Characterizing Phospholamban to Sarco(endo)plasmic Reticulum Ca^{2+}-ATPase 2a (SERCA2a) Protein Binding Interactions in Human Cardiac Sarcoplasmic Reticulum Vesicles Using Chemical Cross-linking*

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Background: The mechanism of PLB inhibition of SERCA2a activity remains unresolved.
Results: Cross-linking Lys^{27} of PLB to SERCA2a in human SR vesicles is conformationally dependent and correlates closely with enzyme inhibition.
Conclusion: PLB stabilizes the $E2$·ATP state, thereby blocking formation of $E1$.
Significance: Mutually exclusive binding of PLB and Ca^{2+} regulates SERCA2a activity in normal and failed human myocardium.

Chemical cross-linking was used to study protein binding interactions between native phospholamban (PLB) and SERCA2a in sarcoplasmic reticulum (SR) vesicles prepared from normal and failed human hearts. Lys^{27} of PLB was cross-linked to the Ca^{2+} pump at the cytoplasmic extension of M4 (at or near Lys^{328}) with the homobifunctional cross-linker, disuccinimidyl glutarate (7.7 Å). Cross-linking was augmented by ATP but abolished by Ca^{2+} or thapsigargin, confirming in native SR vesicles that PLB binds preferentially to $E2$ (low Ca^{2+} affinity conformation of the Ca^{2+}-ATPase) stabilized by ATP. To assess the functional effects of PLB binding on SERCA2a activity, the anti-PLB antibody, 2D12, was used to disrupt the physical interactions between PLB and SERCA2a in SR vesicles. We observed a tight correlation between 2D12-induced inhibition of PLB cross-linking to SERCA2a and 2D12 stimulation of Ca^{2+}-ATPase activity and Ca^{2+} transport. The results suggest that the inhibitory effect of PLB on Ca^{2+}-ATPase activity in SR vesicles results from mutually exclusive binding of PLB and Ca^{2+} to the Ca^{2+} pump, requiring PLB dissociation for catalytic activation. Importantly, the same result was obtained with SR vesicles prepared from normal and failed human hearts; therefore, we conclude that PLB binding interactions with the Ca^{2+} pump are largely unchanged in failing myocardium.

Phospholamban (PLB)[2] is a 52-amino acid, single-span membrane protein that regulates the activity of SERCA2a, the Ca^{2+}-ATPase in cardiac SR (1). SERCA2a is a major Ca^{2+}-handling protein in the heart that actively transports cytosolic Ca^{2+} into the lumen of the SR, inducing myofilament relaxation (2). The dephosphorylated PLB monomer binds to and inhibits Ca^{2+}-ATPase activity by decreasing the apparent Ca^{2+} affinity of the enzyme (3, 4), having little or no effect on the V_{max} of the enzyme measured at saturating Ca^{2+} concentration (1, 5). PLB monomers also oligomerize into noninhibitory homopentamers in the SR membrane (6) (stabilized by transmembrane Leu/Ile zipper interactions) (7), but the functional significance of the pentamer remains unclear. The inhibitory effect of PLB on SERCA2a activity is reversed when PLB is phosphorylated at Ser^{16} (by protein kinase A) or Thr^{17} (by calmodulin-dependent protein kinase) during β-adrenergic receptor stimulation of the heart, increasing the size of the contractile-dependent SR Ca^{2+}-store and the rate of cardiac relaxation (1, 2). Similarly, anti-PLB monoclonal antibodies binding near the phosphorylation site also reverse Ca^{2+} pump inhibition by PLB (5, 8), with functional effects indistinguishable from those of β-adrenergic stimulation (9). As a key regulator of myocardial contractile kinetics, the physical basis of PLB inhibition of SERCA2a activity has long been an active area of investigation, both as it relates to normal cardiac function and to pathophysiological states such as in human heart failure (10).

One obstacle to solving the molecular mechanism of PLB action has been the inability monitor protein binding interactions between PLB and SERCA2a in SR membranes. Numerous biochemical and biophysical techniques have been employed to investigate physical interactions between PLB and SERCA, but nearly all have required cellular expression systems with mutated proteins (11–13) or fusion proteins (14, 15), and/or protein solubilization and reconstitution (16–19) for implementation. Techniques assessing protein-protein interactions have included co-immunoprecipitation (11), chemical cross-linking (12, 13, 17), FRET (14, 16, 18), and two-dimensional

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[2] The abbreviations used are: PLB, phospholamban; DSG, disuccinimidyl glutarate; $E1$, high Ca^{2+}-affinity conformation of Ca^{2+}-ATPase; $E2$, low Ca^{2+} affinity conformation of Ca^{2+}-ATPase; EMCS, N-(ϵ-maleimidocaproyloxy)sulfosuccinimide ester; endo Asp-N, endoproteinase Asp-N; K_{Ca}, Ca^{2+} concentration required for half-maximal effect; K_{ii} concentration giving half-maximal inhibition; M, transmembrane domain; SERCA, sarco-(endo)plasmic reticulum Ca^{2+}-ATPase; SERCA2a, isoform of Ca^{2+}-ATPase in cardiac SR; SR, sarcoplasmic reticulum; SUMO, small ubiquitin-like modifier; TG, thapsigargin.

co-crystallization (2, 19), which have frequently given conflicting results. For example, some studies (including our own) suggest that the Ca^{2+} pump with bound PLB is catalytically inactive, requiring PLB dissociation prior to enzyme activation (11, 12, 13, 15, 17), whereas others maintain that PLB is a subunit of the Ca^{2+} pump that remains attached throughout the catalytic cycle (14, 16, 18). Due to the wide array of techniques utilized, each with its inherent limitations, it has been difficult to reconcile some of these contradictory results. Therefore, a method to study physical interactions between the naturally occurring proteins in their native environment in the SR membrane seems highly desirable.

Chemical cross-linking has been used by our group to study protein binding interactions between PLB and SERCA2a co-expressed in insect cell membranes (13). Using the heterobifunctional (sulfhydryl to amine) cross-linker, EMCS, for example, we showed that the N27C mutant of canine PLB cross-links exclusively to Lys^{328} of the 110-kDa Ca^{2+} pump at the cytoplasmic extension of M4 (20). Notably, only the Ca^{2+}-free, *E*2 conformation of SERCA2a cross-linked to PLB, and the extent of cross-linking was increased significantly by ATP (13, 20). Other cross-linking interactions at different sites between SERCA2a and PLB were subsequently identified, all of which were similarly conformationally dependent (21, 22). Based upon these results, it was proposed that mutually exclusive binding of PLB and Ca^{2+} to the Ca^{2+} pump was the underlying mechanism by which PLB decreases the Ca^{2+} affinity of the enzyme (23, 24). In a series of studies by our group (13, 20–22) and MacLennan and co-workers (12, 25), it was further demonstrated that PLB cross-linking to *E*2 occurred in the groove formed between transmembrane helices M2, M4, and M9 of the Ca^{2+}-ATPase. Residues extending from Gln^{23} (25) to Leu^{52} (21) of PLB were cross-linked to SERCA2a within this groove, providing structural insight into how PLB binding to *E*2 might interfere with the conformational transition to *E*1 required for formation of the high affinity Ca^{2+} binding sites (5). Although these cross-linking studies have provided valuable information with regard to the factors regulating PLB association with the Ca^{2+} pump and effects on enzyme activity, it has yet to be determined whether the naturally occurring proteins in native SR vesicles interact identically to their recombinant counterparts.

Importantly for the work here, human PLB is unique among mammals in that it contains a lysine residue at position 27 instead of asparagine (26). Based upon the studies above with recombinant canine PLB and SERCA2a, we reasoned that it should be possible to cross-link the naturally occurring Lys^{27} of human PLB to SERCA2a directly in native human SR vesicles. Indeed, using human cardiac SR vesicles, we demonstrate highly specific cross-linking of Lys^{27} of PLB to SERCA2a at the cytoplasmic extension of M4. Cross-linking was achieved with the 7.7-Å long, homobifunctional cross-linking reagent, DSG. We went on to investigate the conformational specificity of the PLB to SERCA2a cross-linking interaction and whether normal protein-protein interactions between PLB and SERCA2a are maintained in SR membranes from failing myocardium.

EXPERIMENTAL PROCEDURES

Materials—Cross-linkers were obtained from Pierce. TG was purchased from Sigma. Endo Asp-N sequencing grade was obtained from Roche Applied Science. Protein iodination was performed with Pierce Iodination Tubes. $^{125}I^-$ and $^{45}Ca^{2+}$ were purchased from New England Nuclear. Nitrocellulose membranes and ImmobilonP^SQ sheets were obtained from Bio-Rad and Millipore, respectively.

