The Effects of Mutation on the Regulatory Properties of Phospholamban in Co-Reconstituted Membranes†

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ABSTRACT: Reconstitution into proteoliposomes is a powerful method for studying calcium transport in a chemically pure membrane environment. By use of this approach, we have studied the regulation of Ca^{2+}-ATPase by phospholamban (PLB) as a function of calcium concentration and PLB mutation. Co-reconstitution of PLB and Ca^{2+}-ATPase revealed the expected effects of PLB on the apparent calcium affinity of Ca^{2+}-ATPase (K_{Ca}) and unexpected effects of PLB on maximal activity (V_{max}). Wild-type PLB, six loss-of-function mutants (L7A, R9E, I12A, N34A, I38A, L42A), and three gain-of-function mutants (N27A, L37A, and I40A) were evaluated for their effects on K_{Ca} and V_{max}. With the loss-of-function mutants, their ability to shift K_{Ca} correlated with their ability to increase V_{max}. A total loss-of-function mutant, N34A, had no effect on K_{Ca} of the calcium pump and produced only a marginal increase in V_{max}. A near-wild-type mutant, I12A, significantly altered both K_{Ca} and V_{max} of the calcium pump. With the gain-of-function mutants, their ability to shift K_{Ca} did not correlate with their ability to increase V_{max}. The "super-shifting" mutants N27A, L37A, and I40A produced a large shift in K_{Ca} of the calcium pump; however, L37A decreased V_{max}, while N27A and I40A increased V_{max}. For wild-type PLB, phosphorylation completely reversed the effect on K_{Ca}, but had no effect on V_{max}. We conclude that PLB increases V_{max} of Ca^{2+}-ATPase, and that the magnitude of this effect is sensitive to mutation. The mutation sensitivity of PLB Asn^{34} and Leu^{37} identifies a region of the protein that is responsible for this regulatory property.

The contraction−relaxation cycle in heart muscle relies on the cyclic release and reuptake of sarcoplasmic reticulum (SR)[1] calcium stores. Calcium reuptake into the lumen of the SR is accomplished by Ca^{2+}-ATPase in a dynamically regulated process that involves a second protein, phospholamban (PLB) (*1*, *2*). The reuptake of calcium by Ca^{2+}-ATPase determines the rate of relaxation of the heart and the size of the calcium store that is available for the subsequent contraction. In its dephosphorylated form, PLB is a reversible, calcium-dependent inhibitor of Ca^{2+}-ATPase that modulates relaxation rates and cardiac contractility.

PLB is a small integral membrane protein that physically interacts with the SR Ca^{2+}-ATPase and modulates its calcium transport properties (*3*). Reversible inhibition by PLB is a dynamic process that depends on the cytosolic calcium concentration and the phosphorylation and oligomeric states of PLB. First, PLB inhibition of Ca^{2+}-ATPase is apparent at sub-micromolar calcium concentrations and reversed by micromolar calcium concentrations (*2*). Second, the inhibition is relieved by phosphorylation of PLB by cAMP-dependent protein kinase (*4*) or calcium-calmodulin-dependent protein kinase (*5*). Third, the PLB monomer is thought to be the inhibitory species, and the PLB pentamer is thought to be an inactive storage form (*6*, *7*). The synergistic effect is a modulation of calcium reuptake and cardiac contractility that can be finely tuned in response to activity, stress, or disease.

It is generally agreed that PLB decreases the apparent calcium affinity (K_{Ca}, calcium concentration at half-maximal activity) of Ca^{2+}-ATPase. However, evidence that PLB may alter the maximal activity (V_{max}) of Ca^{2+}-ATPase has been inconsistent. A cysteineless mutant of PLB (*8*) and fragments of PLB (*8*, *9*) have been reported to alter the V_{max} of Ca^{2+}-ATPase. In addition, phosphorylation or trypsin treatment of cardiac SR have been reported to increase V_{max} (*10*, *11*). In a more recent study, wild-type PLB and two gain-of-function mutants, Asn^{27} to Ala (N27A) and Ile^{40} to Ala (I40A), were reported to increase K_{Ca} as well as V_{max} (*12*). By use of a well-defined reconstitution system, these authors addressed the inconsistencies and lack of specificity inherent in previous studies (*8*−*11*). At near physiological lipid-to-protein ratios and physiological molar ratios of PLB and Ca^{2+}-ATPase, a 2-fold stimulation of V_{max} of Ca^{2+}-ATPase was observed (*12*).

If PLB is a physiological activator of the V_{max} of Ca^{2+}-ATPase at saturating calcium concentrations, this behavior

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[1] Abbreviations: Ac-TEV, Active-TEV protease; $C_{12}E_8$, octaethylene glycol monododecyl ether; EYPA, egg yolk phosphatidic acid; EYPC, egg yolk phosphatidylcholine; K_{Ca}, calcium concentration at half-maximal activity; MBP, maltose binding protein; MBP-PLB, maltose binding protein phospholamban fusion protein; PLB, phospholamban; SR, sarcoplasmic reticulum; TEV, tobacco etch virus; V_{max}, maximal activity.

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should be sensitive to mutation of PLB. To test this hypothesis, we have measured the ATPase activities of reconstituted proteoliposomes containing Ca^{2+}-ATPase and a variety of PLB mutants. The reconstitution was performed at a low lipid-to-protein ratio (*13*−*15*) that mimics cardiac SR. The reconstitution approach overcomes some of the difficulties in working with cardiac SR or coexpression systems in that the lipids, PLB, and Ca^{2+}-ATPase are chemically pure and their concentrations can be tightly controlled. In the work reported herein, we have studied wild-type PLB, six loss-of-function mutants of PLB, and three gain-of-function mutants of PLB in co-reconstituted proteoliposomes with Ca^{2+}-ATPase. In addition to decreasing the apparent calcium affinity of Ca^{2+}-ATPase, we show that PLB activates V_{max} of Ca^{2+}-ATPase in a mechanism that is sensitive to PLB mutation. Furthermore, we establish a correlation between Ca^{2+}-ATPase inhibition by PLB and Ca^{2+}-ATPase activation by PLB.

