Three cross-linkable phospholamban (PLB) mutants of increasing inhibitory strength (N30C-PLB < N27A,N30C,L37A-PLB (PLB3) < N27A,N30C,L37A,V49G-PLB (PLB4)) were used to determine whether PLB decreases the Ca\(^{2+}\) affinity of SERCA2a by competing for Ca\(^{2+}\) binding. The functional effects of N30C-PLB, PLB3, and PLB4 on Ca\(^{2+}\)-ATPase activity and E1–P formation were correlated with their binding interactions with SERCA2a measured by chemical cross-linking. Successively higher Ca\(^{2+}\) concentrations were required to both activate the enzyme co-expressed with N30C-PLB, PLB3, and PLB4 and to dissociate N30C-PLB, PLB3, and PLB4 from SERCA2a, suggesting competition between PLB and Ca\(^{2+}\) for binding to SERCA2a. This was confirmed with the Ca\(^{2+}\) pump mutant, D351A, which is catalytically inactive but retains strong Ca\(^{2+}\) binding. Increasingly higher Ca\(^{2+}\) concentrations were also required to dissociate N30C-PLB, PLB3, and PLB4 from D351A, demonstrating directly that PLB antagonizes Ca\(^{2+}\) binding. Finally, the specific conformation of E2 (Ca\(^{2+}\)-free state of SERCA2a) that binds PLB was investigated using the Ca\(^{2+}\)-pump inhibitors thapsigargin and vanadate. Cross-linking assays conducted in the absence of Ca\(^{2+}\) showed that PLB bound preferentially to E2 with bound nucleotide, forming a remarkably stable complex that is highly resistant to both thapsigargin and vanadate. In the presence of ATP, N30C-PLB had an affinity for SERCA2a approaching that of vanadate (micromolar), whereas PLB3 and PLB4 had much higher affinities, severalfold greater than even thapsigargin (nanomolar or higher). We conclude that PLB decreases Ca\(^{2+}\) binding to SERCA2a by stabilizing a unique E2-ATP state that is unable to bind thapsigargin or vanadate.

Calcium transport by SERCA2a, the Ca\(^{2+}\)-ATPase in cardiac SR\(^{2+}\), is regulated by the small inhibitory phosphoprotein PLB. It is generally accepted that PLB inhibits Ca\(^{2+}\)-ATPase activity by decreasing the apparent Ca\(^{2+}\) affinity of the enzyme, with little or no effect on maximal velocity (V\(_{\text{max}}\)) measured at saturating Ca\(^{2+}\) concentration (1, 2). PLB inhibition of Ca\(^{2+}\)-ATPase activity is reversed by phosphorylation of PLB at Ser\(^{16}\) and Thr\(^{37}\) in response to \(\beta\)-adrenergic stimulation, dramatically increasing the rate of Ca\(^{2+}\) uptake into the SR, and enhancing the rates of cardiac relaxation and contraction (1, 2). Yet despite its prominent role in regulating cardiac function, the precise molecular mechanism of PLB inhibition remains unclear. PLB exists as a population of homopentamers and monomers in the SR membrane, the monomer being the active form responsible for Ca\(^{2+}\)-ATPase inhibition (3, 4). Several groups have shown that there is a dynamic equilibrium between PLB pentamers, PLB monomers, and PLB-SERCA heterodimers (5–9). Recent studies with chemical cross-linking have suggested a simple mechanism of PLB inhibition, in which the PLB monomer competes with Ca\(^{2+}\) for binding to SERCA2a by stabilizing a single conformational state of the enzyme (Fig. 1) (7, 10–12). According to this model, PLB stabilizes the low Ca\(^{2+}\) affinity E2 conformation of the Ca\(^{2+}\) pump and blocks the transition to E1, the conformation required for high-affinity Ca\(^{2+}\) binding and ATP hydrolysis (Fig. 1). Thus SERCA2a with PLB bound cannot bind Ca\(^{2+}\) and is catalytically inactive, and PLB must completely dissociate before the enzyme can transition to E1 and initiate Ca\(^{2+}\) transport. By antagonizing formation of E1, PLB significantly decreases the fraction of Ca\(^{2+}\) pumps available to transport Ca\(^{2+}\) at subsaturating Ca\(^{2+}\) concentration. This is manifested as a decrease in the apparent Ca\(^{2+}\) affinity of the Ca\(^{2+}\)-ATPase, the hallmark of PLB inhibition (1, 2).

Ideally, to test the idea that PLB competes with Ca\(^{2+}\) for binding to SERCA2a, Ca\(^{2+}\) binding assays would be used to directly determine whether a population of Ca\(^{2+}\) pumps expressed alone and free from PLB bind Ca\(^{2+}\) with higher affinity than a population of Ca\(^{2+}\) pumps co-expressed with PLB. Unfortunately, accurate measurement of Ca\(^{2+}\) binding affinity with 45Ca\(^{2+}\) requires relatively high expression levels of the Ca\(^{2+}\)-ATPase (13), which is difficult to achieve in recombinant systems (14). As an alternative, we have demonstrated that the Ca\(^{2+}\) affinity of the enzyme is accurately estimated by assaying Ca\(^{2+}\) inhibition of PLB cross-linking to SERCA2a (7, 10). For example, N30C of PLB cross-links to Lys\(^{328}\) of SERCA2a with the heterobifunctional cross-linker KMUS, and PLB cross-linking is inhibited by micromolar Ca\(^{2+}\) concentration over the same concentration range (K\(_{\text{Ca}}\) ~ 0.3 \(\mu\)M) as enzyme activation occurs (K\(_{\text{Ca}}\) ~ 0.3 \(\mu\)M). However, because cross-linking assays

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*This work was supported, in whole or in part, by National Institutes of Health Grant HL49428. 1 To whom correspondence should be addressed: 1800 N. Capitol Ave., Indianapolis, IN 46202. Tel.: 317-962-0095; Fax: 317-962-8259; E-mail: lrjones@iupui.edu. 2 The abbreviations used are: SR, sarcoplasmic reticulum; PLB, phospholamban; SERCA, sarco(endoplasmic reticulum Ca\(^{2+}\)-ATPase); SERCA2a, isoform of Ca\(^{2+}\)-ATPase in cardiac SR; 2D12, anti-PLB monoclonal antibody; SERCA1a, isoform of Ca\(^{2+}\)-ATPase in fast twitch skeletal muscle; E1, high Ca\(^{2+}\) affinity conformation of Ca\(^{2+}\)-ATPase; E2, low Ca\(^{2+}\) affinity conformation of Ca\(^{2+}\)-ATPase; KMUS, N-(maleimidoundecanoyloxy)sulfosuccinimide ester; TNP-ATP, 2(3')-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate; AMPPCP, adenosine 5'-[(β,γ-methylene)triphosphate]; TG, thapsigargin.
cannot be used to assess the Ca$^{2+}$ affinity of SERCA2a expressed alone, the direct effect of PLB on Ca$^{2+}$ affinity has yet to be determined. To overcome this limitation, here we com-
pared the effects of a series of cross-linkable PLB mutants of increasing inhibitory strength on Ca$^{2+}$ binding to the Ca$^{2+}$ pump. If PLB competes with Ca$^{2+}$ for binding to the Ca$^{2+}$-ATPase, then as PLB becomes a stronger inhibitor of enzyme activity, higher concentrations of Ca$^{2+}$ should be required to dissociate it from the Ca$^{2+}$ pump.

