Close Proximity between Residue 30 of Phospholamban and Cysteine 318 of the Cardiac Ca\(^{2+}\) Pump Revealed by Intermolecular Thiol Cross-linking*

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Phospholamban (PLB) is a 52-amino acid inhibitor of the Ca\(^{2+}\)-ATPase in cardiac sarcoplasmic reticulum (SERCA2a), which acts by decreasing the apparent affinity of the enzyme for Ca\(^{2+}\). To localize binding sites of SERCA2a for PLB, we performed Cys-scanning mutagenesis of PLB, co-expressed the PLB mutants with SERCA2a in insect cell microsomes, and tested for cross-linking of the mutated PLB molecules to SERCA2a using 1,6-bismaleimidohexane, a 10-Å-long, homobifunctional thiol cross-linking agent. Of several mutants tested, only PLB with a Cys replacement at position 30 (N30C-PLB) cross-linked to SERCA2a. Cross-linking occurred specifically and with high efficiency. The process was abolished by micromolar Ca\(^{2+}\) or by an anti-PLB monoclonal antibody and was inhibited 50% by phosphorylation of PLB by cAMP-dependent protein kinase. The SERCA2a inhibitors thapsigargin and cyclopiazonic acid also completely prevented cross-linking. The two essential requirements for cross-linking of N30C-PLB to SERCA2a were a Ca\(^{2+}\)-free enzyme and, unexpectedly, a micromolar concentration of ATP or ADP, demonstrating that N30C-PLB cross-links preferentially to the nucleotide-bound, E2 state of SERCA2a. Sequencing of a purified proteolytic fragment in combination with SERCA2a mutagenesis identified Cys\(^{318}\) of SERCA2a as the sole amino acid cross-linked to N30C-PLB. The proximity of residue 30 of PLB to Cys\(^{318}\) of SERCA2a suggests that PLB may interfere with Ca\(^{2+}\) activation of SERCA2a by a protein interaction occurring near transmembrane helix M4.

Ca\(^{2+}\)-ATPase activities or SERCA\(^{1}\) enzymes are 100-kDa integral membrane proteins responsible for the active transport of Ca\(^{2+}\) into the sarco(endo)plasmic reticulum (1). In heart, the predominant Ca\(^{2+}\)-ATPase expressed is SERCA2a (1), which pumps Ca\(^{2+}\) into the SR lumen, causing cardiac muscle relaxation (2). A unique property of cardiac muscle is the regulation of SERCA2a by PLB, a small, single span membrane protein (3, 4), which inhibits the Ca\(^{2+}\)-ATPase by decreasing its apparent affinity for Ca\(^{2+}\) (5). Phosphorylation of PLB at Ser\(^{19}\) by PKA and at Thr\(^{17}\) by Ca\(^{2+}\)/calmodulin-dependent protein kinase relieves the inhibitory action of PLB on SERCA2a, giving an increase in the rate of cardiac muscle relaxation as well as a positive inotropic effect (6, 7). Currently, there is much interest in understanding the molecular mechanism of SERCA2a inhibition by PLB, both as a paradigm for understanding membrane protein interactions and for the potential of targeting drugs to this system to treat heart failure (6, 7).

Phospholamban has an interesting structure (6). Containing only 52 amino acids, the protein is a homopentamer in the membrane held together by Leu/Ile zipper interactions occurring in the transmembrane region (residues 32–52) (8). The cytoplasmic domain (residues 1–31) is highly charged and basic and is postulated to interact with SERCA2a by both electrostatic and hydrophobic interactions (9, 10). PLB mutagenesis studies suggest that the PLB monomer is the active inhibitory species, which dissociates from the pentamer, binds to SERCA2a, and inhibits the Ca\(^{2+}\)-ATPase by direct protein interactions (11–16).

Considerable attention has been given to delineating the three-dimensional structural interactions between PLB and SERCA2a, especially in light of the recent crystal structure determination of SERCA1a (the skeletal muscle isoform) to 2.6-Å resolution (17). An indirect approach for studying protein structure is to use mutagenesis and, by producing loss of function or gain of function PLB mutants, infer sites in both the cytoplasmic (10, 18) and membrane (11, 13, 15) regions of PLB that may be important for regulatory interactions with the Ca\(^{2+}\) pump. Another approach is to use chemical cross-linking to directly identify physical contact points. In an earlier reconstitution study by James et al. (9), Lys\(^{\text{5\text{O}}}\) of PLB was shown to photoaffinity-label to residues 397–400 of SERCA2a. A functional requirement for these SERCA2a residues in transducing PLB inhibition was subsequently demonstrated by Toyofuku et al. (19) using replacement mutagenesis. However, this strategy of purification and reconstitution followed by photoaffinity labeling (9) is difficult to execute with PLB and SERCA2a and has not been duplicated in other laboratories. More recent approaches to address physical interactions between PLB and SERCA2a are cryoelectron microscopy of PLB/SERCA2a co-crystals (20) and assessment of fusion protein interactions (21).

Here we describe direct chemical cross-linking of PLB to SERCA2a in microsomes in situ with no reconstitution required. A Cys-substituted mutant of PLB (N30C-PLB) and
native SERCA2a were co-expressed in SF21 insect cell microsomes (13, 15), and cross-linking of the two molecules was achieved with the homobifunctional thiol probe, BMH (22). N30C-PLB is shown to label SERCA2a specifically and with high efficiency. Coupling occurs at only one of the 26 (13) endogenous Cys residues of SERCA2a, Cys318. Characterization of factors modulating the cross-linking signal gives several new insights into the mechanism of Ca2+ pump inhibition by PLB.

**EXPERIMENTAL PROCEDURES**

**Materials** — BMH was obtained from Pierce. Thapsigargin and cyclosporin A were purchased from Sigma. Mouse recombinant PKA was from Calbiochem, and endo-Asp-N and endo-Lys-C were obtained from Roche Molecular Biochemicals.

