Direct Detection of Phospholamban and Sarcoplasmic Reticulum Ca-ATPase Interaction in Membranes Using Fluorescence Resonance Energy Transfer†

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ABSTRACT: We used fluorescence resonance energy transfer (FRET) to detect and quantify the interaction of the sarcoplasmic reticulum Ca-ATPase (SERCA) with phospholamban (PLB) in membranes. PLB inhibits SERCA only at submicromolar Ca. It has been proposed that relief of inhibition at micromolar Ca is due to dissociation of the inhibitory complex. To test this hypothesis, we co-reconstituted donor-labeled SERCA and acceptor-labeled I40A-PLB (superinhibitory, monomeric PLB mutant) in membranes of defined lipid and protein composition, with full retention of Ca-dependent ATPase activity and inhibitory regulation by PLB. FRET from SERCA to PLB was measured as a function of membrane concentrations of PLB and SERCA, and functional activity was measured on the same samples. The data revealed clearly that the stoichiometry of binding is one PLB per SERCA, and that binding is a strict function of the ratio of total PLB to SERCA in the membrane. We conclude that the dissociation constant of PLB binding to SERCA is far less than physiological PLB membrane concentrations. Binding at low Ca (pCa 6.5), where I40A-PLB inhibits SERCA, was virtually identical to that at high Ca (pCa 5.0), where no inhibition was observed. However, the limiting energy transfer at saturating PLB was less at high Ca, indicating a greater donor–acceptor distance. We conclude that (a) the affinity of PLB for SERCA is so great that PLB is essentially a SERCA subunit under physiological conditions and (b) relief of inhibition at micromolar Ca is due to a structural rearrangement within the SERCA–PLB complex, rather than dissociation.

The Ca-ATPase (SERCA)† of the sarcoplasmic reticulum (SR) removes Ca ions from the cytoplasm to relax muscle. In cardiac muscle, this enzyme is regulated by phospholamban (PLB) (1), which inhibits SERCA at submicromolar Ca2+. This inhibition can be relieved either by elevation of [Ca2+] to the micromolar range or by phosphorylation of PLB by protein kinase A (PKA). This phosphorylation is regulated via β-adrenergic cascades (2) and therefore serves as an adrenergic response element of muscle relaxation. The SERCA–PLB calcium-regulatory system has been implicated in cardiovascular disease (3–9). Recently, the R9C mutation of PLB in humans was directly linked to development of dilated cardiomyopathy (10) and progression to failure of the heart in young adults. A human PLB null phenotype (8), contrary to that of mice (11–13), did not protect against development of heart failure, but instead led to disease in young adults as well. PLB continues to attract attention as a possible target for pharmaceutical intervention (14). Gene therapy in hamsters, in which a pseudophosphorylated PLB (S16E) was introduced in vivo, was successful in preventing succession to heart failure (15). Elucidation of the physical mechanism by which PLB regulates SERCA is needed to evaluate these results and to develop strategies for intervening in human heart disease.

Most insight into the physical interaction of SERCA with PLB has been obtained indirectly, through effects on function. It has often been suggested that decreased inhibition, due to phosphorylation of PLB, micromolar Ca2+, or mutation, is due to dissociation of PLB from SERCA (Kd), in a delicately balanced dynamic binding equilibrium, which also involves oligomeric interactions within PLB (Kd) and...
within SERCA ($K_{d1}$) (Figure 1; 16–20). To test this hypothesis, it is necessary to measure the dissociation constant ($K_{d2}$) for the binding of PLB to SERCA directly and quantitatively, and to determine whether changes in $K_{d2}$ correlate with changes in function.

The SERCA enzyme has been shown to co-immunoprecipitate with coexpressed PLB in detergent solution (21), with the interaction decreased by micromolar Ca$^{2+}$, suggesting that Ca induces dissociation of PLB from SERCA. It was reported that the cytoplasmic domain of PLB can be cross-linked to SERCA in cardiac SR and that elevated Ca$^{2+}$ or phosphorylation of PLB decreases cross-linking (22). Similarly, cross-linking in an expression system (23, 24) showed that PLB is preferentially cross-linked to SERCA at submicromolar [Ca$^{2+}$] in the presence of nucleotide. However, another study showed that PLB cross-links to SERCA in cardiac SR even when [Ca$^{2+}$] is not controlled and is probably saturating the enzyme (25). Cross-linking does not measure $K_{d2}$ quantitatively, since this chemical reaction depends on many factors in addition to binding, and since the cross-linking reaction itself perturbs the equilibrium it is meant to measure.

To measure $K_{d2}$ in a membrane, it is necessary to detect a signal in a functional system that relates directly and quantitatively to this specific protein—protein interaction, and to vary the concentrations of components systematically to define the thermodynamic equilibrium. The PLB–SERCA interaction has been detected previously with spectroscopic probes on either the SERCA enzyme or PLB, but not both simultaneously. Effects of PLB on SERCA have been measured by infrared spectroscopy (26), and time-resolved phosphorescence anisotropy (TPA) has been used to measure SERCA rotational mobility (18, 27–29) and to correlate PLB inhibition of SERCA with aggregation (30). Detailed and quantitative analyses of the dissociation equilibria involved in this system (Figure 1) are needed. Therefore, in the present study, we have used fluorescence resonance energy transfer (FRET) to detect directly the interaction of donor-labeled SERCA with acceptor-labeled PLB in membranes. By using a reconstituted system of defined protein and lipid composition, we have controlled the 2-dimensional membrane concentrations of both proteins. We show through biochemical assays on the same samples that this reconstituted system is fully active and biochemically coupled, and displays normal sensitivity to Ca$^{2+}$, so the spectroscopic results have direct implications for the molecular mechanism of SERCA regulation.

