

Defining the Molecular Components of Calcium Transport Regulation in a Reconstituted Membrane System[†]

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ABSTRACT: Using a chemically defined reconstitution system, we performed a systematic study of key factors in the regulation of the Ca-ATPase by phospholamban (PLB). We varied both the lipid/protein and PLB/Ca-ATPase ratios, determined the effects of PLB phosphorylation, and compared the regulatory effects of several PLB mutants, as a function of Ca concentration. The reconstitution system allowed us to determine accurately not only the PLB effects on K_{Ca} (Ca concentration at half-maximal activity) of the Ca-ATPase, but also the effects on V_{max} (maximal activity). Wild-type PLB (WT-PLB) and two gain-of-function mutants, N27A-PLB and I40A-PLB, showed not only the previously reported increase in K_{Ca} , but also an increase in V_{max} . Specifically, V_{max} increases linearly with the intramembrane PLB concentration, and is approximately doubled when the sample composition approaches that of cardiac SR. Upon phosphorylation of PLB at Ser-16, the K_{Ca} effects were almost completely reversed for WT- and N27A-PLB but were only partially reversed for I40A-PLB. Phosphorylation induced a V_{max} increase for WT-PLB, and a V_{max} decrease for N27A- and I40A-PLB. We conclude that PLB and PLB phosphorylation affect V_{max} as well as K_{Ca} , and that the magnitude of both effects is sensitive to the PLB concentration in the membrane.

Phospholamban (PLB)¹ is a small membrane protein that regulates the Ca-ATPase in cardiac sarcoplasmic reticulum (SR) (1–4). In its unphosphorylated form, PLB inhibits the rate of Ca-pumping and ATP hydrolysis by the Ca-ATPase at subsaturating [Ca]. Upon PLB phosphorylation by cAMP-dependent protein kinase A (PKA) or Ca/calmodulin dependent protein kinase II (CaM kinase II), this inhibition is relieved (2, 4, 5). Although PLB is not present in fast-twitch muscle (6, 7), PLB has been shown to regulate the fast-twitch Ca-ATPase isoform (SERCA1), as well as the cardiac isoform (SERCA2a), in both reconstitution (8–13) and coexpression (14) experiments.

Studies using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and low-angle laser light scattering in SDS solution have shown that wild-type PLB (WT-PLB) is predominantly pentameric, in equilibrium with a minor monomeric fraction (15, 16). Using electron paramagnetic resonance (EPR) spectroscopy, it has been

shown that PLB exists in an average oligomeric size of 3.5 in a lipid membrane, and that upon phosphorylation the average oligomeric size increases to 5.3 (17). That study suggested the existence of a dynamic equilibrium between PLB subunits in the lipid bilayer that is regulated by phosphorylation, and that the regulation of PLB's oligomeric state is critical for its regulation of the Ca-ATPase, with the PLB monomer being the inhibitory species. Alanine-scanning mutagenesis has shown that replacement of single hydrophobic amino acid residues, Leu or Ile, by Ala, at specific sites in the membrane-spanning region, decreases the stability of PLB pentamers on SDS–PAGE (16), and these effects were confirmed in lipid membranes by EPR (17) and fluorescence (19). Fluorescence has also shown that the Ca-ATPase binds preferentially to the monomeric species of PLB (20). In addition, most mutations that destabilize PLB pentamers have been shown to enhance PLB's inhibitory potency, supporting the hypothesis that the active inhibitory species of PLB is the monomer, and that increased oligomeric stability of PLB upon phosphorylation contributes to relief of the inhibition (11, 17, 18, 21, 22).

However, some transmembrane PLB mutants have inhibitory potencies that do not correlate well with their oligomeric states (23, 24), and several mutants outside the transmembrane domain of PLB (e.g., N27A-PLB and N30A-PLB) have enhanced inhibitory potency despite having pentameric stability comparable to that of WT-PLB (21, 22, 25). On the basis of these results and others (26), it has been proposed that the inhibitory potency of PLB depends on both the self-dissociation constant of the PLB pentamer and the association constant of the PLB monomer with the Ca-ATPase (11, 27).

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¹ Abbreviations: PLB, phospholamban; WT-PLB, wild-type phospholamban; N27A-PLB, Asn-27 to Ala mutant of PLB; I40A-PLB, Ile-40 to Ala mutant of PLB; L31A-PLB, Leu-31 to Ala mutant of PLB; C₁₂E₈, octaethylene glycol monododecyl ether; SR, sarcoplasmic reticulum; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; OG, β -octyl glucoside; DOPC, dioleoyl phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; TFE, 2,2,2-trifluoroethanol; TFA, trifluoroacetic acid; PKA, cAMP-dependent protein kinase.

Although it is generally accepted that PLB inhibits the rate of Ca-transport by decreasing the apparent Ca-affinity (increasing K_{Ca} , the Ca concentration at half-maximal activity) of the Ca-ATPase, significant evidence has been accumulated that PLB may also affect V_{max} of the Ca-ATPase at saturating Ca. Synthetic peptides corresponding to the cytoplasmic domain of PLB have been reported to decrease V_{max} (28), although recent work has not confirmed this (10, 29). A PLB mutant that had all three cysteines in the transmembrane domain mutated to alanine was reported to decrease V_{max} of the Ca-ATPase in a reconstituted system (30). In the studies reporting V_{max} effects (28, 30), the molar ratio of PLB or PLB-derived peptides to the Ca-ATPase was at least 100. This is a much higher ratio of PLB to Ca-pump than the numbers previously reported for cardiac SR, which range from 0.2 to 15 mol of PLB per mole of Ca-pump (31–37). There are also several reports that phosphorylation of PLB or treatment of cardiac SR by trypsin can increase V_{max} (38, 39), but these results are not conclusive due to the unspecific nature of these treatments.