Preparation of Human SR Vesicles—Cardiac microsomes enriched in SR ($45,000 \times g$ pellets) were prepared from the left ventricles of normal and failed human hearts according to our standard method for dog hearts (27). Preparations from individual human hearts were made over a period of 2 years from 1993 to 1994 and stored frozen in small aliquots at $-40\,°C$ in 0.25 M sucrose, 30 mM histidine (pH 7.2). Protein concentrations were determined by the Lowry method. Since the date of preparation, enzyme activities have remained stable in the freshly thawed aliquots assayed. Nonfailing left ventricular myocardium was obtained from four unmatched organ donors. Failed left ventricular myocardium was obtained from three transplant recipients with idiopathic dilated cardiomyopathy, who exhibited ejection fractions of 9% or less. Ca^{2+}-ATPase activities, PLB regulatory effects on Ca^{2+} pump activity, and cross-linking results were similar in SR membranes from the different preparations, and results from one representative normal preparation and one representative failed preparation are presented here unless otherwise stated.

PLB and SERCA2a Protein Expression in Sf21 Cells—Sf21 insect cells were co-infected with baculoviruses encoding canine PLB and SERCA2a as described previously (4). Mutagenesis of canine wild-type PLB to N27K-PLB was conducted as described previously (20). Cells were harvested 60 h after co-infection with baculoviruses and homogenized, and membranes were stored frozen in small aliquots at $-40\,°C$ at a protein concentration of 6–10 mg/ml in 0.25 M sucrose, 10 mM MOPS (pH 7.0).

PLB Cross-linking to SERCA2a—Cross-linking reactions were conducted with 11 μg of human SR vesicles or insect cell membranes in 12 μl of buffer. Cross-linking buffer contained 50 mM MOPS (pH 7.0), 3.0 mM $MgCl_2$, 100 mM KCl, 3 mM ATP, and 1 mM EGTA. In some experiments the ATP concentration was varied or the Ca^{2+} pump inhibitor, TG, was added. Ionized Ca^{2+} concentrations were set by addition of $CaCl_2$ to a final concentration of 0.1–1.0 mM. Cross-linking reactions were started by adding cross-linking reagents from concentrated stock solutions in dimethyl sulfoxide. The final cross-linker concentrations were 0.1 mM for EMCS and 5 mM for DSG, except where indicated in the figure legends. Cross-linking was conducted at room temperature for 15 min with EMCS and for 5 min at room temperature with DSG, which gave the maximal cross-linking of PLB to SERCA2a in both cases. Reactions were stopped with 7.5 μl of gel loading buffer containing 15% SDS and 100 mM DTT. Samples were heated to 50 °C for 10 min unless otherwise stated and then subjected to SDS-PAGE and immunoblotting for detection of PLB cross-linked to SERCA2a. In the experiment of Fig. 9, cross-linking was carried out simultaneously with the Ca^{2+} uptake assay and measurement of ^{125}I-

PLB Binding to SERCA2a in Human SR Vesicles

2D12 binding to PLB in SR vesicles, as described in more detail below.

Purification of Human SERCA2a Cross-linked to PLB—SERCA2a cross-linked to PLB was purified to homogeneity from human SR vesicles via sequential monoclonal antibody affinity chromatographies, as described previously (13). The total population of SERCA2a molecules was purified first using the anti-SERCA2a antibody column (2A7-A1), then only those SERCA2a molecules cross-linked to PLB were isolated using the anti-PLB (2D12) antibody column. 50 mg of human SR membranes were first cross-linked in bulk with DSG as described above. Cross-linking was terminated by addition of 200 mM ethanolamine, membranes were spun down, and the pellet was resuspended by addition of 1% SDS followed by 3.5% Triton X-100. Solubilized membrane proteins were then applied to the anti-SERCA2a column. The purified Ca^{2+} pumps that eluted at acid pH were then applied to the anti-PLB column for purification of Ca^{2+} pumps exclusively cross-linked to PLB, exactly as described in Ref. 13.

SDS-PAGE, Immunoblotting, and Antibodies—SDS-PAGE (using 8% polyacrylamide) and immunoblotting were performed as described previously (13). Membrane proteins were transferred to nitrocellulose sheets or Immobilon^{SQ} with similar results. For detection of PLB, blots were probed with anti-PLB monoclonal antibody, 2D12, which recognizes residues 7–13 of PLB. For detection of SERCA2a, blots were probed with anti-SERCA2a monoclonal antibody, 2A7-A1, which recognizes residues 386–396 of SERCA2a (13). Antibody-bound protein bands were visualized with ^{125}I-protein A followed by autoradiography and quantified using a Bio-Rad Personal Fx PhosphorImager. In the experiments of Figs. 8 and 9, antibody-bound bands were visualized directly with ^{125}I-2D12.

For quantification of PLB and SERCA2a in human SR vesicles, pure SERCA2a and PLB protein standards were electrophoresed and processed in parallel with SR membrane samples. Purified, recombinant canine PLB was isolated from insect cell membranes as described previously (28), and the purified SERCA2a standard was isolated from canine cardiac SR vesicles by 2A7-A1 affinity chromatography (13). The protein concentrations of the purified protein standards were determined by absorbance at 280 nM with knowledge of the molar extinction coefficients calculated from the amino acid sequences (29). The results were in good agreement with those determined by the Lowry protein assay.

Three new SERCA2a antibodies were used in this study for peptide mapping purposes. Site-specific antibodies were raised in rabbits against synthetic peptides corresponding to residues 106–120 and 522–533 of SERCA2a. Mouse monoclonal antibody, 2D6, was raised to a synthetic peptide corresponding to residues 319–332 of SERCA2a. All of the sequences above are identical in canine and human SERCA2a (4). Peptides were synthesized with an N-terminal cysteine residue for coupling to maleimide-activated BSA or keyhole limpet hemocyanin (Pierce). The epitope of SERCA2a recognized by 2D6 was further localized to residues 319–323 of the Ca^{2+} pump using PepSpots^{TM} (Jerini Bio Tools) (13).

Peptide Mapping—Approximately 1 μg of purified SERCA2a cross-linked to PLB was incubated with 0.04 μg (1 μl) or 0.24 μg

(6 μl) of endo Asp-N for 30 min at 23 or 37 °C, respectively. Replicate proteolyzed samples were then subjected to SDS-PAGE and immunoblotted with the anti-PLB and the anti-SERCA2a antibodies above.

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$_3$, 3 μg/ml Ca^{2+} ionophore A23187, and 1 mM EGTA. Ionized Ca^{2+} concentrations were set by varying the CaCl$_2$ concentration from 0 to 1.0 mM. Assays were conducted in the presence and absence of 15 μg of the anti-PLB monoclonal antibody, 2D12, which reverses PLB inhibition of SERCA2a activity (5). Ca^{2+}-dependent ATPase activities were determined in a reaction volume of 1 ml containing 32 μg of membrane protein during a 60-min incubation. P$_i$ release from ATP was measured colorimetrically (24).

2D12 Binding to PLB in Human SR Vesicles Measured Simultaneously with 2D12 Effects on PLB Cross-linking and Ca^{2+} Uptake—Ca^{2+} uptake into human cardiac SR vesicles (86.6 μg) was conducted at 37 °C in 450 μl of buffer containing 50 mM MOPS, 100 mM KCl, 3 mM MgCl$_2$, 10 mM potassium oxalate, 5 mM NaN$_3$, 0–46 μg of 2D12, 1 mM EGTA, and 0.1 mM CaCl$_2$ with a trace amount of ^{45}Ca. After a 10-min preincubation at 37 °C, Ca^{2+} uptake reactions were started by adding 45 μl of ATP to a final concentration of 3 mM. 100-μl samples were then taken at 3, 8, and 20 min after initiation of Ca^{2+} uptake and filtered over glass fiber filters to determine the degree of Ca^{2+} oxalate accumulated (27). Filters were rinsed twice with 5 ml of ice-cold saline prior to scintillation counting.

2D12 binding to PLB in SR vesicles was conducted in assay tubes prepared and handled identically to those described for Ca^{2+} uptake, only tracer ^{125}I-2D12 (13) was included with the cold 2D12 and $^{45}Ca^{2+}$ was omitted. After addition of ATP, incubations were conducted for 10 min at 37 °C. For determination of ^{125}I-2D12 binding, three 100-μl aliquots were taken from each reaction tube and filtered over glass fiber filters preblocked with 1% BSA and rinsed as above. ^{125}I retained by the filters was determined by scintillation counting. Control experiments indicated that presoaking the filters in 1% BSA was essential in reducing nonspecific binding of ^{125}I-2D12 to background levels comparable with blank filters receiving no SR vesicle protein. Rabbit skeletal muscle SR vesicles devoid of PLB also retained insignificant ^{125}I-2D12 in control experiments. Antibody binding data analysis was carried out using GraphPad Prism for Macintosh, Version 4.0c (GraphPad Software).

To measure 2D12 effects on PLB cross-linking in the same assay, a third series of identical reaction tubes was prepared only without ^{125}I-2D12 or $^{45}Ca^{2+}$. 10 min after Ca^{2+} uptake initiation by the addition of ATP, the samples were cross-linked with 5 mM DSG for 5 min at room temperature, and cross-linking was stopped with gel loading buffer (15% SDS and 100 mM DTT). Samples were heated to 50 °C for 10 min prior to SDS-PAGE, and immunoblots were probed directly with ^{125}I-2D12, and protein A was omitted (13).