![Figure 1: Model depicting the role of dynamic protein—protein interactions in the regulation of SERCA by PLB (based on refs 16–19). The present study addresses the proposal that inhibitory interactions between SERCA and PLB are diminished by elevated Ca$^{2+}$, resulting in a large increase in the dissociation constant $K_{d2}$.](image)

**MATERIALS AND METHODS**

Reagents. Octaethylene glycol monododecylether (C$_3$E$_8$) and Bioseas SM2 were purchased from Calbiochem (San Diego, CA). Dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). The reagents and equipment for SDS–PAGE (4–20% Tris–glycine Criterion precast polyacrylamide gels and Tris–glycine running buffer) and Western blot analysis (except antibody and [I$^{125}$]protein A) were purchased from Bio-Rad (Richmond, CA). Hybridoma cells expressing anti-PLB antibody 8A3-D5 were purchased from Covance Research Products, Inc. (Denver, PA). Radioactively labeled [I$^{125}$]protein A was obtained originally from Dr. Diana Bigelow, and the antibody was then produced by Covance Research Products, Inc. (Denver, PA). Spectroscopic dyes were purchased from Molecular Probes (Eugene, OR). All other reagents and those used in the coupled enzyme assay for measuring ATP hydrolysis rates were of the highest purity available and purchased from Sigma (St. Louis, MO).

**Peptide Synthesis and Purification of I40A-PLB.** Materials, solvents, instrumentation, and general methods of solid-phase peptide synthesis were essentially as described previously (31–33). In short, the peptide was assembled on Fmoc-Leu-Peg-PS resin (initial load 0.2 mmol/g) by Fmoc chemistry using a PE Biosystems Pioneer peptide synthesis system. All couplings were done in NMP, mediated by HBTU/HOBt/DIEA (4:4:8 (equiv) with respect to peptide–resin). The N-terminal amino group was acetylated using acetic anhydride (34). First, an Fmoc removal step (with 20% piperidine and 2% DBU in NMP) was carried out on 200 mg of peptide resin followed by treatment with 0.5 M acetic anhydride in 10 mM of DMF for 2 h. Final deprotection was performed by treatment with 2 mL of freshly prepared solution (82.5% TFA, 5% phenol, 5% thioanisole, 2.5% 1,2-ethanediol, 5% water) for 6 h at 25 °C (35). The acetylated peptide resin was filtered and cleaved. The cleavage mixture was filtered and washed with 2 mL of the same solution. Combined filtrates were concentrated under nitrogen gas, and precipitated in 30 mL of diethyl ether at 0 °C. Precipitated peptide was collected by centrifugation, and washed three times with ice-cold diethyl ether. Crude peptide was dissolved in 5 mL of TFA and purified by HPLC on a C-18 column (Vydac, 218TP54) that had been equilibrated with 95% water, 2% acetonitrile, and 3% 2-propanol. The peptide was eluted using a linear gradient to a final solvent composition of 5% water, 38% acetonitrile, and 57% 2-propanol (36). Fractions containing peptides were lyophilized to yield 26 mg of I40A-PLB (12% yield based on starting resin).

**Labeling of I40A-PLB.** Acetylated (N-terminus), cleaved, and purified I40A-PLB was labeled with DABCYL-SE on Lys 3. DABCYL was chosen because it lacks fluorescence emission and, therefore, does not interfere with the donor emission signal. A 10 mg sample of acetylated I40A-PLB was dissolved in 100 mM NaHCO$_3$ (pH 9.0) and 0.1% sodium dodecyl sulfate (SDS). A 10-fold molar excess of DABCYL-SE was added from a 100 mM stock solution in DMF, and the sample was incubated for 16 h at 25 °C with shaking. Unreacted DABCYL-SE was removed by HPLC using a diphenyl column (Vydac, 219TP510; 5 μm, 300×30 mm). DABCYL-labeled I40A-PLB (DAB–I40A-
PLB) was eluted in a 30 min, 0–100% A to B gradient with a 2.5 mL/min flow rate (buffer A, 95% H₂O, 2% acetonitrile, 3% 2-propanol, and 0.1% trifluoroacetic acid; buffer B, 5% H₂O, 38% acetonitrile, 57% 2-propanol, and 0.1% TFA). The elution time was approximately 22–25 min. Fractions were lyophilized to yield 6.3 mg of DAB—I40A-PLB (63% yield). The dye concentration in labeled samples was determined by direct sample absorbance in methanol (ε₄₃₃nm = 32000 M⁻¹ cm⁻¹).

Chemical Analysis of Labeled PLB. Mass spectra were acquired with a Bruker Biflex III matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) system equipped with a nitrogen laser (337 nm, 3 ns pulse length) and a microchannel plate detector. Samples were cocrystallized from a stock solution in methanol with the matrix 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), and data were collected in the linear mode, positive polarity, with an accelerating potential of 19 kV. Each spectrum was an accumulation of 100–400 laser shots. Amino acid analysis was used to confirm peptide composition and concentration. Purified DAB—I40A-PLB was stored in methanol in the dark at −80 °C until it was used for reconstitution and Western blots.

SERCA Purification and Labeling. SERCA was purified from skeletal muscle of New Zealand white rabbits in 0.01% C₁₂E₈ by the Reactive-Red method (37) and then flash-frozen and stored in liquid nitrogen after addition of 0.5 mg of lipid (sonicated DOPC and DOPE, 4:1 ratio by weight)/mg of protein. The protein concentration was determined by the Lowry method (38), using bovine serum albumin as the standard. Prior to labeling, detergent was removed by the same method used in reconstitution (see below). SERCA was labeled with IAEDANS (labeling buffer: 80 mM KCl, 5 mM MgCl₂, 1 mM CaCl₂, 20 mM MOPS, pH 6.8) at 18.2 μM enzyme and 273 μM dye for 30 min at 25 °C in the dark. Free dye was removed by centrifugation and washing three times (wash buffer: 20 mM sucrose, 100 mM KCl, 20 mM MOPS, pH 7). The dye concentration in labeled samples was determined from absorbance (ε₃₄₄nm = 6100 M⁻¹ cm⁻¹ (39)) after treatment with 0.1 N NaOH and 1% SDS. Samples of AEDANS—SERCA were flash-frozen and stored in the dark in liquid nitrogen until they were used for reconstitution, ATPase assays, and FRET measurements.