EXPERIMENTAL PROCEDURES

Materials. Octaethylene glycol monododecyl ether ($C_{12}E_8$) was obtained from Barnet Products (Englewood Cliff, NJ). SM2 Biobeads were obtained from Bio-Rad (Hercules, CA). Egg yolk phosphatidylcholine (EYPC) and egg yolk phosphatidic acid (EYPA) were obtained from Avanti Polar Lipids (Alabaster, AL). All the reagents used in the coupled enzyme assay (*16*) for measuring ATPase activity were of highest purity available (Sigma-Aldrich, Oakville, ON Canada). Oligonucleotides and DNA purification kits were purchased from Qiagen (Mississauga, ON Canada), and DNA sequencing was carried out in the DNA Core Services Laboratory (Department of Biochemistry, University of Alberta, Edmonton, AB Canada). Restriction enzymes and DNA modifying enzymes were purchased from Invitrogen (Carlsbad, CA) and New England Biolabs (Pickering, ON Canada). The maltose binding protein (MBP) fusion system plasmid, pMal-c2X, and amylose resin were also purchased from New England Biolabs. Tobacco etch virus (TEV) and Active-TEV (Ac-TEV) proteases were purchased from Invitrogen.

Recombinant PLB Expression and Purification. PLB was expressed and purified from bacterial cell culture, as described in detail elsewhere (*17*, *18*). The human PLB gene was cloned by PCR into the pMal-c2X plasmid to fuse it in frame with MBP and a TEV protease cleavage site. The resultant pM-PLB plasmid encoded in frame MBP, a Factor Xa site, a TEV protease site, and PLB starting at codon two. Cleavage of the fusion protein with TEV protease produced a PLB protein that has the sequence Gly-Ser replacing the initiating Met. Site-directed mutants of PLB were generated directly by PCR if the mutations were near the ends of the gene or by megaprimer mutagenesis (*19*) if near the middle. Products were then cloned into the pM-PLB plasmid, replacing the wild-type gene. The DNA sequences of the PLB genes in all plasmids produced by PCR methodology were confirmed by sequencing. In this way we made the following mutations: L7A, R9E, I12A, N34A, L37A, I38A, I40A, and L42A. The canine PLB mutant N27A was a kind gift from Larry Jones (*14*). The human PLB gene has a Lys residue at position 27.

For expression, *E. coli* DH5α cells were transformed with expression plasmids. A 50-mL starter culture containing LB growth media supplemented with 100 μg/mL ampicillin was inoculated and grown overnight at 37 °C. The starter culture was diluted into 1 L of LB media supplemented with 0.2% glucose and 50 μg/mL ampicillin and grown at 37 °C until $OD_{600} \sim 0.6$. The culture was shifted to 22 °C, and protein expression was induced with 100 μM IPTG. Cells were harvested by centrifugation after 22 h of induction and cell pellets were stored at −20 °C.

Cell pellets were resuspended in 30 mL of lysis buffer (20 mM phosphate buffer, pH 8.0, 120 mM NaCl, 1 mM EDTA, 0.5% (w/v) glycerol, 0.5% (w/v) Triton X-100, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride, 2 μg/mL pepstatin A, 2 μg/mL leupeptin) per gram of cells, and sonicated on ice using a Branson digital sonifier 250. Cell debris was removed by centrifugation. The MBP-fusion protein was purified by affinity chromatography on amylose resin and cleaved with Ac-TEV protease. PLB was purified by reverse-phase high-performance liquid chromatography (HPLC, Varian ProStar HPLC instrument, Palo Alto, CA USA; Zorbax 300 SB-C8 column, Agilent Technologies, Palo Alto, CA) at the Institute for Biomolecular Design (University of Alberta), and analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Voyager-DE Pro mass spectrometer, Applied Biosystems, Foster City, CA) and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing pure recombinant PLB proteins were combined and dried under nitrogen gas. The proteins were resuspended in 60% 2-propanol to a final concentration of approximately 2 mg/mL. The concentration of the protein stock was determined by quantitative amino acid analysis (Beckman System 6300 Amino Acid Analyzer, Beckman Coulter, Fullerton, CA) at the Alberta Peptide Institute (University of Alberta). Finally, the protein stock was subdivided into 100-μg aliquots, lyophilized to dryness, and stored at −80 °C.

Reconstitution of Ca^{2+}-ATPase and Recombinant PLB. Skeletal muscle SR vesicles were prepared from rabbit hind leg (*20*). Rabbit skeletal SR Ca^{2+}-ATPase was purified by Reactive-Red affinity chromatography (*21*). The method for functional reconstitution of Ca^{2+}-ATPase with PLB has been described (*13*, *15*, *22*). Lyophylized recombinant PLB proteins (100 μg) were resuspended in 100 μL of buffer (20 mM imidazole, pH 7.0, 100 mM KCl, 0.02% NaN_3) and incubated at 37 °C to hydrate. An appropriate amount of $C_{12}E_8$ was added to solubilize the PLB. The appropriate amounts of EYPC and EYPA in a 9:1 weight ratio were dried under nitrogen gas to a thin film. Solubilized PLB was added followed by vigorous vortexing to solubilize the lipids. To this suspension of recombinant protein and lipids were added 300 μg of pure Ca^{2+}-ATPase. The final volume was adjusted to 300 μL, and the concentrations were adjusted such that the final weight ratios were 1 protein:1 lipid:2 detergent (final molar ratio of 1 Ca^{2+}-ATPase:6 PLB:150 lipid). The detergent was then removed by incremental addition of 19 mg of wet Biobeads over a 4-h time course at room temperature. The co-reconstituted proteoliposomes containing Ca^{2+}-ATPase and recombinant PLB were removed from Biobeads and purified on a sucrose step gradient (*13*). The concentration of Ca^{2+}-ATPase and PLB within these proteoliposomes was determined by quantitative SDS-PAGE (*14*). The amount of lipid in our co-reconstituted proteoli-

posomes was estimated as previously described (*13*, *23*, *24*).