**Mutagenesis** — Point mutations were introduced into the cDNA encoding the canine isoform of PLB, using the Altered Sites II Mutagenesis System™ (13, 15). All mutations were confirmed by DNA sequencing.

**Protein Co-Expression and Isolation of Microsomes**—Canine SERCA2a and PLB were co-expressed in SF21 insect cells as described (13, 15). Most co-expressions were with wild-type SERCA2a and N30C-PLB (on the Cys-less PLB background). Microsomes were harvested 60 h after initiating baculovirus infections and stored frozen in small aliquots at −40 °C at a protein concentration of 6–10 mg/ml (25).

Cross-linking between N30C-PLB and SERCA2a remained intact after several freeze-thaw cycles of microsomal membranes.

**Cross-linking**—Sulphydryl cross-linking was performed at room temperature using the homobifunctional cross-linking agent BMH (Pierce). The reactions were conducted with 11 μl of microsomal protein in 12 μl of buffer A, consisting of 40 mM MOPS (pH 7.0), 3.2 mM MgCl2, 75 mM KCl, 3 mM ATP, and 1 mM EGTA. Incubations were routinely conducted for 1 h in the presence of 0.1 mM BMH. Reactions were started by addition of 1 μl of BMH from a 1.6 mM stock solution in dimethyl sulfoxide and stopped by adding 7.5 μl of SDS-PAGE sample-loading buffer (13) containing 15% SDS plus 100 μM dithiobenitol. After terminating the reactions, samples were subjected to SDS-PAGE and immunoblotting.

To assess Ca2+ effects on cross-linking, ionized Ca2+ was varied by adding CaCl2 to buffer A (13, 15). When PKA effects on cross-linking were tested, 1 μg of the catalytic subunit was added to 11 μg of microsomes in buffer A, and phosphorylation was conducted for 10 min at room temperature before initiating cross-linking by the addition of BMH. For PKA experiments, the final concentration of BMH was increased to 2.0 mM to compensate for the ∼1.5 mM final concentration of mercaptoethanol contributed by the PKA preparation. In some experiments, ATP in buffer A was replaced by other nucleotides, as indicated.

**SDS-PAGE and Immunoblotting**—SDS-PAGE using 8% polyacrylamide (26) and immunoblotting were performed as described previously (13, 15). For detection of PLB, blots were probed with anti-PLB monoclonal antibody, 2A7-A1, which recognizes residues 386–394 (26) and 2D12, which recognizes residues 7–15 (Jerini Bio Tools). Antibody-binding protein bands were visualized by 125I-protein A and autoradiography and quantified using a Bio-Rad Molecular Imageer Fx. In two experiments, the procedure was modified slightly. In the experiment in which the anti-PLB monoclonal antibody effect on BMH cross-linking was examined (Fig. 4A), the 125I-protein A step was omitted and the immunoblot was probed with 125I-2D12 directly, iodinated using IODO-GEN (Bio-Rad). This was done to eliminate interference from 125I-protein A binding to the 2D12 antibody carried over from the cross-linking samples. In the experiment in which the PKA effect on cross-linking was examined (Fig. 4B), the immunoblot was probed with our anti-PLB monoclonal antibody, 1F1, raised to residues 1–10 of canine PLB, instead of 2D12. In control experiments, we observed that phosphorylation of PLB at Ser6 by PKA partially inhibited 2D12 binding to PLB, apparently due to steric or conformational effects. Phosphorylation of PLB by PKA had no effect on 1F1 binding to PLB.

**Ca2+-ATPase Assay—Ca2+-ATPase activity of microsomes co-expressing SERCA2a and N30C-PLB was measured in the presence of the anti-PLB antibody, 2D12, in buffer containing 50 mM MOPS (pH 7.0), 3 mM MgCl2, 100 mM KCl, 5 mM NaCl, 5 μM μg/ml A23187, 3 mM ATP, and 1 mM EGTA (13, 15). Ionized Ca2+ concentration was varied by adding CaCl2 (13, 15).

**SERCA2a Purification, Protocols, and Isolation of Cross-linked Peptide**—In order to identify which Cys residue of SERCA2a was cross-linked by the cross-linking reagent, the sample was subjected to gel filtration and chromatographies were then used to obtain a purified SERCA2a peptide covalently attached to N30C-PLB, as follows. In round 1, SERCA2a was isolated from the cross-linked microsomal pellet using anti-SERCA (2A7-A1) monoclonal antibody affinity chromatography (Fig. 4), followed by three additional 6-ml rinses with 20 mM MOPS (pH 7.2) and 0.1% Triton X-100 (fractions 5–7). Purified SERCA2a was then eluted in fractions 8–12 with 2D12-A1 affinity beads. A flow-through fraction was collected, and the beads were then washed with four consecutive 6-ml rinses of 20 mM MOPS (pH 7.2), 0.5 mM NaCl, and 0.2% Triton X-100 (fractions 1–4), followed by fractions 8–12 with 2A7-A1 affinity beads. The column was processed identically to that described for round 1, employing 2.8-ml rinses for each fraction. The 6-ml MOPS buffer not cross-linked to N30C-PLB passed freely through the column and were recovered in the flow-through fraction and fraction 1. Only SERCA2a molecules cross-linked to N30C-PLB were retained by the column and recovered in the acidic pH elution fractions (fractions 8–12). Fractions 8–12 were eluted into concentrated MOPS to return the pH to 7.2 as described above.