Reconstitution. AEDANS—SERCA (donor) was co-reconstituted with DAB—I40A-PLB (acceptor), essentially as described (40–42) but adapted to modify the SERCA membrane concentration (molar ratio, SERCA/1000 lipids) and PLB-to-SERCA ratio (molar ratio, PLB/SERCA). The desired amount of DAB—I40A-PLB in methanol and lipids (DOPC and DOPE at a 4:1 weight ratio in chloroform) were solubilized from a stock solution in methanol with the matrix 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), and data were collected in the linear mode, positive polarity, with an accelerating potential of 19 kV. Each spectrum was an accumulation of 100–400 laser shots. Amino acid analysis was used to confirm peptide composition and concentration. Purified DAB—I40A-PLB was stored in methanol in the dark at −80 °C until it was used for reconstitution and Western blots.

ATPase Measurements and Data Analysis. ATPase activity was measured using an NADH-linked, enzyme-coupled microtiter plate assay (200 μL/well) as previously described (32, 43). [Ca²⁺] was controlled by Ca/EGTA buffering by the method of Fabiato and Fabiato (44). The assays were detected at 340 nm on a Spectramax Plus microplate spectrophotometer (Molecular Devices) at 25 °C in the presence of ionophore A23187 to dissipate the Ca gradient building up across the membranes due to ATPase activity. The data were plotted (V vs pCa) and fitted by the Hill equation:

\[ V = \frac{V_{\text{max}}}{1 + 10^{n(pK_{\text{Ca}} - pCa)}} \]  

where V is the initial ATPase rate and n is the Hill coefficient. Data were normalized to the maximal rate, V_{\text{max}}, which was obtained from the fit, and replotted to determine the shift in pK_{Ca}. We did not use PLB phosphorylation as a means of modifying the SERCA—PLB interaction, because phosphorylation of I40A-PLB has been shown to have little or no effect on its inhibition of SERCA (43).

Fluorescence Measurements and Data Analysis. Steady-state fluorescence spectra were obtained in a 5 × 10 mm quartz cuvette with an ISS-K2 fluorescence spectrophotometer (ISS, Champaign, IL) using an argon ion laser (Coherent, Santa Clara, CA) as the source of excitation at 351.1 nm. Each emission spectrum was the average of two scans with a 1 nm step size, and was corrected by subtraction of a corresponding blank using a reconstituted sample containing unlabeled SERCA at the appropriate SERCA membrane concentration and PLB/SERCA ratio. The sample temperature was controlled using a recirculating water bath set to 25 °C. The free calcium concentration, [Ca²⁺], was controlled by Ca/EGTA buffering, calculated by the method of Fabiato and Fabiato (44). Measurements were conducted in low-Ca (pCa 6.5) buffer (50 mM KCl, 5 mM MgCl₂, 0.5 mM EGTA, 210 μM CaCl₂, 50 mM MOPS, pH 7.0), where the inhibitory effect of PLB was maximal, and high-Ca (pCa 4.5) buffer (same as low-Ca buffer, except 489 μM CaCl₂), where the inhibitory effect of PLB was negligible.

The fluorescence resonance energy transfer efficiency (E) was calculated from the fractional decrease of steady-state emission of the donor (F_D) due to the presence of the acceptor (F_A):

\[ E = 1 - \frac{F_{DA}}{F_D} \]  

The donor–acceptor distance R in the saturated SERCA—PLB complex was calculated from

\[ R = R_0(E^{-1} - 1)^{1/6} \]
where the Förster distance $R_o$ of this dye pair was calculated from

$$R_o = 9780(J\kappa^2 n^{-4} \phi_D)^{1/6} \tag{4}$$

where $n$ is the refractive index of protein in aqueous solution (1.33), $\kappa$ is the orientation factor (set to 2/3, corresponding to random orientation), and $\phi_D$ is the donor quantum yield (0.36 for AEDANS (45)). $J$ is the normalized spectral overlap integral of donor emission $F_D(\lambda)$ and acceptor excitation $\epsilon(\lambda)$

$$J = \frac{\int F_D(\lambda) \epsilon(\lambda) \lambda^2 d\lambda}{\int F_D(\lambda) d\lambda} \tag{5}$$

and was calculated by numerical integration using a Microcal Origin 7.0 template.

Time-resolved fluorescence decays were detected to obtain more detailed information about FRET. Decays were obtained in the same 5 x 10 mm quartz cuvette and using identical samples on an instrument developed in collaboration with Dakota Technologies, Inc. (Fargo, ND). This instrument uses a solid-state pulsed laser (frequency-tripled Nd:YAG, 355 nm), operating at a rate of 10 kHz, and acquires the entire fluorescence waveform after each laser pulse, at a time resolution of 0.2 ns/data point. The instrument response function was measured with a light-scattering sample (water) in the same cuvette used for sample measurements and was used in data analysis to determine fluorescence lifetimes (45). Excited-state lifetimes ($\tau_i$) and mole fractions ($x_i$) were obtained by fitting the lifetime-dependent fluorescence exponential

$$F(t) = F_o \sum_{i=1}^{n} x_i e^{-t/\tau_i} \tag{6}$$

to decays with peak intensity $F_o$. The energy transfer efficiency $E$ was calculated as the fractional decrease of the donor lifetime. A direct comparison with steady-state data was made by calculating the fractional decrease in the average lifetime $\langle \tau \rangle = \sum \tau_i x_i$.