Earlier mutagenesis studies showed that the inhibitory function of PLB was enhanced by point mutations that either increased PLB monomer formation by destabilizing the PLB pentamer (e.g. L37A (3, 4)), or otherwise enhanced PLB monomer binding interactions with the Ca$^{2+}$ pump (e.g. N27A (15) and V49G (16, 17)). As these gain-of-function PLB mutants increased the $K_{ca}$ of enzyme activation more than wild-type PLB, they were termed “supershifters” (3). In the present study, supershifting mutations were combined with the cross-linking mutation, N30C, to create two new PLB mutants, PLB3 (N27A,N30C,L37A-PLB) and PLB4 (N27A,N30C,L37A,V49G-PLB) (Fig. 2). PLB3 and PLB4 are predicted to be strongly inhibitory compared with N30C-PLB (which has a normal inhibitory strength (7)), while remaining cross-linkable to the Ca$^{2+}$ pump, thus allowing their physical interactions with SERCA2a to be measured simultaneously with their functional effects on enzyme activity. These cross-linkable PLB mutants of increasing inhibitory potency were used to demonstrate directly that PLB decreases the actual Ca$^{2+}$ binding affinity of the enzyme. Our results indicate that PLB supershifters act by stabilizing a single conformation of SERCA2a, the nucleotide-bound $E_2$ state, forming a ternary complex ($E_2$:ATP:PLB) that is highly resistant to traditional Ca$^{2+}$ pump inhibitors like TG and vanadate, which also act at $E_2$ (18, 19). In addition, using these superinhibitory PLB mutants we gained new insights on what effect, if any, PLB has on the $V_{max}$ of enzyme activity (20–23), and whether the catalytically inactive SERCA mutant, D351A (14), has substantially enhanced Ca$^{2+}$ binding affinity, as was reported earlier (24), but not subsequently confirmed (25).

**EXPERIMENTAL PROCEDURES**

**Materials**—The cross-linking agent KMUS was purchased from Pierce. $[\gamma-^{32}P]$ATP was obtained from PerkinElmer Life Sciences, and thapsigargin and sodium orthovanadate were purchased from Sigma.

**Mutagenesis and Baculovirus Production**—Mutation of canine SERCA2a and PLB cDNAs was conducted as described previously (4). For consistency with previous cross-linking studies, N30C-PLB was made on the Cys-less PLB background, in which Cys residues 36, 41, and 46 were mutated to Ala (7, 10). N30C-PLB has been previously well characterized, and is fully functional with an inhibitory potency similar to wild-type PLB (7, 10). In control experiments,
identical results were obtained when N30C-PLB was made on the wild-type PLB background with Cys residues 36, 41, and 46 unaltered (data not shown). cDNAs encoding PLB3 and PLB4 were generated on the wild-type PLB cDNA background inserted in the transfection vector pVL1393, using the QuikChange™ XL-Gold system (Stratagene). D351A was made similarly using canine cardiac SERCA2a cDNA as the template (10). All mutated cDNAs were confirmed by DNA sequencing of the plasmid vectors. Baculoviruses encoding mutated proteins were generated as described previously with BaculoGold™ (Pharmengen) linearized baculovirus DNA (10).

Protein Expression and Characterization—SF21 insect cells were co-infected with baculoviruses encoding PLB and SERCA2a as described previously (4). Viral titers were adjusted to give an expression level of PLB to SERCA2a of ~4:1, as used in previous publications (7, 10–12, 17). Cells were harvested 60 h after co-infection, washed with phosphate-buffered saline, and homogenized with a Polytron for 90 s at 15,000 × g. Crude microsomal pellets were then collected by centrifuging at 48,000 × g for 20 min. Microsomes were re-suspended at a protein concentration of 6–10 mg/ml in 0.25 m sucrose, 10 mM MOPS (pH 7.0) and stored frozen in small aliquots at −40 °C. Protein concentrations were determined by the Lowry method. PLB and SERCA2a contents in the membrane samples were determined by quantitative Western blotting with monoclonal antibodies 2D12 and 2A7-A1, respectively (7). Only membranes expressing PLB and SERCA2a at a molar ratio of ~4:1 were used for further analyses. As shown in Fig. 3, all PLB mutants were predominantly monomeric on SDS-PAGE. The low pentamer stability of N30C-PLB made on the Cys-less PLB background was reported previously (7).

Ca\(^{2+}\)-ATPase Assay—Ca\(^{2+}\)-ATPase activities were measured at 37 °C in buffer containing 50 mM MOPS (pH 7.0), 100 mM KCl, 3 mM MgCl\(_2\), 3.0 mM ATP, 5 mM NaN\(_3\), and 3 μg of the Ca\(^{2+}\) ionophore, A23187, and 1 μg of EGTA. Ionized Ca\(^{2+}\) concentrations were set by varying the CaCl\(_2\) concentration from 0 to 1.2 mM. Assays were conducted in the presence and absence of the anti-PLB monoclonal antibody, 2D12, which reverses PLB inhibition of SERCA2a (11, 26). Ca\(^{2+}\)-dependent ATPase activities were determined in a reaction volume of 1 ml containing 50–100 μg of membrane protein during a 30–60-min incubation. P\(_i\) release from ATP was measured colorimetrically (7). Maximal Ca\(^{2+}\)-ATPase activities ranged between 15 and 25 μmol of P\(_i\)/mg of protein/h for all samples, which is ~25–40% of the maximal Ca\(^{2+}\)-ATPase activity typically reported for dog cardiac SR vesicles (27). In some Ca\(^{2+}\)-ATPase assays, small aliquots were taken from the assay tubes during the incubations, to simultaneously measure PLB cross-linking to SERCA2a (see below).

PLB Cross-linking to SERCA2a—In most experiments, cross-linking of N30C of PLB to Lys\(^{328}\) of SERCA2a with KMUS was conducted identically as previously described (10). Cross-linking reactions were conducted with 11 μg of membrane protein in 12 μl of buffer. The final concentrations of PLB and SERCA2a in the cross-linking tubes were 1.2 and 0.3 μM, respectively. Standard cross-linking buffer contained 50 mM MOPS (pH 7.0), 0.3 mM MgCl\(_2\), 100 mM KCl, 3 mM ATP, and 1 mM EGTA with zero to 1.2 mM added CaCl\(_2\). In some experiments, ATP concentrations were varied, or different nucleotides were used, as indicated in the figure legends. In the experiments with SERCA2a inhibitors TG and vanadate, TG was added from a 59 mM stock solution in ethanol, and sodium orthovanadate was added from a 15 mM stock solution in H\(_2\)O. Cross-linking reactions were started by adding 0.75 μl of 1.6 mM KMUS dissolved in Me\(_2\)SO (final KMUS concentrations 0.1 mM), and the incubations were conducted for 2 min at room temperature. Reactions were stopped by adding 7.5 μl of gel loading buffer containing 15% SDS and 100 mM dithiothreitol. The samples were subjected to SDS-PAGE, and Western blotting was performed with the anti-PLB antibody, 2D12, using 125I-protein A for PLB visualization. In the experiment of Fig. 6, blots were probed directly with 125I-2D12, and protein A was omitted (11). Radioactive signals (representing SERCA2a with bound PLB) were quantified using a Bio-Rad Personal FX Phosphimager. For economy of space, only the region of the autoradiographs containing PLB cross-linked to SERCA2a is displayed, except for the experiment shown in Fig. 7, A and B, in which the entire autoradiograph is shown.

For the data depicted in Figs. 4, 6, and 7, to assess Ca\(^{2+}\) effects on PLB cross-linking to SERCA2a, experiments were conducted at 37 °C in the same buffer used for measurement of Ca\(^{2+}\)-ATPase activity, as described above. 15 min after initiation of Ca\(^{2+}\)-ATPase reactions with ATP, 80–μl aliquots containing 8 μg of membrane protein were taken from Ca\(^{2+}\)-ATPase assay tubes and cross-linked with 1 mM KMUS for 15 s, giving the maximal cross-linking obtainable at each Ca\(^{2+}\) concentration tested. Reactions were stopped with gel loading buffer, and samples were then processed as described above. Cross-linking of PLB to D351A to assess Ca\(^{2+}\) affinity was determined under identical conditions.

