30–50% at all sites examined, suggesting that phosphorylation substantially decreases the binding affinity of PLB for SERCA2a at low Ca$^{2+}$ concentration. Cross-linking at the two sites, N30C/PLB and L31C-PLB/T317C, was especially sensitive to Ca$^{2+}$ after PLB phosphorylation, suggesting that at these two sites local conformational changes in PLB also attenuated cross-linking, as noted above. All of these results were consistent with the decreased $K_{Ca}$ values obtained in Ca$^{2+}$-ATPase assays after phosphorylation of each PLB mutant (Fig. 1 and Table 1).

Cross-linking of $^{32}$P-labeled PLB to SERCA2a—To confirm our cross-linking results with phosphorylated PLB, we labeled the protein at Ser16 with $^{32}$P using PKA and then monitored cross-linking of phosphorylated PLB to SERCA2a directly by detection of $^{32}$P. This approach eliminated the possibility of detecting any incompletely phosphorylated PLB cross-linking to SERCA2a in the experiments. As expected, when reactions were conducted in the absence of Ca$^{2+}$ (1st lane), cross-linking of $^{32}$P-labeled PLB to SERCA2a occurred at both cytoplasmic (Fig. 4A) and transmembrane (Fig. 4B) sites, thus confirming directly that phosphorylated PLB remains capable of binding to the E2 conformation of SERCA2a. Moreover, micromolar Ca$^{2+}$ completely dissociated $^{32}$P-labeled PLB from SERCA2a at all cross-linking sites (Fig. 4, 2nd to 10th lanes). $K_{Ca}$ values for inhibition of cross-linking of $^{32}$P-labeled PLB to SERCA2a were also substantially decreased (40–60%) for phosphorylated PLB relative to dephosphorylated PLB at all sites (Table 2), again demonstrating that PLB phosphorylated at Ser16 by PKA has decreased binding affinity for SERCA2a.

2D12 Effect on PLB Cross-linking to SERCA2a—In contrast to phosphorylation of PLB by PKA, 2D12 gives virtually complete reversal of PLB inhibition of Ca$^{2+}$-ATPase activity (Fig. 1 and Table 1). Moreover, in previous studies, 2D12 was shown to inhibit cross-linking of PLB to SERCA2a nearly completely at
several cytoplasmic residues (14–16), when reactions were conducted in the absence of Ca²⁺ favoring E₂ and maximal cross-linking. Based on these results, we proposed that binding of 2D12 to PLB blocks PLB binding to SERCA2a. Here we tested this idea in more detail, also analyzing cross-linking interactions occurring at transmembrane residues of PLB.

Cross-linking of PLB to E₂ of SERCA2a in the absence of Ca²⁺ was measured over a range of 2D12 concentrations approximating levels of PLB expressed in insect cell microsomes (Fig. 5). Inhibition of cross-linking of PLB to SERCA2a occurred at both cytoplasmic (Fig. 5A) and transmembrane (Fig. 5B) residues, with as little as 1 µg of 2D12 included in the assays. At higher 2D12 concentrations, inhibition of cross-linking became virtually complete, ranging between 80 and 100%. Approximately 2 µg of 2D12, corresponding to an antibody concentration of 1 µM in the cross-linking assays, produced half-maximal inhibition of cross-linking for all mutants tested, except for L31C-PLB and I48C-PLB, which were somewhat more sensitive to the antibody. Quantitative immunoblotting demonstrated that the concentration of PLB in the cross-linking assays ranged between 2 and 4 µM for all mutants tested, thus demonstrating that an approximately equimolar ratio of 2D12 to PLB inhibited cross-linking by 80% or greater. As expected, the low level of residual cross-linking detected at some sites in the presence of 2D12 was totally eliminated by micromolar Ca²⁺, as depicted in Fig. 5, C and D, for several sites. These results showed that 2D12 binding to residues 7–13 of PLB is sufficient to inhibit cross-linking at both cytoplasmic and transmembrane residues of PLB, thus strongly suggesting that 2D12 blocks PLB binding to the Ca²⁺ pump nearly completely.