In round 2, fractions 8–12 from round 1 containing purified SERCA2a were pooled and subjected to anti-PLB (2D12) monoclonal antibody affinity chromatography (28). Pooled fractions 8–12 from round 1 were re-equilibrated in 1% SDS, 3.5% Triton X-100 and then loaded over 2.8 ml of 2D12 affinity beads. The column was processed identically to that described for round 1, employing 2.8-ml rinses for each fraction. The 6-ml MOPS buffer not cross-linked to N30C-PLB was retained by the column and were recovered in the flow-through fraction and fraction 1. Only SERCA2a molecules cross-linked to N30C-PLB were eluted from the column and recovered in the acidic pH elution fractions (fractions 8–12). Fractions 8–12 were eluted into concentrated MOPS to return the pH to 7.2 as described above.

In round 3, the purified, cross-linked product from round 2 was proteolyzed, and the cross-linked peptide was isolated. Fractions 8–12 from round 2 in 90 mM MOPS, 18 mM glycine, and 0.1% Triton X-100 (pH 7.2) were pooled and Amicon-concentrated to 1 ml. 8 μg of endo-Asp-N was then added, and proteolysis was conducted for 4 h at 37 °C. Digestion was terminated by adding 10 mM EDTA, and then 20 μg of endo-Lys-C was added, and the proteolysis continued overnight at 37 °C. The next morning, endo-Lys-C digestion was terminated by the addition of 0.3 mM 1-chloro-3-tosylamido-7-aminoo-2-heptanone, followed by the addition of 1% SDS and placement of the sample in a boiling water bath for 10 min. 3.5% Triton X-100 was then added, and the SERCA2a peptide cross-linked to N30C-PLB was isolated using anti-PLB monoclonal antibody affinity chromatography as described in round 2 above.

**Peptide Sequencing**—Peptides were subjected to SDS-PAGE and transferred to Immobilon-P™ (Millipore Corp.) for sequencing. Sequencing was performed with an Applied Biosystems 492 Protein Sequencer at the Laboratory for Macromolecular Structure (Purdue University, W. Lafayette, IN).

**RESULTS**

**BMH-induced Cross-linking of N30C-PLB to SERCA2a**—To screen for residues of PLB interacting with SERCA2a, we performed Cys-scanning mutagenesis of PLB and co-expressed the PLB mutants with wild-type SERCA2a in SF21 microsomes (13, 15). Cross-linking of the mutated PLB molecules to SERCA2a was then tested using the homobifunctional thiol cross-linking reagent, BMH.
agent, BMH, a 10-Å-long probe (22). Taking advantage of the fact that PLB devoid of Cys residues is fully functional (23, 24), we made the Cys substitutions on the Cys-less PLB background, which is native PLB with its three endogenous cysteines (residues 36, 41, and 46) changed to alanine.

Fig. 1 shows cross-linking results obtained when single Cys replacements at residues 30–41 of PLB were scanned for cross-linking to SERCA2a. (Upstream and downstream PLB mutations are indicated at the top of the autoradiograph. PLB/SER, PLB cross-linked to SERCA2a; PLB, pentameric through monomeric mobility forms of PLB.) B, immunoblot of identical samples probed with anti-SERCA2a monoclonal antibody, 2A7-A1. SER, SERCA2a protein.

Time Course and Concentration Dependence of Cross-linking—Maximal cross-linking of N30C-PLB to SERCA2a by 0.1 mM BMH in buffer A was achieved at an incubation time of 60 min; half-maximal cross-linking occurred at 15 min (Fig. 2A). The mobility of the SERCA2a band decreased gradually with increasing cross-linking (Fig. 2B), but due to the broadness of the Ca\(^{2+}\)/H\(_{11001}\) pump band and the absence of clear doublet formation, the mobility shift could not be used to accurately assess the percentage of Ca\(^{2+}\)/H\(_{11001}\)-ATPase molecules cross-linked, which is considerable (see Fig. 8). When cross-linking was conducted for 60 min in buffer A, half-maximal cross-linking of N30C-PLB to SERCA2a occurred at a BMH concentration of 20–30 \(\mu\)M; maximal cross-linking was achieved at 100 \(\mu\)M BMH (Fig. 2C). Twenty-fold higher concentrations of BMH (2 mM) gave no additional cross-linking (data not shown).

Under the conditions of SDS-PAGE utilized, N30C-PLB (expressed on the Cys-less PLB background) migrated primarily as a monomer (Fig. 2, A and C, lane 1). BMH induced the rapid dimerization of PLB monomers, a process that was already complete at an incubation time of 5 min in the presence of 0.1 mM BMH (Fig. 2A, lane 2, PLB\(_{2}\)), when only 14% of the maximal level of PLB-SERCA2a heterodimers had formed, or at an incubation time of 60 min in the presence at 10 \(\mu\)M BMH (Fig. 2C, lane 2, PLB\(_{2}\)), when only 6% of the maximal level of PLB-SERCA2a heterodimers had formed. We found that even without cross-linking agents, N30C-PLB could retain pentamers on SDS-PAGE but that these pentamers were unstable and dissociated readily at low concentrations of SDS (Fig. 2D). With only 0.2% SDS in the sample loading buffer prior to electro-
FIG. 2. Time course and concentration dependence of BMH-induced cross-linking of N30C-PLB to SERCA2a. A and B, identical sets of microsomes co-expressing N30C-PLB and SERCA2a were incubated with 0.1 mM BMH in buffer A for the times indicated (top). A, the immunoblot was probed with the anti-PLB antibody, 2D12; B, the immunoblot was probed with the anti-SERCA2a antibody, 2A7-A1. C, microsomes from the same preparation incubated for 1 h with different concentrations of BMH (top) in buffer A. The immunoblot was probed with the anti-PLB antibody. D, microsomes from the same preparation incubated for 1 h in buffer A but without BMH. Sample-loading buffer was added, yielding the final SDS concentrations indicated (top), and the immunoblot was probed with the anti-PLB antibody. In the experiments depicted in A–C, the final SDS concentration was 5.8%.