To determine the V_{max} effects accurately, it is necessary to perform Ca-ATPase measurements on membranes having identical concentrations of Ca-pump but varying concentrations of PLB, both unphosphorylated and phosphorylated. This is difficult in cardiac SR, where the “no-PLB” control is usually approximated by phosphorylating PLB or by treatment with an anti-PLB antibody, which have been shown to only partially reverse the PLB effect (21, 24). In addition, neither in SR nor in coexpression systems can sample composition be varied. In the present study, we have solved these problems by using a reconstitution system in which intramembrane concentrations of lipid, PLB, and Ca-ATPase are well defined and varied systematically, and we have measured Ca-ATPase activity in each case as a function of ionized calcium concentration, so that K_{Ca} and V_{max} can be measured accurately. We have used WT-PLB and its mutants N27A-PLB [pentameric gain-of-function mutant (40)], and I40A-PLB [monomeric gain-of-function mutant (22)] to explore the possible roles of oligomeric stability on these effects.

EXPERIMENTAL PROCEDURES

Reagents. Octaethylene glycol monododecyl ether ($C_{12}E_8$), β -octylglucoside (OG), and Biobeads SM2 were purchased from CALBIOCHEM (San Diego, CA). Dioleoyl phosphatidylcholine (DOPC) and dioleoyl phosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). The reagents for SDS–PAGE (10–20% gradient Tris-tricine Ready Gels polyacrylamide precast minigels and the Tris-tricine gel running buffer) were from Bio-Rad Laboratories (Richmond, CA). Anti-PLB monoclonal antibody (1D11) and anti-phosphoserine PLB polyclonal antiserum were produced as described previously (41). All the other reagents used in the coupled enzyme assay for measuring the ATP hydrolysis rates were of highest purity available and were purchased from Sigma (St. Louis, MO).

PLB Expression and Purification. Point mutations were introduced in the canine isoform of PLB cloned into the BglIII site of the pVL1393 shuttle vector using the QuikChange site-directed mutagenesis kit (Stratagene). The mutated PLB-pVL1393 constructs were cotransfected into Sf21 insect cells

using the BaculoGold transfection kit (Pharmingen). A plaque assay was used to isolate recombinant baculovirus clones, which were further individually amplified and confirmed for PLB expression by immunoblotting using the 1D11 anti-PLB monoclonal antibody. Recombinant PLB molecules were expressed in Sf21 insect cells and purified by immunoaffinity chromatography using the 1D11 monoclonal antibody as previously described (10). The concentration of PLB was determined by the Amido Black assay (42). Purified PLB was stored at -20° at a protein concentration of 1.5–2.5 mg/mL, in a buffer (designated “OG-buffer”) containing 88 mM MOPS, 18 mM glycine, and 0.92% OG at pH 7.0. In certain experiments, the OG-buffer was replaced with the organic solvent trifluoroethanol (TFE) using HPLC with a 300×7.8 mm DeltaPak C4 semipreparative column (Waters). Elution was carried out with a solvent gradient starting with 100% water containing 0.1% trifluoroacetic acid (TFA) (solvent A) and ending with a solvent containing 95% 2-propanol, 5% water, and 0.1% TFA (solvent B), at a flow rate of 1.0 mL/min. PLB eluted between 60 and 75% of solvent B. PLB fractions were collected, the solvent was evaporated, and PLB was resuspended in 100% TFE at a final concentration of about 2 mg/mL.

Co-Reconstitution of Ca-ATPase and PLB. Rabbit skeletal sarcoplasmic reticulum Ca-ATPase was purified from SR using the Reactive-Red affinity chromatography method (43). Protein concentration was determined by the Amido Black method (42). The functional co-reconstitution of the Ca-ATPase with PLB was carried out as described in our previous reports (10, 11), adapted for the systematic variation of protein and lipid ratios. Briefly, the required amount of PLB (5 to 25 μ g) in 20 μ L of OG buffer was lyophilized, redissolved in 160 μ L of chloroform containing 25 μ L of TFE and the required quantities of DOPC/DOPE (20% DOPE by weight). The organic solvent was dried under nitrogen, and the residual solvent was removed by pumping the sample under vacuum. The dried film of lipid and PLB was hydrated with 100 μ L of 20 mM imidazole (pH 7.0) by vortexing thoroughly, followed by a 30 s sonication. The resulting vesicles composed of lipid and PLB were adjusted to 20 mM imidazole (pH 7.0) 0.1 M KCl, 5 mM $MgCl_2$, and 10% glycerol. $C_{12}E_8$ was then added at a detergent/lipid weight ratio of 2, followed by addition of 40 μ g of purified Ca-ATPase in a final volume of 200 μ L. Finally, the detergent was removed by incubation with Biobeads SM2 (25 mg of beads/mg of detergent) for 3 h at room temperature. The resultant Ca-ATPase/PLB/lipid vesicles were separated from the Biobeads, and immediately assayed for ATP hydrolysis activity.