It should be pointed out that the heterobifunctional cross-linking agent KMUS reacts irreversibly with Lys\(^{328}\) of SERCA2a and N30C of PLB whether the two proteins are bound or not. If the two proteins are bound when the cross-linker is added, then PLB is irreversibly cross-linked to SERCA2a by a single KMUS molecule. If the proteins are not bound when the cross-linker is added, then N30C of PLB can react with one KMUS molecule, and Lys\(^{328}\) of SERCA2a can react with a second KMUS molecule, thus blocking additional cross-linking of the two proteins as new PLB-SERCA2a complexes are formed. Therefore, the amount of PLB-SERCA2a complex detected by chemical cross-linking in this study is essentially a “snapshot” of the amount of PLB-SERCA2a complex present at the time at which the cross-linker is added.

Phosphorylation of E1-Ca\(_{2+}\) by [γ\(^{32}\)P]ATP—Phosphorylation of SERCA2a using [γ\(^{32}\)P]ATP was conducted by incubating 11 μg of membrane protein in 12 μl of buffer containing 50 mM MOPS (pH 7.0), 3.0 mM MgCl\(_2\), 100 mM KCl, 1 mM EGTA, and 0–1.2 mM CaCl\(_2\). Phosphorylation was initiated by adding a final concentration of 200 μM [γ\(^{32}\)P]ATP and conducted for 5 s at room temperature. Reactions were terminated with 7.5 μl of acidic gel loading buffer (pH 2.4) containing 3% lithium dodecyl sulfate, and lithium dodecyl sulfate-PAGE was conducted under acidic conditions as recently described (17). After electrophoresis, proteins were transferred to nitrocellulose and the
radioactive acylphosphoprotein bands were visualized by autoradiography and quantified with the Fx Phosphoimager.

RESULTS

**Ca**\(^{2+}\) Activation of **Ca**\(^{2+}\)-ATPase Activity and **Ca**\(^{2+}\) Inhibition of **PLB** Cross-linking—In the present study, SERCA2a was expressed alone in SF21 insect cells, or co-expressed with the three cross-linkable PLB mutants, N30C-PLB, PLB3, and PLB4, which were designed to be of increasing inhibitory strength. Prior to functional analyses, protein expression levels were quantified by Western blotting, and **Ca**\(^{2+}\)-ATPase activities were then corrected for small variability in SERCA2a expression between preparations (±20%). Fig. 3 demonstrates that similar levels of SERCA2a and PLB were co-expressed in the different membrane preparations.

SERCA2a expressed alone exhibited typical ATP hydrolysis, with half-maximal activation of **Ca**\(^{2+}\)-ATPase activity occurring at 0.16 μM **Ca**\(^{2+}\) (\(K_{Ca} = 0.16\) μM), and maximal enzyme activity reached at the saturating **Ca**\(^{2+}\) concentration of 1–2 μM (Fig. 4A and Table 1). At **Ca**\(^{2+}\) concentrations greater than 2 μM, substantial back inhibition of the enzyme by **Ca**\(^{2+}\) was observed (28, 29). Co-expression of SERCA2a with N30C-PLB

![Image 67x416 to 283x734]

FIGURE 3. Amido Black staining and immunoblot of SERCA2a co-expressed with N30C-PLB, PLB3, and PLB4. SERCA2a and N30C-PLB, PLB3, or PLB4 were co-expressed in SF21 insect cells. Membrane samples (11 μg) were then subjected to SDS-PAGE, transferred to nitrocellulose, and the nitrocellulose sheet stained with Amido Black (left panel). The nitrocellulose sheet was then cut in half, and the upper portion was probed with the anti-SERCA2a antibody, 2A7-A1, and the lower half was probed with the anti-PLB antibody, 2D12, followed by \(^{125}\)I-protein A (right panel). Control experiments showed that the 2D12 antibody bound with equal strength to all three PLB mutants (data not shown).

![Image 283x734]

FIGURE 4. **Ca**\(^{2+}\) activation of **Ca**\(^{2+}\)-ATPase activity and **Ca**\(^{2+}\) inhibition of **cross-linking**. SERCA2a was expressed alone or co-expressed with N30C-PLB, PLB3, or PLB4 in SF21 cells and SERCA2a and PLB expression levels were determined by Western blotting. Panel A depicts **Ca**\(^{2+}\)-ATPase activities of membrane fractions measured as described under “Experimental Procedures.” Enzyme activities were normalized to expression levels of SERCA2a expressed alone. The gray line intersecting the ordinate indicates the 50% \(V_{max}\) value determined for SERCA2a expressed alone. Panel B shows cross-linking of the PLB mutants to SERCA2 determined under identical conditions as the **Ca**\(^{2+}\)-ATPase assay. Aliquots were taken from the **Ca**\(^{2+}\)-ATPase assay and cross-linked for 15 s with 1 mM KMUS at 37 °C. Samples were then subjected to SDS-PAGE and immunoblotting with the anti-PLB antibody, 2D12. Protein bands in the upper panel show SERCA2a cross-linked with the PLB monomer. PLB cross-linking is quantified in the graph below. The graph in panel C was derived from the data in panels A and B. The percent of maximal PLB cross-linking to SERCA2a (determined in the absence of **Ca**\(^{2+}\)) was calculated at each **Ca**\(^{2+}\) concentration for each PLB mutant, and then plotted against the percent inhibition of **Ca**\(^{2+}\)-ATPase activity by PLB obtained at the same **Ca**\(^{2+}\) concentration. The percent inhibition of **Ca**\(^{2+}\)-ATPase activity by PLB at each **Ca**\(^{2+}\) concentration was calculated by dividing the **Ca**\(^{2+}\)-ATPase activity of membranes expressing SERCA2a plus PLB by the **Ca**\(^{2+}\)-ATPase activity of membranes expressing SERCA2a alone, and multiplying by 100.
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In Table 1, the $K_c$ values for Ca$^{2+}$-ATPase activity and phosphorylation of E1-Ca$_2$ with $[\gamma^{32}P]$ATP, and $K_i$ values for Ca$^{2+}$ inhibition of PLB cross-linking are shown. SERCA2a was expressed alone or co-expressed with N30C-PLB (N30C), PLB3, or PLB4 in insect cell microsomes. Ca$^{2+}$-ATPase activities and cross-linking were determined under identical conditions in the presence and absence of the anti-PLB antibody, 2D12, as described in the text. ND, not determinable.

**TABLE 1**

$K_c$ values (μM) for Ca$^{2+}$-ATPase activity and phosphorylation of E1-Ca$_2$ with [γ$^{32}$P]ATP, and $K_i$ values (μM) for Ca$^{2+}$ inhibition of PLB cross-linking

SERCA2a was expressed alone or co-expressed with N30C-PLB (N30C), PLB3, or PLB4 in insect cell microsomes. Ca$^{2+}$-ATPase activities and cross-linking were determined under identical conditions in the presence and absence of the anti-PLB antibody, 2D12, as described in the text. ND, not determinable.

| Protein expressed | Ca$^{2+}$-ATPase activity | E1-Ca$_2$ phosphorylation | $K_i$, values, cross-linking |
|-------------------|--------------------------|---------------------------|-----------------------------|
|                   | − 2D12 | + 2D12 | − 2D12 | + 2D12 |
| SERCA2a | 0.16 ± 0.01 | 0.16 ± 0.02 | 0.11 ± 0.02 | ND |
| +N30C | 0.33 ± 0.02 | 0.19 ± 0.00 | 0.36 ± 0.04 | ND |
| +PLB3 | 0.53 ± 0.05 | 0.26 ± 0.02 | 1.16 ± 0.19 | 0.88 ± 0.02 |
| +PLB4 | 0.70 ± 0.04 | 0.56 ± 0.01 | 2.05 ± 0.25 | 1.80 ± 0.17 |

$K_c$ values shown in parentheses were calculated based upon the maximal activity determined for SERCA2a expressed alone (see "Results"). Results are mean ± S.E. of two to six determinations.