RESULTS

SR vesicles were prepared from the left ventricles of three failing and five nonfailing human hearts, and the protein content of SERCA2a and PLB in the preparations was measured via

quantitative immunoblotting. Results obtained from one representative normal and one failed heart are shown in Fig. 1. Similar levels of SERCA2a (anti-SER) and PLB (anti-PLB) were detected in the two preparations (Fig. 1*A*), consistent with our earlier reports (33–35). The characteristic heat-induced dissociation of the PLB pentamer into monomers in the presence of SDS is also apparent in the *leftmost lanes* of the anti-PLB immunoblot in Fig. 1*A*.

Using purified PLB and SERCA2a protein standards (Fig. 1*B*), the molar ratio of PLB to SERCA2a was determined to be 1.37 ± 0.3 and 1.18 ± 0.2 mol of PLB/mol of Ca^{2+} pump in normal and failed membranes, respectively (mean $\pm$ S.E. from 10 determinations). For either type of preparation, the ~110-kDa Ca^{2+}-ATPase accounted for ~10% of the total SR protein and is therefore clearly visible on the Amido Black-stained nitrocellulose transfer in Fig. 1*A* (*lanes 1* and *2*). However, despite being approximately equimolar with the Ca^{2+} pump, due to its small molecular mass (~6 kDa), PLB accounted for only ~0.7% of the total SR protein and is not visible on the protein-stained nitrocellulose. No evidence for a lower mobility SUMOylated form of the Ca^{2+}-ATPase (~150 kDa) (30) was noted in SR vesicles prepared from any of the human hearts.

Cross-linking PLB to SERCA2a in Human SR Vesicles—Human PLB differs from canine PLB at only two residues; in human PLB, Asp^2 is a glutamic acid, and Asn^{27} is a lysine (Fig. 2*A*). Based upon earlier co-expression studies in which canine N27C-PLB cross-linked to Lys^{328} of SERCA2a with the cysteine to lysine cross-linker EMCS (20), we predicted that the natural Lys^{27} of human PLB should also be cross-linkable to Lys^{328} of SERCA2a with a homobifunctional, lysine-specific cross-linking reagent. To test this hypothesis, human cardiac microsomes and insect cell microsomes co-expressing canine N27C-PLB and SERCA2a, were incubated with the homobifunctional, amine-specific, NHS ester, DSG (7.7 Å), or the heterobifunctional (sulfhydryl to amine) cross-linker, EMCS (9.4 Å). Cross-linking was conducted in the absence of Ca^{2+} and presence of ATP, shown previously to yield optimal chemical coupling between PLB and SERCA2a. Cross-linked PLB/SERCA2a heterodimers (PLB/SER) were detected using the anti-PLB monoclonal antibody, 2D12. Strong cross-linking between PLB and SERCA2a was observed in the human SR vesicles using DSG, whereas no cross-linking occurred with EMCS (Fig. 2*B*). Alternately, canine N27C-PLB cross-linked to SERCA2a only with EMCS and not with DSG. In both the human and insect cell microsomes, PLB cross-linked exclusively to the Ca^{2+} pump, indicating that the protein-protein interaction between PLB and SERCA2a detected by cross-linking is highly specific.

Next, to verify that Lys^{27} of PLB cross-links to SERCA2a with DSG, we tested for cross-linking of canine PLB with Asn^{27} changed to Lys (N27K-PLB). Fig. 2*C* shows that under the same cross-linking conditions with a range of cross-linker concentrations (0.5–5 mM DSG), wild-type canine PLB did not cross-link to the Ca^{2+} pump, whereas canine PLB substituted with Lys^{27} did. This result confirms that Lys^{27} allows cross-linking of human PLB to SERCA2a in native SR vesicles.

Cross-linking Quantification and Localization—To quantify the extent of PLB cross-linking to SERCA2a in human SR vesicles and to gain information on the site cross-linked in
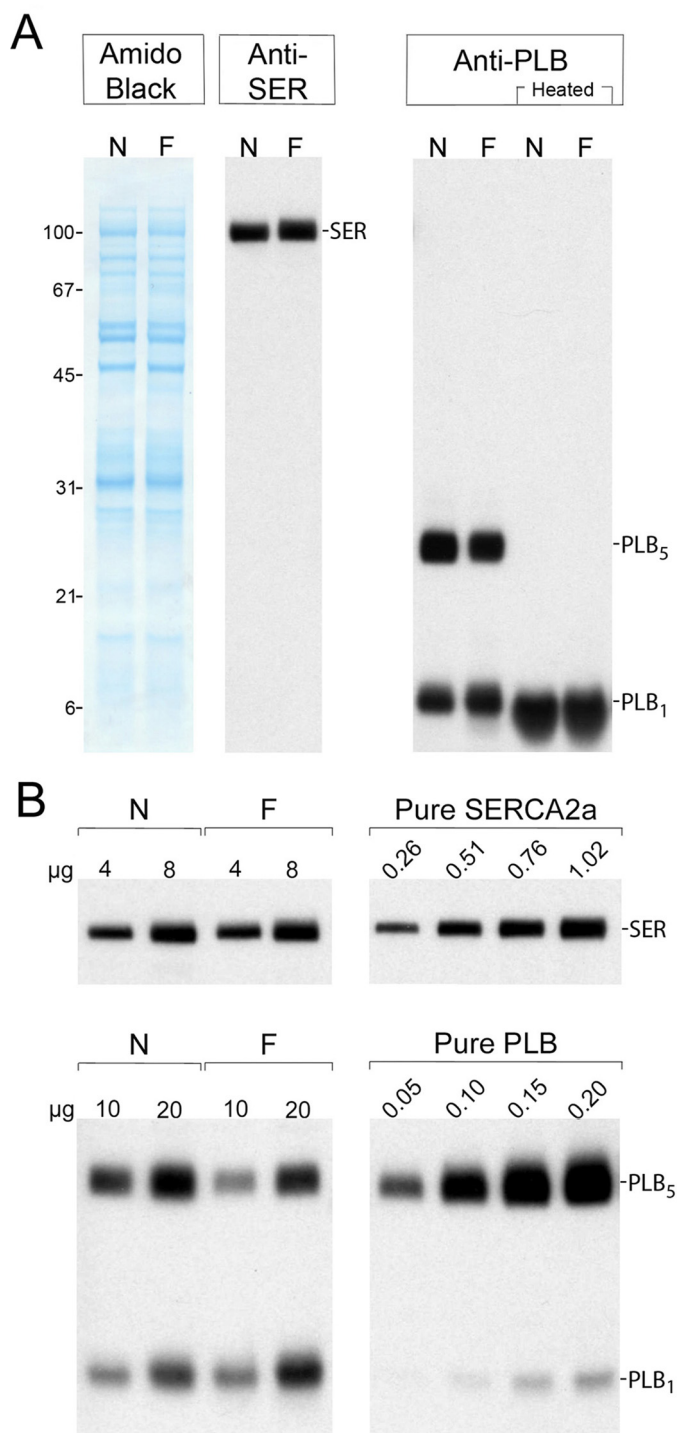