ATPase Activity Measurements. Ca-ATPase activity was measured by an enzyme-linked assay performed in microtiter plates (200 μ L total volume in each well) as described previously (13). Each well contained 0.2–0.6 μ g of Ca-ATPase (1–3 μ L of vesicles, optimized for sensitivity at each particular Ca concentration), 50 mM Imidazole, pH 7.0, 0.1 M KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM phosphoenol pyruvate, 2.5 mM ATP, 0.2 mM NADH, 2 IU of pyruvate kinase, 2 IU of lactate dehydrogenase, and 1–2 μ g of calcium ionophore (A23187). Each assay was done in triplicate at each of 12 different free calcium concentrations. Free calcium ion concentrations (designated Ca in this paper) were calculated according to the method of Fabiato and

Fabiato (44). The decay of NADH absorbance was monitored at 340 nm to determine the rates of ATP hydrolysis. The activity assays were carried out in the presence of calcium ionophore A23187 such that no transported Ca is retained in the vesicles, thus allowing the ATPase to operate freely, in the absence of generated transmembrane Ca gradients. With this assay method, ATP hydrolysis by the Ca-pump is not limited by the finite Ca capacity of the reconstituted vesicles, and the permeability of the lipid vesicles does not act as a confounding variable. The assays were performed at 25 °C in a SPECTRAMax PLUS microplate spectrophotometer (Molecular Devices). We report the Ca-pump ATPase activity in international units (IU), which are defined as micromoles of substrate hydrolyzed (mg Ca-pump)⁻¹ min⁻¹.

Phosphorylation of PLB. PLB was phosphorylated using the catalytic subunit of PKA (from bovine brain, Sigma). The phosphorylation reaction mix contained 25 mM MOPS, pH 7.0, 5 mM MgCl₂, 0.5 mM EGTA, 0.92% OG, 1 mg/mL PLB, 1000 IU/mL protein kinase A catalytic subunit, and 1 mM ATP. The reaction mix was incubated at 30 °C for 10 h. The efficiency of phosphorylation using this method was tested in a sample reaction by incorporating radioactive phosphate using γ -³²P-ATP. Quantitation of radioactivity in PLB bands cut from SDS-PAGE yielded phosphorylation levels of $\geq 90\%$.

Data Analysis. The pK_{Ca} and V_{max} values were calculated by fitting the ATPase data (V vs pCa) to the Hill equation

$$V = \frac{V_{max}}{1 + 10^{-n(pK_{Ca} - pCa)}} \quad (1)$$

where V is the initial rate of ATP hydrolysis and n is the Hill coefficient. Data below are either plotted as V vs pCa or as V/V_{max} vs pCa , where V_{max} was obtained from the fit to the Hill equation.

RESULTS

Effects of PLB and Its Mutants on Ca-ATPase Activity as a Function of Lipid/Ca-ATPase. The functional effects of recombinant WT-PLB and three PLB mutants were studied by measuring the Ca-dependence of ATPase activity (Figure 1). The mutants tested initially were N27A-PLB (pentameric, gain-of-function) and I40A-PLB (monomeric, gain-of-function). Purified PLB and Ca-ATPase were reconstituted at a molar ratio of 10, and with varying lipid/Ca-ATPase molar ratios. The lipid-to-Ca-ATPase weight ratios were 5, 10, 20, 30, and 40, corresponding to lipid/Ca-ATPase molar ratios of 700 ("low"), 1400, 2800, 4200, and 5600 ("high"). For simplicity, only the data for low and high lipid/Ca-ATPase are shown in Figure 1, but the effects on K_{Ca} (the Ca concentration required for 50% activity) and V_{max} (the maximal activity, observed at saturating Ca) are summarized in Figure 2 for all lipid/Ca-ATPase values. WT-PLB, N27A-PLB, and I40A-PLB increased both K_{Ca} (Figure 2A) and V_{max} (Figure 2B). The K_{Ca} shifts caused by N27A- and I40A-PLB were significantly greater than that caused by WT-PLB, consistent with the previously reported gain-of-function properties of N27A-PLB and I40A-PLB in heterologous coexpression systems (22, 34, 24) or in SR isolated from transgenic animals (45).

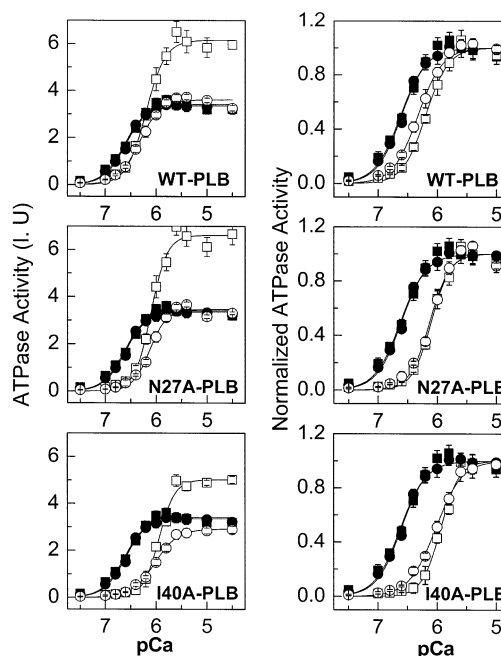


FIGURE 1: ATP hydrolysis rates as a function of ionized calcium concentration. The Ca-ATPase was reconstituted in the absence (■ ●) and in the presence (□ ○) of WT-PLB, N27A-PLB, and I40A-PLB at lipid/Ca-ATPase molar ratios of 700 (■ □) and 5600 (● ○). The raw data (left) was normalized to the V_{max} values calculated from the fits to eq 1 (curves) and plotted as normalized ATPase activity vs pCa (right). Each data point represents mean \pm SEM ($n \geq 3$).