Analysis of FRET due to Specific Binding. The SERCA–PLB interaction can be described by the dissociation equilibrium

$$\text{SERCA–PLB} \leftrightarrow \text{SERCA} + \text{PLB} \tag{7}$$

This assumes that the stoichiometry of the SERCA–PLB complex is 1:1, consistent with previously reported models (46–48), and observations that the PLB monomer is the inhibitory species (16, 17). It has been established previously that 140A-PLB is monomeric (17, 43). Therefore, the dissociation equilibrium (eq 7) is not complicated by linkage to the monomer–oligomer equilibrium of PLB ($K_{d2}$ in Figure 1). The dissociation constant $K_{d2}$ for this equilibrium is defined as

$$K_{d2} = \frac{[\text{SERCA}][\text{PLB}]}{[\text{SERCA–PLB}]} \tag{8}$$

and the fraction of SERCA molecules with bound PLB ($x_B$) is

$$x_B = \frac{[\text{SERCA–PLB}]}{[\text{SERCA}][\text{SERCA–PLB}]} = \frac{[\text{PLB}]}{K_{d2} + [\text{PLB}]} \tag{9}$$

The binding of PLB to SERCA can be measured by FRET due to specific binding ($E_{SB}$):

$$E_{SB} = x_B E_{max} \tag{10}$$

where $E_{max}$ is the maximal FRET value that describes the asymptote of the specific binding hyperbola.

We expect high-affinity binding and hence ligand depletion, so that a significant fraction of the proteins added is actually in the SERCA–PLB complex. Therefore, the concentrations of free ligand and free enzyme must be expressed using mass balance as

$$[\text{PLB}] = [\text{PLB}_{tot} - [\text{PLB}_{bound}] \text{ and } [\text{SERCA}] = [\text{SERCA}_{tot} - [\text{SERCA}_{bound}] \tag{11a,b}$$

where $[\text{PLB}_{tot}]$ and $[\text{SERCA}_{tot}]$ are the concentrations of PLB and SERCA added during reconstitution, respectively. [PLB] in eq 9 can then be expressed using a quadratic equation in terms of total protein concentrations

$$[\text{PLB}] = \frac{-b + (b^2 - 4ac)^{1/2}}{2a} \tag{12}$$

with coefficients $a = 1$, $b = [\text{SERCA}_{tot}] - [\text{PLB}_{tot}] + K_{d2}$, and $c = -K_{d2}[\text{PLB}_{tot}]$.

When all specific PLB binding sites on SERCA are occupied with acceptor-labeled PLB, FRET is equal to $E_{max}$ and the fraction of SERCA molecules bound is equal to 1. Therefore, FRET due to specific binding at any fraction bound can be expressed as

$$E_{SB} = E_{max} \frac{1 - [\text{SERCA}]}{[\text{SERCA}_{tot}]} = E_{max} \left(1 - \frac{[\text{SERCA}_{tot} - [\text{PLB}_{tot}] + [\text{PLB}]}{[\text{SERCA}_{tot}]} \right) \tag{13}$$

Specific binding interaction between donor-labeled SERCA and acceptor-labeled PLB was analyzed using eq 13 (right side) substituted with eq 12.

When the fraction of SERCA molecules bound is equal to 1, the distance between the donor and acceptor in each complex should be the same, assuming homogeneity in complex formation. Therefore, $E_{max}$ was used to calculate the donor–acceptor distance $R$ (eq 3).

Analysis of Nonspecific FRET. In addition to the FRET caused by specific binding of PLB, to SERCA, we calculated the additional FRET due to the random arrangement of acceptor-labeled PLB in the membrane. As described by Fung and Stryer (49), this FRET depends on the concentration of acceptors in the membrane, and on the distance of closest approach ($R_c$) between the donor and acceptor. An approximate version of this theory, which is accurate when
**RESULTS**

**Characterization of Labeled Proteins.** The dye-to-protein molar ratio of AEDANS–SERCA was calculated to be 0.95 ± 0.09. The labeled enzyme, reconstituted into lipid membranes, retained 70–80% of its Ca-dependent ATPase activity and all of its sensitivity to regulation by PLB. Mass spectrometry of DAB–I40A-PLB confirmed that both synthesis and labeling were successful and yielded the correct product. The major peak of mass spectrometry was at 6330.50 Da [M + H] (theoretical: 6333.94 Da), confirming that the peptide was synthesized completely, with a DABCYL molecule bound to the terminal amino group of Lys 3. Analysis of unlabeled, acetylated I40A-PLB showed a major peak at 6080.0 Da [M + H] (theoretical: 6081.54 Da). Amino acid analysis was consistent with the expected peptide composition. Results from amino acid analysis and direct sample absorbance verified a dye-to-protein ratio of 0.98 ± 0.1.

To determine the effect of the DABCYL label on I40A-PLB inhibitory function, purified SERCA was co-reconstituted with either unlabeled I40A-PLB or DAB–I40A-PLB at a membrane concentration of 1.4 SERCAs/1000 lipids and 10 PLBs/SERCA (mol/mol). SERCA ATPase activity (Figure 2) was determined using an NADH-linked enzymatic assay (32, 43). SERCA reconstituted alone exhibited the expected Ca sensitivity, with a half-maximal pCa value (pK_{Ca} in eq 1) of 6.81 ± 0.02, and a maximal ATPase activity (V_{max} in eq 1) of 3.21 ± 0.06 IUs (μmol of ATP mg^{-1} min^{-1}).
at micromolar free Ca. Unlabeled I40A-PLB decreased the apparent Ca affinity of SERCA as expected, to $pK_{Ca} = 6.12 \pm 0.02$ when co-reconstituted with unlabeled I40A-PLB (43).

