Synthetic Phosphopeptides Enable Quantitation of the Content and Function of the Four Phosphorylation States of Phospholamban in Cardiac Muscle*

Background: Phosphorylation of phospholamban regulates cardiac calcium transport, but the content and function of the four phosphorylation states of phospholamban are unknown.

Results: Synthetic phosphopeptides solved both problems.

Conclusion: The phosphorylation states were quantified in normal and hypertrophic pig hearts, and each has a distinct effect on calcium transport.

Significance: This information is needed for improved diagnosis and treatment of heart failure.

We have studied the differential effects of phospholamban (PLB) phosphorylation states on the activity of the sarcoplasmic reticulum Ca-ATPase (SERCA). It has been shown that unphosphorylated PLB (U-PLB) inhibits SERCA and that phosphorylation of PLB at Ser-16 or Thr-17 relieves this inhibition in cardiac sarco-lasmic reticulum. However, the levels of the four phosphorylation states of PLB (U-PLB, P16-PLB, P17-PLB, and doubly phosphorylated 2P-PLB) have not been measured quantitatively in cardiac tissue, and their functional effects on SERCA have not been determined directly. We have solved both problems through the chemical synthesis of all four PLB species. We first used the synthetic PLB as standards for a quantitative immunoblot assay, to determine the concentrations of all four PLB phosphorylation states in pig cardiac tissue, with and without left ventricular hypertrophy (LVH) induced by aortic banding. In both LVH and sham hearts, all phosphorylation states were significantly populated, but LVH hearts showed a significant decrease in U-PLB, with a corresponding increase in the ratio of total phosphorylated PLB to U-PLB. To determine directly the functional effects of each PLB species, we co-reconstituted each of the synthetic peptides in phospholipid membranes with SERCA and measured calcium-dependent ATPase activity. SERCA inhibition was maximally relieved by P16-PLB (the most highly populated PLB state in cardiac tissue homogenates), followed by 2P-PLB, then P17-PLB. These results show that each PLB phosphorylation state uniquely alters Ca$^{2+}$ homeostasis, with important implications for cardiac health, disease, and therapy.

To develop improved therapies for heart failure, the major health problem for aging Americans, it is essential to develop analytical procedures to assess cardiac physiology quantitatively. During muscle relaxation, the sarcoplasmic reticulum calcium ATPase (SERCA) actively transports Ca$^{2+}$ into the sarcoplasmic reticulum. The resulting electrochemical gradient supplies most of the driving force for passive Ca$^{2+}$ efflux into the cytosol during contraction (1). In cardiac sarcoplasmic reticulum, SERCA forms a complex with its 52-amino acid peptide inhibitor, phospholamban (PLB) (1–4). It has been shown that an increase in the ratio of unphosphorylated phospholamban (U-PLB) to SERCA (by overexpression of PLB, decreased PLB phosphorylation, or decreased SERCA expression) decreases the apparent Ca$^{2+}$ affinity of SERCA (i.e. increases $K_{Ca}$, the free Ca$^{2+}$ concentration corresponding to half-maximal SERCA activation) and contributes to contractile dysfunction (5, 6). Phosphorylation at Ser-16 and/or Thr-17 partially reverses this inhibition by decreasing $K_{Ca}$ (5, 7–10). Spectroscopic studies show that PLB phosphorylation induces structural rearrangement within the PLB-SERCA complex without dissociating PLB, which is essentially a subunit of SERCA under physiological conditions (11–14). Thus, PLB phosphorylation is a molecular switch that regulates SERCA activity. However, this is a complex switch because there are four distinct PLB phosphorylation states: U-PLB (no phosphorylation, dephosphorylated by protein phosphatase-1) (15–17), P16-PLB (phosphorylated by PKA (18)), P17-PLB (phosphorylated by calmodulin-dependent kinase II, CaMkII), and 2P-PLB (phosphorylated by both kinases). Their regulation of SERCA depends on two factors: (a) the concentration of each state and (b) the potency of each state as a SERCA-bound inhibitor.

It has been proposed that the concentration of each PLB phosphorylation state differs with the etiology of heart disease. Qualitative Western blots have suggested that P16-PLB and P17-PLB concentrations decrease in dilated cardiomyopathy...
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(19) and that P17-PLB concentration increases in ischemia (20) and acidosis (21). 2P-PLB has not been measured in either healthy or failing hearts. Conflicting reports suggest that left ventricular hypertrophy (LVH) decreases, increases, or has no effect on PLB phosphorylation (22). Moreover, these trends can vary with age (23) and gender (24). A complete phosphorylation profile for each age group, gender, and etiology of heart failure could aid diagnosis and guide rational design of disease-specific drug or gene therapies (25–27), targeted toward specific PLB phosphorylation states. Such a phosphorylation profile could also evaluate protein phosphatase-1, CaMKII, and PKA as targets for small molecules (28, 29). Assessing the role of PLB phosphorylation states in cardiac pathology and treatment requires quantification of their concentrations and functions. These goals require purified standards for each of the four species and antibodies that are at least partially specific for each of them (30). Antibodies to 2P-PLB have become available only recently, and none of the PLB antibodies that are selective for other phosphorylation states are completely specific (30). Thus, the concentrations of the four species have not been determined previously.