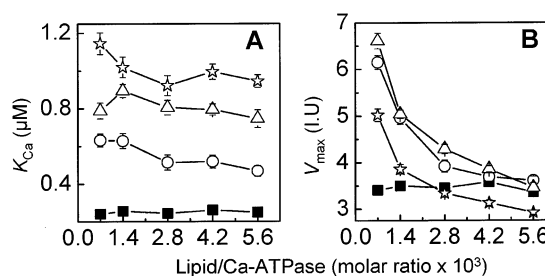


FIGURE 2: Dependence of ATPase parameters on the molar ratio of lipid to Ca-ATPase. The pump was reconstituted in the absence (■), and in the presence of WT-PLB (○), N27A-PLB (△), and I40A-PLB (☆), at a ratio of 10 PLB molecules per Ca-ATPase. The K_{Ca} and V_{max} values were calculated by fitting the data from Figure 1 to the Hill equation (eq 1). Each data point is mean \pm SEM ($n \geq 3$).

For WT-PLB, N27A-PLB, and I40A-PLB, we observed two effects that correlate with the increase of lipid/Ca-ATPase: a slight decrease in the K_{Ca} effect (Figure 2A), and a large decrease in the V_{max} effect (Figure 2B). The V_{max} for N27A-PLB and WT-PLB (both pentameric) were essentially the same, while I40A-PLB (monomeric) induced a smaller increase in V_{max} at low lipid/Ca-ATPase and actually inhibited V_{max} slightly at high lipid/Ca-ATPase (Figure 2B).

Controls That Rule Out Reconstitution Artifacts. The results reported here for the highest lipid/Ca-ATPase ratio are consistent with previously reported effects of PLB co-reconstituted with the Ca-ATPase using a similar lipid/Ca-ATPase, where no V_{max} effects were observed (10, 11). However, since it is widely accepted that PLB increases K_{Ca} of the Ca-ATPase without affecting V_{max} (4), our observation of substantial V_{max} enhancement by PLB and its gain-of-function mutants, at the lower lipid/protein levels, requires

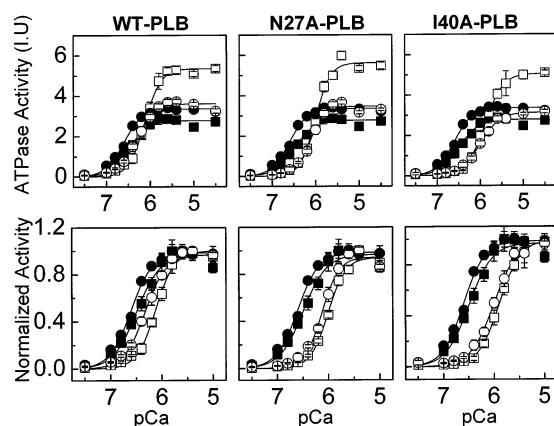


FIGURE 3: Effect of HPLC-purified PLBs on the activity of Ca-ATPase. The pump was reconstituted in the absence (■ ●) and presence (□ ○) of the given PLB, at low (■ □) and high (● ○) lipid/Ca-ATPase ratios, at a PLB/Ca-ATPase molar ratio of 10. Curves are best fits to the Hill equation (eq 1). Each data point is mean \pm SEM ($n = 3$).

extra scrutiny to ensure that this finding is not a consequence of uncontrolled factors in the reconstitution procedure. First, note that variation in lipid/Ca-ATPase had no significant effect on the Ca-ATPase in the absence of PLB, as measured by either K_{Ca} (Figure 2A) or V_{max} (Figure 2B). Second, we prepared mutant L31A-PLB, which has been reported to be a loss-of-function mutant, i.e., it had no effect on K_{Ca} in a coexpression system (40). When L31A-PLB was reconstituted and assayed under the conditions of Figure 1, this mutant had no significant effect on either K_{Ca} or V_{max} , indicating that the effects caused by the other PLB variants are not the result of systematic errors induced by the reconstitution procedure.

Because PLB samples contained OG (0.92%), and $C_{12}E_8$ was added during reconstitution (at a detergent/lipid weight ratio of 2), samples having different final lipid/protein ratios were exposed to different concentrations of the two detergents during the reconstitution procedure. This was a potential concern because previous studies have shown that the precise ratio of detergent to lipid used can be critical in the final activity of reconstituted Ca-ATPase (46). We took several steps to ensure that this was not the source of functional differences. First, in the standard reconstitution procedure described above, all samples contained the same amount of OG, regardless of the amount of PLB added. Second, we carried out reconstitutions using PLB stock solutions in $C_{12}E_8$, and using either $C_{12}E_8$ or OG to solubilize the lipid. We observed no detergent-dependent effect on the final Ca-ATPase activities, particularly on V_{max} . Third, we performed reconstitutions with PLB samples that had been purified by HPLC to replace the detergent with organic solvent (trifluoroethanol, TFE) and obtained results (Figure 3) that were essentially the same as those reported in Figure 1. Therefore, we conclude that the V_{max} increase is caused by PLB, not by variations in detergent mixtures during reconstitution.