**DISCUSSION**

Here we have shown that phosphorylation of PLB by PKA decreases its binding affinity for SERCA2a, as revealed by the decreased cross-linking between the two proteins occurring at multiple sites distributed throughout both cytoplasmic and transmembrane domains of PLB. In the absence of Ca²⁺, when SERCA2a was predominantly in the E₂ conformation, essentially 100% of Ca²⁺ pump molecules contained bound PLB, regardless of the phosphorylation state of PLB. This is because both dephosphorylated and phosphorylated PLB exhibited binding affinities for SERCA2a sufficient to nearly completely saturate the enzyme in the Ca²⁺-free state (see Scheme 1). However, at intermediate Ca²⁺ concentrations allowing formation of some E₁, a decreased binding affinity of phosphorylated PLB for SERCA2a became unmasked, as reflected by the ~2-fold reduction of $K_\text{Ca}$ values for inhibition of cross-linking of phosphorylated PLB to SERCA2a at all sites (Table 2). We did note that at the two adjacent sites, N30C/Cys318 and L31C/T317C, the cross-linking of phosphorylated PLB to SERCA2a was substantially inhibited even in the absence of Ca²⁺, which we attribute to local conformational changes in PLB induced by phosphorylation interfering with PLB binding at these two sites.

In the case of the anti-PLB antibody, 2D12, however, PLB to SERCA2a cross-linking was virtually completely abolished by
2D12 at all sites, even when conducted in the absence of Ca\(^2+\) and any enzyme turnover. 2D12 binding to cytoplasmic residues 7–13 of PLB in domain IA (1) was sufficient to block cross-linking at the transmembrane sites at the C terminus of PLB (17), which are located at least 30 Å away from the site of antibody binding. Therefore, it seems likely that 2D12 binding to PLB physically blocks its interaction with SERCA2a, rather than just decreasing its binding affinity for the enzyme as is the case with PKA phosphorylation. Binding of 2D12 to PLB most likely obstructs PLB access to its binding site on SERCA2a, as once cross-linked to SERCA2a the 2D12 epitope still remains accessible, at least after denaturing SDS-PAGE and immunoblotting, as demonstrated in this study and previously (14–17). This latter observation supports the idea that in native membranes PLB monomers are interchanging with SERCA2a on a continual basis (4), and points out that 2D12 must have a much higher binding affinity for free PLB monomers than PLB monomers do for SERCA2a. The observed effects of both PKA phosphorylation and 2D12 binding on cross-linking were entirely consistent with Ca\(^2+\)-ATPase data for all of the different mutants used and with previous results with SR vesicles (11–13). PKA phosphorylation of PLB partially reversed Ca\(^2+\) pump inhibition by weakening its binding interaction with SERCA2a at both cytoplasmic and transmembrane domains. 2D12, on the other hand, totally reversed SERCA2a inhibition, by completely disrupting PLB binding to SERCA2a. Interestingly, this mechanism to explain the greater effect of the antibody versus PKA phosphorylation to relieve SERCA2a inhibition was proposed by Tada and co-workers (10) more than a decade ago, but could not be tested directly because of the lack of a method to measure the binding interaction between PLB and SERCA2a. Here, we overcame this hurdle with use of recombinant proteins and chemical cross-linking.

The results with PKA phosphorylation of PLB strongly support our model of PLB inhibition of SERCA2a in which we proposed that the binding of Ca\(^2+\) to E1 and PLB to E2 is mutually exclusive (14–17). Micromolar Ca\(^2+\) totally inhibited the binding of phosphorylated and dephosphorylated PLB to SERCA2a at all domains (Fig. 3), suggesting the complete dissociation of both forms of PLB from SERCA2a, in contrast to recent claims of others (21, 22, 24, 25). Our results show that phosphorylated PLB, however, cannot compete with Ca\(^2+\) for binding to SERCA2a nearly as well as dephosphorylated PLB (Table 2). Therefore, at intermediate Ca\(^2+\) concentrations, fewer phosphorylated PLB molecules bind to SERCA2a than dephosphorylated PLB molecules. These results in more PLB-free SERCA2a molecules actively transporting Ca\(^2+\), which is manifested as an increase in the apparent affinity of the enzyme for Ca\(^2+\). Previous cross-linking results have demonstrated that PLB binds to the E2 conformation of SERCA2a (14–16) in the groove between transmembrane domains 2 and 9 (17, 19). It is easy to understand how either dephosphorylated or phosphorylated PLB binding to this region of SERCA2a could block enzyme turnover, because in order for ATP hydrolysis to occur, PLB must first exit its E2-binding site to allow the substantial movement in transmembrane domain 2 required for formation of E1 and the high affinity Ca\(^2+\)-binding sites (29).