FIG. 3. Ca²⁺-inhibition of N30C-PLB cross-linking to SERCA2a. A, anti-PLB immunoblot showing Ca²⁺ effect on N30C-PLB cross-linking to SERCA2a. Microsomes co-expressing N30C-PLB and SERCA2a were incubated for 1 h in buffer A in the presence (+) and absence (−) of 0.1 mM BMH. CaCl₂ was included in buffer A to yield the ionized Ca²⁺ concentrations indicated (top). B, plots of Ca²⁺ inhibition of the cross-linking signal and of Ca²⁺-activation of Ca²⁺-ATPase activity. PLB/SERCA2a cross-linked bands (PLB/SER) shown in A were quantified and plotted. Ca²⁺-ATPase activity of the same microsomal preparation was measured in the presence and absence of the anti-PLB monoclonal antibody 2D12 (∆ Ab) and is also plotted. All activities are expressed as percentage of the maximal activity. Maximal Ca²⁺-ATPase activity was 16.3 μmol of P/mg of protein/h.
phoresis, N30C-PLB was mostly pentameric on SDS-PAGE; with 1.4% or higher concentrations of SDS in the sample loading buffer, the protein was mostly monomeric, with some dimers (Fig. 2D). These results suggest that in intact microsomal membranes, N30C-PLB is predominantly a pentamer. Cross-linking between PLB monomers preassembled as pentamers is expected to be a very rapid process (29). Cross-linking of PLB monomers to SERCA2a, in contrast, is a slower process. It should be pointed out that even after rapid homodimer formation by PLB monomers within pentamers, there should always be at least one uncross-linked monomer per pentamer that is free to dissociate from the complex and interact with SERCA2a. Using purified PLB and SERCA2a as standards, we calculated a molar ratio of 4:1 for N30C-PLB to SERCA2a in Sf21 microsomes. A molar ratio of 2:1 for the naturally occurring proteins in dog cardiac SR vesicles was found.

Ca\(^{2+}\) Effect on Cross-linking—PLB inhibits the Ca\(^{2+}\)-ATPase at low ionized Ca\(^{2+}\) concentration by decreasing the apparent affinity of the enzyme for Ca\(^{2+}\) (5). No inhibition is typically observed at high ionized Ca\(^{2+}\) concentration (6). This suggests that Ca\(^{2+}\) may disrupt the physical interaction between PLB and SERCA2a (9, 30). Fig. 3A demonstrates that Ca\(^{2+}\) inhibited cross-linking of N30C-PLB to SERCA2a in concentration-dependent fashion. Quantification of the cross-linking signal revealed that half-maximal inhibition occurred at 0.13 μM Ca\(^{2+}\), with complete inhibition achieved at 1 μM Ca\(^{2+}\) or greater (Fig. 3B). Half-maximal activation of the Ca\(^{2+}\)-ATPase activity of the same microsomes assayed in the absence of BMH occurred at a Ca\(^{2+}\) concentration of 0.28 μM (Fig. 3B). The addition of the anti-PLB monoclonal antibody shifted the Ca\(^{2+}\) activation curve to the left, giving half-maximal activation of Ca\(^{2+}\)-ATPase activity at 0.12 μM Ca\(^{2+}\). This characteristic antibody response (5, 13, 15, 31) demonstrates that N30C-PLB was well coupled functionally to the Ca\(^{2+}\)-ATPase in Sf21 microsomes. Inhibition of N30C-PLB cross-linking to SERCA2a occurred over the same Ca\(^{2+}\) concentration range that was required for activation of ATP hydrolysis (Fig. 3B). This is consistent with PLB binding to the low Ca\(^{2+}\) affinity or E2 conformation of the Ca\(^{2+}\)-ATPase (5), the conformation that predominates in the absence of Ca\(^{2+}\) (32). Ca\(^{2+}\) had no effect on dimerization of PLB monomers (Fig. 3A, PLB\(_1\)).

Anti-PLB Antibody and Phosphorylation Effects—Since the anti-PLB monoclonal antibody reverses the inhibitory effect of PLB on the Ca\(^{2+}\) pump (6, 13, 15), it was of interest to test if it also prevented the cross-linking reaction. Cross-linking of N30C-PLB to SERCA2a by BMH, measured at low ionized Ca\(^{2+}\) concentration, was essentially eliminated by the 2D12 monoclonal antibody (Fig. 4A). At the same time, the antibody had no effect on the rapid dimerization of PLB monomers induced by BMH (PLB\(_2\)).

Phosphorylation of PLB at Ser\(^{16}\) by PKA (3) also reverses Ca\(^{2+}\) pump inhibition by PLB, although not as completely as anti-PLB monoclonal antibodies (33, 34). Phosphorylation of N30C-PLB by PKA inhibited PLB cross-linking to SERCA2a by 52 ± 3.2% (mean ± S.D. from three determinations) (Fig. 4B). Heat-inactivated PKA had no effect on cross-linking. A phosphorylation-induced mobility shift (35) in PLB dimers was apparent (Fig. 4B, PLB\(_2\)), demonstrating that efficient phosphorylation of N30C-PLB at Ser\(^{16}\) had occurred.