Conceivably, Ca-ATPase regulation by PLB could be sensitive to the orientation of the pump in the reconstituted lipid vesicles, and it is plausible that this can be the cause of the systematic PLB-dependent variation in V_{max} . Therefore, we examined the orientation of the Ca-ATPase by tryptic digestion assay (46). The extent of Ca-ATPase trypsinolysis

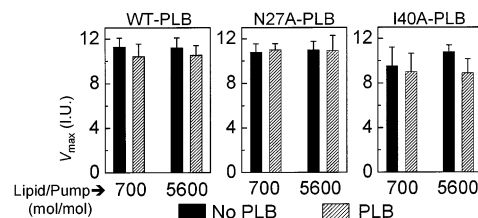


FIGURE 4: ATPase activity at maximally activating calcium concentrations. The V_{max} of Ca-ATPase reconstituted in the absence (filled bars) and presence (hatched bars) of the indicated PLB derivative, at low and high lipid/Ca-ATPase ratios, measured in the presence of 0.2% nonionic detergent $C_{12}E_8$. Each bar represents mean \pm SEM ($n \geq 3$).

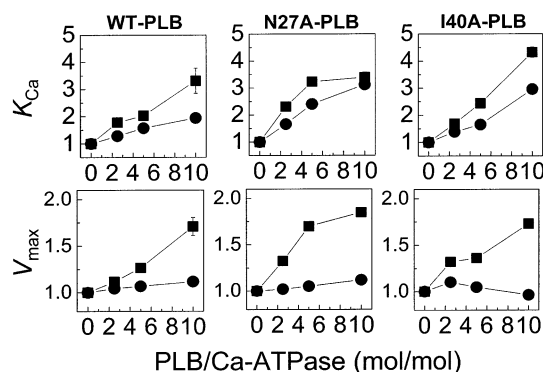


FIGURE 5: Effects of varying PLB/Ca-ATPase molar ratio on K_{Ca} and V_{max} . Ca-ATPase was co-reconstituted with PLB at low (700, ■) and high (5600, ●) lipid/Ca-ATPase. V_{max} and K_{Ca} were obtained by fitting data of the type represented in Figure 1 to the Hill equation (eq 1). The K_{Ca} and V_{max} values represented are normalized to the control (no PLB) values. Each data point is mean \pm SEM ($n \geq 3$).

was similar at low and high lipid/Ca-ATPase ratios, whether reconstituted in the absence (control) or presence of PLB. This indicates a similar Ca-ATPase orientation in vesicles reconstituted under different lipid/Ca-ATPase and PLB/Ca-ATPase conditions.

It is possible that, at low lipid/Ca-ATPase ratios, PLB has a protecting effect on the Ca-ATPase function (stability) by preventing irreversible inactivation during the reconstitution. This would result in the type of V_{max} effects reported in Figures 1, 2, and 3. To test this hypothesis, we measured the Ca-ATPase activity at saturating Ca^{2+} concentrations (pCa 4.5) in the presence of $C_{12}E_8$, which is a nonionic detergent that has been shown to uncouple the functional effects of PLB on the Ca-ATPase (47). It is well-known that solubilizing amounts of $C_{12}E_8$ cause an approximately 3-fold activation of the Ca-ATPase (48), an effect that was reproduced with our SR samples. The results in Figure 4 show that $C_{12}E_8$ treatment restores all reconstituted samples to the same final activity (similar to the activity of purified Ca-ATPase at the same $C_{12}E_8$ concentration), regardless of the lipid/Ca-ATPase ratio or PLB species present. We conclude that the PLB-induced V_{max} enhancement (Figures 1, 2, and 3) is not due to protection against irreversible denaturation.

Variation of PLB/Ca-ATPase. Figure 5 shows the dependence of K_{Ca} and V_{max} on PLB/Ca-ATPase. For all three functional PLB variants, K_{Ca} increases with PLB/Ca-ATPase at both low and high lipid/Ca-ATPase (Figure 5 top), while V_{max} increases only at low lipid/Ca-ATPase (Figure 5 bottom). A similar increase in the K_{Ca} shift as a function of increasing PLB/Ca-ATPase at high (5600) lipid/Ca-ATPase

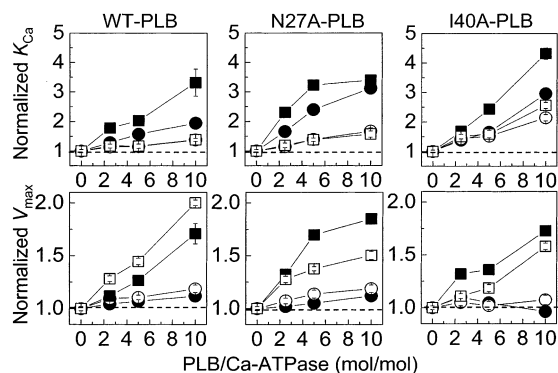


FIGURE 6: Effects of PLB phosphorylation on V_{\max} and K_{Ca} . Effect of phosphorylated PLB (\square , \circ) in comparison with unphosphorylated PLB (\blacksquare , \bullet) on K_{Ca} (top panels) and V_{\max} (bottom panels), as a function of varying PLB/Ca-ATPase molar ratios reconstituted at low (\blacksquare , \square) and high (\bullet , \circ) lipid/Ca-ATPase. The K_{Ca} and V_{\max} values at each PLB/Ca-ATPase ratio were normalized to the corresponding control (no PLB) values. The data for unphosphorylated PLB was normalized from Figure 5. The broken line represents the control (basal level in the absence of PLB). The K_{Ca} values for the no-PLB control ranged from 0.210 to 0.270 μ M and 0.300 to 0.370 μ M for unphosphorylated and phosphorylated PLBs, respectively. The V_{\max} values for the no-PLB control ranged from 2.50 to 3.50 IU and 3.30 to 4.00 IU for unphosphorylated and phosphorylated PLBs, respectively. Each data point represents mean \pm SEM ($n \geq 3$).

has been reported previously (11). In the case of N27A-PLB, both K_{Ca} and V_{\max} effects appear to saturate with increasing PLB/Ca-ATPase at low lipid/Ca-ATPase, but there is no suggestion of saturation for WT-PLB or I40A-PLB.

