Comparable Levels of Ca-ATPase Inhibition by Phospholamban in Slow-Twitch Skeletal and Cardiac Sarcoplasmic Reticulum†

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ABSTRACT: Alterations in expression levels of phospholamban (PLB) relative to the sarcoplasmic reticulum (SR) Ca-ATPase have been suggested to underlie defects of calcium regulation in the failing heart and other cardiac pathologies. To understand how variation in PLB expression relative to that of the Ca-ATPase can modulate calcium transport, we have investigated the inhibition of the Ca-ATPase by PLB in native SR membranes from slow-twitch skeletal and cardiac muscle and in reconstituted proteoliposomes. Quantitative immunoblotting in combination with affinity-purified protein standards was used to measure protein concentrations of PLB and of the Ca-ATPase. Functional inhibition of the Ca-ATPase was determined from both the calcium concentrations for half-maximal activation ($Ca_{1/2}$) and the shift in the calcium concentrations following release of PLB inhibition (i.e., $\Delta Ca_{1/2}$) by incubation with monoclonal antibodies against PLB, which are equivalent to phosphorylation of PLB by cAMP-dependent protein kinase. We report that equivalent levels of PLB inhibition and antibody-induced activation ($\Delta Ca_{1/2} = 0.25 \pm 0.02$ μM) are observed in SR membranes from slow-twitch skeletal and cardiac muscle, where molar stoichiometries of PLB expressed per Ca-ATPase vary, respectively, from 0.9 ± 0.1 to 4.1 ± 0.8. Similar levels of inhibition to those observed in isolated SR vesicles were observed using reconstituted proteoliposomes following co-reconstitution of affinity-purified Ca-ATPase with PLB. These results indicate that total expression levels of one PLB per Ca-ATPase result in full inhibition of the Ca-ATPase and, based on the measured K_D (140 ± 30 μM), suggests one PLB complexed with two Ca-ATPase molecules is sufficient for full inhibition of activity. Therefore, the excess PLB expressed in the heart over that required for inhibition suggests a capability for graded responses of the Ca-ATPase activity to endogenous kinases and phosphatases that modulate the level of phosphorylation necessary to relieve inhibition of the Ca-ATPase by PLB.

The sarcoplasmic reticulum (SR)[1] Ca-ATPase plays a major role in muscle contraction, providing the rate-limiting resequestration of cytosolic calcium into the SR lumen allowing relaxation and resetting of the cytosolic calcium gradient for the next cycle of excitation-contraction coupling (*1*). Coexpressed with the SERCA2a isoform of the Ca-ATPase in slow-twitch skeletal and cardiac muscle is a 52 amino acid membrane protein, phospholamban (PLB), which acts to partially inhibit the Ca-ATPase until phosphorylated at Ser-16 or Thr-17 by cAMP-dependent protein kinase (PKA) or calcium-calmodulin dependent protein kinase, respectively (*2*). Phosphorylation of PLB is manifested by shifts in the calcium dependence of Ca-ATPase activity toward lower calcium concentrations (higher apparent calcium affinity). In transgenic animals, ablation of PLB results in enhanced rates of relaxation that are essentially insensitive to PKA activation by isoproterenol, indicating that PLB is a major target in vivo of the β-adrenergic cascade that is responsible for regulation of rates of muscle relaxation (*3*, *4*). While altered expression of PLB relative to that of the Ca-ATPase has been implicated in deficits in contractile performance in the senescent heart as well as other cardiac pathologies, quantitative information regarding optimal PLB and Ca-ATPase concentrations and how their changes affect heart function is currently not available (*5*, *6*). Several studies have shown that altering expression of PLB protein in myocytes and transgenic mouse models results in alterations in apparent calcium affinities of the Ca-ATPase; however, altering expression of SERCA2a has no effect on the

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[1] Abbreviations: BAPTA, glycine,*N*,*N*′(1,2-ethanediylbis(oxy-2,1-phenylene))bis(*N*-(carboxymethyl)); BCA, bicinchoninic acid; $Ca_{1/2}$, calcium concentration at half-maximal activation; $C_{12}E_8$, polyoxyethylene 8-lauryl ether; $C_{12}E_9$, polyoxyethylene 9-lauryl ether; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDL, extensor digitorum longus; NBT/BCIP, nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate p-toluidine; mAb, monoclonal antibody; MOPS, 3-(*n*-morpholino)propane-sulfonic acid; PKA, cAMP-dependent protein kinase; PLB, phospholamban; PVDF, polyvinylidene fluoride; SERCA1, Ca-ATPase fast-twitch isoform; SERCA2a, Ca-ATPase cardiac/slow-twitch isoform; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum.

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apparent calcium affinity of the Ca-ATPase (*7*−*14*). While these individual protein manipulations result in similar PLB to SERCA2a ratios relative to wild-type levels, wild-type levels have not been defined in terms of molar stoichiometries of these two proteins.