Increased the $K_c$ value for enzyme activation ~2-fold, from 0.16 to 0.33 μM, with little or no effect on the $V_{max}$ of the enzyme measured at 1–2 μM Ca$^{2+}$ (Fig. 4A and Table 1). The effect of N30C-PLB on enzyme activity observed here is identical to the effect of wild-type PLB reported previously (4). In contrast to N30C-PLB, PLB3 and PLB4 had large effects on both the $K_c$ of enzyme activity and on $V_{max}$. The $K_c$ values were increased 3.3- (0.53 μM Ca$^{2+}$) and 4.4-fold (0.70 μM Ca$^{2+}$) by PLB3 and PLB4, respectively, when calculated based on the highest Ca$^{2+}$-ATPase activity achieved by SERCA2a co-expressed with these two mutants, which occurred at 2 μM Ca$^{2+}$. It should be noted that at 2 μM Ca$^{2+}$ concentration, the $V_{max}$ of the enzyme was inhibited by ~30% by both PLB3 and PLB4, relative to the same amount of SERCA2a expressed alone or with N30C-PLB (Fig. 4A). Nevertheless, complete reversal of Ca$^{2+}$-ATPase inhibition by PLB3 and PLB4 did occur at much higher Ca$^{2+}$ concentrations (in the range of 100–200 μM), Ca$^{2+}$ concentrations at which significant back inhibition of the enzyme occurred. Thus, in Ca$^{2+}$-ATPase assays, SERCA2a co-expressed with PLB3 or PLB4 can never achieve its maximal turnover rate, even though very high Ca$^{2+}$ concentrations do completely reverse Ca$^{2+}$-ATPase inhibition by the supershifters. To correct for back inhibition of the enzyme by Ca$^{2+}$, we also calculated the $K_c$ values for SERCA2a co-expressed with N30C-PLB, PLB3, and PLB4 using the $V_{max}$ value for SERCA2a expressed alone (Fig. 4A, gray lines intersecting the abscissa) ($K_c$ values). When calculated by this method, the $K_c$ values for SERCA2a co-expressed with N30C-PLB, PLB3, and PLB4 were 0.35, 1.16, and 2.05 μM (Table 1, parentheses). These corrected $K_c$ values indicate that 2.2-, 5.5-, and 9.3-fold higher Ca$^{2+}$ concentrations are required to half-maximally activate SERCA2a co-expressed with N30C-PLB, PLB3, and PLB4, respectively.

Next, the relative binding affinities of the PLB mutants for SERCA2a were estimated by measuring Ca$^{2+}$ inhibition of PLB cross-linking to the enzyme. All three PLB mutants cross-linked only to the cardiac Ca$^{2+}$ pump expressed in insect cell membranes, and all cross-linking results reported here depict the PLB monomer bound to SERCA2a (7, 10) (see "Experimental Procedures"). In the absence of Ca$^{2+}$, strong cross-linking of all three PLB mutants to Lys$^{328}$ of SERCA2a was observed, and cross-linking was completely eliminated by increasing Ca$^{2+}$ concentration (Fig. 4B). The $K_i$ values for Ca$^{2+}$ inhibition of N30C-PLB, PLB3, and PLB4 cross-linking to SERCA2a were 0.35, 0.88, and 1.8 μM Ca$^{2+}$, respectively (Table 1). These $K_i$ values for Ca$^{2+}$ inhibition of cross-linking agree closely with the $K_c$ values determined for half-maximal activation of Ca$^{2+}$-ATPase activity. This demonstrates a strong correlation between PLB binding to E2, and decreased Ca$^{2+}$ affinity of SERCA2a determined by the Ca$^{2+}$-ATPase assay. This conclusion is strengthened by plotting the percent maximal PLB cross-linking to SERCA2a (determined at each Ca$^{2+}$ concentration covering the Ca$^{2+}$ concentration range from 0.12 to 200 μM), against the percent inhibition of Ca$^{2+}$-ATPase activity determined at the same Ca$^{2+}$ concentrations (Fig. 4C). For all three PLB mutants, regardless of inhibitory strength, there was strong correlation ($r^2 = 0.97$) between the extent of PLB cross-linking to SERCA2a and degree of enzyme inhibition. These data strongly suggest that SERCA2a with bound PLB is catalytically inactive, and point to competitive binding of PLB and Ca$^{2+}$ as the mechanism of enzyme inhibition.

Consistent with the Ca$^{2+}$-ATPase results, similar shifts in $K_c$ by the different PLB mutants were observed when Ca$^{2+}$ stimulation of phosphoenzyme formation from ATP was monitored (Fig. 5). The $K_c$ for SERCA2a expressed alone was 0.11 μM, whereas when co-expressed with N30C-PLB, PLB3, and PLB4, the $K_c$ values were 0.36, 1.16, and 2.05 μM (Table 1), respectively. These $K_c$ values are nearly identical to the $K_i$ values determined for Ca$^{2+}$ inhibition of PLB cross-linking (gray lines). These results are particularly significant due to the fact that back inhibition of the Ca$^{2+}$ pump is not a factor when phosphoenzyme formation from $[\gamma^{32}P]$ATP is monitored, therefore, no correction for loss of enzyme turnover at high Ca$^{2+}$ concentrations is required when $K_c$ values are estimated by this method.

**Effect of 2D12 on Ca$^{2+}$-ATPase Activity and PLB Cross-linking**—The anti-PLB monoclonal antibody, 2D12, recognizes residues 7–13 of PLB and reverses PLB inhibition of the Ca$^{2+}$ pump by physically disrupting PLB binding to SERCA2a (11). 2D12 reverses enzyme inhibition by wild-type PLB (4, 26) and N30C-PLB virtually completely (11), but only partially reverses the effects of several supershifting PLB mutants on Ca$^{2+}$-ATPase activity (28). This suggests that the PLB supershifters may bind more tightly to the Ca$^{2+}$ pump than wild-type PLB or...
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N30C-PLB, but this has not been demonstrated directly. Therefore, to confirm tighter binding of the PLB supershifters, and to show that the $\text{Ca}^{2+}$ affinity of the enzyme is restored commensurate with dissociation of PLB from SERCA2a, we measured the effect of 2D12 on N30C-PLB, PLB3, and PLB4 cross-linking to SERCA2a simultaneously with $\text{Ca}^{2+}$-ATPase activity.

$\text{Ca}^{2+}$-ATPase activity of SERCA2a co-expressed with N30C-PLB, PLB3, and PLB4 was measured in the presence of 2D12 (2.2 $\mu$M), a concentration sufficient to completely saturate PLB (Fig. 6, A–C). As shown previously (11), addition of 2D12 restored the $\text{Ca}^{2+}$ affinity of SERCA2a co-expressed with N30C-PLB nearly completely, decreasing the $K_\text{Ca}$ from 0.33 to 0.19 $\mu$M $\text{Ca}^{2+}$, compared with 0.16 $\mu$M $\text{Ca}^{2+}$ for SERCA2a expressed alone (Table 1). On the other hand, 2D12 only partially restored the $\text{Ca}^{2+}$ affinity of the enzyme co-expressed with the superinhibitory mutants PLB3 and PLB4, shifting the $K_\text{Ca}$ values from 0.53 to 0.26 $\mu$M for PLB3, and from 0.70 to 0.36 $\mu$M for PLB4. Also, whereas 2D12 had little or no effect on the $V_{\text{max}}$ of the enzyme co-expressed with N30C-PLB, 2D12 increased the $V_{\text{max}}$ of the enzyme co-expressed with PLB3 and PLB4 significantly. In the presence of the antibody at 1–2 $\mu$M $\text{Ca}^{2+}$, $\text{Ca}^{2+}$ pumps co-expressed with PLB3 and PLB4 achieved 80–95% of the maximal activity of $\text{Ca}^{2+}$ pumps expressed alone (gray lines).