The labeling of I40A-PLB with DABCYL-SE on Lys 3 had no effect on the inhibitory activity of I40A-PLB (Figure 2). The rightward shift of the Ca activation curve caused by labeled I40A-PLB (to $pK_{Ca} = 6.11 \pm 0.02$) was essentially identical to that of unlabeled I40A-PLB. The label also had no effect on the shape of the curve; the Hill coefficient in the presence of labeled I40A-PLB (2.08 ± 0.15; $n$ in eq 7) indicated cooperativity, as did those obtained for the control (1.70 ± 0.14) and with unlabeled I40A-PLB (2.07 ± 0.15). Maximal ATPase activity ($V_{max} = 3.62 \pm 0.10$ IUs with unlabeled I40A-PLB) was essentially unaffected by the label (3.48 ± 0.07 IUs). Therefore, DAB–I40A-PLB is suitable for studying the binding interaction of SERCA and PLB in a functional membrane system. At pCa 6.5 (low Ca), the total inhibition of SERCA by either unlabeled or labeled I40A-PLB is greater than 80% (Figure 2). At pCa 5.0 (high Ca), there is no inhibition by either labeled or unlabeled I40A-PLB.

**Western Blots of Reconstituted Samples.** When a dissociation constant is measured, it is necessary to obtain an accurate measurement of the concentration of ligand present. It has been reported previously (26, 40), using a different reconstitution method that involves a centrifugation step and a greater amount of Bio beads, that PLB can be lost during the reconstitution procedure. Therefore, Western blot analysis of reconstituted samples was conducted to determine whether PLB was lost during reconstitution. We used anti-PLB antibody 8A3-D5 and purified DAB–I40A-PLB as standard (concentration determined using amino acid analysis). The detection range of this method was linear from 0 to 100 ng of PLB, and samples were diluted so that this range was not exceeded. Control experiments with lipids and SERCA showed that antibody detected only PLB, and was not altered by lipids or SERCA. We determined that the PLB content of the reconstituted samples used for fluorescence and activity measurements was quantitatively identical to the initial content. We conclude that the protein compositions of the reconstituted samples are accurate.

**Fluorescence Data Analysis.** FRET measurements were conducted on co-reconstituted samples containing AEDANS–SERCA (SERCA from here on) and DAB–I40A-PLB (PLB from here on). Both the ratios of these proteins and their respective membrane concentrations were varied to measure the dependence of binding on protein concentrations in the membrane. The amount of PLB present in the membrane was adjusted for incorrectly oriented PLB (facing into the vesicle, away from the SERCA cytoplasmic domain). In reconstituted samples, the SERCA enzyme is oriented asymmetrically, with the cytoplasmic domains protruding from the vesicle, while PLB is oriented symmetrically, with approximately half of the molecules oriented right-side-out, and the other half inverted (53). SERCA interacts preferentially with correctly oriented PLB (26). Furthermore, if the acceptor-labeled cytoplasmic domain of PLB is on the opposite side of the membrane from the donor-labeled cytoplasmic domain of SERCA, the distance between the donor and acceptor is too great (greater than 60 Å) for these pairs to participate in FRET. Therefore, incorrectly oriented PLB is not part of either the biochemical or the spectroscopic data sets collected here, and the amount of correctly oriented PLB participating in inhibition and FRET is half of that added during reconstitution. All data were corrected accordingly.

Spectral overlap of donor emission and acceptor absorbance (Figure 3) is moderate, and the dye pair has a Förster distance (eq 4) of 32 Å (45). Thus, at distances greater than 45 Å, the FRET contribution is less than 10%. The dimensions of a SERCA molecule are 55–90 Å across the cytoplasmic domains (54, 55), so FRET is only detectable when the proteins actually interact physically in the membrane, and FRET dissipates quickly as soon as PLB dissociates from the enzyme. For the same reason, contribution to FRET by acceptors bound to inverted PLB molecules (across the membrane) is also insignificant.

**FRET at Low Ca (pCa 6.5).** Steady-state FRET (eq 2) was measured at low Ca (pCa 6.5) in co-reconstituted membranes from SERCA to PLB (Figure 4). The PLB/SERCA ratio was varied from 0 to 20, and the membrane concentration of SERCA ranged from 1.4 to 0.18 SERCA/1000 lipids. Energy transfer increased sharply upon addition of PLB, and began to change more gradually when the concentration of SERCA ranged from 1.4 to 0.18 SERCA/1000 lipids. Energy transfer increased sharply upon addition of PLB, and began to change more gradually when the expected stoichiometry of the complex was exceeded. The curves did not overlap at low [PLB$_{tot}$], but only at PLB membrane concentrations of greater than 2 PLBs/1000 lipids.

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**FIGURE 4:** Total FRET at low Ca (pCa 6.5) due to SERCA–PLB interaction as a function of the total membrane concentration of PLB. SERCA membrane concentrations were 1.4 (●), 0.71 (●), and 0.18 (▲) SERCA/1000 lipids. Experiments were conducted at 25 °C, in buffer containing 50 mM KCl, 5 mM MgCl$_2$, 50 mM MOPS (pH 7.0), 0.5 mM EGTA, and 210 µM CaCl$_2$. Data points represent the mean ± standard deviation of two or more experiments. Curves show the best fits of eq 16.
was at such high affinity that this (0.012 PLB/1000 lipids. This indicates that the binding specific FRET data should be replotted as ESB vs the of PLB to SERCA is essentially stoichiometric PLB concentration range studied here, the specific binding in the membrane.

this high-affinity specific binding by dilution of the proteins cardiac SR membranes in the membrane used in these experiments, which in turn is

FRET (ESB) (Figure 5). When the dissociation constant was as determined by the fits of eq 16, and replotted as specific binding that depends on the dissociation equilibrium (eq 7).

FRET binding curves were corrected for nonspecific FRET from the specific component that depends on the dissociation equilibrium (eq 7).