Even less is understood regarding the in vivo role of PLB expression levels in slow-twitch skeletal muscle. For example, an early study of SR isolated from canine slow-twitch muscle suggested that PLB was not tightly coupled to the Ca-ATPase (*15*). This suggestion was based on the observation that Ca-ATPase activity in SR from slow-twitch skeletal muscle was only weakly activated upon incubation with activating anti-PLB antibodies, in contrast to the robust stimulation observed by the same treatment of canine cardiac SR; identical PLB expression levels relative to the Ca-ATPase were reported for skeletal and cardiac SR (*15*). More recently, using a genetically modified mouse model, it was demonstrated that ablation of PLB resulted in identical (ca. 30%) decreases in the half-time of relaxation for both slow-twitch soleus muscle and the heart; quantitation of the loss of PLB in each tissue by ablation relative to the wild-type mouse was not determined (*16*−*21*). While differences in calcium handling among different species, tissues and in genetically modified mice might account for some of these apparent discrepancies, unambiguous interpretation of the results of these studies will also require knowledge of the in vivo concentrations of both PLB and SERCA2a. Such estimates have been made, usually tangentially, and the wide range of reported values for PLB to Ca-ATPase molar ratios, i.e., from 1:3 to 9:1 in slow-twitch skeletal muscle, and from 1:1 to 12:1 in the heart, probably reflect the fact that few studies have explicitly addressed a comparison of heart and skeletal muscle by quantitative methods (*15*, *22*−*26*).

Therefore, to more clearly define the relationship between PLB expression and regulation of the Ca-ATPase, we have first made quantitative measurements of PLB and SERCA2a protein levels in SR membranes isolated from skeletal and cardiac muscle from wild-type tissues using quantitative immunoblotting. Second, we have correlated these stoichiometries with the corresponding inhibition of the Ca-ATPase by PLB and activation by anti-PLB antibody based on measurements of the calcium dependence of Ca-ATPase activity. Our results demonstrate an equivalent inhibition of the Ca-ATPase by PLB expressed in either slow-twitch skeletal or cardiac muscle, in which stoichiometries of PLB relative to the Ca-ATPase differ by approximately 4-fold. Equivalent results are observed using reconstituted proteoliposomes that contain only PLB and the Ca-ATPase proteins. In all cases, nearly maximal inhibition of the Ca-ATPase by PLB is observed at a stoichiometry of 1 mol of PLB/mol of Ca-ATPase.

EXPERIMENTAL PROCEDURES

Materials. Primary monoclonal antibodies against SERCA1 from rabbit fast-twitch skeletal muscle SR and SERCA2a from canine cardiac SR were from Affinity Bioreagents (Golden, CO). Goat anti-mouse IgG-alkaline phosphatase conjugated secondary antibody, nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine (NBT/BCIP) substrate kit, and the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL). Reagents for gel electrophoresis and electroblotting, Biobeads (SM2), Protein Sequencing polyvinylidene fluoride (PVDF) membranes, and prestained molecular weight markers were purchased from Bio-Rad (Hercules, CA). Immobilon-P PVDF membrane were from Millipore (Bedford, MA). BAPTA [glycine, *N*,*N*′(1,2-ethanediylbis(oxy-2,1-phenylene))bis(*N*-(carboxymethyl)] and Calcium Green were obtained from Molecular Probes (Eugene, OR). All other reagents and products were from Sigma (St. Louis, MO).

Preparation of Cardiac and Skeletal SR. Cardiac SR membranes were isolated from the ventricles of hearts from a New Zealand White rabbit or 4−6 month old Fisher 344 rats, essentially as previously described with the exception that MOPS was substituted as the buffer (*27*). SR was isolated from freshly obtained hearts that were never frozen. SR vesicles were suspended in a medium of 20 mM MOPS (pH 7.0), 100 mM KCl, 10 mM dithiothreitol (DTT), and 0.3 M sucrose and stored at −70 °C. To determine if the ratio of PLB to SERCA2a was dependent on the specific SR membrane preparation, an alternative method was used to prepare cardiac SR isolated from five rat heart ventricles (*28*, *29*). After the final purification step, SR vesicles were suspended in a medium containing 0.3 M sucrose, 5 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 30 mM KH_2PO_4 (pH 7.0), and 0.5 mM DTT. Protein concentrations of cardiac SR were assessed by the Amidoschwarz method using bovine serum albumin as the standard (*30*); the accuracy of Amidoschwarz assay was confirmed by amino acid analysis.

SR from freshly obtained rat or rabbit skeletal muscle was prepared as described previously (*31*), with minor modifications (*32*). For all preparations, isolated SR vesicles were suspended in a medium consisting 20 mM MOPS (pH 7.0) and 0.3 M sucrose and stored at −70 °C. Protein concentrations of skeletal SR preparations were determined using the BCA assay following the addition of 0.1% SDS and using bovine serum albumin as the standard; the accuracy of this assay was confirmed by amino acid analysis.

Co-reconstitution of the Ca-ATPase with PLB. Affinity-purified Ca-ATPase was reconstituted in the presence of variable amounts of PLB into lipids extracted from SR membranes, with minor modifications to established protocols (*33*, *34*). Prior to reconstitution, 1.1 mg of extracted SR lipids was dried under nitrogen gas and kept in a vacuum desiccator overnight. The dried SR lipids were suspended in approximately 0.5 mL of reconstitution buffer [20 mM MOPS (pH 7.0), 0.1 M KCl, 0.1 mM $CaCl_2$, and 0.3 M sucrose] and solubilized by adding 1.7 mM octyl glucoside. Separately, 200 μg (1.8 nmol) of purified Ca-ATPase was mixed with 2, 6, and 11 nmol of PLB solubilized in 4 mg/mL $C_{12}E_9$ in a final volume of 100 μL. This mixture was then combined with the solubilized SR lipids, resulting in a final volume of 0.6 mL containing 1.4 mM octyl glucoside and 0.7 mg/mL $C_{12}E_9$. To remove the detergent, 100 mg of SM-2 Bio-Beads (BioRad, Richmond, CA) was added, and the solution was incubated with gentle stirring for 1 h. Two more 100 mg aliquots of Bio-Beads were added at 1 h intervals. The resulting reconstituted vesicles were removed from the Bio-Beads and concentrated by centrifugation at 300000g_{max} for 15 min. The final lipid-to-protein ratio was 750 phospholipids per Ca-ATPase. Molar ratios of PLB

relative to the Ca-ATPase were determined using quantitative immunoblotting.