Nucleotide Requirement—Cross-linking of N30C-PLB to SERCA2a exhibited a remarkable and unexpected requirement for adenine nucleotides. ATP and ADP dramatically stimulated heterodimer formation (Fig. 5A). No significant cross-linking was observed in the presence of AMP, adenosine, or adenine at concentrations as high as 3 mM, indicating that a nucleotide with at least two phosphates was required for effective coupling (Fig. 5A). Identical cross-linking signals were obtained at 0.3 and 3.0 mM concentrations of ATP or ADP, suggesting that the nucleotide effect was saturable. In fact, the intense cross-linking signal imparted by ATP or ADP made it easy to estimate apparent SERCA2a nucleotide affinities by performing cross-linking isotherms (Fig. 5B). Half-maximal stimulation of cross-linking by ATP and ADP occurred at concentrations of 21.8 ± 8.1 and 36.8 ± 9.0 μM, respectively (means ± S.D. from five determinations). These apparent nucleotide affinities are similar to those previously measured for SERCA1a using an equilibrium radioligand-binding assay (36). The nonhydrolyzable ATP analogs, AMP-PCP and AMP-PNP, also allowed efficient cross-linking (data not shown). Nucleotides had no effect on homodimerization of PLB monomers (Fig. 5A, PLB\(_2\)).

Thapsigargin and Cyclopiazonic Acid Inhibition—Thapsigargin and cyclopiazonic acid are specific inhibitors of SERCA enzymes that act by forming dead end complexes with the E2 conformation of the Ca\(^{2+}\) pump (37, 38). Both thapsigargin (Fig. 6A) and cyclopiazonic acid (Fig. 6B) inhibited BMH-induced cross-linking of N30C-PLB to SERCA2a in dose-dependent fashion. Half-maximal inhibition of cross-linking occurred at 0.2 μM for thapsigargin and at 2.5 μM for cyclopiazonic acid. Neither inhibitor had an effect on dimerization of PLB monomers (Fig. 6, PLB\(_2\)).

Cross-linking Site Localization—to localize the site of SERCA2a cross-linked to N30C-PLB, we purified the cross-linked ATPase from Sf21 microsomes and isolated and sequenced the proteolytic peptide that contains the covalently linked Cys residues. To accomplish this, 166 mg of microsomal protein co-expressing SERCA2a and N30C-PLB were cross-
linked with 0.1 mM BMH in buffer A on a large scale (see “Experimental Procedures”). Microsomes were then solubilized in detergent, and the two cross-linked proteins were purified and processed as described below.

Round 1 of the purification used anti-SERCA2a monoclonal antibody affinity chromatography to purify the Ca\(^{2+}\)-ATPase to homogeneity in one step from detergent-solubilized, BMH-treated microsomes. Immunoblotting with the anti-SERCA2a antibody demonstrated that all of the Ca\(^{2+}\)-ATPase was retained by the column and specifically eluted at acidic pH (Fig. 7B). The Coomassie Blue-stained gel revealed that the Ca\(^{2+}\)-ATPase was purified to virtual homogeneity in fractions 9–11 (Fig. 7A). Immunoblotting the same fractions with the anti-PLB antibody showed that the unattached PLB monomers and
dimers (PLB₁ and PLB₂) passed freely through the column in the flow-through (FT) fraction and wash fractions 1 and 2, whereas only PLB cross-linked to SERCA2a was retained by the column and eluted at acidic pH (Fig. 7C, 9–11).

In round 2, the Ca²⁺-ATPase purified from round 1 was subjected to anti-PLB monoclonal antibody affinity chromatography to separate the uncross-linked SERCA2a molecules from those covalently attached to N30C-PLB. The anti-PLB immunoblot shows that all SERCA2a molecules cross-linked to N30C-PLB were retained by the column and eluted at acidic pH (Fig. 8C). The Coomassie Blue-stained gel (Fig. 8A) and the anti-SERCA2a immunoblot (Fig. 8B) show that a substantial amount of uncross-linked Ca²⁺-ATPase molecules was recovered in the FT fraction and wash fraction 1. Quantification of immunoblot signals from four separate purifications demonstrated that 41.3 ± 12.8% (mean ± S.D.) of the Ca²⁺-ATPase molecules in Sf21 microsomes were cross-linked to N30C-PLB by BMH.

In round 3, cross-linked Ca²⁺-ATPase molecules exclusively (recovered from fractions 8–12 in round 2) were subjected to sequential proteolysis with endo-Asp-N and endo-Lys-C, and the SERCA2a limit peptide was isolated by a second cycle of anti-PLB monoclonal antibody affinity chromatography. This could be accomplished because the epitope recognized by the anti-PLB antibody (residues 7–13 of PLB) resided downstream from Asp² and Lys³ of PLB, the only PLB residues cleaved by the two proteases (see Fig. 10). Fig. 9 shows that purified
Fig. 9. Purification of SERCA2a proteolytic peptide cross-linked to N30C-PLB using anti-PLB monoclonal antibody affinity chromatography (round 3). Fractions 8–12 from round 2, containing exclusively Ca\(^{2+}\)-ATPase molecules cross-linked to N30C-PLB, were pooled (Pre-Digest) and digested with endo Asp-N (AspN) followed by endo Lys-C (LysC). The protease-treated sample (Load) was applied to the anti-PLB monoclonal antibody (2D12) column, flow-through (FT) and wash (Wash) fractions were collected, and the purified SERCA2a peptide cross-linked to N30C-PLB was eluted at acidic pH (pH 2.4) in fractions 9 and 10 (red asterisk). The immunoblot shown was probed with the anti-PLB monoclonal antibody, 2D12.