FIGURE 1. **SDS-PAGE and immunoblotting of SR vesicles prepared from normal (*N*) and failed (*F*) human hearts.** *A*, 20 μg of membrane protein was electrophoresed per gel *lane*, and the samples were transferred to nitrocellulose for protein staining or incubation with antibodies. One replicate set was stained with Amido Black (*left*). A second set was probed with the anti-SERCA2a antibody, 2A7-A1 (*middle*), and the third set was probed with the anti-PLB antibody, 2D12 (*right*), followed by ^{125}I-protein A and autoradiography. In the third set, the last two samples were heated to 50 °C for 10 min prior to SDS-PAGE. PLB_5 and PLB_1 designate the pentameric and monomeric forms of PLB, respectively. *B*, autoradiograph depicting typical immunoblotting results used for SERCA2a and PLB quantification in human SR vesicles. Micrograms of pure PLB and SERCA2a protein standards run are indicated in the *upper margins*.
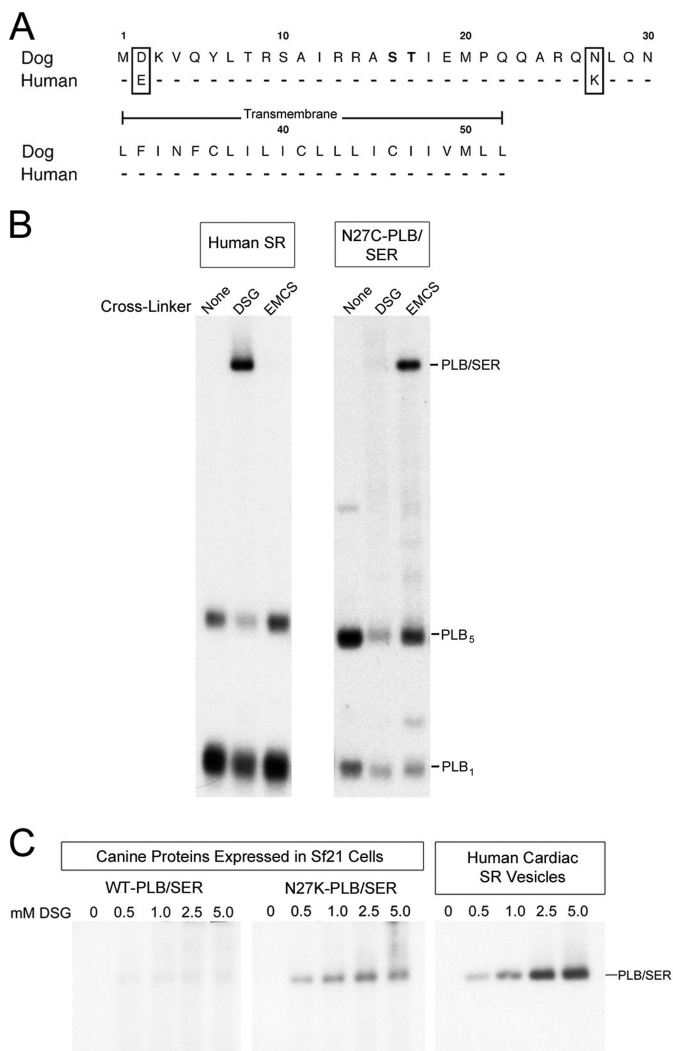
FIGURE 2. **Lys[27] of human PLB facilitates cross-linking to SERCA2a in SR vesicles.** *A*, amino acid sequences of human and canine PLB. Residues 1–30, containing the two phosphorylation sites (*bold*), project into the cytoplasm. *B*, 11 μg of human SR vesicles (*left*) or Sf21 membranes co-expressing canine SERCA2a and N27C-PLB (*right*) were cross-linked with DGS or EMCS. Samples were then subjected to SDS-PAGE and immunoblotting with 2D12. The high molecular mass band (PLB/SER) is SERCA2a cross-linked to the PLB monomer. Samples were not heated to 50 °C prior to SDS-PAGE to allow visualization of the PLB pentamer. *C*, human SR vesicles (*right*), or Sf21 membranes co-expressing canine SERCA2a and either wild-type canine PLB (*left*) or canine PLB with Asn[27] mutated to lysine (N27K) (*middle*) were cross-linked with 0–5 mM DSG, subjected to SDS-PAGE, and probed with 2D12.

SERCA2a, we purified the cross-linked PLB/SERCA2a heterodimers. Sequential monoclonal antibody chromatographies were used for the purification (13, 20). 50 mg of human SR vesicles were cross-linked with DSG, solubilized in detergent, and then passed over an anti-SERCA2a (2A7-A1) monoclonal antibody column. This allowed purification of the Ca^{2+} pump protein to homogeneity while removing the free PLB monomers not cross-linked to the enzyme (Fig. 3). Fig. 3*A* shows the Coomassie Blue-stained gel of selected fractions from the first purification and demonstrates that the purified Ca^{2+} pump was eluted from the column at acidic pH (pH 2.4). Immunoblotting the same fractions with the anti-SERCA2a antibody (Fig. 3*B*, 2A7-A1) demonstrates that all the Ca^{2+}-ATPase was bound to the column prior to acid elution at pH 2.4. The anti-PLB anti-

body panel (Fig. 3*C*, 2D12) shows that the uncross-linked PLB monomers (PLB_1) passed freely through the column and were recovered in the column flow-through fraction, whereas all of the PLB cross-linked to SERCA2a (PLB/SER) was retained and eluted at pH 2.4.

Next, to separate PLB-free Ca^{2+} pumps from Ca^{2+} pumps cross-linked to PLB, a second round of purification was done with the anti-PLB (2D12) column. The purified Ca^{2+}-ATPase from the first purification (*Input*) was loaded onto the anti-PLB column. The PLB-free Ca^{2+} pumps passed though the column and were recovered in the flow-through fraction and fraction 1, as visible on both the Coomassie Blue-stained gel (Fig. 4*A*) and the 2A7-A1 immunoblot (Fig. 4*B*). All of the Ca^{2+} pump molecules cross-linked to PLB were retained by the 2D12 column and eluted at pH 2.4, visible on the 2D12 immunoblot (Fig. 4*C*). Quantification of immunoblots from four separate purifications, which included SR vesicles isolated from both normal and failed hearts, indicated that 23.6 $\pm$ 5.1% of the Ca^{2+}-ATPase molecules in human SR vesicles were cross-linked to PLB with DSG (mean $\pm$ S.E.).

To gain information on the region of SERCA2a cross-linked to Lys[27] of PLB, peptide mapping of the purified PLB/SERCA2a heterodimers was performed. PLB/SERCA2a was digested with endo Asp-N, and the proteolytic fragments were analyzed by immunoblotting using the anti-PLB antibody (2D12) and four different anti-SERCA2a antibodies recognizing different regions of the Ca^{2+} pump (Fig. 5). Because human PLB contains no aspartic acid residues, any Ca^{2+} pump fragment generated that is cross-linked to PLB will remain anchored to the intact PLB molecule.

Immunoblotting with the 2D12 antibody shows that digestion with the highest endo Asp-N concentration (6 μl) generated two major proteolytic fragments with molecular masses of $\sim$23 and 19 kDa (Fig. 5, *2D12 panel*). The Ca^{2+} pump fragments detected using the anti-SERCA2a monoclonal antibody, 2A7-A1 (to residues 386–396), as well as the polyclonal antibodies recognizing residues 106–120 or 522–533, were distinctly different in mobility from those detected by 2D12, indicating that PLB was not cross-linked to the Ca^{2+} pump near these sites. However, the SERCA2a fragments detected by the 2D6 antibody, recognizing residues 319–323 of the Ca^{2+} pump, were identical in mobility to those detected by 2D12. Therefore, Lys[27] of PLB is cross-linked to the same fragments recognized by the 2D6 antibody.

Analysis of the human Ca^{2+} pump sequence indicates that digestion with endo Asp-N could generate a fragment spanning residues 254–369 (containing the 2D6 epitope), which when combined with the molecular mass of PLB (6 kDa), results in a cross-linked peptide with molecular mass of $\sim$18.6 kDa. This is consistent with the size of the lower, major cross-linked peptide detected by both the 2D12 and 2D6 antibodies (*asterisks*). Importantly, in an earlier study we showed that proteolytic digestion of canine SERCA2a cross-linked to N27C-PLB generated the same two major fragments detected in the present study, and protein sequencing confirmed that the smaller fragment ($\sim$18.6 kDa) started at residue Asp[254] of the Ca^{2+} pump (20). Residues 254–369 of human SERCA2a, which includes trans-
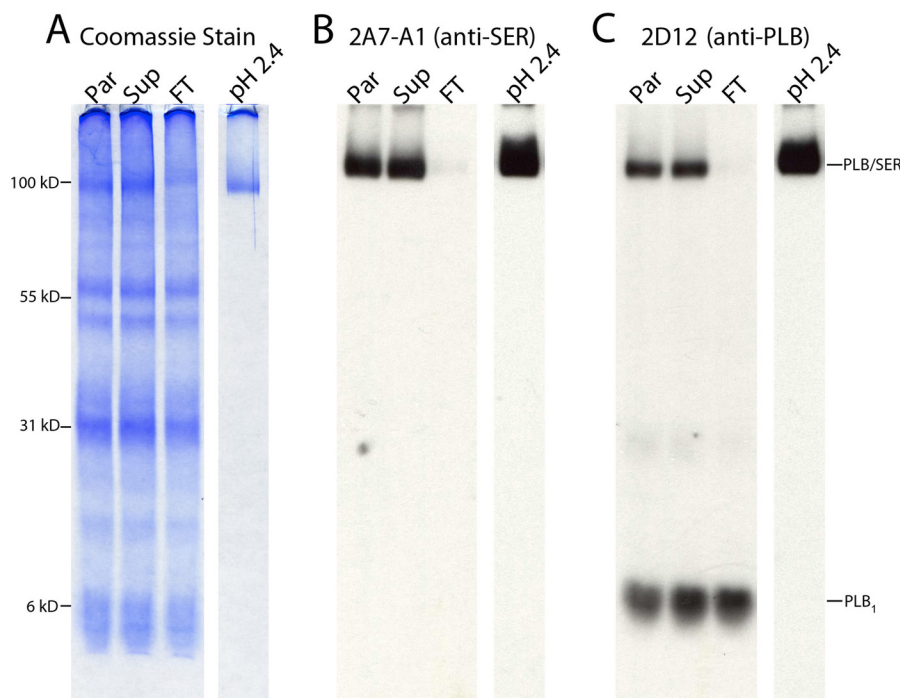
FIGURE 3. **Purification of SERCA2a from human SR vesicles using anti-SERCA2a monoclonal antibody (2A7-A1) affinity chromatography.** PLB and SERCA2a in human SR vesicles were cross-linked with DSG on large scale, solubilized in detergent, and then the Ca^{2+}-ATPase was purified by monoclonal antibody affinity chromatography using 2A7-A1. *Par*, parent microsomal fraction; *Sup*, detergent supernatant fraction; *FT*, column flow-through fraction; *pH 2.4*, peak acid-elution fraction. *A*, Coomassie Blue-stained gel showing selected fractions from the purification. *B* and *C*, immunoblots of the same fractions probed with the anti-SERCA2a antibody, 2A7-A1, or the anti-PLB antibody, 2D12, respectively.
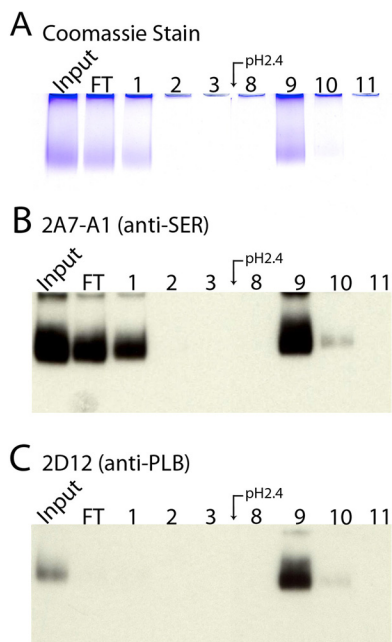