Purification of Protein Standards. SERCA1 and SERCA2a isoforms of the Ca-ATPase were affinity purified from rabbit fast-twitch (soleus) skeletal SR and pig cardiac SR, respectively, using Reactive Red 120 dye coupled to agarose beads, as previously described (*35*, *36*). Following elution, the Ca-ATPase was reconstituted in the presence of exogenously added phosphatidylcholine and phosphatidic acid (10:1), essentially as previously described (*36*), and was stored at −70 °C. Recombinant PLB was expressed and purified from *Escherichia coli* as previously described (*37*). In the final step, the protein was eluted in 25 mM Tris (pH 8.0), 0.5 M KCl, and 1% $C_{12}E_9$, and the purified protein was stored at −70 °C. On the basis of densitometric analysis of Coomassie blue-stained gels, purified PLB and both Ca-ATPase isoforms exhibited greater than 98% purity (*36*, *37*). Protein concentrations of purified proteins were assessed using the BCA protein assay, using bovine serum albumin as the standard. Additionally, the concentration of PLB was confirmed by amino acid analysis.

Electrophoresis and Western Immunoblotting of SR Proteins. SR proteins were separated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) using a 5% separating gel for assessing Ca-ATPase content or a 13% separating gel for assessing PLB content, according to the method of Laemmli (*38*). For densitometric analysis of SR protein content, gels were stained with Coomassie blue. For Western immunoblotting, electrophoretically separated SR proteins were transferred to polyvinylidene fluoride (PVDF) membranes, according to the method of Towbin et al. (1979). For optimal transfer of SERCA1 or SERCA2a, SR proteins were electrophoretically transferred for 2 h at 90 V (4 °C) to a 0.45 μm Immobilon-P PVDF membrane. For optimal transfer of PLB, SR proteins were transferred for 5 h at 90 V (4 °C) to a 0.2 μm Protein Sequencing Grade PVDF membrane.

Primary monoclonal mouse antibodies raised against SERCA 1 (IgG1, clone IIH11) from rabbit fast-twitch skeletal muscle SR and SERCA2a (IgG2a, clone 2A7-A1) raised against canine cardiac SR were used for the identification of these Ca-ATPase isoforms on Western blots. SERCA1 and SERCA2a antibodies were diluted at 1:20000 and 1:5000, respectively, before incubation with protein-blotted PVDF membranes. Antibody reactions were visualized using goat anti-mouse IgG-alkaline phosphatase conjugated secondary antibody, diluted at 1:1000 before incubation with the blot. Color development was accomplished using the NBT/BCIP substrate kit. Mouse monoclonal antibodies directed against recombinant PLB (clone 8A3) were prepared in this laboratory from hybridoma cells derived from ascites fluid using standard protocols. This antibody against PLB, subtyped as IgG, was diluted at 1:50000 before incubation with protein-blotted PVDF membranes. The 8A3 PLB antibody recognizes both the native (based on reaction on slot blots and with an ELISA assay, data not shown) and linear forms, including monomeric and oligomeric species (based on reaction with Western immunoblotting), of the PLB protein. Visualization of the antibody reaction was accomplished using the same system as outlined for SERCA1 and SERCA2a antibodies.

Slot-Blot Immunoassays. SR proteins were adsorbed to PVDF membranes using a slot-blot apparatus (Bio-Dot SF, Bio-Rad, Hercules, CA). The type of PVDF membrane for specific proteins and primary and secondary antibodies, as well as their dilutions, were the same as those used for Western immunoblotting. The immunoreaction of protein standards, tissue homogenates, and SR vesicle preparations to specific antibodies was quantified by densitometric analysis of scanned slot-blots using the Sigma Scan software. The relative amount of immunoreaction is designated as absorbance units, which is the product of the reaction density and area. SERCA1, SERCA2a, and PLB content in SR preparations and homogenates were determined from the slope within the linear range of absorbance units as a function of protein load and compared with slopes from purified protein standards on the same PVDF membrane. All protein standards exhibited a linear response with their respective antibody between 3 and 60 ng of protein. This procedure was adapted from methods described by Kranias and coworkers for evaluating the relative content of PLB and SERCA2 in SR preparations (*40*).