Effects of PLB Phosphorylation. WT-, N27A-, and I40A-PLB were phosphorylated using the catalytic subunit of PKA. Phosphorylated PLB was then co-reconstituted with the Ca-ATPase. Alternatively, PLB phosphorylation was carried out after reconstitution of unphosphorylated PLB and the Ca-ATPase. The latter method of PLB phosphorylation was less effective in reversing the inhibitory effects compared to the former, presumably because phosphorylation was less complete. Therefore, the effects of the former method are shown in Figure 6, normalized to control (no PLB) values to facilitate comparison.

Upon phosphorylation, the K_{Ca} effects were almost completely reversed in the case of WT-PLB (Figure 6, top left panel) and N27A-PLB (Figure 6, top middle panel) at all PLB/Ca-ATPase and lipid/Ca-ATPase ratios tested. However, only about half of the K_{Ca} effects were reversed upon phosphorylation of I40A-PLB (Figure 6, top right panel). Effects of phosphorylation on V_{\max} were substantially less than the effects on K_{Ca} , and were only observed at low lipid/protein levels. WT-PLB showed an increase in V_{\max} , while both gain-of-function mutants showed a decrease in V_{\max} (Figure 6).

DISCUSSION

To better characterize the molecular mechanism of Ca-pump regulation by PLB, we have measured the functional effects of wild type and mutant PLBs in an in vitro reconstitution system of controlled composition, which was varied systematically. ATP hydrolysis rates were measured as a function of free Ca concentration, at varying lipid/Ca-ATPase and PLB/Ca-ATPase ratios, and using both unphosphorylated and phosphorylated PLB. N27A- and I40A-PLB

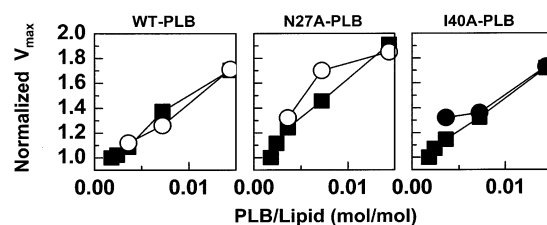


FIGURE 7: Normalized V_{\max} values of Ca-ATPase as a function of PLB/lipid ratio, at varying lipid/Ca-ATPase molar ratios (\blacksquare) and PLB/Ca-ATPase molar ratios (\circ). The V_{\max} values of Ca-ATPase at varying molar ratios of PLB/Ca-ATPase were normalized to the V_{\max} values obtained in the absence of PLB, and the V_{\max} values of Ca-ATPase at varying molar ratios of lipid/Ca-ATPase were normalized to the V_{\max} values obtained at the high lipid/Ca-ATPase.

decreased the apparent Ca-affinity (increased K_{Ca}) more potently than WT-PLB (Figures 1 and 2). These results are consistent with the previously reported increase in inhibitory potency caused by the N27A and I40A mutations (22, 40). Also consistent with previous reports using cardiac SR (4), we found a phosphorylation-dependent increase in Ca-affinity (decrease in K_{Ca}) when the pump was co-reconstituted with WT-PLB (Figure 6). These results validate the use of this reconstitution system, which offers a distinct advantage over other systems (cardiac SR or heterologous coexpression systems) in that it allows precise control over the sample composition, especially over PLB/Ca-ATPase and lipid/protein ratios. Therefore, co-reconstitution allows a more rigorous and quantitative study of the equilibria underlying the PLB–Ca-ATPase functional interactions.

Effect of PLB on V_{\max} . At the highest lipid/protein levels studied (5600 lipids/Ca-ATPase), PLB has no effect on V_{\max} . This is consistent with our previous reconstitution studies, which were done at a very high lipid/protein level and showed effects of PLB only on K_{Ca} (10, 11, 49). However, as the lipid/Ca-ATPase molar ratio is decreased, there is a substantial increase in V_{\max} due to PLB (Figure 2). When lipid/Ca-ATPase has decreased to 700, which is still more than the value of 200–300 in cardiac SR (50, 51), WT-PLB has doubled V_{\max} , a large increase in V_{\max} was induced by N27A-PLB, and a moderate increase in V_{\max} was induced by I40A-PLB (Figure 2). The loss-of-function mutant L31A-PLB does not affect V_{\max} , further validating the observed V_{\max} effects for the PLB variants that affect K_{Ca} . V_{\max} also increases with the PLB/Ca-ATPase ratio (Figure 5). Therefore, since a decrease in lipid/Ca-ATPase (at constant PLB/Ca-ATPase) and an increase in PLB/Ca-ATPase (at constant lipid/Ca-ATPase) both increase the concentration of PLB in the plane of the membrane (PLB/lipid), we replotted the combined results of Figures 2 and 5 in a single graph in which the ordinate is the concentration of PLB in the membrane, expressed as the molar ratio of PLB/lipid (Figure 7). The resulting plot shows that the V_{\max} increase correlates consistently with increasing PLB concentration in the membrane.