FIGURE 4. **Purification of SERCA2a cross-linked to PLB using anti-PLB monoclonal antibody affinity chromatography.** SERCA2a purified as indicated in Fig. 3 (*Input*) was subjected to anti-PLB monoclonal antibody affinity chromatography. *A*, Coomassie Blue stained gel of selected fractions. *B* and *C*, immunoblots of the same fractions probed with 2A7-A1 and 2D12, respectively.

membrane segments M3 and M4, contain four Lys residues at which PLB could potentially cross-link: Lys^{262}, Lys^{328}, Lys^{329}, and Lys^{352}. (Lys^{297} is also contained within this fragment, but is not is not a likely candidate as it is located on the opposite face of the membrane from Lys^{27} of PLB.) These

peptide mapping results are consistent with our earlier findings with recombinant N27C-PLB, which was shown to cross-link exclusively to Lys^{328} of the Ca^{2+} pump (20). Based upon these results we conclude that Lys^{27} of human PLB cross-links at (or near) Lys^{328} of the Ca^{2+} pump located within the cytoplasmic extension of M4.

Effects of Ca^{2+}, TG, and ATP on PLB Cross-linking—To test whether cross-linking interactions between PLB and SERCA2a in SR vesicles are conformationally dependent, we measured the effects of Ca^{2+}, TG, and ATP on protein coupling by DSG. SR vesicles from normal and failed human ventricles were analyzed.

Ca^{2+} effects on PLB cross-linking to SERCA2a are shown in Fig. 6, *A* and *B*. In the absence of Ca^{2+}, comparable cross-linking of PLB to SERCA2a was detected in both normal and failed SR vesicles. Cross-linking was completely inhibited by increasing the Ca^{2+} concentration over the micromolar range at which SERCA2a binds Ca^{2+} (Fig. 6B). The mean K_i values for Ca^{2+} inhibition of cross-linking were 0.11 ± 0.01 and 0.10 ± 0.01 μM Ca^{2+} for SR membranes from normal and failed hearts, respectively (mean $\pm$ S.E. from seven determinations). These results demonstrate that PLB does not cross-link to SERCA2a in the *E*1 conformation in SR vesicles.

TG effects on cross-linking, measured in the absence of Ca^{2+}, are shown in Fig. 6, *C* and *D*. Cross-linking was completely inhibited by TG in normal and failed membranes (Fig. 6C). The K_i values for TG inhibition of cross-linking were 0.18 ± 0.04 and 0.16 ± 0.04 μm, respectively (mean $\pm$ S.E. from four determinations). It should be noted that the concentration of SERCA2a protein present in the cross-linking assays was
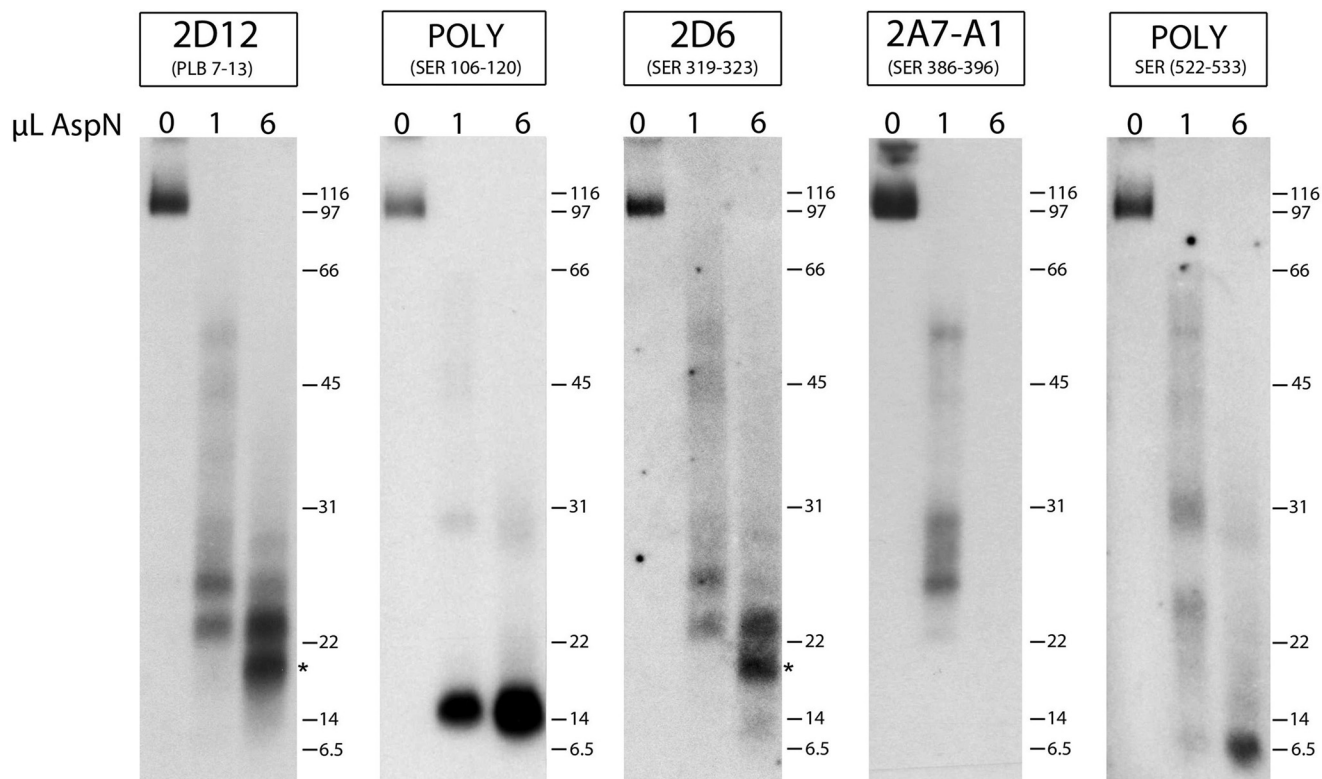
FIGURE 5. **Proteolytic mapping of PLB cross-linked to SERCA2a in human SR vesicles.** Purified SERCA2a cross-linked to PLB (1 μg) was proteolyzed with 0.04 μg (1 μl) or 0.24 μg (6 μl) of endo Asp-N. Replicate proteolyzed samples were subjected to SDS-PAGE and immunoblotted with the anti-PLB antibody, 2D12, or one of four different anti-SERCA2a antibodies recognizing different regions of the Ca^{2+} pump. *POLY*, polyclonal rabbit antiserum.

0.6–0.8 μM, within the range of TG concentration required to inhibit cross-linking completely.

Fig. 7 shows the effect of increasing ATP concentration on PLB cross-linking to the Ca^{2+} pump (measured in the absence of Ca^{2+}). For both normal and failed SR preparations, cross-linking was stimulated 2–3-fold by ATP; half-maximal stimulation of cross-linking occurred at 109.4 $\pm$ 17.1 μM ATP for membranes from normal hearts and 99.4 $\pm$ 15.4 μM ATP for membranes from the failed hearts (mean $\pm$ S.E. from five determinations). Thus, the affinity of SERCA2a for ATP at the modulatory ATP binding site (13, 31) is comparable in membranes from normal and failed hearts.

Effect of 2D12 on Ca^{2+}-ATPase Activity and PLB Cross-linking—The anti-PLB antibody, 2D12, reverses PLB inhibition of SERCA2a activity in SR vesicles (5, 9) and has been shown to disrupt the physical interaction between the two proteins in our recombinant system (23). It was of interest to know whether 2D12 inhibits PLB cross-linking to SERCA2a in SR vesicles.