Calcium-Dependent ATPase Activity. Calcium-dependent ATPase activity was measured at 25 °C from the initial rate of release of inorganic phosphate from vesicles made leaky to calcium by the addition of the calcium ionophore A23187 (*41*) using microtiter plates as outlined in Penney (*42*). The reaction medium contained 2 μg of SR protein/mL in 25 mM MOPS (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 4 μM A23187, and variable amounts of calcium. Concentrations of free calcium were measured as previously described (*43*). PLB inhibition was reversed by addition of 2 μg/mL of antibody (8A3) against PLB or 2 μg/mL cAMP-dependent protein kinase (PKA) and 1 μM cAMP. Calcium concentrations for half-maximal activation ($Ca_{1/2}$) of the Ca-ATPase were determined from plots of the calcium concentration-dependence of ATPase activity fit to the Hill equation using Origin 4.1 (Microcal, Northhampton, MA) software. Values for antibody induced shifts in $Ca_{1/2}$, i.e., $\Delta Ca_{1/2}$ from SR having both SERCA1 and SERCA2a isoforms were corrected to reflect the $\Delta Ca_{1/2}$ value for the SERCA2a Ca-ATPase activity only, using immunoblots to quantitate the relative molar proportions of SERCA1 and SERCA2a present.

Statistical Analysis. Differences between SR preparations were tested for statistical significance using the Student's t-test analysis, with the level of significance set at $p \leq 0.05$. Statistical analysis was performed on a personal computer using the Numbers Crunching Statistical System 6.0.1 for Windows (NCSS, Kaysville, UT).

RESULTS

Selectivity of Antibody Binding. A key component to this study is the reliable and consistent quantitation of molar ratios of PLB relative to either the SERCA2a or SERCA1 isoforms of the Ca-ATPase in both isolated SR membranes and reconstituted proteoliposomes. The SERCA2a isoform of the Ca-ATPase can be resolved from SERCA1 on SDS−PAGE based on its slightly faster mobility as illustrated by Figure 1. Lane 1 shows the clear resolution of SERCA2a in the presence of even a large excess of SERCA1 from SR isolated from mixed (fast and slow) fibers. Thus, determination of

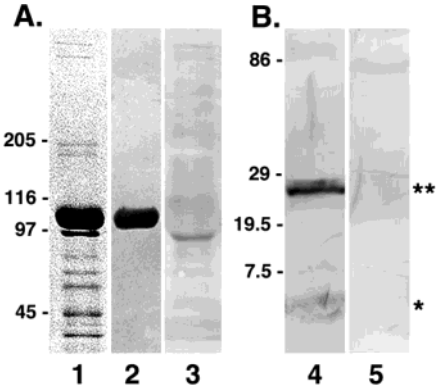

FIGURE 1: Immunodetection of SERCA2a and PLB. (A) Lane 1 represents Coomassie blue staining of SR proteins from rat hindlimb skeletal muscle separated using a 5% (Laemmli) SDS−PAGE. Lanes 2 and 3 represent the same rat skeletal SR proteins blotted onto PVDF membranes and stained using as a primary antibody against SERCA1 (lane 2) or SERCA2a (lane 3). Relative migration of molecular mass standards are indicated on the left, and are based on myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), and carbonic anhydrase (29 kDa). (B) SR proteins isolated from rat cardiac (lane 4) and fast-twitch skeletal muscle (lane 5) were electrophoretically separated on 13.5% Laemmli SDS−PAGE. After blotting onto PVDF membranes, blots were visualized using primary antibodies against PLB, where expected mobilities are indicated for major oligomers of PLB, i.e., pentamers (**) and monomers (*). Relative migration of molecular mass standards are indicated, is based on prestained markers: bovine serum albumin (86 kDa), soybean trypsin inhibitor (29 kDa), lysozyme (19.5 kDa), and aprotinin (7.5 kDa).

the proportional representation of each isoform in the presence of the other is possible, as previously demonstrated (*44*). Identification of these bands is confirmed by specific detection of each band by antibodies directed against SERCA1 or SERCA2a; additional confirmation was previously obtained using amino acid analysis (*44*, *45*). Moreover, using purified SERCA2a or SERCA1, no cross-reaction is detected from SERCA1 or SERCA2a antibody, respectively (data not shown). The monoclonal antibody raised against recombinant PLB expressed in *E. coli* detects both the predominant pentameric as well as the monomeric PLB species (Figure 1B). No antibody reaction was detected in SR isolated from rabbit fast-twitch muscle, which is devoid of PLB protein. Thus, antibodies raised against both SERCA1 and SERCA2a isoforms of the Ca-ATPase and PLB are specific for these proteins, permitting their use in combination with affinity-purified protein standards to measure the concentrations of these proteins in membrane preparations.

Abundance of the Ca-ATPase and PLB in SR. We have measured the concentrations of both SERCA2a and PLB in slow-twitch soleus and cardiac (ventricular) muscle, using affinity-purified SERCA2a and PLB as protein standards. Immunoreaction of anti-SERCA2a in SR from rabbit cardiac and soleus muscle as a function of protein load indicates similar expression levels of SERCA2a in these two tissues (Figure 2a), where SERCA2a represents 40 ± 6 and 41 ± 7% of the total protein mass. However, detection with an antibody raised against PLB indicates that there is substantially greater expression of PLB in cardiac than in soleus muscle (Figure 2b), where PLB represents 8 ± 1 and 1.8 ± 0.2% of the total protein mass. Correcting for differences in the molecular masses of SERCA2a (109 477 g/mol) and PLB (6123 g/mol), the calculated molar ratio of PLB per Ca-ATPase is 3.5 ± 0.6 and 0.8 ± 0.2 in rabbit cardiac and soleus muscle. Replicate determinations from multiple SR preparations isolated from either cardiac (ventricular) or skeletal (soleus) rat muscles indicate respective molar stoichiometries of 4.7 ± 0.8 and 1.0 ± 0.2 mol of PLB/mol of the SERCA2a isoform of the Ca-ATPase. These results indicate that similar levels of SERCA2a and PLB expression are observed in cardiac (about 4.1 ± 0.8) and slow-twitch skeletal muscle (about 0.9 ± 0.1) from both rabbit and rat, suggesting that the 4-fold greater expression of PLB in cardiac muscle is a general feature of these tissues (Table 1). Furthermore, the result for cardiac SR agrees favorably with earlier determinations in porcine cardiac SR, where the molar ratio of PLB per Ca-ATPase was estimated to be 4.2 ± 0.8 based on phosphoprotein determinations using γ-^{32}P-ATP (*26*).