To discuss the potential relevance of this observation to physiology, it is important to determine whether the concentrations of PLB in Figure 7 are comparable to that of cardiac SR. Although a wide range of values have been reported for the molar ratio of PLB to Ca-ATPase, a value of four has been reported in the most rigorous estimate to date, in which purified PLB and Ca-pump were used as standards in quantitative immunoblots (37). Since cardiac

SR contains approximately 200–300 phospholipids per Ca-ATPase (50, 52), the concentration of PLB in the cardiac SR membrane is approximately 0.016 PLB/lipid (mol/mol). Thus, the right edge of each plot in Figure 7 corresponds to a PLB concentration in the membrane that is comparable to that of cardiac SR. Therefore, Figure 7 suggests that under physiological conditions in the heart, V_{\max} of the Ca-ATPase should be quite sensitive to small changes in the PLB concentration.

Relationship to Previous Results on Cardiac SR. Most studies of Ca-ATPase regulation in cardiac SR have used either PLB phosphorylation or antibodies to reverse the effects of PLB on the Ca-ATPase, and have supported the conclusion that PLB regulates the Ca-ATPase in cardiac SR mainly by increasing K_{Ca} , with little or no effect on V_{\max} (4). However, inspection of the literature shows that the effect of PLB on V_{\max} has received very little direct study, and that the most thorough studies of this question do support the hypothesis that PLB can affect V_{\max} in cardiac SR (28, 38, 39, 53). Phosphorylation by PKA or trypsin treatment of cardiac SR has been reported to increase V_{\max} and decrease K_{Ca} of the Ca-ATPase (38), suggesting that PLB inhibits the ATPase activity of cardiac SR by increasing K_{Ca} and decreasing V_{\max} of the Ca-ATPase, and that phosphorylation of PLB relieves inhibition of both K_{Ca} and V_{\max} . However, the reported PKA-dependent increase in V_{\max} was small and occurred only when sucrose gradient purification of SR was carried out prior to phosphorylation (53), and it is unclear whether the trypsin effect was specific for PLB. It has been suggested that direct phosphorylation of the Ca-ATPase in cardiac SR is associated with increased V_{\max} (54), but further investigation suggested that this was an artifact arising from the incubation conditions during the phosphorylation reaction (55).

Thus, previous evidence of PLB affecting the V_{\max} of the Ca-ATPase has been inconclusive, due to the complexity of the membranes in which it has been studied. Isolating the direct effect of PLB on V_{\max} in cardiac SR has remained elusive, because any attempt to remove or dissociate PLB from SR to measure the activity of the unregulated Ca-pump (i.e., in the absence of PLB) is complicated, and treatments (trypsin, detergent) to dissociate PLB from the Ca-ATPase have been known to also alter Ca-pump function (47). In the present study, we have addressed this problem directly, using a well-defined reconstitution method, and we have shown that PLB increases V_{\max} of the Ca-ATPase in reconstituted membranes. We carried out a series of control studies to ensure that the observed effects are not due to uncontrolled aspects of the reconstitution process (Figures 3 and 4). We found that V_{\max} is clearly increased by PLB and its mutants, when the concentration of PLB in the membrane is comparable to that in cardiac SR. We also found that PLB phosphorylation by PKA actually increases V_{\max} slightly for WT-PLB (Figure 6 *left*). When a similar increase in V_{\max} was reported previously in cardiac SR upon PLB phosphorylation, it was suggested that phosphorylation activates by relieving PLB-induced inhibition of the Ca-ATPase (38). However, the present results suggest that, instead, phosphorylation increases V_{\max} by enhancing the PLB-induced activation of the Ca-ATPase.

Previous Results with Synthetic Peptides. Previously, using synthetic peptides corresponding to the isolated cytoplasmic

domain (residues 1–31) or transmembrane domain (residues 28–47), it was reported that the cytoplasmic domain decreased V_{\max} and the transmembrane domain increased K_{Ca} of the purified cardiac SR Ca-ATPase (28). However, these effects required extremely high (nonphysiological) molar ratios of peptide/Ca-ATPase (60–120 for the cytoplasmic domain peptide, over 400 for the transmembrane domain peptide). Similarly, a synthetic PLB mutant, in which all the cysteines were replaced with alanine, was reported to decrease V_{\max} and increase K_{Ca} , but only at a PLB/Ca-ATPase ratio of 100 (30). In contrast, the PLB/Ca-ATPase ratios explored in the present study (2.5–10) bracket the physiologically relevant ratio of 4, recently reported for cardiac SR (37).

Previous Coexpression Results. In several previous studies, the functional effects of PLB were studied by coexpressing it with Ca-ATPase in Sf21 or HEK-293 cells (21, 22, 25, 40, 56). In most of these cases, the protein levels were not quantitated, so only K_{Ca} effects could be measured (21, 22, 25, 40, 56). In one coexpression study, the protein levels were quantitated on immunoblots, and no effect of PLB on V_{\max} was observed (57). However, the molar ratio of PLB to pump in that study was 1.5, which is substantially below typical levels of about 4 in cardiac SR (37), and below the PLB/Ca-ATPase level where V_{\max} effects are clearly observed in the present study (Figure 5). The V_{\max} effects also depend on the lipid/protein ratio (Figures 6 and 7), which has not been quantitated in coexpression systems.