The effects of 2D12 on PLB cross-linking during the same assay are shown in Fig. 6, D–F. Consistent with previous results, 2D12 inhibited cross-linking of N30C-PLB to the $\text{Ca}^{2+}$ pump nearly completely in the absence of $\text{Ca}^{2+}$ and at all $\text{Ca}^{2+}$ concentrations tested (7, 11). This explains why $\text{Ca}^{2+}$ pump inhibition by N30C-PLB is 30% or less at each $\text{Ca}^{2+}$ concentration tested when $\text{Ca}^{2+}$-ATPase activity was measured in the presence of 2D12. On the other hand, PLB3 and PLB4 cross-linking to SERCA2a in the absence of $\text{Ca}^{2+}$ was substantially reduced by addition of 2D12, but not eliminated altogether (25 and 53% maximal cross-linking persisted, respectively). Even in the presence of the antibody, $\text{Ca}^{2+}$ concentrations of 1 $\mu$M or higher were required to completely dissociate PLB3 and PLB4 from the $\text{Ca}^{2+}$ pump (Fig. 6, E and F). These cross-linking results agree well with the results of the $\text{Ca}^{2+}$-ATPase assays, which showed that the enzyme was significantly inhibited by PLB3 and PLB4 even in the presence of 2D12. In experiments not shown, the binding affinity of PLB for 2D12 was determined to be 0.1 $\mu$M. Therefore, we conclude that the binding affinities of PLB3 and PLB4 for SERCA2a must be very high, at least within the range at which PLB binds 2D12.

Effect of $\text{Ca}^{2+}$ on PLB Cross-linking to D351A—To test directly for competition between PLB and $\text{Ca}^{2+}$ for binding to SERCA2a, we took advantage of the D351A pump mutant, D351A. During catalysis, Asp351 is phosphorylated by ATP to form the high-energy acylphosphoprotein intermediate, $E_1$–P–Ca$_2$ (Fig. 1). Replacement of aspartic acid at this position renders the enzyme catalytically inactive (14, 30). Although inactive at the site of ATP hydrolysis, D351A retains the ability to bind $\text{Ca}^{2+}$ and maintains the thermodynamic equilibrium between $E_1$ and $E_2$ (14, 24, 25). Therefore, if PLB acts by stabilizing $E_2$ and shifting the $E_1$–Ca$_2$ ↔ $E_2$–PLB equilibrium away from $E_1$, then this effect should be fully reproducible with D351A. The advantage of using D351A for these experiments is that enzyme turnover is prevented; hence the system is at equilibrium with respect to $\text{Ca}^{2+}$ binding (Fig. 1). Consistent with previous results with SERCA1a, we first confirmed that the D351A mutant made from SERCA2a exhibited no $\text{Ca}^{2+}$-ATPase activity, and was not phosphorylatable by [$\gamma$-32P]ATP to form $E_1$–P (30), nor by Pi to form $E_2$–P (14) (data not shown).

Next, the affinity of D351A for $\text{Ca}^{2+}$ was compared with that of wild-type SERCA2a by measuring $\text{Ca}^{2+}$ inhibition of N30C-PLB cross-linking. In the absence of $\text{Ca}^{2+}$, D351A and wild-type SERCA2a bound comparable amounts of N30C-PLB (Fig. 7, A and B). However, a strikingly lower $\text{Ca}^{2+}$ concentration was sufficient to disrupt N30C-PLB cross-linking to D351A ($K_i = 18 \text{ nm}$) compared with wild-type SERCA2a ($K_i = 280 \text{ nm}$) (Fig. 7C). In fact, the $\text{Ca}^{2+}$ affinity of D351A determined by this method (18 nm) is ~9-fold higher than the $\text{Ca}^{2+}$ affinity of wild-type SERCA2a estimated by the $\text{Ca}^{2+}$-ATPase assay (0.16 $\mu$M in Table 1). Assuming that N30C-PLB decreases the $\text{Ca}^{2+}$ affinity of D351A by ~2-fold (as it does for wild-type SERCA2a), the $\text{Ca}^{2+}$ affinity of D351A expressed alone is likely even higher than this, in the range of 10 nm. This remarkably high $\text{Ca}^{2+}$ affinity for D351A was first reported by MacIntosh et al. (24), but subsequently not confirmed (25) (see "Discussion"). Fig. 7A also points out the highly specific nature of the PLB to the SERCA2a cross-linking reaction, with PLB cross-linking exclusively to the $\text{Ca}^{2+}$ pump protein expressed in Sf21 membranes.
Ca\(^{2+}\) inhibition of N30C-PLB, PLB3, and PLB4 cross-linking to D351A was then measured (Fig. 7D). As predicted from results with wild-type SERCA2a, progressively higher concentrations of Ca\(^{2+}\) were also required to dissociate N30C-PLB (\(K_i = 18 \pm 3\) nM), PLB3 (\(K_i = 131 \pm 25\) nM), and PLB4 (\(K_i = 234 \pm 23\) nM) from D351A (means ± S.E. from 4 determinations). Fig. 7D demonstrates unambiguously that the super-shifting PLB mutants inhibit Ca\(^{2+}\) binding to D351A.

Effects of TG and Nucleotides on PLB Cross-linking—To confirm the relative binding affinities of the PLB mutants for SERCA2a, and to gain additional insights on the specific conformation of the Ca\(^{2+}\) pump that binds PLB, we determined the effects of TG and nucleotides on PLB cross-linking to SERCA2a, measured in the absence of Ca\(^{2+}\). It was shown previously that N30C-PLB binds preferentially to the E2 state of SERCA2a stabilized by bound nucleotide (7, 10), and that TG antagonizes formation of this state.

When measured in the absence of ATP, TG potently inhibited the cross-linking of all three PLB mutants to SERCA2a (Fig. 8, A and B). The \(K_i\) values for TG inhibition of N30C-PLB, PLB3, and PLB4 cross-linking to SERCA2a were low and similar (0.07, 0.07, and 0.10 μM, respectively) (Table 2), and within the range of Ca\(^{2+}\) pumps present within the reaction tubes (~0.3 μM). Thus, in the absence of nucleotide, under conditions favoring formation of E2 (absence of Ca\(^{2+}\)), TG binds virtually stoichiometrically to the Ca\(^{2+}\) pump (18, 31), whether co-expressed with N30C-PLB, PLB3, or PLB4. However, addition of 3 mM ATP to the assay tubes dramatically increased the concen-
igration of TG required to inhibit PLB3 and PLB4 cross-linking to SERCA2a. The $K_i$ values (Fig. 8, A and C) increased from 0.07 to 4.9 μM for PLB3, and from 0.10 to 7.8 μM for PLB4, whereas for N30C-PLB, addition of ATP only increased the $K_i$ value from 0.07 to 0.23 μM (Table 2). That is a remarkable 70-(PLB3) and 78-fold (PLB4) decrease in TG binding affinity induced by ATP when supershifting PLB mutants are present. It should be pointed out that the concentration of PLB present in the reaction tubes was ~1.0 μM, which is considerably lower than the concentration of TG required to significantly inhibit cross-linking of PLB3 and PLB4 to the Ca$^{2+}$-ATPase in the presence of ATP (Fig. 8C). Thus the affinity of the two PLB supershifters for E2-ATP must be even greater than the affinity of TG for E2-ATP, which is within the nanomolar range or lower (18, 31). The same results with PLB3 or PLB4 were obtained whether membranes were preincubated with TG for 5 or 60 min prior to initiation of the cross-linking reactions with KMUS, indicating that the supershifters prevent formation of a dead-end complex by TG (18) under these conditions.