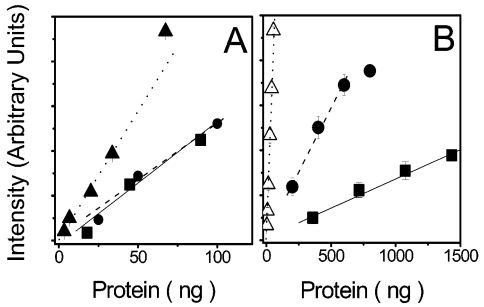

FIGURE 2: Expression Levels of SERCA2a and PLB. Densitometric analysis of immunoresponse against (panel A) SERCA2a and (panel B) PLB in SR vesicles isolated from rabbit cardiac (●) and slow-twitch soleus (■) muscles. Standard curves (▲, △) were generated using either affinity-purified SERCA2a (A) or PLB (panel B) as described in Experimental Procedures. Values represent the mean of duplicate measurements for each protein load. Least-squares fits to the data indicate the following slopes, which were used in the determination of relative abundances of SERCA2a and PLB. For SERCA2a: affinity-purified standard, 4.1 ± 0.3; cardiac SR, 1.7 ± 0.2; soleus SR, 1.7 ± 0.3. For PLB: affinity-purified standard, 3.6 ± 0.3; cardiac SR, 0.28 ± 0.03; soleus SR, 0.064 ± 0.006.

Table 1: Stoichiometry of PLB:SERCA2A from Cardiac and Skeletal SR Isolated from Rat and Rabbit Muscles[a]

preparation	SERCA 2a (fmol/ng SR)[b]	phospholamban (fmol/ng SR)[c]	PLB/SER (mol/mol)
rat SR			
cardiac[d]	1.2 ± 0.4	6.3 ± 2.9	4.7 ± 0.8
soleus[e]	1.8 ± 0.3	1.6 ± 0.1	1.0 ± 0.2
rabbit SR			
cardiac[f]	3.7 ± 0.5	12.8 ± 1.1	3.5 ± 0.6
soleus[f]	3.7 ± 0.7	2.9 ± 0.4	0.8 ± 0.2

[a] Content of PLB and SERCA2a was determined from their relative antibody reaction compared to the immunoreaction of PLB or SERCA2a standard proteins. Values are expressed as mean (±SE) of multiple (two to four) measurements for each SR preparation. The value and errors for the molar ratio of PLB to SERCA2a (PLB/SER) represent the mean of molar ratios and propagated errors determined from individual preparations (*63*). From a Student's *t*-test, the pooled results from both species reveals a significant ($p = 0.008$) difference in the relative stoichiometry of PLB to SERCA2a between cardiac (4.1 ± 0.8) and slow-twitch skeletal (0.9 ± 0.1) muscle. [b] Determined based on a molecular weight of 109 477 g/mol for pig SERCA2a (*64*). [c] Determined based on a molecular weight of 6123 g/mol for pig PLB (*65*). [d] $n = 4$ preparations. [e] $n = 2$ preparations. [f] $n = 1$ preparation, errors are standard deviations of three separate measurements.

Functional Inhibition of the Ca-ATPase by PLB. The calcium-concentration dependence of ATPase activity was determined for multiple preparations of SR from skeletal and

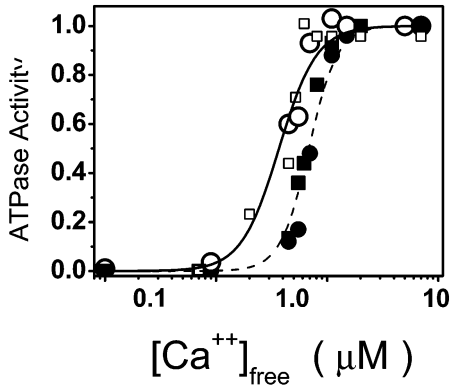

FIGURE 3: Calcium dependence of the activation of cardiac and skeletal membranes. Calcium-dependent ATPase activities were measured for SR isolated from rabbit slow-twich soleus (□, ■) or cardiac (○, ●) muscle with no addition (■, ●) or following incubation with a monoclonal antibody 8A3 against PLB (□, ○). Initial rates of ATPase activity were measured at 25 °C in a medium containing 2 μg of SR protein/mL, 25 mM MOPS (pH 7.0), 0.1 M KCl, 5 mM $MgCl_2$, 5 mM ATP, 4 μM A23187, 0.1 mM EGTA, and sufficient calcium to provide the indicated free calcium concentrations. Activities were normalized relative to maximal velocities, and represent the mean for two different preparations, each measured in duplicate. Dashed and solid lines represent the nonlinear least-squares fits to the Hill equation for the data obtained in the absence and presence of antibody, respectively.