As mentioned earlier, there are conflicting reports on the effect of phosphorylation of PLB on its binding to SERCA2a. Using photoaffinity labeling of detergent-solubilized proteins, James et al. (20) reported that PKA phosphorylation of PLB abolished cross-linking of Lys\(^3\) of PLB to Lys\(^400\) of SERCA in the absence of Ca\(^2+\). From these data it was concluded that phosphorylated PLB does not bind to SERCA2a, which is opposite to the conclusions reached here and previously (14). However, as we have pointed out (15, 17), Lys\(^3\) of PLB does not appear to cross-link to any residue of SERCA2a in native membranes, and three-dimensional crystallographic data (29) demonstrate that it is highly unlikely that the N terminus of PLB could reach Lys\(^400\) of SERCA2a (30), the cross-linking site detected by James et al. (22). Thus, many of the conclusions made in this study (20) can be viewed skeptically. Using immunoprecipitation of detergent-solubilized proteins, Asahi et al. (23) found no effect of PKA phosphorylation of PLB on its association with SERCA2a and concluded that physical interactions between PLB and the Ca\(^2+\) pump were unaffected by phosphorylation. However, in these studies, detergent solubilization and immunoprecipitation were conducted under conditions in which the Ca\(^2+\) pump readily denatures, making it possible that nonspecific interactions may have been detected by Asahi et al. (23). In a more recent study from the same group, an apparently opposite effect was noted, in which PKA phosphorylation of PLB abolished the cross-linking of N27C, N30C, and V49C of PLB to E2 of SERCA1a with copper-phenanthroline (19). In the present work, we were unable to reproduce this result, even when using copper-phenanthroline as the cross-linking reagent (data not shown). Figs. 2–4 clearly show that Ser\(^16\)-phosphorylated PLB readily cross-links to E2 of SERCA2a at N27C, N30C, and V49C of PLB. It should be noted that the commercially available catalytic subunit preparation used by both groups contains appreciable DTT. We observed that extensive dialysis of the catalytic subunit preparation was required to remove DTT prior to use, to prevent scavenging of cross-linking reagents and an artifactual diminution of cross-linking intensity. In this more recent study, it is unclear if this precaution was taken (19).

Most recently, fluorescence probe and spin-labeling studies of purified proteins reconstituted into liposomes have been interpreted to suggest that phosphorylation of PLB has no effect on its binding affinity for SERCA2a (21, 24, 25). However, in the same systems, Ca\(^2+\) also had no effect on the binding affinity between PLB and SERCA1a (21, 22), unlike the highly specific, micromolar Ca\(^2+\) effect promoting total PLB dissociation we consistently observed here and in several recent studies (14–17). The advantages of studying physical associations between PLB and SERCA2a in native membranes versus in reconstituted systems have been recently discussed (17). In none of the conflicting reports above measuring physical interactions between PLB and SERCA was it concluded that phosphorylation of PLB weakens its binding affinity for SERCA2a while heightening sensitivity to Ca\(^2+\), which is the mechanism our results strongly suggest.

Finally, it is well known that in addition to phosphorylation at Ser\(^16\) by PKA, phospholamban is also phosphorylated at Thr\(^17\) by calmodulin kinase II during β-adrenergic stimulation of the heart (7). In vitro, phosphorylation of PLB at Thr\(^17\) also stimu-
lates SERCA2a activity by increasing its apparent Ca\(^{2+}\) affinity (1, 8). In fact, in most studies simultaneous phosphorylation of PLB by PKA (at Ser\(^{16}\)) and calmodulin kinase II (at Thr\(^{17}\)) gives an additive effect on Ca\(^{2+}\)-ATPase activation (31–33), although nonadditivity was noted in one report (34). It will be interesting to use our cross-linking method to determine whether PLB phosphorylated at Thr\(^{17}\) also has a decreased binding affinity for SERCA2a, and whether dual site phosphorylation of PLB completely reverses PLB binding to SERCA2a like 2D12.

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