Phosphorylation of PLB was performed prior to reconstitution. PLB was solublized as described above. The catalytic subunit of protein kinase A (Sigma-Aldrich, St. Louis, MO) was added to a concentration of 100 units/mg of PLB, and the reaction incubated at 30 °C for 3 h. Phosphorylation was confirmed by MALDI-TOF mass spectrometry and SDS-PAGE.

ATPase Activity Measurements. ATPase activity of the co-reconstituted vesicles was measured by a coupled-enzyme assay (*16*). All co-reconstituted membranes were matched to controls, and each assay was performed multiple times ($n = 3-10$ for PLB-containing samples, $n = 26$ for matched Ca^{2+}-ATPase controls) over a range of free calcium concentrations from 0.1−10 μM. Approximately 5 μg of Ca^{2+}-ATPase in the co-reconstituted proteoliposomes were added to assay buffer (50 mM imidazole, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM phosphoenolpyruvate, 2.4 mM ATP, 0.18 mM NADH, 9.6 units/mL pyruvate kinase, 9.6 units/mL lactate dehydrogenase). The reaction was started by the addition of vesicles and calcium ionophore (A23187) to 0.75 mL of assay buffer. The absorbance of NADH was monitored at 340 nm at an assay temperature of 25 °C. Data were plotted as ATPase specific activity (μmol of ATP hydrolyzed mg^{-1} of Ca^{2+}-ATPase min^{-1}) vs pCa. The K_{ca} and V_{max} values were calculated based on fitting the data to the Hill equation using Sigma Plot software (SPSS Inc., Chicago, IL). Errors shown are the standard error of the mean for a minimum of three independent measurements. Comparisons of the K_{ca} and V_{max} parameters were carried out using ANOVA (between-subjects, one-way analysis of variance) followed by Tukey's HSD post hoc test for pairwise comparisons.

RESULTS

There has been a long history of reconstitution as a method for studying the functional properties of the SR Ca^{2+}-ATPases (*25*−*27*). Reconstitution has proven to be an effective mimic of native SR membranes, where pure Ca^{2+}-ATPase and lipids (EYPC and EYPA) are reincorporated into membrane vesicles at low lipid-to-protein ratios (*13*). Indeed, these same methods were applied herein, and we obtained a fully active preparation of reconstituted Ca^{2+}-ATPase proteoliposomes. Measurement of ATP hydrolysis rates yielded a maximal activity of 3.94 ± 0.07 μmol mg^{-1} min^{-1} ($n = 26$) at saturating calcium concentrations (2.5 μM Ca^{2+}). This activity level was consistent with previous observations and served as a control for all further studies in the presence of PLB.

Co-Reconstituted Proteoliposomes. At low lipid-to-protein ratios, co-reconstitution has proven to be a useful approach in structural (*14*), and functional (*12*−*15*, *28*) studies of the inhibitory complex of Ca^{2+}-ATPase and PLB. The co-reconstituted proteoliposomes used herein were characterized for their stoichiometries of lipid-to-protein and PLB-to-SERCA (Table 1). In the co-reconstitution procedure, 1 mg of lipid was included per mg of protein. This corresponded to a lipid-to-protein molar ratio of approximately 150-to-1. After co-reconstitution and purification, the proteoliposomes contained on average 0.79 ± 0.01 mg of lipid per mg of protein ($n = 40$), and this ratio was similar for all the PLB proteins. Thus, the reconstitution procedure resulted in proteoliposomes with a lipid-to-protein molar ratio of approximately 120-to-1. In addition, we measured the PLB-to-SERCA molar ratio obtained in our co-reconstituted proteoliposomes by quantitative SDS-PAGE (*14*). The co-reconstitution procedure resulted in proteoliposomes with a PLB-to-SERCA molar ratio of 4.63 ± 0.22 ($n = 52$), and this ratio was unaffected by PLB mutation. Therefore, we conclude that our reconstituted proteoliposomes are identical to those previously characterized (*13*−*15*, *28*) and that they are suitable for studying the effects of mutation on the regulatory properties of PLB.

Table 1: Lipid and Protein Ratios in Co-Reconstitutions of Ca^{2+}-ATPase and PLB[a]

	lipid/protein (w/w)	PLB/Ca^{2+}-ATPase (mol PLB/mol Ca^{2+}-ATPase)
Ca^{2+}-ATPase	0.76 ± 0.05	
	Phospholamban	
wild type	0.84 ± 0.02	4.66 ± 0.48
	Cytoplasmic Loss of Function Mutants	
L7A	0.83 ± 0.01	5.45 ± 0.50
R9E	0.79± 0.04	4.75 ± 1.09
I12A	0.74 ± 0.01	5.41 ± 0.76
	Transmembrane Loss of Function Mutants	
N34A	0.77 ± 0.01	4.58 ± 0.38
I38A	0.79 ± 0.02	4.55 ± 0.39
L42A	0.85 ± 0.01	5.38 ± 0.50
	Gain of Function Mutants	
N27A	0.74 ± 0.04	4.89 ± 0.87
L37A	0.74 ± 0.1	3.68 ± 1.25
I40A	0.85 ± 0.02	6.88 ± 0.60
average of all co-reconstitutions	0.79 ± 0.01[b]	4.63 ± 0.22

[a] Lipid to protein weight ratios and molar ratios of PLB to Ca^{2+}-ATPase are the mean ± SEM ($n \geq 3$). [b] Stoichiometry of approximately 116 mol lipid per mole Ca^{2+}-ATPase.

Wild-Type Phospholamban. The PLB produced using the MBP fusion system replaces Met^1 of PLB with the sequence Gly-Ser, making the recombinant protein 53 amino acids in length. This recombinant PLB sequence has been shown to behave identically to wild-type PLB (*29*) and is hereafter referred to as recombinant wild-type PLB. The functional effects of our recombinant wild-type PLB were tested in co-reconstituted membranes with Ca^{2+}-ATPase (Figure 1). Wild-type PLB decreased K_{Ca} by 0.26 pCa units, consistent with previously reported effects of PLB on Ca^{2+}-ATPase using co-reconstitution (*22*). However, contrary to our expectations, we observed that wild-type PLB increased V_{max} of Ca^{2+}-ATPase to 7.46 ± 0.10 μmol mg^{-1} min^{-1} ($n = 10$; 1.89-fold increase over control). The decrease in K_{Ca} was reversed by phosphorylation, while the stimulation of V_{max} remained at the same level as with unphosphorylated PLB.