Fig. 8 shows Ca^{2+} stimulation of Ca^{2+}-ATPase activity measured in SR vesicles prepared from normal (*A*) and failed (*B*) human ventricles, determined in the presence and absence of the 2D12 antibody. In the absence of 2D12, the K_{Ca} values for half-maximal enzyme activation were 0.24 and 0.20 μM Ca^{2+} for normal and failed membranes, respectively. In the presence of 2D12, the K_{Ca} values were leftward-shifted about 2-fold, from 0.24 to 0.13 μM Ca^{2+} for the normal SR vesicles and from 0.20 to 0.12 μM Ca^{2+} for the failed SR vesicles. No effect of 2D12 on maximal Ca^{2+}-ATPase activity, measured at saturating

Ca^{2+} concentration ($\sim$1 μM), was noted for SR membranes prepared from normal and failed hearts, consistent with previous results by Movsesian *et al.* (32).

Fig. 8*C* shows the effect of 2D12 on PLB cross-linking to SERCA2a measured at 0.03 μM Ca^{2+}, the lowest Ca^{2+} concentration used in the Ca^{2+}-ATPase assay. 2D12 abolished PLB cross-linking to SERCA2a in membranes prepared from both normal and failed hearts. These results demonstrate that 2D12 activation of Ca^{2+}-ATPase activity is associated with PLB dissociation from the Ca^{2+} pump in SR vesicles.

Effect of 2D12 on Ca^{2+} Transport and PLB Cross-linking in Human SR Vesicles—Functional effects of 2D12 were analyzed in greater detail using the more sensitive Ca^{2+} uptake assay and correlated with PLB cross-linking. The effect of increasing 2D12 concentration on Ca^{2+} uptake into human SR vesicles, measured at 0.03 μM Ca^{2+}, is shown in Fig. 9*A*. In the absence of 2D12, Ca^{2+} uptake was low and barely detectable at 30 nM Ca^{2+} (*filled squares*). Addition of 2D12 strongly stimulated Ca^{2+} uptake. For example, 0.6 μg of 2D12 added to 14.5 μg of SR vesicles (*filled circles*) gave a 5-fold stimulation of Ca^{2+} uptake, producing 20% of the maximal response. At 1.7–7.5 μg of added 2D12, stimulation of Ca^{2+} uptake reached saturation, giving a striking 18-fold increase in Ca^{2+} uptake.

2D12 binding to PLB in SR vesicles, measured in the same assay, is shown in Fig. 9*B*. Antibody binding saturated over the same concentration range at which Ca^{2+} uptake was stimulated. The K_d determined for 2D12 binding to PLB (42.5 nM) corresponds to an added 2D12 concentration of 0.8 μg. Fig. 9*C* shows the effect of 2D12 on PLB cross-linking in the same assay.
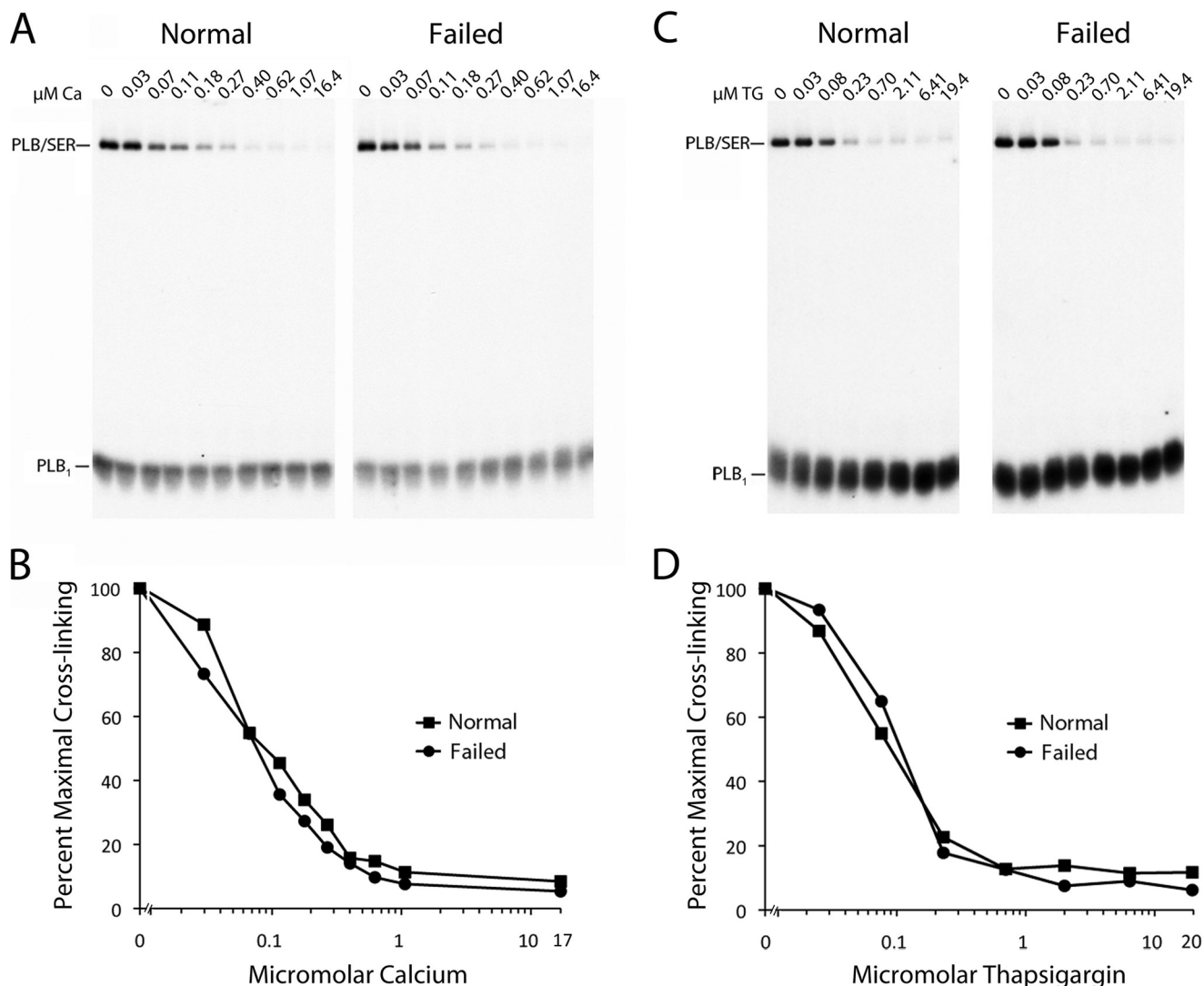
FIGURE 6. **Effect of Ca^{2+} and TG on PLB cross-linking to SERCA2a in SR membranes from normal and failed human ventricles.** *A* and *C*, autoradiographs showing concentration dependence of Ca^{2+} (*A*) and TG (*C*) inhibition of PLB cross-linking to SERCA2a in normal and failed human SR vesicles. *B* and *D*, results depicted graphically.

2D12 inhibited PLB cross-linking to SERCA2a over the identical concentration range at which Ca^{2+} uptake was stimulated and 2D12 binding to PLB occurred. Fig. 9*D* is a composite plot showing the average results from five separate assays conducted with both nonfailing and failing human cardiac SR preparations. Clearly, there is a strong correlation between 2D12 binding to PLB, stimulation of Ca^{2+} uptake, and inhibition of cross-linking. These results strongly suggest that dissociation of PLB from the Ca^{2+} pump is associated with SECA2a activation.

DISCUSSION

In the present study, we demonstrated highly specific, robust cross-linking between PLB and SERCA2a in SR vesicles prepared from normal and failed human hearts. Cross-linking was monitored for the first time between the naturally occurring proteins in the SR membrane, concurrently with PLB effects on Ca^{2+}-ATPase activity and Ca^{2+} transport. Our results provide important insights into the physical mechanism of Ca^{2+} pump inhibition by PLB.