Previously, protein phosphatase-1, PKA, and CaMKII have been used in efforts to phosphorylate PLB selectively at Ser-16 or Pro-17, but complete and exclusive phosphorylation at one site has not been achieved, so the only clear conclusion was that both P16-PLB and P17-PLB decrease SERCA inhibition, compared with U-PLB (8, 31–37). The effect of double phosphorylation (2P-PLB) is controversial. In one study, addition of a second phosphate increased $pK_{Ca}$ (38), whereas in two other studies, it had no effect (8, 39). Thus, the inhibitory potencies of the four PLB phosphorylation states are yet to be determined.

In the present study, we have used solid-phase peptide synthesis to produce pure samples of each of the four PLB phosphorylation states, allowing the accurate quantification of both the concentration and potency of each. To measure concentrations, the four synthetic peptides were used as standards to quantify antibody selectivity, enabling the accurate determination of the mole fractions of all four species by immunoblot, despite the imperfect specificities of the four antibodies. To demonstrate that the method can report site-specific changes in phosphorylation, we assayed pig hearts with and without LVH induced by aortic banding. To measure the potency of each PLB phosphorylation state, $pK_{Ca}$ was measured after each of the four synthetic peptides was co-reconstituted with SERCA, guaranteeing complete, exclusive, and site-specific phosphorylation, as well as identical PLB/SERCA stoichiometry.

**EXPERIMENTAL PROCEDURES**

**Synthesis of the Four Phosphorylation States of PLB**—The sequences of human U-PLB, P16-PLB, P17-PLB, and 2P-PLB were prepared using Fmoc solid-phase peptide synthesis with or without incorporation of phosphorylated amino acids at specific sites (13, 40, 41). Phosphorylation was accomplished by incorporation of Fmoc-Ser(PO(OBzl)OH)-OH for P16-PLB and Fmoc-Thr(PO(OBzl)OH)-OH for P17-PLB during peptide synthesis. 2P-PLB was prepared by incorporation of both phosphorylated amino acids at position Ser-16 and Thr-17 (13, 41).

Phospholamban was characterized by amino acid analysis and mass spectrometry (41).

SERCA1a was purified from rabbit skeletal muscle using reactive red in 0.1% octaethylene glycol monododecyl ether (C12E8) (42, 43), and the protein concentration was measured with the Pierce BCA assay (13, 41). SERCA1a, the fast-twitch skeletal muscle isofrom, substitutes quantitatively for SERCA2a, the cardiac isofrom, with respect to regulation by PLB (44).

**Production of LVH**—All experiments were performed in accordance with the animal use guidelines of the University of Minnesota, and the experimental protocol was approved by the University of Minnesota Research Animal Resources Committee. The investigation conformed to the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH publication no. 85–23, revised 1985). Procedures for producing and analyzing the porcine model of LVH secondary to aortic banding have been described previously (45, 46). Briefly, Yorkshire pigs at 45 days of age were anesthetized with inhaled isoflurane (2% v/v), intubated, and ventilated with a respirator. A left thoracotomy was performed in the third intercostal space, and the ascending aorta, ~1.5 cm above the aortic valve, was mobilized and encircled with a polylethylene band 2.5 mm in width. Whereas left ventricle (LV) and distal aortic pressures were simultaneously measured, the band was tightened until a 40-mm Hg peak systolic pressure gradient was achieved across the narrowing. The chest was then closed in layers, the pneumothorax was evacuated, and the animals were allowed to recover (45). Left ventricular hypertrophy occurred progressively as the area of aortic constriction remained fixed in the face of normal body growth. Two months after banding, animals were returned to the laboratory for study. Five size-matched normal pigs were studied as controls.

**Myocardial Tissue Sample Preparation**—After the terminal physiological study (~8 weeks after aortic banding), each heart was cross-sectioned into five short-axis rings. Odd-numbered rings were used for histological studies, which revealed clear evidence for LVH, and even-numbered rings were frozen for molecular biological analysis. For analysis, every LV short axis ring was divided into 12 across the LV wall according to the coronary anatomy. Tissues were embedded in Tissue-Tek OCT compound (Fisher Scientific) and frozen in liquid nitrogen-cooled isopentane (45, 46). Frozen tissue sections were stored at −80 °C. Frozen tissue was homogenized in 10 mM NaHCO3, 10 mM Tris-HCl (pH to 7.2 with KOH), 0.8 M benzamidine, 1 mg/liter aprotinin, 1 mg/liter leupeptin, 1 mM PMSE, and 1 mg/liter pepstatin A, using the Navy bead kit from Midwest Scientific. The homogenate was centrifuged at 4 °C for 5 min at speed 12 with a bullet blender (Midwest Scientific). The pellet was discarded, and the supernatant was frozen at −80 °C. Protein concentration was determined by the Pierce BCA assay.