Cytoplasmic Loss-of-Function Mutations. We tested the effects of cytoplasmic domain mutations on the ability of PLB to decrease K_{Ca} and increase V_{max} of Ca^{2+}-ATPase (Figure 2A). Three well-characterized cytoplasmic mutants were chosen, namely, Leu^7 to Ala (L7A), Arg^9 to Glu (R9E), and Ile^{12} to Ala (I12A) (*30*). All of the PLB mutants were able to form pentamers. In general, the cytoplasmic mutants had a variable impact on PLB function, despite their classification as loss-of-function mutations. The PLB mutants retained the ability to decrease the calcium affinity of Ca^{2+}-

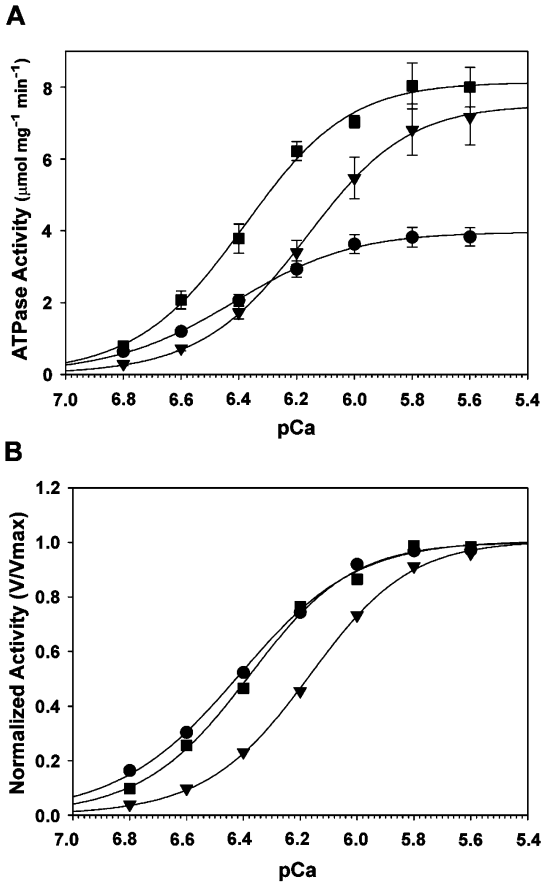

FIGURE 1: ATPase activity as a function of free calcium concentration. The Ca^{2+}-ATPase was co-reconstituted in the absence (●) and in the presence (▼) of recombinant wild-type PLB or in the presence (■) of phosphorylated recombinant wild-type PLB. (A) The data are plotted as ATPase specific activity (μmol mg^{-1} min^{-1}) vs pCa, and each data point is the mean ± SEM ($n = 26$ for (●), $n = 10$ for (▼), $n = 4$ for (■)). The K_{ca} values were calculated by fitting the data to the Hill equation and are as follows: 6.43 pCa units for Ca^{2+}-ATPase alone, 6.17 pCa units for Ca^{2+}-ATPase and recombinant wild-type PLB, and 6.40 pCa units for Ca^{2+}-ATPase and phosphorylated PLB. (B) The ATPase activities were normalized to the V_{max} calculated from fitting the data in (A) to the Hill equation and plotted against pCa.

ATPase, albeit to a lesser extent than wild type (Figure 3A). The exception was the R9E mutant, which had a significant impact on PLB function. The L7A mutant decreased K_{Ca} by 0.13 pCa units, the R9E mutant decreased K_{Ca} by 0.06 pCa units, and the I12A mutant decreased K_{Ca} by 0.19 pCa units. The PLB mutants also retained the ability to increase the maximal activity of Ca^{2+}-ATPase, with the R9E mutant again having the least effect (Figure 3B). The L7A mutant increased V_{max} to 7.83 ± 0.15 μmol mg^{-1} min^{-1} ($n = 5$; 1.99-fold increase over control), the R9E mutant increased V_{max} to 5.58 ± 0.19 μmol mg^{-1} min^{-1} ($n = 5$; 1.42-fold increase over control), and the I12A mutant increased V_{max} to 6.79 ± 0.35 μmol mg^{-1} min^{-1} ($n = 4$; 1.72-fold increase over control). As a group, mutations in the cytoplasmic domain of PLB demonstrated a significant ability to alter K_{Ca} and V_{max}, similar to wild-type PLB. Only the charge reversal of the R9E mutant diminished the inhibitory properties of PLB and reduced the effect on maximal activity. In terms of the observed K_{Ca} and V_{max}, the PLB mutant R9E had a high statistical probability for being distinct from wild-type PLB, as well as the PLB mutants L7A and I12A (Figure 3).

Transmembrane Loss-of-Function Mutations. We also tested the effects of transmembrane domain mutations on the ability of PLB to decrease K_{Ca} and increase V_{max} of Ca^{2+}-ATPase (Figure 2B). Three well characterized transmembrane mutants were chosen, namely, Asn^{34} to Ala (N34A), Ile^{38} to Ala (I38A), and Leu^{42} to Ala (L42A) (*6*). All of the PLB mutants were able to form pentamers. These PLB residues are located on one face of PLB's transmembrane helix proposed to interact with Ca^{2+}-ATPase and preserve PLB oligomerization. The mutant PLB species decreased the calcium affinity of Ca^{2+}-ATPase, with the exception of the N34A mutation that completely abolished PLB function (Figure 3A). The N34A mutation had no effect on K_{Ca}, while the mutations I38A and L42A both decreased K_{Ca} by 0.12 pCa units. The mutant PLB species also increased V_{max}, with the N34A mutation having the least effect (Figure 3B). The mutation N34A increased V_{max} to 4.73 ± 0.18 μmol mg^{-1} min^{-1} ($n = 6$; 1.20-fold increase over control), the mutation I38A increased V_{max} to 5.83 ± 0.20 μmol mg^{-1} min^{-1} ($n = 5$; 1.48-fold increase over control), and the mutation L42A increased V_{max} to 6.32 ± 0.32 μmol mg^{-1} min^{-1} ($n = 5$; 1.60-fold increase over control). The N34A mutation was the most detrimental to the inhibitory properties of PLB and produced only a small increase in maximal activity. In terms of the observed K_{Ca} and V_{max}, the PLB mutant N34A had a high statistical probability for being distinct from wild-type PLB (Figure 3).