Effects of PLB Mutation. The results from mutant PLB provide further insight into the relationship of PLB structure and function. The increased potency of two well-studied gain-of-function mutants (N27A and I40A) and the lack of potency of a previously characterized loss-of-function mutant (L31A) agree with previous findings in coexpression systems, validating the reconstitution system. The two gain-of-function mutants are more potent than WT-PLB in their effects on K_{Ca} , but not in their V_{\max} effects, where their potency is comparable to WT-PLB (Figures 2 and 5).

Although it has been clearly shown that Ca-ATPase inhibition can be reversed by phosphorylation of WT-PLB (4), the reversal of inhibition caused by PLB mutants has been studied only using anti-PLB antibody (11, 21, 24). Here, we show that phosphorylation of PLB reverses at least part of the K_{Ca} effects for all mutants under all conditions (Figure 6, top row). However, the extent of K_{Ca} reversal by phosphorylation varied among the PLBs tested: the reversal of K_{Ca} was almost complete in the case of WT- and N27A-PLB, but only partial in the case of I40A-PLB. This difference may be related to the lower oligomeric stability of I40A-PLB (58). Evidence has been presented suggesting that the monomeric form of PLB is more inhibitory than the oligomer (17, 22) and that the reversal of inhibition by phosphorylation is due, at least in part, to increased stability of the PLB oligomer (17). Thus, it is possible that phosphorylation of I40A-PLB causes incomplete reversal of inhibition because it causes incomplete stabilization of the oligomer.

The effect of mutation on the phosphorylation dependence of V_{\max} is also intriguing. First, the effect of phosphorylation on V_{\max} (compare open and closed squares in the bottom row of Figure 6) is much less dramatic than its effect on K_{Ca} (compare open and closed squares in the top row of

Figure 6). Second, these data suggest that phosphorylation *increases* V_{\max} of the Ca-ATPase in the presence of WT-PLB, and *decreases* it in the presence of both gain-of-function mutants (Figure 6). This phosphorylation-induced increase in V_{\max} for WT-PLB is consistent with the previously reported V_{\max} increase in cardiac SR upon phosphorylation by PKA (53). Our conclusion that phosphorylation enhances the V_{\max} effects of WT-PLB implies that phosphorylation does not completely dissociate PLB from the Ca-ATPase, which further supports a previous report using spectroscopically labeled PLB to measure its interaction with the Ca-pump (59). The observation that phosphorylation of N27A- and I40A-PLB decreased V_{\max} suggests that these mutants interact with the Ca-ATPase in a manner that is different from that of WT-PLB. Similarly, the observation that the pattern of mutational effects is qualitatively different for K_{Ca} (N27A-PLB similar to WT-PLB) and for V_{\max} (N27A-PLB similar to I40A-PLB) suggests that these two kinetic parameters depend on different structural features.

Relevance to Pathophysiology. The present study indicates that Ca-ATPase function in the heart should be sensitive to the relative levels of PLB, Ca-ATPase, and lipid in cardiac SR. Indeed, Figure 7 suggests that the membrane concentration of PLB (PLB per lipid) is of fundamental importance. Studies of transgenic mice support the proposal that the PLB/Ca-ATPase ratio is physiologically important in the heart (60). This may be particularly true under pathological conditions when levels of PLB and Ca-ATPase are substantially altered. For example, changes in thyroid function have opposite effects on the levels of PLB and Ca-ATPase (61), while in idiopathic dilated cardiomyopathy PLB/Ca-ATPase is increased 3-fold (36). The present study indicates that these changes could have substantial effects on both V_{\max} and K_{Ca} of the Ca-ATPase in the heart.

Sarcolipin (SLN), which is highly homologous to PLB and is the only Ca-ATPase regulator to have its high-resolution structure determined in lipid environments (62), has been shown to regulate the skeletal SR Ca-ATPase by increasing both K_{Ca} and V_{\max} , as determined in both coexpression experiments (63) and co-reconstitution experiments (63, 64). Chronic low-frequency stimulation of rabbit fast-twitch muscle has been shown to reduce the level of SLN while also reducing V_{\max} of the Ca-ATPase (63, 64). In the present study, a decrease in the effective concentration of PLB in the membrane also correlates with a decrease in V_{\max} of the Ca-ATPase. Thus, it is possible that the PLB/Ca-ATPase ratio is regulated under certain pathophysiological conditions to modulate V_{\max} of the Ca-ATPase as a compensatory mechanism.

Conclusions. Using a chemically defined reconstitution system, we have determined the dependence of Ca-ATPase activity on the intramembrane concentrations of PLB and Ca-ATPase for WT-PLB and several functional mutants, with and without PLB phosphorylation. By controlling these protein concentrations, we were able to determine quantitatively not only the decrease in apparent Ca sensitivity caused by PLB, but also the increase in V_{\max} of the Ca-ATPase. The dependence of V_{\max} on the PLB membrane concentration suggests that the interaction between PLB and pump is in dynamic equilibrium in the membrane, and provides new insight into the potential importance of PLB content as a pathophysiological variable in the heart. The observed

differences among the inhibitory patterns of PLB mutants shed new light on the complex relationship between PLB structure and regulatory function. The reconstitution system described here will be a valuable tool in further chemically controlled studies of the interaction between PLB and Ca-pumps, where the physical and functional aspects of this interaction can be measured on the same sample.

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