FRET binding curves were corrected for nonspecific FRET as determined by the fits of eq 16, and replotted as specific FRET (ESB) (Figure 5). When the dissociation constant was linked in the global analysis (\(\chi^2 = 8.6 \times 10^{-4}\)), \(K_d\) was 0.018 ± 0.012 PLB/1000 lipids. This indicates that the binding was at such high affinity that this \(K_d\) value should be considered an upper bound for the actual \(K_d\). Thus, in the PLB concentration range studied here, the specific binding of PLB to SERCA is essentially stoichiometric. Therefore, the specific FRET data should be replotted as ESB vs the ratio of ligand (PLB) to enzyme (SERCA).

Specific FRET data at low Ca (pCa 6.5) were replotted as a function of the ratio of total PLB to SERCA in the membrane (Figure 6). The curves now overlap at all SERCA membrane concentrations. This indicates tight stoichiometric binding, with \(K_d\) much lower than the concentration of PLB in the membrane used in these experiments, which in turn is much lower than the physiological concentration of PLB in cardiac SR membranes (56). It was not possible to attenuate this high-affinity specific binding by dilution of the proteins in the membrane.

with the membrane concentration of PLB. According to the fit, the distance of closest approach \(R_C\) was 26 ± 3 Å (eq 15). This validates the use of the simplified theory for nonspecific FRET in the membrane (eq 14), since \(R_C\) is greater than 0.7\(R_C\) (50). By fitting of eq 16, it was possible to resolve nonspecific FRET from the specific component that depends on the dissociation equilibrium (eq 7).

The distance of closest approach (\(R_C\)) was 27 ± 2 Å, and the quality of nonspecific quenching, was virtually identical to those obtained at low Ca (Figure 4), suggesting that high-affinity binding occurred at high Ca in the same manner as that at low Ca. The nonspecific FRET component was observed at PLB membrane concentrations of greater than 2 PLBs/1000 lipids, where it was also observed at low Ca. The distance of closest approach (\(R_C\)) was 26 ± 3 Å. After correction for nonspecific FRET, the remaining FRET due to specific binding interaction was essentially identical at all SERCA membrane concentrations, and the stoichiometric (extremely-high-affinity) binding observed at low Ca was therefore also observed at high Ca.

**FRET at High Ca (pCa 5.0).** Having established that binding of PLB to SERCA at low Ca is of very high affinity and stoichiometric in nature, we conducted FRET experiments at pCa 5.0 with the same samples, to test the hypothesis that the SERCA–PLB complex dissociates at high Ca due to an increase in \(K_d\) (Figure 1). Surprisingly, the FRET results at high Ca (Figure 7) were nearly identical to those obtained at low Ca (Figure 4), suggesting that high-affinity binding occurred at high Ca in the same manner as that at low Ca. The nonspecific FRET component was observed at PLB membrane concentrations of greater than 2 PLBs/1000 lipids, where it was also observed at low Ca. The distance of closest approach (\(R_C\)) was 27 ± 2 Å, and therefore the quality of nonspecific quenching, was virtually identical to that at low Ca (\(R_C\) = 26 ± 3 Å). After correction for nonspecific FRET, the remaining FRET due to specific binding interaction was essentially identical at all SERCA membrane concentrations, and the stoichiometric (extremely-high-affinity) binding observed at low Ca was therefore also observed at high Ca.

**Stoichiometry of the SERCA–PLB Complex.** FRET due to specific binding only depended on the ratio of total PLB to SERCA in the membrane (Figure 8) at both low and high Ca. The stoichiometry of the complex was determined by extrapolating straight lines from the slope of initial and final data points. As indicated by the dashed arrow in Figure 8, the stoichiometry of the PLB–SERCA complex in a functional membrane system is 1.0 ± 0.1 PLB/SERCA under both activating and inhibitory Ca concentrations.
When specific FRETs due to specific binding were compared at low and high Ca (Figure 8), a small but significant difference in maximal energy transfer (obtained from fitting of eq 16) was noted. $E_{\text{max}}$ was $0.45 \pm 0.02$ at low Ca, and $0.40 \pm 0.01$ at high Ca. On the basis of the Förster distance of 32 Å (Figure 3) and using eq 3, this corresponds to a distance of $33.1 \pm 0.4$ and $34.2 \pm 0.2$ Å at low and high Ca, respectively. Therefore, with respect to the donor–acceptor distance of the labeling sites used here, there was a small but significant structural change in the complex in the transition from low to high Ca. It is clear, however, that the complex did not dissociate at high Ca, as shown by saturation of specific FRET at both low and high Ca.

**Time-Resolved Fluorescence Analysis.** We conducted time-resolved fluorescence experiments on the same samples to confirm and clarify the findings. Time-resolved data supported the findings of steady-state analysis at both low and high Ca concentration. Average fluorescence lifetimes of decays were consistent with steady-state FRET data. The SERCA membrane concentration did not affect the average decay in the absence of PLB, shown by essentially identical decays at 1.4 and 0.18 SERCA/1000 lipids (Figure 9). Upon addition of 0.5 PLB/SERCA, fluorescence decayed faster than in the absence of PLB, indicating FRET, and the decays from two different SERCA membrane concentrations were identical, supporting the conclusion from steady-state data that the interaction is stoichiometric and specific at this low ratio of PLB to SERCA (Figure 8). At an excess of PLB over SERCA, the decay at 1.4 SERCAs/1000 lipids was clearly faster than that at 0.18 SERCA/1000 lipids (Figure 9), consistent with the presence of more nonspecific FRET at the former and less at the latter SERCA concentration. When eq 6 was fitted to the data, it was generally found that more exponentials were needed for satisfactory fits at higher SERCA membrane concentration, suggesting that the nonspecific FRET observed under these conditions arises from a distribution of distances between the donor and acceptor due to nonspecific binding of PLB to SERCA. These results confirm the above interpretation of steady-state FRET.