Gain-of-Function Mutations. Last, we tested the effects of gain-of-function mutations, the so-called "super-shifters", on the ability of PLB to decrease K_{Ca} and increase V_{max} of Ca^{2+}-ATPase (Figure 2C). Three well-characterized mutants were chosen. The Leu^{37} to Ala (L37A) and Ile^{40} to Ala (I40A) PLB mutants are monomeric (*6*). The pentameric mutant Asn^{27} to Ala (N27A) of canine PLB was also used (*14*); human PLB has a lysine residue at this position. All three mutants produced a large shift in K_{Ca} with I40A being the most inhibitory PLB species (Figure 3A). The N27A mutant decreased K_{Ca} by 0.30 pCa units, the L37A mutant decreased K_{Ca} by 0.31 pCa units, and the I40A mutant decreased K_{Ca} by 0.41 pCa units. The mutant PLB species also altered V_{max}, with the L37A mutant having the atypical behavior of decreasing V_{max} (Figure 3B). The N27A mutant increased V_{max} to 6.55 ± 0.24 μmol mg^{-1} min^{-1} ($n = 4$; 1.66-fold increase over control), the L37A mutant decreased V_{max} to 2.23 ± 0.05 μmol mg^{-1} min^{-1} ($n = 3$; 1.77-fold decrease compared to control), and the I40A mutant increased V_{max} to 6.26 ± 0.09 μmol mg^{-1} min^{-1} ($n = 6$; 1.59-fold increase over control). As a group, the super-shifting mutations imparted an enhanced ability to decrease K_{Ca} and a variable ability to alter V_{max}. In terms of the observed K_{Ca} and V_{max}, the PLB mutant L37A had a high statistical probability for being distinct from wild-type PLB (Figure 3).

Correlation between K_{Ca} and V_{max}. It is obvious from the above data that a correlation exists between the ability of the PLB mutant species to decrease K_{Ca} and their ability to increase V_{max}. For the loss-of-function mutants, plotting K_{Ca} versus V_{max} yielded a reasonable fit to a line (Figure 3C; $r = 0.83$). At one end of the scale, the transmembrane loss-of-function mutant N34A had K_{Ca} and V_{max} values that were similar to control (Ca^{2+}-ATPase in the absence of PLB). At

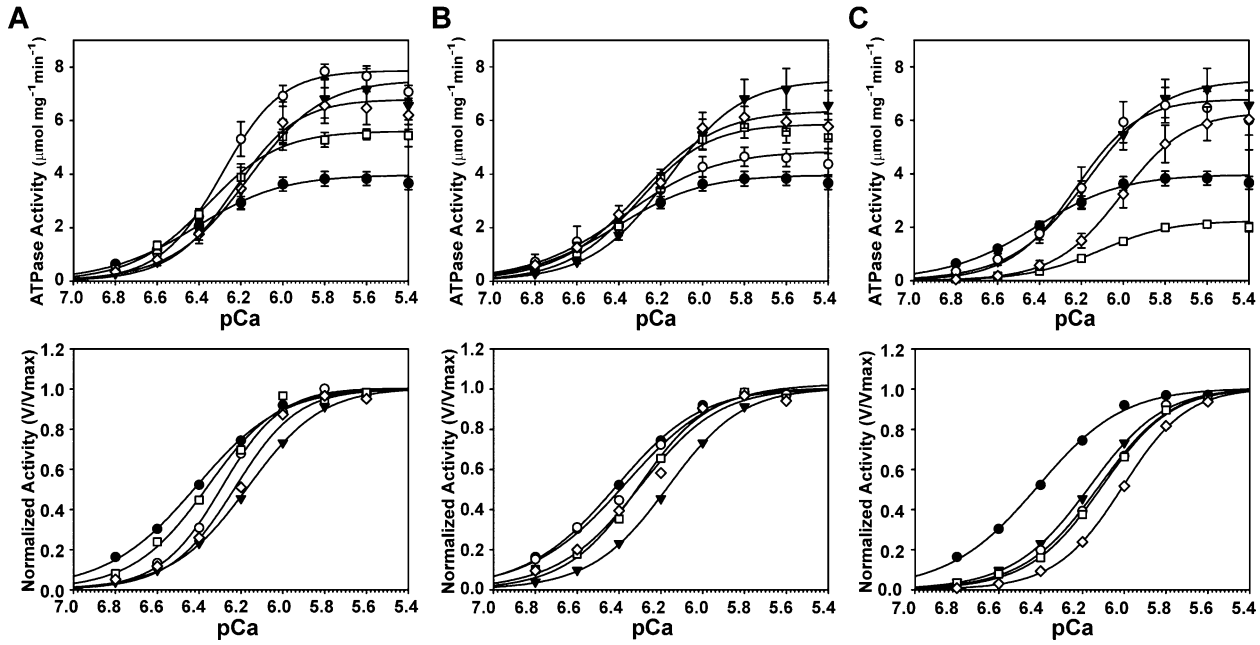

FIGURE 2: ATPase specific activity as a function of free calcium concentration for PLB mutants. (A) The Ca^{2+}-ATPase was co-reconstituted in the absence (●) and in the presence of cytoplasmic loss-of-function mutants (L7A (○), R9E (□), I12A (◊)). (B) The Ca^{2+}-ATPase was co-reconstituted in the absence (●) and in the presence of transmembrane loss-of-function mutants (N34A (○), I38A (□), L42A (◊)). (C) The Ca^{2+}-ATPase was co-reconstituted in the absence (●) and in the presence of gain-of-function mutants (N27A (○), L37A (□), I40A (◊)). In the top panels, the ATPase specific activity (μmol mg^{-1} min^{-1}) were plotted vs pCa, and each data point is the mean ± SEM ($n = 3-6$). The bottom panel shows the specific activities normalized to the V_{max} calculated from fitting the data in the top panels to the Hill equation. For comparison, the wild-type PLB (▼) ATPase specific activities and normalized activities from Figure 1 are also shown in each plot.