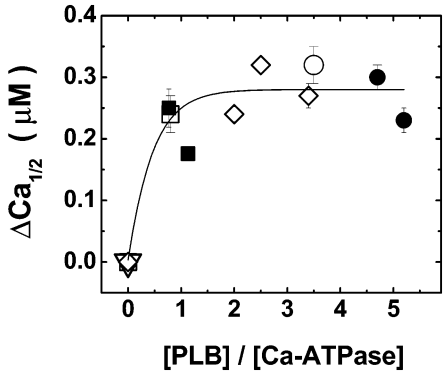

FIGURE 4: Molar stoichiometry of PLB and inhibition of the Ca-ATPase. Relationship between shift in the half-point of the calcium dependence of ATP hydrolysis activity ($\Delta Ca_{1/2}$) and molar stoichiometry of total PLB per SERCA2a isoform of the Ca-ATPase in SR isolated from fast-twitch (▽), slow-twitch soleus (□, ■) and cardiac (○, ●) muscle from rat (■, ●) or rabbit (▽, □, ○). Comparable measurements were made using reconstituted proteoliposomes containing the SERCA1 isoform of the Ca-ATPase (◇).

cardiac muscle of both rat and rabbit, fitting data from each sample to the Hill equation. ATPase assays were performed with saturating amounts of the calcium ionophore, A23187, to negate any prep-to-prep differences in membrane leakiness. The approximate 2-fold ionophore stimulation for these preparations indicates that native vesicle integrity was retained through the multiple steps of differential centrifugation involved in membrane isolation. Plotting together multiple sets of ATPase activity data obtained from both ventricular and slow-twitch soleus muscle from the rabbit indicates a similar extent of inhibition of the Ca-ATPase by PLB (Figure 3, solid symbols) despite very different levels of expressed PLB.

Separate fits to the data indicate values of the calcium concentration associated with half-maximal activation ($Ca_{1/2}$) of 0.67 and 0.76 μM for slow-twitch skeletal and cardiac SR, respectively. Incubation with a monoclonal anti-PLB antibody relieves PLB inhibition quantitatively the same as does PKA activation, producing shifts in the calcium dependence of ATPase activity toward lower calcium concentrations without observed changes in maximal activity (V_m) (Figure 3). These observed shifts in calcium dependence of Ca-ATPase activity are equivalent for the SR isolated from cardiac as compared with slow-twitch (soleus) muscle (open symbols), where $Ca_{1/2}$ values are 0.40 and 0.45 μM for slow-twitch and cardiac muscle, respectively. Similarly, activity data obtained from individual samples of rat slow-twitch skeletal and cardiac SR was fit to the Hill equation and values for the individual *shifts* in the calcium-concentration necessary for half-maximal activation $\Delta Ca_{1/2}$ following incubation with anti-PLB antibodies were correlated with the corresponding values of molar ratios of PLB to SERCA2a (Figure 4). This plot shows that despite the scatter in data from different samples, $\Delta Ca_{1/2}$ values vary relatively little as a function of these PLB-to-Ca-ATPase ratios, already having attained near maximal levels at one mol PLB per mol Ca-ATPase. Similar $\Delta Ca_{1/2}$ values were observed in SR vesicles isolated from rat EDL (data not shown) muscle.

To determine whether structural differences between cardiac and slow-twitch muscle that may result in differences in co-purified proteins influence the observed results, we utilized reconstituted membranes containing only Ca-ATPase and PLB, measuring both inhibition by PLB and antibody activation correlated with PLB and Ca-ATPase stoichiometries. SERCA1, which is regulated by PLB identically to SERCA2a in reconstituted membranes, in intact skeletal muscle and in the heart, was used in these experiments as the source of Ca-ATPase due to its greater stability which facilitates a fully functional preparation (*18*, *36*, *46*). Both Ca-ATPase and PLB were co-reconstituted with extracted SR lipids resulting in membranes having a lipid content (0.8 mg/mg) similar to that of native SR. These membranes were tightly sealed and fully functional based on their high rates of calcium-dependent ATPase activity and extent of ionophore stimulation identical to that in native membranes. On the basis of immunoblot determinations, these reconstituted proteoliposomes provided molar ratios of PLB:SERCA ranging from 2.4 to 3.4, with $\Delta Ca_{1/2}$ values comparable to those obtained from native SR from either skeletal or cardiac muscle (Figure 4). Thus, data from both native SR membranes and reconstituted proteoliposomes demonstrates a comparable level of Ca-ATPase inhibition by PLB, where the presence of one or more PLB per Ca-ATPase results in comparable shifts in the calcium-dependence of enzyme activation (having an average value of $\Delta Ca_{1/2} = 0.26 \pm 0.02$ μM).

DISCUSSION

Summary of Results. We have measured the concentrations of both PLB and the Ca-ATPase in SR isolated from cardiac and slow-twitch skeletal muscle from two rodent species. Notably, we find that PLB is expressed in approximately equimolar amounts to the Ca-ATPase in SR isolated from slow-twitch muscle; in contrast, PLB is expressed in 4-fold molar excess of the Ca-ATPase in SR isolated from cardiac SR (Figure 2). In this respect, this study represents an explicit and quantitative comparison of skeletal muscle and heart with

respect to PLB and Ca-ATPase protein expression within the same species. Measurements of the calcium concentration dependence of Ca-ATPase activity in the presence or absence of antibody activation allows the extent of inhibition by PLB and its reversal to be assessed in the same membrane sample. Specific reversal of PLB inhibition is important in distinguishing specific effects related to normal regulatory PLB−Ca-ATPase interactions from nonspecific inhibitory effects that may also alter the $\Delta Ca_{1/2}$ value.