**ATPase Activity.** SERCA ATPase activity (Figure 10) was measured on the same reconstituted and labeled samples that were used for fluorescence measurements at pCa 6.5 and 5.0. At pCa 6.5, PLB inhibits SERCA, whereas there is no inhibition at pCa 5.0 (Figure 2). No inhibition of SERCA by PLB was observed at pCa 5.0, as expected. In fact, there was a slight increase in activity in the presence of PLB at pCa 5.0, as reported previously (43). At pCa 6.5, SERCA ATPase activity was inhibited to the same extent at all SERCA membrane concentrations by PLB (Figure 10), suggesting that inhibition is dependent on the ratio of total PLB to SERCA, and not the membrane concentration of PLB, consistent with specific FRET measurements. Inhibition increased sharply upon addition of PLB, and continued to increase in stoichiometric fashion (curves overlapped) even beyond the 1:1 stoichiometry of the SERCA–PLB complex. This suggested the presence of a nonspecific component of inhibition.

**TPA of SERCA Co-Reconstituted with I40A-PLB.** To assess the role of SERCA self-association ($K_d$ in Figure 1) in the mechanism of PLB-dependent inhibition, we conducted TPA measurements of phosphorescent-labeled SERCA co-reconstituted with I40A-PLB (Figure 11). PLB caused significant increases in anisotropy, indicating SERCA aggregation, consistent with previous observations in cardiac SR (30). Although FRET showed that the specific binding interaction between SERCA and PLB is saturated at a ratio greater than 1 PLB/SERCA (Figure 8), TPA showed aggregation of SERCA that continued to increase in virtually linear fashion (on the basis of the final anisotropy evaluation) from 2.5 to 5 PLBs/SERCA added. This aggregation probably accounts for the continued increase in inhibition at high PLB/SERCA ratios (Figure 10).
Kd2 much lower than the membrane protein concentrations allowed us to test directly the hypothesis that the relief of inhibition is due to the failure to vary the membrane concentration of PLB (Figure 10) without dissociating PLB from SERCA. We conclude that this hypothesis must be revised (Figure 12).

Tight, Ca-Independent Binding of PLB to SERCA. We have shown directly that the SERCA-PLB complex remains intact at saturating [Ca\(^{2+}\)]. FRET measurements showed that specific binding of PLB to SERCA at both inhibitory and activating [Ca\(^{2+}\)] is stoichiometric (Figure 8). 140A-PLB remained bound to SERCA at pCa 5.0 despite complete reversal of inhibition, so reversal of inhibition does not require dissociation of PLB. Co-immunoprecipitation of PLB with SERCA 1a or 2a (21) was reduced at saturating Ca concentration. However, the enzyme is inactive in the detergent conditions used, and correlation of physical and biochemical measurements is difficult. In the present study, done in functional membranes, Ca clearly relieves inhibition (Figure 10) without dissociating PLB from SERCA (Figures 7 and 8).

An 8-fold dilution of the proteins in the membrane (from a SERCA membrane concentration of 1.4 SERCA/1000 lipids to one of 0.18 SERCA/1000 lipids) had no effect on the specific binding interaction: specific FRET as a function of the ratio of total PLB to SERCA was virtually identical at all SERCA membrane concentrations. Therefore, PLB binds to SERCA with very high affinity (Figure 12), with Kd2 much lower than the membrane protein concentrations used in these experiments and therefore much lower than the physiological membrane concentrations of these proteins (56). Since binding was too tight to measure the dissociation constant, and since there is at least 1 PLB/SERCA in both slow-twitch skeletal and cardiac SR membranes (56–59), we conclude that each SERCA molecule under normal physiological conditions has a PLB molecule specifically bound to it. In effect, all SERCA molecules are permanently under adrenergic control, regardless of whether the interaction is strongly inhibitory (low Ca) or moderately activating (high Ca) (Figure 2). This serves as one mechanism of access to the large cardiac reserve in most animals. We propose that, under physiological conditions, PLB should therefore be considered a subunit of SERCA that is bound at both low (nanomolar) and high (micromolar) Ca\(^{2+}\) concentration (Figure 12).

It is conceivable that superinhibitory I40A-PLB binds to SERCA with greater affinity than WT-PLB, and that WT-PLB interactions with SERCA are decreased at high Ca. It is also possible that the labeled PLB has greater affinity for SERCA than does unlabeled PLB. If the PLB–PLB interaction were of comparable affinity to the PLB–SERCA interaction, equilibria involving Kd1 and Kd2 (Figure 1) would be in competition, and dissociation of the SERCA–PLB complex would be more likely. However, it has been shown that PLB oligomers dissociate in the presence of SERCA (60), indicating that the interaction between SERCA and the PLB monomer is dominant.

Other, less direct, evidence is consistent with Ca-independent binding of PLB to SERCA. Cross-linking experiments conducted under reportedly uncontrolled Ca conditions (25), and therefore presumably at saturating Ca concentration, detected proximity between PLB and SERCA. Infrared spectroscopy showed that the effects of PLB on SERCA were the same at low and high Ca (26).

Despite clear evidence for stoichiometric binding of PLB to SERCA, plots of specific FRET as a function of the ratio of total PLB to SERCA (Figure 8) display a degree of curvature. Since we conclude that the membrane concentration of PLB is much greater than Kd2, it is expected instead that a sharp kink should be observed at the point of stoichiometry of the SERCA–PLB complex (1:1) as illustrated by the straight lines in Figure 8. The most likely source of curvature is heterogeneity in the composition of vesicles, an artifact of reconstitution. Simulations of the data in Figure 8 indicate that the observed curvature can be accounted for by assuming that the actual ratio of PLB to SERCA in individual vesicles varies by about 30% within a single preparation. Curvature could also arise from aggregation of the enzyme (18, 27–30), which is likely to produce a heterogeneous system in which some PLB binding sites are less accessible than others, or from cooperativity within a preexisting oligomeric SERCA complex (46, 61).