the other end of the scale, the cytoplasmic loss-of-function mutant I12A had a K_{Ca} and V_{max} that were similar to wild-type PLB. Arguably, the pentameric super-shifting mutant N27A also fit this correlation; however, the monomeric super-shifting mutants L37A and I40A deviated from this behavior.

Inhibition by Thapsigargin. The accurate measurement of maximal activity of Ca^{2+}-ATPase is sensitive to the quantitation of active enzyme for calculating specific activities. While quantitative SDS-PAGE provides an accurate measure of total Ca^{2+}-ATPase in our co-reconstituted membranes, the amount of functional enzyme may vary between preparations of co-reconstituted proteoliposomes. To address this, titration curves for inhibition of Ca^{2+}-ATPase by thapsigargin at saturating calcium concentrations were measured in the absence and presence of wild-type PLB. For instance, an appropriate amount of thapsigargin that will inhibit half of the Ca^{2+}-ATPase present should also reduce the observed activity by half, given the high affinity and stoichiometric nature of Ca^{2+}-ATPase inhibition by thapsigargin (*31*). Despite the nearly 2-fold difference in V_{max} of Ca^{2+}-ATPase in the absence and presence of PLB, the thapsigargin inhibition profiles were identical (data not shown). Moreover, the two methods for quantitation of Ca^{2+}-ATPase, SDS-PAGE and thapsigargin inhibition, yielded identical results for calculation of specific activities.

DISCUSSION

As a critical regulatory component of cardiac SR, PLB has been the subject of extensive mutagenesis and biochemical characterization. Scanning-alanine mutagenesis of the primary sequence of PLB has provided insight into the importance of individual amino acids in the functional interaction with Ca^{2+}-ATPase (*6*, *30*). Coexpression in mammalian cell culture has proved to be an effective tool in these studies, and every amino acid in the primary sequence of PLB has been mutated. However, no one has been entirely successful in expanding these studies to reconstituted systems, where the molecular mechanism of calcium pump regulation by PLB can be characterized in a well-controlled membrane environment. One reason has been the difficulty in obtaining sufficient quantities of many different PLB mutants for a thorough investigation under reconstitution conditions. Our success at expressing and purifying multi-milligram quantities of many mutant forms of PLB from bacterial cell culture (*17*) led us to investigate the ability of PLB to regulate Ca^{2+}-ATPase in co-reconstituted proteoliposomes.

Indeed, one of the remaining uncertainties in the regulation of Ca^{2+}-ATPase by PLB is the persistent reports in the literature regarding the effect of PLB on the maximal activity of the pump (*8*−*12*). Notably, a recent study used reconstitution to examine the dependence of maximal activity on the molar ratio of PLB, Ca^{2+}-ATPase, and lipids for several PLB species (*12*). These authors provided a convincing argument for an effect of PLB on the maximal activity of Ca^{2+}-ATPase. Specifically, PLB increased the calcium pump maximal activity as the membrane composition approached that found in cardiac SR. Moreover, the loss-of-function mutant Leu^{31} to Ala (L31A) had no effect on the V_{max} of Ca^{2+}-ATPase, indicating that this was not due to the reconstitution procedure itself.

To further characterize the importance of individual amino acids in the functional interaction of PLB with Ca^{2+}-ATPase, we have measured the functional effects of wild-type PLB and nine PLB mutants in reconstituted proteoliposomes. ATP