**TABLE I**

Sequence analysis of PLB/SERCA2a peptide

The BMH cross-linked peptide of approximate M\(_r\) = 16,000 indicated by the red asterisk in Fig. 11 was sequenced. pmol of SERCA2a and N30C-PLB amino acids recovered at each cycle are listed. AA, amino acid; Xaa, residue unidentified.

| Cycle no. | SERCA2a | Phospholamban |
|-----------|---------|---------------|
|           | AA      | Amount | AA | Amount |
| 1         | Asp\(^{254}\) | 19.5 | Val\(^{t}\) | 9.1 |
| 2         | Glu     | 10.2 | Gln   | 7.5 |
| 3         | Phe     | 7.1  | Tyr    | 6.5 |
| 4         | Gly     | 11.5 | Leu    | 7.1 |
| 5         | Glu     | 6.7  | Thr    | 6.3 |
| 6         | Gin     | 5.8  | Arg    | 5.8 |
| 7         | Leu     | 6.0  | Ser    | 3.2 |
| 8         | Ser     | 3.4  | Ala    | 4.3 |
| 9         | Lys\(^{a}\) | 2.6 | Ile    | 4.7 |
| 10        | Val     | 3.4  | Arg    | 4.4 |
| 11        | Ile     | 5.3  | Arg    | 5.0 |
| 12        | Ser     | 1.7  | Ala    | 3.6 |
| 13        | Leu     | 2.5  | Ser    | 2.5 |
| 14        | Ile     | 3.5  | Thr    | 2.3 |
| 15        | XXX     | —     | Ile    | 4.8 |
| 16        | Ile     | 4.8  | Glu    | 1.9 |
| 17        | Ala     | 1.5  | Met    | 1.0 |
| 18        | Val\(^{271}\) | 1.8 | Pro\(^{t1}\) | 1.4 |

SERCA2a cross-linked to N30C-PLB was readily digested by two proteases, giving a limit peptide of ~16-kDa molecular mass that was still recognized by the anti-PLB antibody. This peptide bound quantitatively to the anti-PLB antibody column and was eluted in pure form at acidic pH (Fig. 9, asterisk). The anti-SERCA2a antibody did not recognize the peptide (data not shown), demonstrating that its epitope (residues 386–396) had been removed by the proteases.

Edman degradation of the cross-linked peptide purified in round 3 gave two different residues per sequence cycle as expected (Table I), consistent with one PLB molecule cross-linked per Ca\(^{2+}\)-ATPase molecule. The readable PLB sequence aligned with Val\(^{t}\)--Pro\(^{t1}\) of intact PLB, and the readable Ca\(^{2+}\)-ATPase sequence matched with Asp\(^{254}\)--Val\(^{271}\) of intact SERCA2a. It was not possible to obtain readable sequence beyond 18 cycles for either protein. From analysis of the data, however, it was possible to conclude that either Cys\(^{318}\), Cys\(^{344}\), or Cys\(^{349}\) was the residue of SERCA2a cross-linked to PLB (Fig. 10). Cys\(^{386}\) in transmembrane helix M3 could be excluded because readable sequence ran past this residue, and in another sequencing run an interior peptide was isolated beginning at Asp\(^{281}\). The cross-linked SERCA2a peptide had to terminate at Lys\(^{352}\) or earlier, because endo Lys-C digestion alone removed the epitope recognized by the anti-SERCA2a antibody at residues 386–396, and there are no lysines between residue 352 and residues 386–396. The sum of the molecular masses of the cross-linked peptides schematized in Fig. 10 is consistent with their combined molecular masses of 16 kDa estimated by SDS-PAGE (Fig. 9); however, it is possible that Cys\(^{344}\) and Cys\(^{349}\) were not retained in the cross-linked peptide due to the two potential endo-Lys-C cleavage sites at residues 328 and 329.

**SERCA2a Mutagenesis**—To distinguish which of the three Cys residues of SERCA2a, Cys\(^{318}\), Cys\(^{344}\), or Cys\(^{349}\), was the one cross-linked to N30C-PLB, we individually replaced each Cys residue with Ala and tested for loss of cross-linking function. For completeness, the two cysteines bordering these residues, Cys\(^{306}\) and Cys\(^{364}\), were also replaced. Each SERCA2a mutant was co-expressed with N30C-PLB in Sf21 microsomes and tested for cross-linking to N30C-PLB by BMH. Fig. 11A shows that only SERCA2a with the C318A mutation failed to cross-link to N30C-PLB after the addition of BMH. Fig. 11B shows that all of the SERCA2a mutants expressed well, although the maximal Ca\(^{2+}\)-ATPase activity was reduced somewhat for some of the mutants (see the legend to Fig. 11). These results demonstrate that Cys\(^{318}\) of SERCA2a is the amino acid cross-linked to N30C-PLB (Fig. 12).

**DISCUSSION**

Here we have demonstrated a highly specific cross-linking interaction between residue 30 of N30C-PLB and Cys\(^{318}\) of the canine cardiac Ca\(^{2+}\) pump. Agents known to disrupt the functional interaction between PLB and the Ca\(^{2+}\) pump, like Ca\(^{2+}\) (5, 6), the anti-PLB antibody (13, 31, 33), and PLB phosphorylation by PKA (33, 34), also disrupted the cross-linking interaction between the two molecules. Other agents, specifically adenine nucleotides, were shown to be essential for cross-linking. Identification of both cross-linking enhancers and inhibitors suggests that the cross-linking process accurately reported con-
formational changes in the SERCA2a molecule involved in the binding and dissociation of PLB. It is interesting that only Cys\textsuperscript{318} of SERCA2a cross-linked to the maleimide probe at -tached to PLB. Numerous previous studies have detected labeling of multiple Cys residues (residues 344, 364, 377, 471, 498, 614, 636, 670, and 674) of the Ca\textsuperscript{2+}-ATPase with different sulfhydryl-reacting compounds, including maleimides (39, 40). To our knowledge, however, this is the first report of any covalent modification of Cys\textsuperscript{318} of the Ca\textsuperscript{2+}-pump. This implies that accessibility to Cys 318 of SERCA2a is normally restricted but can be allowed provided that PLB intercalates with the structure and delivers the covalent labeling probe. The efficiency of cross-linking by BMH was high. At least 40% of Ca\textsuperscript{2+}-ATPase molecules in Sf21 microsomes were coupled to N30C-PLB by the 10-Å-long probe.