The reconstitution system is optimal for measuring the binding of two membrane proteins under defined conditions, because membrane protein concentrations and ratios can be varied precisely. Kd2 was previously estimated to be 0.22 PLB/1000 lipids (26) at a concentration comparable to 1.4 SERCA/1000 lipids, whereas we obtain an upper bound for Kd2 that is at least 10 times lower. This overestimate of Kd2 is due to the failure to vary the membrane concentration of SERCA in that study (26), so it was not possible to detect the stoichiometric, high-affinity binding detected here.

Our results shed light on physical aspects of successful gene therapy targeting the SERCA–PLB system in hamsters (15), which attenuated progression to heart failure. During protein synthesis, SERCA and PLB are inserted into membranes, where they may become available for immediate interaction. Once PLB is bound to SERCA, the complex is unlikely to dissociate due to the very-high-affinity interaction. If, upon insertion into the membrane, a competing mutant of PLB is present that binds to SERCA first, WT-PLB may be permanently displaced from specific SERCA binding sites.
in effect preventing deleterious effects of PLB on SERCA implicated in heart failure (3–9).

SERCA–PLB Complex Stoichiometry and Structure. We have shown here that the stoichiometry of the SERCA–PLB complex is 1:1, consistent with most other models (i.e., refs 47 and 48), including the model of two PLB molecules bound to a SERCA homodimer (46). The SERCA–PLB complex has been proposed to consist of one PLB molecule per SERCA dimer (56), but that model was based on an erroneously high estimate for the value of $K_D$ as explained above. The best estimate for the expected donor–acceptor distance in the Ca-free crystal structure is approximately 40 Å (SERCA Cys 674 to Lys 400; on the basis of PLB Lys 3 cross-linking to SERCA residues 397–400; 22). The distance measured with FRET in the present study is approximately 7 Å shorter; this difference can easily be accounted for by structural dynamics, uncertainty of probe conformations, or the distance separating PLB Lys 3 from SERCA Lys 400 that was probably bridged by the cross-linker (22). The observed distance is not consistent with the hypothesis that PLB binds to SERCA with its cytoplasmic domain in contact with the membrane surface; rather, our observations are consistent with the conclusion that SERCA induces a large conformational change in PLB, lifting the cytoplasmic domain substantially above the membrane surface (62).

Ca-Dependent Structural Change within the SERCA–PLB Complex. What does happen when [Ca$^{2+}$] relieves inhibition? Our results show that the maximum energy transfer efficiency at saturating PLB ($E_{max}$) is significantly less at high Ca (Figure 8), indicating a structural change within the SERCA–PLB complex induced by micromolar Ca$^{2+}$. This is consistent with the evidence from spectroscopy (63) and from crystallography (54, 55) that there is a substantial conformational change within SERCA in the transition from low to high Ca. On the basis of the change in $E_{max}$, the donor–acceptor distances at low and high Ca (33.1 ± 0.4 and 34.2 ± 0.2 Å, respectively) display a small but significant change. This is consistent with the small change predicted in the distance between Cys 674 and Lys 400, from the Ca-bound to the Ca-free SERCA crystal structures (54, 55). A larger change, more characteristic of large domain movements thought to occur during the SERCA enzymatic cycle (i.e., refs 54, 55, and 64), would presumably be detected with a donor probe at another site on SERCA. Conformational changes in the SERCA–PLB complex upon Ca binding probably explain the observed Ca-dependent cross-linking of PLB to the enzyme (22–24). For cross-linking to occur efficiently, distances between sites must match the length of the cross-linker, and alignment of residues to be cross-linked must be sterically favorable. Rotational or translational helix or domain movement containing the cross-linking residues may be sufficient to prevent cross-linking if the distance between sites becomes too great or short, alignment of the residues is unfavorable, or at least one of the residues becomes inaccessible to the cross-linker due to steric hindrance. Cross-linking was surprisingly dependent on the presence of nucleotide (23, 24), which may induce SERCA conformations that favor cross-linking to PLB.

Nonspecific Binding and Inhibition. FRET measurements showed a nonspecific component that became prevalent at PLB membrane concentrations that exceeded the stoichiometry of the SERCA–PLB complex (Figures 4 and 7), and this was accounted for by assuming random arrangement of PLB in the membrane (49). Proximity to various acceptors at a range of distances would give rise to numerous different energy-transfer events, and this was confirmed by the complexity of time-resolved fluorescence decays (Figure 9). Nonspecific FRET may also arise due to SERCA aggregation (Figure 11), which has been shown previously to occur in the presence of PLB and other inhibitors (18, 27–30). Acceptor-labeled PLB may become trapped in nonspecific sites that lead to FRET, in addition to specifically bound PLB. This is more likely to occur at high protein concentrations in crowded membranes. After correction for the nonspecific FRET component, all remaining FRET overlapped, indicating high-affinity, stoichiometric binding due to a specific interaction (Figure 8).

SERCA aggregation (Figure 11) probably helps to explain some of the complexity in the inhibition data (Figure 10). A correlation between aggregation and inhibition is observed upon addition of PLB (30) and other inhibitors (27–29, 65–68), some of which are likely to be nonspecific. Aggregation probably serves as part of the mechanism of control of SERCA activity by PLB. It is clear from Figure 10 that there are remaining interactions that are not detected by FRET, but still cause inhibition. The various inhibitory mechanisms of PLB on SERCA are likely to be more complex than can be detected here, and deserve further attention.

Conclusions and Future Work. We have shown that FRET can be used to detect and quantitate the binding interaction of PLB and SERCA in intact membranes, and that this interaction is so strong that PLB is effectively a subunit of the SERCA enzyme under physiological conditions. The SERCA–PLB complex has a stoichiometry of 1:1 in a functional membrane system at both activating and inhibitory Ca concentrations. While micromolar Ca$^{2+}$ does not dissociate PLB from SERCA, our FRET data strongly suggest that it does change the structure of the SERCA–PLB complex, and this internal structural change is probably the mechanism by which inhibition is relieved. Further experiments will be needed to understand the effects of nucleotide, phosphorylation, and mutation on the structure of the SERCA–PLB complex.

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