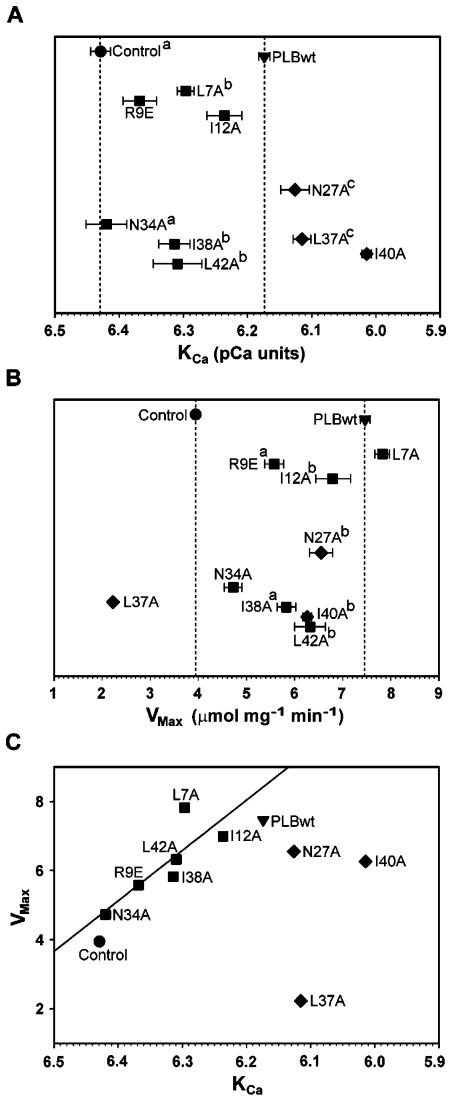

FIGURE 3: (A) The effect of PLB mutations on the calcium affinity of Ca^{2+}-ATPase. The Ca^{2+}-ATPase was co-reconstituted in the absence (Control (●)) and in the presence of recombinant wild-type PLB (PLB_{wt} (▼)), six loss-of-function PLB mutants (L7A, R9E, I12A, N34A, I38A, L42A (■)), and three gain-of-function PLB mutants (N27A, L37A, I40A (♦)). K_{Ca} is the calcium concentration at half-maximal ATPase activity. The *vertical dashed line* on the *left* represents the K_{Ca} value for Ca^{2+}-ATPase in the absence of PLB ($K_{Ca} = 6.43$), and the *vertical dashed line* on the *right* represents K_{Ca} in the presence of wild-type PLB ($K_{Ca} = 6.17$). Each data point is the mean ± SEM ($n = 3-6$). An analysis of variance was calculated between K_{Ca} values for all PLB mutants, and significant variance was found. The K_{Ca} values were then compared using Tukey's HSD post hoc test and were found to be statistically different at a level of $p < 0.01$ except where marked with the same superscript (a, b, or c); that is, the K_{Ca} values for N34A and control are marked with the superscript a and are not different. (B) The effect of PLB mutations on the maximal activity of Ca^{2+}-ATPase. The V_{max} values of the Ca^{2+}-ATPase co-reconstituted in the absence and in the presence of recombinant wild-type PLB and the nine PLB mutants are plotted using the same symbols as in (A). The *vertical dashed line* on the *left* represents the V_{max} value for Ca^{2+}-ATPase in the absence of PLB ($V_{max} = 3.94$), and the *vertical dashed line* on the *right* represents V_{max} in the presence of wild-type PLB ($V_{max} = 7.46$). An analysis of variance was calculated between the V_{max} values for all PLB mutants and significant variance was found. The V_{max} values were then compared using Tukey's HSD post hoc test and were found to be statistically different at a level of $p < 0.01$ except where marked with the superscript a or b. (C) The V_{max} plotted as a function of the K_{Ca}.

hydrolysis rates were measured as a function of free calcium concentration for each PLB species at fixed molar ratios of PLB, Ca^{2+}-ATPase, and lipids. Our findings with recombinant wild-type PLB are consistent with recent findings using reconstitution (*12*). At physiological lipid-to-protein ratios, PLB inhibits Ca^{2+}-ATPase at low calcium concentrations (decreases K_{Ca}) and activates Ca^{2+}-ATPase at high calcium concentrations (increases V_{max}). Phosphorylation of PLB relieves the inhibition, yet it has no effect on the activation. These effects occur at approximate molar ratios of 120 lipids to 5 PLB to 1 Ca^{2+}-ATPase, conditions that simulate cardiac SR. In contrast, the effect of PLB on Ca^{2+}-ATPase maximal activity was not observed at the high lipid-to-protein used in earlier reconstitutions (*22*).

The accurate measurement of maximal activity of Ca^{2+}-ATPase is sensitive to both the free calcium concentration while measuring ATP hydrolysis and the quantitation of active enzyme for calculating specific activities. First, to overcome variability in the free calcium concentration, all co-reconstituted membranes were matched to controls, and ATPase activities were measured over the full range of calcium concentrations (0.1−10 μM). The V_{max} was determined by fitting the Hill equation to the average dataset. Second, to address the quantitation of functional Ca^{2+}-ATPase in the co-reconstituted proteoliposomes, inhibition by thapsigargin was used as an indicator of active enzyme in the absence and presence of PLB. Thapsigargin is a high-affinity, conformation-dependent inhibitor of Ca^{2+}-ATPase (*31*). Quantitation of Ca^{2+}-ATPase for calculation of specific activity yielded identical results, whether based on SDS-PAGE or thapsigargin inhibition. Consequently, we were confident that our co-reconstituted membranes provided an accurate measure of Ca^{2+}-ATPase regulation by PLB.

With this in mind, our data can be summarized by two simple observations. First, we have provided new insight into the importance of individual amino acids in the functional interaction between PLB and the Ca^{2+}-ATPase. The PLB mutation N34A is a loss-of-function mutation that affects neither K_{Ca} nor V_{max} of Ca^{2+}-ATPase. The PLB mutation L37A is a gain-of-function mutation that has an enhanced effect on K_{Ca} and the contrary effect of depressing V_{max} of Ca^{2+}-ATPase. In terms of the observed V_{max}, the PLB mutants N34A and L37A showed the greatest decrease from wild-type PLB (63 and 30% of wild-type V_{max}, respectively). Taken together, these mutations identify a region of PLB that is responsible for stimulation of Ca^{2+}-ATPase maximal activity. In support of this observation, the loss-of-function PLB mutant L31A has been reported by others (*12*) to have no significant effect on either K_{Ca} or V_{max}. In contrast, all other mutations tested exhibited an ability to enhance V_{max}, albeit to variable degrees. Second, a strong correlation exists between the ability of PLB mutants to decrease K_{Ca} and increase V_{max}. Three so-called "super-shifting" mutants deviated from this trend, and the L37A mutant appeared to uncouple effects on K_{Ca} from effects on V_{max}. Therefore, we conclude that the activation of Ca^{2+}-ATPase maximal activity by PLB is mutation sensitive, depends on interactions involving amino acids Leu^{31}, Asn^{34}, and Leu^{37} of PLB, and is proportional to PLB inhibition.