Mechanism of PLB Inhibition in SR Vesicles—It is largely accepted that PLB inhibits SERCA2a activity by lowering Ca^{2+} affinity, having little or no effect on the V_{max} of the enzyme measured at saturating Ca^{2+} concentration (1). This inhibitory effect of PLB is manifested as a rightward shift in the Ca^{2+} curve of enzyme activation and has been observed in SR vesicles prepared from all mammalian species examined to date, including SR vesicles prepared from canine (5) and human hearts (32). As a mechanism to explain this effect of PLB on Ca^{2+} affinity, it has long been proposed that PLB competes with Ca^{2+} for binding to SERCA2a (1). However, due to an inability to easily monitor protein binding interactions between the wild-type proteins, it was, until now, difficult to test this hypothesis directly in SR vesicles. Here, we took advantage of the observation that Lys[27] of human PLB is cross-linkable to SERCA2a at the cytoplasmic extension of M4, to test for competition between PLB and Ca^{2+} for binding to SERCA2a in human SR vesicles. Indeed, PLB cross-linking to SERCA2a was completely inhibited by Ca^{2+} over the same micromolar concentration range required for formation of *E*1 and activation of ATP hydrolysis. This result

confirms in human SR vesicles that PLB cross-links exclusively to $E2$. In our present work, Ca^{2+}-sensitive cross-linking of PLB to SERCA2a in SR vesicles was demonstrated at a single point of interaction, but it should be pointed out that identical effects of Ca^{2+} were demonstrated previously at numerous cross-linking sites between PLB and SERCA2a, located on either side of the lipid bilayer (12, 13, 20–25).
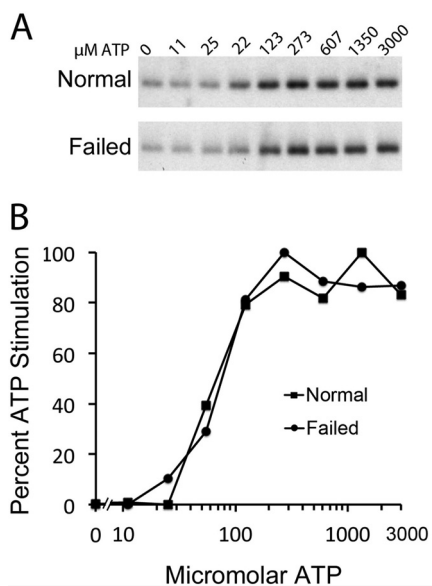

FIGURE 7. **ATP effect on cross-linking.** *A*, ATP stimulation of PLB cross-linking to SERCA2a in normal and failed human SR vesicles in the absence of Ca^{2+}. *B*, percentage of ATP stimulation of cross-linking *versus* ATP concentration plotted graphically.

To demonstrate directly in human SR vesicles that SERCA2a with PLB bound is catalytically inactive (12, 13, 24, 25), we used the 2D12 antibody to disrupt the physical interaction between the two proteins at very low Ca^{2+} concentration. Importantly, we observed a strong correlation between 2D12-induced dissociation of PLB from $E2$, and 2D12 stimulation of Ca^{2+}-ATPase activity and Ca^{2+} uptake (Figs. 8 and 9). Collectively, these results strongly support the model of mutually exclusive binding of PLB to $E2$ and Ca^{2+} to $E1$ as the molecular mechanism of PLB inhibition.

To verify further the specificity of the Lys^{27} cross-link identified in human SR vesicles, we tested the effects of other allosteric modifiers on the cross-linking interaction. As demonstrated previously with the recombinant proteins (13, 20, 24), TG binding to the Ca^{2+} pump induced a conformational state that was incompatible with PLB binding ($E2 \cdot TG$), whereas ATP binding to the modulatory nucleotide binding site induced a state that favored PLB binding. Therefore, we conclude that PLB competes with Ca^{2+} for binding to SERCA2a in SR vesicles by stabilizing the enzyme in a unique $E2 \cdot ATP$ state.

Molar Content of PLB and SERCA2a in SR Vesicles and Role of Pentamer—In this work, we determined that PLB is present in slight molar excess over SERCA2a in human SR vesicles. The molar ratio of PLB to SERCA2a determined here (about 1.3:1) is considerably lower than the 5:1 ratio (PLB to SERCA2a) previously reported for mammalian SR vesicles (36).

The molar stoichiometry determined here allows us to draw two new conclusions about the protein-protein interactions occurring between PLB and SERCA2a in the SR membrane. First, the affinity of the PLB monomer for $E2$ is
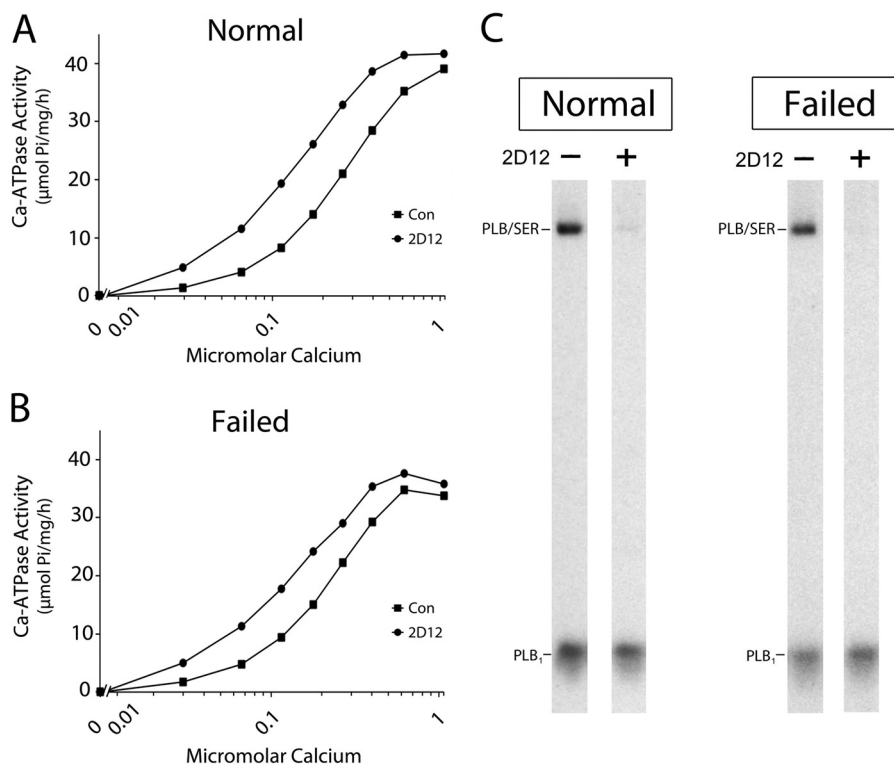

FIGURE 8. **2D12 effects on Ca^{2+}-ATPase activity and PLB cross-linking.** *A* and *B*, Ca^{2+}-ATPase activity was measured in SR vesicles from normal (*A*) and failed (*B*) hearts in the presence and absence of the 2D12 antibody. *C*, effect of 2D12 on PLB cross-linking to SERCA2a at 0.03 μm Ca^{2+} concentration. Cross-linking samples contained 11 μg of SR vesicles in the presence or absence of 5 μg of 2D12.
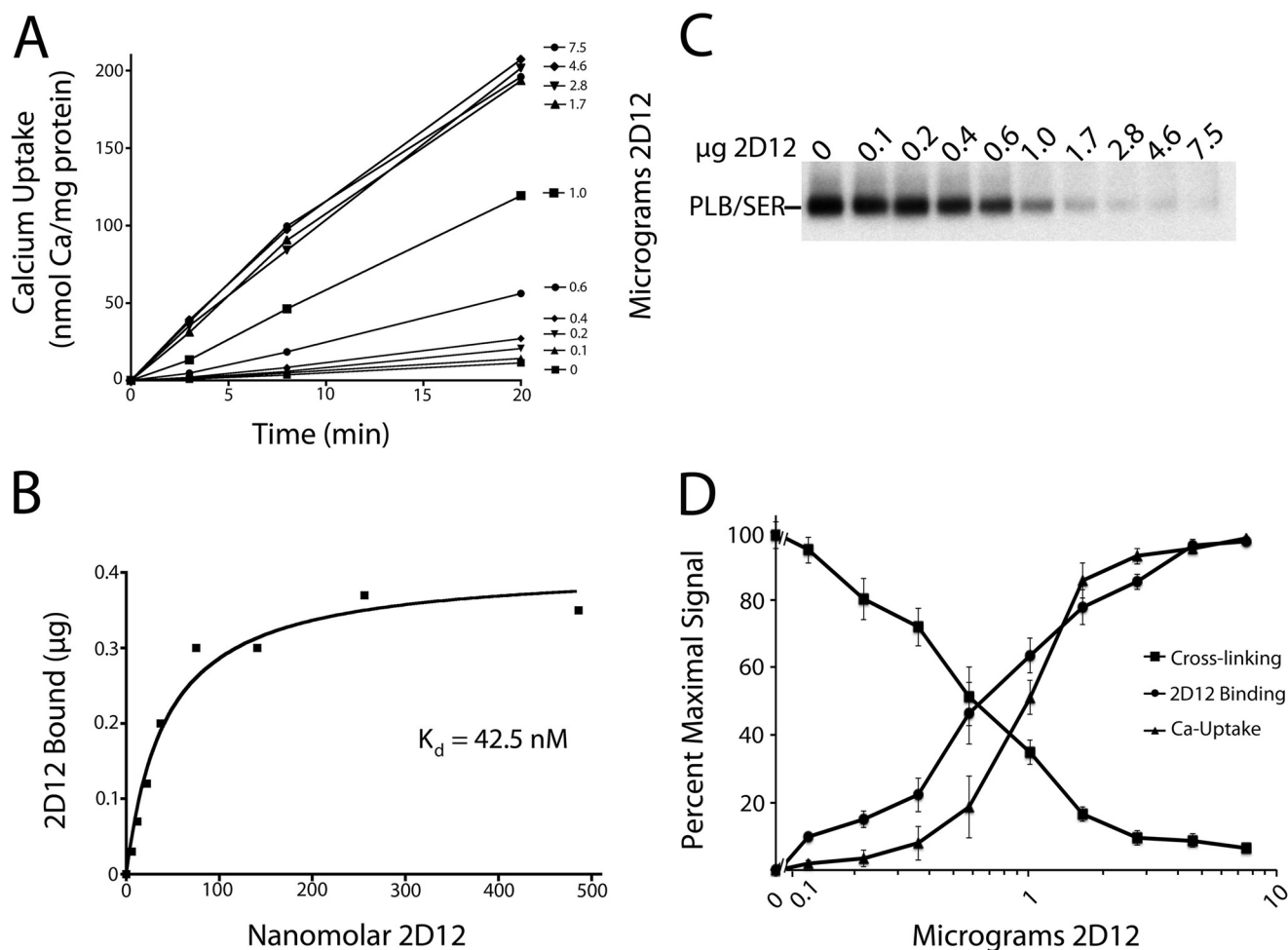
FIGURE 9. **2D12 Binding to PLB in human SR vesicles measured simultaneously with 2D12 effects on PLB cross-linking and Ca^{2+} uptake.** *A*, ^{45}Ca^{2+} uptake into human cardiac SR vesicles (14.5 μg) was measured at 37 °C in the presence of 1 mM EGTA and 0.1 mM CaCl$_2$, and 0–7.5 μg of 2D12 antibody (indicated in *right margin*). *B*, ^{125}I-2D12 binding to PLB was measured in assay tubes prepared and handled identically to those described for Ca^{2+} uptake, only trace ^{45}Ca^{2+} was omitted. On the *abscissa*, the free 2D12 (non–protein-bound) concentration is plotted. *C*, 2D12 effect on PLB cross-linking to SERCA2a measured in assay tubes prepared and handled identically to those described for Ca^{2+} uptake (*A*) and 2D12 binding (*B*), only omitting radionuclides. *D*, composite plot showing averaged results conducted as in *A–C* (means ± S.E. (*error bars*) from five separate experiments conducted with normal and failed membranes).