We have observed equivalent levels of Ca-ATPase inhibition and activation in SR from the heart as compared with that from slow-twitch skeletal muscle, indicating that coexpression of only a single mole of PLB per mole of Ca-ATPase is sufficient for essentially maximal inhibition (Figures 3 and 4). Moreover, equivalent levels of inhibition are observed following co-reconstitution of affinity-purified Ca-ATPase with PLB (Figure 4), indicating the same specific structural coupling between PLB and the Ca-ATPase irrespective of the tissue source or presence of other proteins. Therefore, previous results by Kranias and Schneider and co-workers demonstrating that ablation of PLB produces identical effects on rates of muscle relaxation in skeletal muscle and heart can be readily understood as a direct result of the identical PLB−Ca-ATPase interaction in both muscle types (*16*−*18*).

PLB and Ca-ATPase Concentrations and Regulation of the Ca-ATPase. The concentrations of PLB and SERCA2a measured in wild-type rat and rabbit hearts can provide insight into previous studies in which PLB and SERCA2a concentrations were individually altered with quite different functional effects (*7*, *10*−*14*). For example, specific overexpression of SERCA2a in mouse hearts was reported in two independent transgenic lines to result in SERCA2a protein concentrations that were 1.31- and 1.54-fold of wild-type; another transgenic line with a single gene copy of SERCA2a resulted in 65% wild-type SERCA2a protein levels (*11*, *13*). No variation was observed in the apparent calcium affinity for the Ca-ATPase in SR from either of these lines; however, the maximal velocity of the Ca-ATPase was altered in each line, in accordance with the increased or decreased expression of SERCA2a. Extrapolation of the in vivo PLB and SERCA2a concentrations measured in the present study to these transgenic lines would suggest that corresponding molar ratios of PLB-to-SERCA2a are 3.2, 2.6, and 6.1 mol of PLB/mol of SERCA2a, respectively. Thus, with PLB-to-SERCA2a ratios greater than one and within the range observed in the present study (Figure 4), the extent of change in protein expression in these transgenic hearts would be not be expected to affect the apparent calcium affinity of the Ca-ATPase, as was the case in these previous studies (*11*, *13*). In contrast, overexpression of PLB in both a transgenic mouse and in adenoviral-infected rat cardiomyocytes demonstrated significant changes in the apparent calcium affinity of the Ca-ATPase relative to wild-type (*7*, *10*, *12*, *14*). In the case of PLB overexpression substantially higher levels of protein expression were achieved, i.e., up to 4.7-fold PLB relative to wildtype in transgenic mice and 2.8-fold in rat cardiomyocytes, corresponding-to-molar ratios of 18.8 and 11.2 PLB/SERCA2a, respectively. While the functional and structural effects of such a large additional density of PLB within the SR membrane has not been tested, previous work has shown that addition of similar amounts

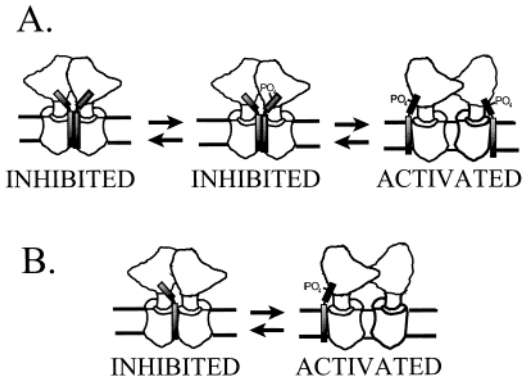

FIGURE 5: Model depicting structural coupling between PLB and Ca-ATPase oligomers in cardiac (A) and slow-twitch skeletal (B) muscle. For simplicity, only key regulatory complexes are shown rather than the full extent of molecular heterogeneity within the bilayer. Ca-ATPase-PLB complexes are shown in which the Ca-ATPase is depicted as a dimer, based on chemical derivatization, EPR, and FRET experiments (*26*, *55*, *59*). In skeletal muscle (B), PLB−Ca-ATPase complexes are depicted as one PLB per Ca-ATPase dimer, based on the measured K_D (140 ± 30 μM) for PLB binding to the Ca-ATPase, which indicates that in the presence of one total PLB per Ca-ATPase, only 0.69 ± 0.15 mol of PLB is bound to each Ca-ATPase (*61*). Thus only one PLB per Ca-ATPase dimer provides the full extent of regulation, consistent with chemical inactivation studies showing that modification of one PLB results in loss of activation of two Ca-ATPase molecules (*55*). In cardiac SR (A), PLB−Ca-ATPase complexes consisting of two PLBs per ATPase dimer are expected to predominate with excess PLB, not bound to the Ca-ATPase, also occurring (not shown) (*52*). Release of Ca-ATPase inhibition requires that all bound PLB molecules be phosphorylated in a quaternary complex with the Ca-ATPase, as demonstrated previously (*26*, *55*, *60*−*62*). Thus only the right-hand figures in panels A or B are activated Ca-ATPase. Conformational rearrangements depicted for Ca-ATPase polypeptide chains associated with the phosphorylation of PLB are consistent with measured alterations in the spatial arrangement and rotational dynamics of Ca-ATPase polypeptide chains (*26*, *59*). PLB remains associated with the Ca-ATPase following phosphorylation, as demonstrated by immunoprecipitation, fluorescence anisotropy, spin-label EPR, and NMR measurements (*62*, *66*, *67*). In its unphosphorylated state, PLB associates with transmembrane and cytosolic sites on two Ca-ATPase polypeptide chains within an oligomeric complex, and functions to increase the energetic barrier associated with calcium activation, entailing both (i) increased rigidity of Ca-ATPase helices and (ii) restricted domain motions within the nucleotide domain that are both coupled to membrane-spanning helices and important to catalytic function (*26*, *56*−*61*).