The activation of Ca^{2+}-ATPase maximal activity by PLB did not depend on pentameric assembly, as the monomeric "super-shifting" mutant I40A also increased V_{max}. The

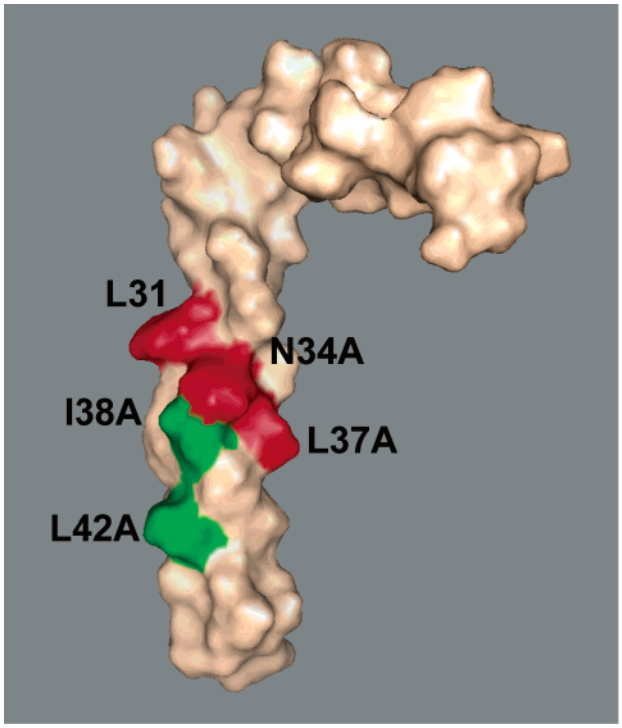

FIGURE 4: The amino acids Leu^{31}, Asn^{34}, and Leu^{37} in the primary sequence of PLB form a surface patch on the face of PLB's transmembrane helix proposed to interact with Ca^{2+}-ATPase. A surface representation of PLB (PDF accession code 1FJK (*43*)) is shown. Amino acids identified as important for activation of Ca^{2+}-ATPase (Leu^{31}, Asn^{34}, and Leu^{37} of PLB) are shown as red surfaces. Amino acids that had no effect on activation of Ca^{2+}-ATPase (Ile^{38}, Ile^{40}, and Leu^{42} of PLB) are shown as green surfaces. Ile^{40} of PLB is on the opposite face of PLB's transmembrane helix and is not seen in this view.

activation of Ca^{2+}-ATPase by the various PLB mutants did not correlate with monomeric or pentameric forms of PLB, yet the mutants that had lost this ability did correspond to a region on one face of PLB's transmembrane helix. The amino acids Leu^{31}, Asn^{34}, and Leu^{37} of PLB are important for Ca^{2+}-ATPase activation; however, the L31A and N34A mutants are pentameric, and the L37A mutant is monomeric. Similarly, the amino acids Asn^{27}, Ile^{38}, Ile^{40}, and Leu^{42} of PLB are not critical for Ca^{2+}-ATPase activation, where the N27A, I38A, and L42A mutants are pentameric and the I40A mutant is monomeric. The one obvious feature of amino acids Leu^{31}, Asn^{34}, and Leu^{37} is that they form a surface patch on the face of PLB's transmembrane helix proposed to interact with Ca^{2+}-ATPase (Figure 4). The amino acids Ile^{38} and Leu^{42} are on this same face of PLB's transmembrane helix, yet they have little effect on Ca^{2+}-ATPase activation. We conclude that the ability of PLB to activate Ca^{2+}-ATPase localizes to a structural domain of PLB and does not depend on PLB oligomerization.

In structural terms, how can PLB be both an inhibitor and an activator? To inhibit Ca^{2+}-ATPase at limiting calcium and activate Ca^{2+}-ATPase at saturating calcium, PLB must be associated with the pump in both conditions. Indeed, evidence suggests that PLB may remain associated with Ca^{2+}-ATPase even in the absence of inhibition (*32*−*34*). With regards to the PLB binding site, cross-linking studies have identified regions of the nucleotide-binding domain and transmembrane helices M2/M4 of Ca^{2+}-ATPase that contribute to binding (*35*−*38*). This effort has been aided by numerous structures for Ca^{2+}-ATPase (*39*−*42*) and PLB (*43*, *44*), and the inhibitory complex has been modeled accordingly (*35*, *37*). What about the structural basis for Ca^{2+}-ATPase activation by PLB? In terms of an interaction site involving Leu^{31}, Asn^{34}, and Leu^{37} of PLB, the proximal residue Asn^{30} has been cross-linked to Cys^{318} and Lys^{328} of Ca^{2+}-ATPase (*35*, *36*). This suggests that a physical interaction between the cytoplasmic portion of M4 and PLB might be key to increasing Ca^{2+}-ATPase maximal activity.

To increase V_{max}, PLB likely acts on a rate-limiting step in the calcium transport cycle. One possible explanation is that PLB lowers the transition-state energy required for a rate-limiting conformational change. The E_1P-to-E_2P transition has been identified as a rate-limiting step (*45*, *46*); however, there are several other slow steps in the calcium transport cycle. Large conformational changes accompany the E_1P-to-E_2P transition involving all three cytoplasmic domains and transmembrane helices M1 to M6 (*39*−*42*, *47*). Furthermore, PLB binding is enhanced by ATP (*34*−*36*), suggesting that PLB can interact with a nucleotide bound conformation of Ca^{2+}-ATPase. Nonetheless, the structure of the inhibitory complex may be quite distinct from the available structures for Ca^{2+}-ATPase (*39*−*42*) and PLB (*43*, *44*), as suggested by models for the inhibitory complex (*14*, *37*, *48*). A complete understanding of the molecular mechanism of PLB regulation will await a structure of the complex with Ca^{2+}-ATPase.

If PLB is a physiological activator of Ca^{2+}-ATPase, one would expect this behavior to be sensitive to PLB mutation. Indeed, the amino acids Leu^{31}, Asn^{34}, and Leu^{37} of PLB are important for activation of Ca^{2+}-ATPase, as measured in co-reconstituted proteoliposomes. These three residues lie on one face of PLB's transmembrane helix (Figure 4 (*43*, *44*)) supporting the notion that part of one helical face forms the Ca^{2+}-ATPase interaction site responsible for activation. In general, there is a relationship between PLB's ability to decrease the calcium affinity and increase the maximal activity of Ca^{2+}-ATPase, where PLB mutations that effect inhibition have an equivalent effect on activation. Nevertheless, mutation of Leu^{37} demonstrates that inhibition of Ca^{2+}-ATPase can be enhanced in the absence of activation, effectively uncoupling these two behaviors. Given these findings, ample evidence has been presented for PLB as an activator of calcium pumping in the SR. Future research should include an exhaustive study of Ca^{2+}-ATPase activity in response to PLB mutation in co-reconstituted proteoliposomes.

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