Like ATP, ADP also dramatically increased the $K_i$ value for TG inhibition of PLB cross-linking to the Ca$^{2+}$-ATPase, whereas AMP had no significant effect (Fig. 9A demonstrated with PLB4). These results confirm previous findings that both ATP and ADP, but not AMP, stabilize the E2 state that favors PLB binding (7). We then measured the binding affinity of SERCA2a for ATP determined at different concentrations of TG (Fig. 9B). Successively higher concentrations of ATP were required to stimulate PLB4 cross-linking to the Ca$^{2+}$-ATPase when the concentration of TG was increased. In the absence of TG, the affinity of SERCA2a for ATP was 9 μM; in the presence of 6.4 μM TG, the affinity of the enzyme for ATP was decreased 10-fold, to ~100 μM (Table 3). These $K_{ATP}$ values for SERCA2a measured in the absence...
of Ca\(^{2+}\) agree well with those in previous reports, and confirm for SERCA2a that TG significantly reduces the affinity of the enzyme for ATP at the modulatory nucleotide-binding site (32–35). Collectively, these results demonstrate that PLB binds to a single conformation of SERCA2a, E2 with bound nucleotide, and this state is distinct from the E2 conformation binding TG (see “Discussion”).

Similar nucleotide effects on PLB binding to D351A were noted. The \(K_i\) values for TG inhibition of cross-linking of all three PLB mutants to D351A were low and similar when assessed in the absence of ATP, but dramatically increased for PLB3 and PLB4 when ATP was included (Table 2). TG also substantially decreased the ATP binding affinity of Asp351 (Fig. 9C and Table 3). Interestingly, the affinity of D351A for ATP was only about 2-fold greater than the affinity of wild-type SERCA2a for ATP (Table 3), which is substantially lower than the ATP binding affinity of D351A made from SERCA1a (24, 25) (see “Discussion”).

**Vanadate Effects on PLB Cross-linking**—According to results above with TG, the binding affinities of N30C-PLB and the supershifters for the E2 state of SERCA2a are much higher than previously predicted (36). Therefore, to confirm these surprising results, we used a second lower affinity Ca\(^{2+}\) pump inhibitor, vanadate, to estimate the binding affinities of the PLB mutants for SERCA2a. Vanadate inhibits the Ca\(^{2+}\)-ATPase with micromolar affinity, and like TG is proposed to bind preferentially to the nucleotide-free, E2 conformation of the Ca\(^{2+}\) pump (19). Fig. 10 shows that in the absence of Ca\(^{2+}\) and ATP, vanadate inhibited cross-linking of all three PLB mutants to SERCA2a. However, significantly higher concentrations of vanadate were required to inhibit PLB3 (\(K_i = 46 \mu\text{M}\)) and PLB4 (\(K_i = 380 \mu\text{M}\)) cross-linking to SERCA2a, relative to N30C-PLB (\(K_i = 1.6 \mu\text{M}\), Fig. 10B). Moreover, maximal cross-linking of PLB3 and PLB4 to SERCA2a could only be inhibited by 80 and 60% at 1 mM vanadate, the highest concentration tested. When 36 \(\mu\text{M}\) ATP was included in the buffer, the \(K_i\) for vanadate inhibition of N30C-PLB cross-linking to SERCA2a was increased 125-fold, from 1.6 (no nucleotide) to 200 \(\mu\text{M}\) vanadate (36 \(\mu\text{M}\) ATP), and cross-linking of PLB3 and PLB4 to SERCA2a became nearly completely vanadate resistant (Fig. 10C). At 3 \(\mu\text{M}\) ATP, vanadate failed to inhibit cross-linking of any PLB mutant to the Ca\(^{2+}\)-ATPase (data not shown). Thus, results with vanadate also show that PLB binds with surprisingly high affinity to the E2 state of SERCA2a when nucleotide is present.

**DISCUSSION**

In the present study, chemical cross-linking was used to monitor protein-protein interactions between the cardiac Ca\(^{2+}\) pump and PLB mutants of increasing inhibitory strength to investigate the physical basis of enzyme inhibition by PLB. Using these cross-linkable PLB mutants, new insights were gained on the effect of PLB on the Ca\(^{2+}\) pump (19). Fig. 10 shows that in the absence of Ca\(^{2+}\) and ATP, vanadate inhibited cross-linking of all three PLB mutants to SERCA2a. However, significantly higher concentrations of vanadate were required to inhibit PLB3 (\(K_i = 46 \mu\text{M}\)) and PLB4 (\(K_i = 380 \mu\text{M}\)) cross-linking to SERCA2a, relative to N30C-PLB (\(K_i = 1.6 \mu\text{M}\), Fig. 10B). Moreover, maximal cross-linking of PLB3 and PLB4 to SERCA2a could only be inhibited by 80 and 60% at 1 mM vanadate, the highest concentration tested. When 36 \(\mu\text{M}\) ATP was included in the buffer, the \(K_i\) for vanadate inhibition of N30C-PLB cross-linking to SERCA2a was increased 125-fold, from 1.6 (no nucleotide) to 200 \(\mu\text{M}\) vanadate (36 \(\mu\text{M}\) ATP), and cross-linking of PLB3 and PLB4 to SERCA2a became nearly completely vanadate resistant (Fig. 10C). At 3 \(\mu\text{M}\) ATP, vanadate failed to inhibit cross-linking of any PLB mutant to the Ca\(^{2+}\)-ATPase (data not shown). Thus, results with vanadate also show that PLB binds with surprisingly high affinity to the E2 state of SERCA2a when nucleotide is present.

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**DISCUSSION**

In the present study, chemical cross-linking was used to monitor protein-protein interactions between the cardiac Ca\(^{2+}\) pump and PLB mutants of increasing inhibitory strength to investigate the physical basis of enzyme inhibition by PLB. Using these cross-linkable PLB mutants, new insights were gained on the effect of PLB on the Ca\(^{2+}\) affinity of the enzyme, the binding affinity of PLB for the Ca\(^{2+}\) pump, and the specific conformation of SERCA2a required for PLB binding.

**Effect of PLB on Ca\(^{2+}\) Binding Affinity and \(V_{\text{max}}\)**—The hallmark of PLB regulation of SERCA2a is its ability to decrease the apparent Ca\(^{2+}\) affinity of the Ca\(^{2+}\)-ATPase, while having little or no effect on the \(V_{\text{max}}\) of the enzyme measured at saturating

| Protein expressed | \(K_{\text{TG}}\) values (\(\mu\text{M}\)) for TG inhibition of PLB cross-linking to Ca\(^{2+}\)-ATPase |
|-------------------|--------------------------------------------------|
|                   | No Nuc | + ATP |
| SERCA2a           |       |       |
| +N30C             | 0.07 ± 0.02 | 0.23 ± 0.05 |
| +PLB3             | 0.07 ± 0.01 | 4.87 ± 0.53 |
| +PLB4             | 0.10 ± 0.01 | 7.78 ± 1.0 |
| D351A             |       |       |
| +N30C             | 0.07 ± 0.005 | 0.17 ± 0.04 |
| +PLB3             | 0.08 ± 0.02 | 13.3 ± 5.2 |
| +PLB4             | 0.11 ± 0.02 | 24.3 ± 1.9 |

![A](image1.png)