much stronger than previously appreciated (12); and second, it is likely that an important function of the PLB pentamer is to aid in complete reversal of SERCA2a inhibition at high Ca^{2+} concentration.

These two conclusions are supported by the following reasoning: at low Ca^{2+} concentration, SERCA2a is almost completely catalytically inactive due to the fact that nearly all of the Ca^{2+} pumps have PLB bound (as evidenced by the dramatic stimulation of Ca^{2+} uptake induced by the 2D12 antibody at nanomolar Ca^{2+} concentration, Fig. 9). Because most of the PLB is bound to SERCA2a at very low Ca^{2+} concentration, the pool of PLB pentamers in the SR membrane must be nearly depleted. This suggests that the transmembrane contacts stabilizing the PLB-*E*2 interaction are actually much stronger than the Leu/Ile zipper contacts stabilizing the PLB pentamer (7). However, despite the high affinity of the PLB monomer for *E*2 (in the range of vanadate binding to *E*2) (24), PLB inhibition of SERCA2a activity is still completely reversed at saturating Ca^{2+} concentration (Fig. 8), when Ca^{2+} binding to *E*1 induces the complete dissociation of PLB from the Ca^{2+} pump (Fig. 6).

Therefore, self-assembly of PLB monomers into noninhibitory pentamers may help to reverse Ca^{2+} pump inhibition when Ca^{2+} is not limiting and pentamer formation is expected to be at its greatest.

Physical Association between PLB and SERCA2a in SR Membranes from Failed and Nonfailed Hearts—In the present study, we found no difference in K_{Ca} values for activation of Ca^{2+}-ATPase activity in SR membranes prepared from normal and failed human hearts. Using chemical cross-linking, we went on to demonstrate that physical interactions between PLB and SERCA2a are identical in SR membranes prepared from normal and failed hearts. In both cases, PLB binds exclusively to *E*2, and dissociation of PLB from *E*2 is required for SERCA2a activation. Moreover, the molar ratio of PLB to SERCA2a is similar in SR membranes from normal and failed human hearts. These results suggest that PLB is a reasonable protein target for developing drugs to treat heart failure. A drug that acts like 2D12, preventing PLB binding to SERCA2a, might be expected to produce large increases in cardiac contraction and relaxation in failed human myocardium (9).

Although not the focus of this study, in light of our current results, we feel it important to address the very recent study by Kho *et al.* (30) in which it was suggested that SUMOylation of SERCA2a in human hearts by SUMO1 increases Ca^{2+} pump stability and Ca^{2+}-ATPase activity by enhancing ATP binding affinity (30). The authors also suggested that the level of SUMOylation of SERCA2a is reduced in failing human hearts and that restoration of SERCA2a SUMOylation may be a viable means of treating heart failure (30). However, in our present study with normal and failed human hearts, no 150-kDa mobility form that might correspond to SUMOylated SERCA2a was detected. Nor was a slower mobility form of SERCA2a detected in numerous previous immunoblotting studies quantifying the Ca^{2+}-ATPase in normal and failed human myocardium (35). Also in contrast to Kho *et al.* (30), here we observed similar ATP binding affinities for SERCA2a in membranes from normal and failed myocardium (Fig. 7). It should be pointed out that in the study by Kho *et al.* (30), ATP affinity of SERCA2a was assessed to an enzyme solubilized in Triton X-100 and 10 mM EDTA, a condition known to promote rapid denaturation of the Ca^{2+} pump. Therefore, we believe the role of SERCA2a SUMOylation in normal and failing human myocardium should be viewed cautiously.

Functionality of Lys^{27}—An earlier mutagenesis study demonstrated that lysine substitution at Asn^{27} of PLB (creating human PLB) modestly augments PLB inhibitory function, increasing the K_{Ca} for SERCA2a activation slightly more than PLB with Asn^{27} (37). Subsequently, in a study in which human PLB was expressed in transgenic mice, Kranias and co-workers concluded that lysine at position 27 makes PLB superinhibitory, inducing heart failure and cardiac remodeling in transgenic mice (38, 39). Because of these effects observed in mice, novel functions for PLB in human myocardium were proposed (38, 39). However, in the present study, we determined that human PLB (with Lys^{27}) inhibits SERCA2a activity to the same extent as canine PLB, which has Asn^{27}. In human SR vesicles, the K_{Ca} value for Ca^{2+}-ATPase activation was determined to be 0.22 ± 0.03 μM; in dog SR vesicles, the K_{Ca} value for Ca^{2+}-ATPase activation was 0.27 ± 0.01 μM. In the presence of the 2D12 antibody, the K_{Ca} values were 0.13 ± 0.01 and 0.12 ± 0.01 μM for human and canine cardiac SR vesicles, respectively (mean $\pm$ S.E. from five separate preparations). Clearly, no superinhibitory effect of human PLB was noted. Likewise, using our co-expression system with Sf21 cells, we observed that human PLB (with Lys^{27}) decreased the apparent Ca^{2+} affinity of SERCA2a to the same extent as canine PLB (with Asn^{27}) (data not shown). Therefore, we conclude that PLB with Asn^{27} or Lys^{27} behaves virtually identically with respect to its ability to inhibit SERCA2a activity in SR membranes.

CONCLUSIONS

In summary, we have demonstrated with the simple system of human SR vesicles that PLB cross-links exclusively to the *E*2 state of SERCA2a. Cross-linking occurs at the inhibitory binding site originally characterized in more complicated recombinant systems (12, 13, 20–25). Importantly, because the naturally occurring Lys^{27} of human PLB (unique to human PLB) is cross-linkable near the natural Lys^{328} (at the cytoplasmic exten-

sion of M4) of SERCA2a, we were able, for the first time, to characterize PLB to SERCA2a binding interactions using native proteins in the SR milieu, without the need for mutant or fusion proteins (11–15), or protein solubilization and reconstitution (16–19). Cross-linking PLB to SERCA2a in human SR vesicles exhibited conformational constraints identical to those observed previously using recombinant proteins, including sensitivity to Ca^{2+}, TG, ATP, and the 2D12 antibody. Most importantly, stimulation of Ca^{2+}-ATPase activity and Ca^{2+} ion transport correlated closely with inhibition of PLB cross-linking to SERCA2a, which strongly suggests that enzyme activation requires PLB dissociation. It was recently suggested that when the enzyme binds Ca^{2+}, PLB may leave the M2/M4/M9 binding site and move to an alternate binding location in the Ca^{2+} pump (14, 19), but an accessory binding site has yet to be identified with the cross-linking approach (12, 13, 20–25). Regardless of whether or not PLB binds at a secondary, noninhibitory site, it seems clear that the cross-linking interactions detected here and previously reflect genuine protein-protein interactions that are that closely associated with functionality of the Ca^{2+} pump.

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