Residue 30 of PLB and Cys\textsuperscript{318} of SERCA2a are both situated close to the cytoplasmic face of the SR membrane, approximately 2 residues removed from the lipid bilayer (1, 3, 41). The proximity of residue 30 of PLB to Cys\textsuperscript{318} of SERCA2a suggests that PLB has the potential to perturb Ca\textsuperscript{2+} binding to SERCA2a by interference at Ca\textsuperscript{2+}-binding site 2 (32), which contains Gln\textsuperscript{309} in M4 as a critical Ca\textsuperscript{2+}-liganding residue only 9 amino acids distant from Cys\textsuperscript{318} (Fig. 12). Cys\textsuperscript{318} of SERCA2a is also directly adjacent to Leu\textsuperscript{319}, which when mutated to Arg in SERCA1a, drastically slows the Ca\textsuperscript{2+} binding transition from E\textsubscript{2} to E\textsubscript{1} (42), the same kinetic step Cantilina et al. (5) proposed was decreased 10-fold by PLB binding to SERCA2a. Thus, this region of SERCA2a, at the boundary between M4 and its cytoplasmic extension (42), has the potential to be a key regulatory target for PLB action. Likewise, point mutations at the complementary interaction domain of PLB, between residues 27 and 31, can either enhance or attenuate SERCA2a inhibition (11, 18), demonstrating the importance of this region of PLB in SERCA2a regulation. Consistent with chemical localization of residue 30 of PLB close to Cys\textsuperscript{318} of SERCA2a, localization of PLB in co-crystals with the Ca\textsuperscript{2+}-ATPase by cryoelectron microscopy suggests that PLB may enter the SR membrane near the cross-linking site at Cys\textsuperscript{318} (20). In contrast, Asahi et al. (43) recently proposed that residue 30 of PLB
interacts directly with Asp\textsuperscript{813} of SERCA1a (corresponding to Asp\textsuperscript{812} of SERCA2a). However, this interpretation was based on indirect results from a co-immunoprecipitation assay, and attempts to directly cross-link the two molecules were unsuccessful. Asp\textsuperscript{812} of SERCA2a is located in the M6/M7 loop of the transmembrane region of PLB (residues 32–52) is colored yellow. Only residues 29–52 of PLB are depicted. The SERCA2a topology diagram is adapted from Ref. 32, critical residues of SERCA2a binding Ca\textsuperscript{2+} are located in the lipid membrane (M). N, nucleotide binding domain; P, phosphorylation domain; A, actuator domain (17).

Cross-linking of N30C-PLB to Cys\textsuperscript{318} of SERCA2a was completely prevented by high ionized Ca\textsuperscript{2+} concentration. A similar result was obtained earlier by James et al. (9), who used a radioactive PLB-phoat affinity probe to show that Ca\textsuperscript{2+} prevents cross-linking of Lys\textsuperscript{397} of PLB to residues 397–400 of purified SERCA2a. Binding of SERCA2a to PLB was also inhibited by Ca\textsuperscript{2+} in a recent co-immunoprecipitation study (30). Thus, there is general agreement that Ca\textsuperscript{2+} blocks the binding interaction between PLB and SERCA2a, which is consistent with the idea that PLB interacts specifically with the E2 (Ca\textsuperscript{2+}-free), not the E1 (Ca\textsuperscript{2+}-bound), conformation of the Ca\textsuperscript{2+}-ATPase (5). However, there is disagreement on the issue of whether other agents that block the functional interaction between SERCA2a and PLB also block the physical interaction. Here we demonstrated that both the anti-PLB antibody and phosphorylation of PLB by PKA inhibited cross-linking of residue 30 of PLB to Cys\textsuperscript{318} of SERCA2a. James et al. (9) showed that PKA phosphorylation of PLB decreased cross-linking of Lys\textsuperscript{397} of PLB to SERCA2a, and, in a more recent study, the binding interaction between fusion proteins containing residues 1–26 of PLB and residues 331–726 of SERCA2a was inhibited by PKA phosphorylation of the PLB fusion peptide or by an anti-PLB monoclonal antibody (21). Thus, there is considerable evidence that both phosphorylation of PLB and anti-PLB monoclonal antibodies inhibit the binding interaction between PLB and SERCA2a, at least as it occurs between residues 1 and 30 of PLB and SERCA2a (Refs. 9 and 21; this work). Asahi et al. (14, 30), however, noted no effect of phosphorylation of PLB or of anti-PLB antibodies on co-immunoprecipitation of PLB with the Ca\textsuperscript{2+}-ATPase. Again, with use of this system, it is difficult to rule out the occurrence of nonspecific protein interactions.