**FIGURE 8. TG effect on PLB cross-linking.** A, autoradiographs showing concentration dependence of TG inhibition N30C-PLB, PLB3, and PLB4 cross-linking to SERCA2a, measured in the absence (+ATP) and presence (−ATP) of 3 mM ATP. B, and C, graphs of TG inhibition of cross-linking, determined in the absence and presence of 3 mM ATP, respectively.
Ca\textsuperscript{2+} concentration (1, 2). However, whether PLB increases the \(K\text{Ca}\) of \(\text{Ca}\textsuperscript{2+}/\text{H}\text{11001}\)-ATPase activation by decreasing the actual \(\text{Ca}\textsuperscript{2+}/\text{H}\text{11001}\) binding affinity of the enzyme (7, 12, 22, 37), or by affecting one or more catalytic steps in the reaction cycle (23, 26) has remained unclear. Here we addressed this question directly, using cross-linkable PLB mutants of increasing inhibitory potency (PLB4 > PLB3 > N30C-PLB). We showed that successively higher \(\text{Ca}\textsuperscript{2+}\) concentrations were required to both activate the enzyme co-expressed with N30C-PLB, PLB3, and PLB4 and to dissociate N30C-PLB, PLB3, and PLB4 from the \(\text{Ca}\textsuperscript{2+}\) pump. Moreover, there was a direct correlation between the degree of PLB binding to SERCA2a and the extent of PLB inhibition of \(\text{Ca}\textsuperscript{2+}/\text{H}\text{11001}\)-ATPase activity at all \(\text{Ca}\textsuperscript{2+}\) concentrations tested with all three PLB mutants (Fig. 4C). These results strongly suggest that PLB competes with \(\text{Ca}\textsuperscript{2+}\) for binding to the \(\text{Ca}\textsuperscript{2+}/\text{H}\text{11001}\)-ATPase, and that SERCA2a with PLB bound is catalytically inactive. Competition between PLB and \(\text{Ca}\textsuperscript{2+}\) for binding to SERCA2a was confirmed using the \(\text{Ca}\textsuperscript{2+}\) pump mutant D351A, which retains \(\text{Ca}\textsuperscript{2+}\) binding, but cannot hydrolyze ATP. Progressively higher \(\text{Ca}\textsuperscript{2+}\) concentrations were also required to dissociate the increasingly potent PLB mutants from D351A. Thus at each \(\text{Ca}\textsuperscript{2+}\) concentration tested, progressively more \(E_2\) PLB was formed by the increasingly inhibitory PLB mutants, meaning that less \(E_1\) was available for \(\text{Ca}\textsuperscript{2+}\) binding (Fig. 7D). Therefore, by stabilizing the enzyme in a \(\text{Ca}\textsuperscript{2+}\)-free state, PLB decreases \(\text{Ca}\textsuperscript{2+}\) binding to the pump and alters the kinetics of enzyme activation by \(\text{Ca}\textsuperscript{2+}\).

We also confirmed that PLB molecules of normal inhibitory strength (N30C-PLB) do not significantly affect the \(V\text{max}\) of the \(\text{Ca}\textsuperscript{2+}/\text{H}\text{11001}\)-ATPase (1). This is contrary to conclusions of several recent studies in which PLB was reported to either decrease (22) or increase (20, 21, 23) the \(V\text{max}\) of the \(\text{Ca}\textsuperscript{2+}/\text{H}\text{11001}\)-ATPase. Using our viral constructs and the 2D12 antibody, Waggoner et al. (22) recently noted a modest reduction (\(-20\%) in the \(V\text{max}\) of SERCA2a co-expressed with wild-type PLB compared with SERCA2a expressed alone. This is in disagreement with an earlier study in which no effect on \(V\text{max}\) was noted (37). We believe that the modest reduction in \(V\text{max}\) observed by Waggoner et al. (22) is more apparent than real. Fig. 4A points out that when \(\text{Ca}\textsuperscript{2+}/\text{H}\text{11001}\)-ATPase activities are carefully corrected for \(\text{Ca}\textsuperscript{2+}\) pump expression levels, there is little or no inhibition of the enzyme at saturating \(\text{Ca}\textsuperscript{2+}\) concentrations when SERCA2a is co-expressed with PLB mutants of normal inhibitory potency (N30C-PLB). Moreover, this relief of \(\text{Ca}\textsuperscript{2+}/\text{H}\text{11001}\)-ATPase inhibition at saturating \(\text{Ca}\textsuperscript{2+}\) concentration is entirely

\begin{table}[h]
\centering
\begin{tabular}{lcc}
\hline
\textbf{TG (mM)} & \textbf{SERCA2a} & \textbf{D351A} \\
\hline
0 & 9.0 ± 1.5 & 4.0 ± 0.9 \\
0.23 & 34.0 ± 7.8 & 15.3 ± 2.7 \\
2.11 & 65.0 ± 10.4 & 24.0 ± 1.5 \\
6.41 & 103 ± 12.0 & 46.0 ± 4.0 \\
\hline
\end{tabular}
\caption{\(K\text{ATP}\) values (mM) for ATP stimulation of PLB4 cross-linking to the \(\text{Ca}\textsuperscript{2+}/\text{H}\text{11001}\)-ATPase, determined at different TG concentrations. Results are the mean ± S.E. of three to five determinations.}
\end{table}
consistent with the complete dissociation of N30C-PLB from SERCA2a observed at 1–2 μM Ca\(^{2+}\) by chemical cross-linking (Fig. 4B). The studies in which wild-type PLB and some other PLB mutants were reported to actually increase the \(V_{\text{max}}\) of the Ca\(^{2+}\)-ATPase were all conducted with the purified rabbit skeletal muscle enzyme co-reconstituted with purified PLB from detergent solution (20, 21, 23). In this case, it is possible that enzyme protection by PLB during the reconstitution process may have artifically affected the results, as was recently suggested (23). Regardless, in multiple studies using cellular expression systems, no increase in \(V_{\text{max}}\) by PLB has been noted (1, 2).

The results shown here illustrate how PLB is perfectly poised to regulate cardiac contractile kinetics in intact myocardium (Fig. 4). Ca\(^{2+}\) concentrations within the cardiomyocyte range from nanomolar to 1–2 μM (38), and the affinity of PLB for SERCA2a allows it to associate and dissociate from the enzyme over the same Ca\(^{2+}\) concentration range at which contractile force develops. At low cytosolic Ca\(^{2+}\) concentrations at which myofilament contractile force is low, the affinity of PLB for the Ca\(^{2+}\) pump is high and enzyme inhibition by PLB is substantial, but still reversible by phosphorylation by protein kinase A (1, 2) or by the 2D12 antibody (39). At high Ca\(^{2+}\) concentrations yielding peak contractile force, PLB is completely dissociated from the Ca\(^{2+}\) pump and the enzyme is maximally active. However, for the supershifting PLB mutants the situation is different. By virtue of their very high binding affinities for SERCA2a, the supershifters remain significantly bound to the Ca\(^{2+}\) pump and continue to inhibit the enzyme at Ca\(^{2+}\) concentrations that are normally saturating. At Ca\(^{2+}\) concentrations high enough to dissociate these potent PLB molecules from the Ca\(^{2+}\) pump (10–200 μM Ca\(^{2+}\)) (Fig. 4), significant back inhibition of the enzyme occurs. Thus, maximal Ca\(^{2+}\)-ATPase activity can never be realized when SERCA2a is co-expressed with potent PLB supershifters, even after phosphorylation of PLB by protein kinase A or after addition of the 2D12 antibody. This may explain why transgenic mice over-expressing the most potent PLB supershifters develop heart failure and premature death (16).

**D351A**—Using PLB as a reporter molecule, we were able to estimate the Ca\(^{2+}\) and nucleotide binding affinities of D351A relative to wild-type SERCA2a, and make comparisons with previous determinations made for the skeletal muscle enzyme (SERCA1a). In an earlier study, MacIntosh et al. (24) used 8-Ni\(^{3+}\)-TNP-ATP photolabeling to measure the Ca\(^{2+}\) and ATP binding affinities of D351A (rabbit skeletal isoform) expressed in COS membranes. The authors found that relative to wild-type Ca\(^{2+}\)-ATPase, D351A had an extraordinarily high affinity for both Ca\(^{2+}\) (>10-fold increase) and ATP (20–100-fold increase) (24).

They postulated that Ala substitution at Asp351 significantly increases the ATP affinity of the Ca\(^{2+}\)-ATPase by relieving electrostatic repulsion between the γ-phosphate of ATP and Asp351 of the wild-type enzyme. Moreover, they proposed that mutationally induced conformational changes at the site of ATP binding within the cytoplasmic head group were transmitted to the Ca\(^{2+}\) binding sites located at the membrane, substantially increasing the Ca\(^{2+}\) affinity of the enzyme. The very high ATP affinity of D351A, but not the high Ca\(^{2+}\) affinity, was confirmed in a subsequent study by Marchand et al. (25), also with SERCA1a. In this later report, ATP and Ca\(^{2+}\) binding affinities were determined for the purified enzyme in detergent solution.