(up to 20 mol/mol of Ca-ATPase) of the hydrophobic peptide, melittin, to SR results in aggregation and concomitant inhibition of the Ca-ATPase (*47*, *48*). Thus, the higher amounts of total protein in the membrane may result in shifts in $Ca_{1/2}$ values that are due to nonspecific inhibition of the Ca-ATPase unrelated to normal PLB−Ca-ATPase interactions. For none of these experimental models was there data showing specific reversal of PLB inhibition by activating antibodies, kinases, or isoproterenol which could rule out the possibility of PLB aggregation as a means of inhibition.

Model of PLB-Ca-ATPase Interactions. Results from previous studies in relationship with the current study suggest a molecular model which explains how differences in total expression of PLB relate to differences in individual populations of PLB and their functional roles (Figure 5). Multiple populations of PLB have been shown to be present in the membrane, e.g., one, involving PLB unbound to the Ca-ATPase, which in wild-type PLB exhibits a propensity to

form oligomeric complexes, and another, comprising PLB associated with the Ca-ATPase (*50*−*55*). PLB mutants that lack the ability for aggregation have been shown to be capable of full inhibition of the Ca-ATPase, suggesting that a monomeric form of PLB regulates Ca-ATPase function (*56*). The results of the present study demonstrates that the presence of one (unphosphorylated) PLB per Ca-ATPase provides the full extent of regulation represented by the $Ca_{1/2}$, (Figure 4). The K_D for PLB binding to the Ca-ATPase, measured from both activity and spectral signals, is 140 ± 30 μM; thus the molar fraction of PLB bound to the Ca-ATPase corresponds to 0.69 ± 0.15 mol, suggesting that one PLB inhibits two Ca-ATPase molecules (*61*). This latter suggestion is consistent with data showing that chemical derivatization of one PLB results in loss of PKA-induced activation of two Ca-ATPase molecules (*55*). Moreover, the close apposition of the Ca-ATPase in dimeric arrangement within the membrane has been suggested by the strong propensity of the Ca-ATPase for dimers under crystalline conditions and more directly by rotational dynamics measurements of the Ca-ATPase in cardiac SR membranes (*26*, *68*).

Thus, in slow-twitch skeletal muscle, the most abundant PLB−Ca-ATPase complex is expected to be one PLB associated with two Ca-ATPase molecules (Figure 5B). In contrast, in the heart, having an average of 4 PLB expressed/Ca-ATPase, the most abundant complex would be a heterotetramer of two PLB and two ATPase molecules, with additional unbound PLB occurring in self-associating oligomers within the membrane. Therefore, the Ca-ATPase in skeletal muscle should be quite sensitive to the phosphorylation state of PLB, where each phosphorylation event would relieve inhibition of two Ca-ATPase molecules. On the other hand, the molar excess of PLB in the heart would provide a buffer against kinase activity in two ways. First, the abundance of PLB-ATPase heterotetramers would ensure that phosphorylation of two PLB would be required to relieve inhibition of two Ca-ATPases. In addition, the additional PLB would provide empty substrates for kinase phosphorylation which result in no functional effects as a result of their not being associated with Ca-ATPase. Therefore, equivalent activation of the Ca-ATPase in the heart as compared with that in skeletal muscle should require greater kinase action, essentially providing the heart with a more graded response to adrenergic signaling than in skeletal muscle. Several studies measuring the phosphorylation status of PLB in the heart and skeletal muscle have lent support for the suggestion that the Ca-ATPase in skeletal muscle is more readily activated. For example, measurements of endogenous phosphorylation of PLB isolated from skeletal muscle have shown that PLB is fully phosphorylated and insensitive to further in vitro activation by PKA (*17*). In contrast, PLB isolated from the heart, under resting conditions, exhibits minimal levels of phosphorylation and following isoproterenol perfusion, in vivo, only 20% of the isolated PLB was phosphorylated (*49*).

Conclusions and Future Directions. PLB associates with the Ca-ATPase in both slow-twitch and cardiac muscle, and fully inhibits the Ca-ATPase at molar stoichiometries of total PLB expression that are equivalent to the Ca-ATPase, where less than one PLB is bound per ATPase. Differences in PLB expression levels are expected to modify the sensitivity of the Ca-ATPase to kinase activation or phosphatase inactivation. Future measurements should identify how changes in the stoichiometry of PLB affect the sensitivity of the Ca-ATPase to CaM-kinase and PKA dependent signaling in both the normal and failing heart.

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