Cross-linking of N30C-PLB to Cys\textsuperscript{318} of SERCA2a required the presence of ATP or ADP, in addition to a Ca\textsuperscript{2+}-free enzyme. This result suggests that PLB interacts most productively with the nucleotide-bound E2 state, a conformation of the Ca\textsuperscript{2+}-ATPase that has been previously characterized (36, 44, 45). The nucleotide requirement for PLB cross-linking to SERCA2a is remarkably similar to that required to elicit fluorescence enhancement of SERCA1a in the absence of Ca\textsuperscript{2+} (36). For both processes, ATP and ADP have maximal effects at micromolar concentrations, and AMP and adenosine are virtually without effect (36). The data here show that at least two phosphates on the nucleotide are required to induce the E2 state of SERCA2a that allows cross-linking to PLB. A specific interaction with this E2 conformation of the Ca\textsuperscript{2+} pump by PLB explains part of its inhibitory mechanism, because stabilization of E2 is expected to retard the transition to E1 associated with occupancy of the high affinity Ca\textsuperscript{2+}-binding sites (5). Accompanying this E2 to E1 transition is a large conformational change in the enzyme, which is required to bring the terminal phosphate of ATP close to Asp\textsuperscript{H531} during ATP hydrolysis (17, 46). PLB could interfere with this conformational change by direct interactions at M4, by preventing movement of the M4 helix associated with Ca\textsuperscript{2+} binding, which has recently been suggested to accompany the E2 to E1 transition (46). It cannot be determined from this study, however, or from previous studies assessing Ca\textsuperscript{2+} effects on PLB/SERCA2a interactions (9, 30) whether PLB actually binds to and dissociates from SERCA2a with each cycle of Ca\textsuperscript{2+} transport or whether PLB remains bound to SERCA2a through multiple transport cycles, while sensing conformational changes in the Ca\textsuperscript{2+}-ATPase. ATP hydrolysis by the Ca\textsuperscript{2+} pump is a rapid process occurring on a time scale of milliseconds, and it could be that the off rate for PLB binding is too slow to allow dissociation of PLB with each transition from E2 to E1. Instead, the cross-linking interaction may be reporting a time-averaged conformation of the Ca\textsuperscript{2+}-ATPase, wherein the E2 conformation with bound nucleotide is the one most perturbed by N30C-PLB and the one ideally spatially positioned for efficient chemical coupling to SERCA2a.
binding affinity of SERCA2a directly, perhaps by physical interactions occurring near Glu309. In an earlier study, we could detect no effect of PLB on equilibrium Ca\textsuperscript{2+}-binding to SERCA2a (5). However, in that study, nucleotides were not included in the Ca\textsuperscript{2+}-binding assay. The cross-linking results presented here demonstrate that nucleotides may be required for certain physical interactions between PLB and SERCA2a. Experiments are currently in progress to test whether PLB does lower the Ca\textsuperscript{2+} binding affinity of SERCA2a directly but only in the presence of adenine nucleotides. In fact, a requirement for nucleotides for an effect on Ca\textsuperscript{2+} binding can be deduced from kinetic observations of the functional interactions between PLB and SERCA2a (47). Asahi et al. (30) recently noted that 5 mM ATP gave a 2-fold increase in co-immunoprecipitation of SERCA2a with PLB; Kimura and Inui (21) and James et al. (9) detected no ATP effects on PLB/SERCA2a interactions with their systems.

The specific SERCA inhibitors thapsigargin and cyclopiazonic acid potently inhibited cross-linking of N30C-PLB to SERCA2a interactions with their systems. No reconstitution is required for analysis of the cross-linking system described here has several attractive features that will be useful for future studies assessing PLB/SERCA2a interactions. Both proteins are expressed in Sf21 microsomes in large quantities, functionally intact, and biochemically coupled. No reconstitution is required for analyzing mechanistically meaningful cross-linking interactions. In addition, sufficient protein is easily expressed for purification and detailed biochemical analyses including direct protein sequencing as described previously. Using this system in combination with other cross-linkers, we have recently identified additional cross-linking sites of PLB that covalently attach to SERCA2a. These will be reported in subsequent papers.

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REFERENCES

1. MacLennan, D. H., Rice, W. J., and Green, N. M. (1997) J. Biol. Chem. 272, 28615–28619

2. Bers, D. M. (2002) Nature 415, 198–205

3. Simmerman, H. K. B., Collins, J. H., Theibert, J. L., Wegener, A. D., and Jones, L. R. (1986) J. Biol. Chem. 261, 13333–13341

4. Fujii, K. J., Ueno, A., Kitano, K., Tanaka, S., Kojima, M., and Tada, S. (1987) J. Clin. Invest. 79, 301–304

5. Cantillana, T., Sagara, Y., Inui, M., and Jones, L. R. (1993) J. Biol. Chem. 268, 17018–17025

6. Simmerman, H. K. B., Kobayashi, Y. M., Autry, J. M., and Jones, L. R. (1996) J. Biol. Chem. 271, 5941–5946

7. James, P., Inui, M., Tada, M., Chiesi, M., and Carafoli, E. (1989) Nature 342, 467–465

8. Toyofuku, T., Kuryzidowski, K., Tada, M., and MacLennan, D. H. (1994) J. Biol. Chem. 269, 3088–3094

9. Kimura, Y., Kuryzidowski, K., Tada, M., and MacLennan, D. H. (1997) J. Biol. Chem. 272, 15061–15064

10. Cornea, R. L., Jones, L. R., and Stokes, D. L. (2001) Biophys. J. 81, 884–884

11. Autry, J. M., and Jones, L. R. (1997) Biochemistry 36, 2960–2967

12. Kimura, Y., and Inui, M. (2002) Mol. Pharmacol. 61, 667–673

13. Green, N. S., Reisler, E., and Houk, K. N. (2001) Protein Sci. 10, 1293–1304

14. Toyofuku, T., Kobayashi, Y., and Jones, L. R. (1999) Biochem. J. 347, 337 (abstr.)

15. Cornea, R. L., Autry, J. M., Chen, Z., and Jones, L. R. (2000) J. Biol. Chem. 275, 41487–41494

16. Reddy, L. G., Autry, J. M., Jones, L. R., and Thomas, D. D. (1999) J. Biol. Chem. 274, 7649–7655

17. Toyoshima, C., Nakasako, M., Nomura, H., and Oagawa, H. (2000) Nature 405, 647–655

18. Kimura, Y., Kuryzidowski, K., Tada, M., and MacLennan, D. H. (1994) J. Biol. Chem. 269, 3088–3094

19. Reddy, L. G., Jones, L. R., Cala, S. E., and O’Brian, J. J. (2001) J. Biol. Chem. 276, 35328–35335

20. Xue, Z., Rice, W. J., and Stokes, D. L. (2002) J. Biol. Chem. 277, 201–211

21. Mahaney, J., Barlow, A., Hanakez, E., Hoffman, J., and Muchnik, T. (1999) Arch. Biochem. Biophys. 372, 408–413