Here, using PLB cross-linking to estimate Ca\(^{2+}\) affinity, we also noted an extremely high Ca\(^{2+}\) affinity for D351A, this time using the cardiac muscle isoform (SERCA2a). Our results indicate that D351A has a Ca\(^{2+}\) affinity at least 10 times higher than wild-type SERCA2a (Fig. 7A). This result is consistent with the earlier findings of MacIntosh et al. (24), but inconsistent with the results of Marchand et al. (25). It is well known that non-ionic detergents like C\(_{12}\)E\(_{8}\) and dodecylmaltoside substantially decrease the Ca\(^{2+}\) binding affinity of SERCA pumps (40, 41), which may explain the failure of Marchand et al. (25) to detect an increase in Ca\(^{2+}\) affinity for D351A.

Regarding ATP affinity, we determined a \(K_d\) value of 9 μM for the wild-type enzyme, which is well within the range reported by other investigators for ATP binding at the low-affinity modulatory binding site of E2 measured in the absence of Ca\(^{2+}\).
For D351A, we noted a modest 2.3-fold increase in ATP affinity relative to wild-type SERCA2a ($K_d = 4.0 \mu M$), in contrast to the two studies above that reported a much higher nucleotide binding affinity for D351A measured under similar conditions (24, 25). However, our results appear to be consistent with the recently determined crystal structure of the E2(TG)-AMPPCP complex, representing E2 with ATP bound at the modulatory site (34). According to this structure, ATP fits more loosely into the modulatory site (ATP binding site in E2) relative to the catalytic site. When ATP is bound to E2, the γ-phosphate is 9 Å away from the phosphorylation site, making the electrostatic repulsion between the γ-phosphate and the negatively charged Asp$^{351}$ much less pronounced than what occurs when ATP is bound to E1 (34). Thus ATP affinity at the modulatory site may be less affected by the D351A mutation because the γ-phosphate of ATP does not interact closely with Asp$^{351}$ when ATP is bound here. Nevertheless, our results with D351A demonstrate that there is long-range communication between the catalytic site and the Ca$^{2+}$ binding sites, and removal of the negative charge at Asp$^{351}$ strikingly enhances the Ca$^{2+}$ binding affinity at the two Ca$^{2+}$ binding sites in the membrane.

E2-ATP Conformation—Early studies showed that in the absence of Ca$^{2+}$, the intrinsic tryptophan fluorescence of SERCA was substantially increased by the nucleotides, ATP and ADP, but was unaffected by AMP (32). This nucleotide-induced increase in fluorescence intensity was completely inhibited by TG, which was subsequently shown to reduce the affinity of the Ca$^{2+}$-ATPase for ATP through uncompetitive inhibition (33–35). Similarly, PLB cross-linking to SERCA2a occurs in the absence of Ca$^{2+}$, is enhanced by ATP and ADP, but inhibited by TG (7, 11). Based upon these similarities, it was suggested that the physiological state detected by changes in fluorescence induced by nucleotide binding to E2 (32–35) is the unique E2-ATP state that binds PLB (7).

In a recent study by Jensen et al. (34), it was suggested that TG stabilizes the fully protonated H$_n$E2 state of the Ca$^{2+}$ pump, and that ATP binding at the modulatory site stimulates deprotonation of E2, initiating the transition to E1. Here, using ATP stimulation of PLB cross-linking to measure ATP binding at different TG concentrations, we confirmed the ATP affinities of E2 and E2-TG reported previously (32–35). Moreover, we showed that ATP dramatically increases the resistance of the E2-PLB complex to TG, shifting the $K_i$ values for TG inhibition of cross-linking by 100–200-fold for the supershifters PLB3 and PLB4 (Table 2). Thus, the PLB supershifters and ATP interact synergistically at E2, stabilizing an E2-ATP-PLB ternary complex that is remarkably resistant to TG. These results suggest that the E2-ATP state detected by ATP-induced changes in Trp fluorescence (32–35) and by chemical cross-linking of PLB (7, 11), may be the deprotonated E2-ATP state with ATP bound at the modulatory site. Moreover, TG may inhibit formation of this specific conformation, not by blocking ATP binding, but by hindering ATP-stimulated deprotonation of the enzyme. Consistent with this interpretation, PLB does not bind to the P$_i$ (17) or vanadate (Fig. 10) bound forms of the enzyme, both of which interact with the protonated H$_n$E2 state like TG (42). Also as observed with TG, ATP strongly enhances PLB cross-linking to SERCA2a in the presence of P$_i$ (17) and vanadate (Fig. 10), being able to compete for P$_i$ (43) or vanadate binding (19, 44) to the Ca$^{2+}$-ATPase.

Given the reputation of TG as an extremely potent, irreversible inhibitor of the Ca$^{2+}$-ATPase (18, 31), we were surprised to discover that TG did not disrupt the ternary complex between the PLB supershifters and E2-ATP, even when membranes were preincubated for up to 1 h with greater than stoichiometric concentrations of TG. Moreover, under these conditions favoring E2-ATP, PLB3 and PLB4 bind even more tightly to SERCA2a than does TG, the highest affinity SERCA inhibitor identified to date (18, 31). Crystallographic studies have revealed that TG binds to E2 in a cavity formed between transmembrane helices M3, M5, and M7, near the cytoplasmic membrane surface (45). This is on the opposite face from the PLB binding site, which is predicted to extend along the groove formed between transmembrane helices M2, M4, and M9, based on cross-linking results (7, 17, 36). Our results suggest that binding of PLB at its site must drastically distort the TG binding pocket. Nonetheless, under enzyme turnover conditions, TG is the more powerful SERCA inhibitor. In the presence of Ca$^{2+}$, the catalytic activity is completely inhibited by TG through formation of a dead-end complex (18), whereas Ca$^{2+}$-ATPase inhibition by the PLB supershifters remains reversible, albeit at very high Ca$^{2+}$ concentrations (Fig. 4). It should be pointed out that TG binding to E1-Ca$^{2+}$ as well as to E2 has been noted in many studies (18, 34, 46–48), and that the ability of TG to bind to different conformational states of SERCA may contribute to its apparently irreversible effect on Ca$^{2+}$-ATPase activity.

The overall conclusion of this work is that PLB inhibits Ca$^{2+}$ binding to SERCA2a by stabilizing the enzyme in a Ca$^{2+}$-free E2 state. Clearly, PLB binding has long-range conformational effects on both the cytoplasmic domains and the transmembrane domain, and these effects may be even more profound with the PLB supershifters. ATP binding to E2 accelerates the E2 to E1-Ca$^{2+}$ transition by stimulating H$^+$/Ca$^{2+}$ cation exchange (34), while at the same time inducing structural changes that promote PLB binding. So, is the conformation of SERCA2a that binds to PLB really deprotonated E2-ATP, or Ca$^{2+}$-free E1 (35), or perhaps something in between (47)? Until the crystal structure of PLB-bound SERCA2a is determined we have no way of knowing. It was recently suggested that TG “rigidifies” the transmembrane domain of the Ca$^{2+}$ pump, making it unresponsive to conformational changes occurring within the cytosolic domain (35). It is this ability of TG to fix the transmembrane helices that has enabled the Ca$^{2+}$-free, TG-bound enzyme to be crystallized, providing valuable structural information about the Ca$^{2+}$-ATPase in different E2 states (45). However, all of the E2 structures determined to date have been in the presence of irreversible inhibitors like TG or cyclopiazonic acid (49), and it is unclear how closely these inhibitor-bound structures resemble other, perhaps more physiological states of the enzyme (35). It is therefore our long-term goal to crystallize the Ca$^{2+}$ pump complexed with PLB to provide a structure of E2 stabilized by a reversible inhibitor that is physiologically active in the heart. Here, we have shown that in the absence of Ca$^{2+}$, the binding affinities of the supershifters are
severalfold higher than even TG, making the goal of crystallizing the Ca^{2+}-free enzyme stabilized by PLB3 or PLB4 seem plausible.

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