**Electrophoresis and Immunoblot**—Samples were dissolved in Laemmli buffer (Bio-Rad) with 5% β-mercaptoethanol, loaded onto a 10–20% Tris-Tricine gel (Bio-Rad), and separated by SDS-PAGE at constant voltage (120 V) at 25 °C for 90 min. Proteins were transferred to 0.45-micron Immobilon-FL PVDF membranes (Millipore) in Towbin transfer buffer (47) for 50 min at constant current (300 mA), blocked overnight in pure Odyssey blocking buffer (LI-COR Biosciences), and rinsed for 1...
Quantification of Four Phosphorylation States of PLB—

Quantification of the four phosphorylation states of PLB in the coin-reconstituted system or in pig cardiac homogenates was accomplished by an extension of our previous method for quantification of two species (30), using the antibodies given in Table 2. Antibodies entirely specific for each of these four species are not available, so it is necessary to start with four antibodies having partial selectivity, determine this selectivity quantitatively using purified synthetic standards, run the unknown on the same four blots, and then solve a system of four equations with four unknowns to determine the composition of the unknown sample (Equation 1),

\[ l_i = \sum \epsilon_{ij} c_j i = 1, \ldots 4; j = 1, \ldots 4 \] (Eq. 1)

where \( l_i \) refers to the primary antibody, and \( j \) refers to the phosphorylation state of the standard, as defined in Table 2. Thus, \( \epsilon_{ij} \) is the slope of the standard curve from phosphorylation state \( j \) (obtained using purified synthetic standards) from a blot using primary antibody \( i \), and \( c_j \) is the concentration of the phosphorylation state \( j \) of PLB in the sample. The result is a 4 × 4 matrix of simultaneous equations, which was solved in MATLAB to determine the unknown concentration \( c_j \). The mole fraction of each PLB phosphorylation state \( (X_j) \) was calculated from Equation 2.

\[ X_j = \frac{c_j}{\sum c_i} (j = 1, \ldots 4) \] (Eq. 2)

To validate the method, it was applied to mixtures of synthetic standards containing known concentrations of each phosphorylation state (Table 1). The PLB primary antibodies were all based on epitopes that exclude residues 2 and 27 (the only differences between human and pig sequences), so the method is equally valid for analysis of both species. Secondary antibodies

Ab800CW (goat anti-mouse), Ab800CW (donkey anti-goat), and Ab680LT (goat anti-rabbit), were obtained from Li-COR Biosciences. Quantification of SERCA for co-reconstitutions was accomplished by SDS-page, stained with Coomassie Brilliant Blue, using purified SERCA1a.

**ATPase Assay**—Ca-ATPase activity was measured after co-reconstitution of SERCA with each of the four synthetic PLB standards (13, 41, 48–50). PLB and SERCA were co-reconstituted in dioleoyl-phosphatidylcholine and dioleoyl-phosphatidylethanolamine, at a molar ratio of 4:1 (51) and at a molar ratio of PLB/SERCA/lipid of 5/1/700 and 10/1/700. Both ratios yielded indistinguishable functional results, indicating that SERCA was saturated by PLB in each case. Ca-ATPase activity was measured as a function of \([Ca^{2+}]\) using an NADH-coupled, enzyme-linked ATPase assay (42, 52) at a temperature of 25 °C. Each data set was fitted by the Hill equation,

\[ V = \frac{V_{\text{max}}/(1 + 10^{-n_{H}p_{KCa} - p_{Ca}})}{n_H} \] (Eq. 3)

where \( V_{\text{max}} \) is the maximum ATPase rate (at saturating \([Ca^{2+}]\), \( p_{Ca} \) is the negative log of \([Ca^{2+}]\), \( p_{KCa} \) is the \( p_{Ca} \) value corresponding to half-maximal SERCA activation (where \( V = 0.5V_{\text{max}} \)), and \( n_H \) is the Hill coefficient. Inhibitory potency of each PLB species is defined as \( -\Delta p_{KCa} \), the decrease in \( p_{KCa} \) compared with that measured for SERCA in the absence of PLB.

**Statistical Analysis**—For validation of the method, the accuracies of \( X_{PLB} \) values, performed on known mixtures of standards, were calculated as \( \Delta X_{PLB} = X_{PLB} \) (apparent) - \( X_{PLB} \) (known). Precision is expressed as S.E./mean. \( X_{PLB} \) in pig homogenates and \( p_{KCa} \) in the co-reconstitutions were compared using one-way analysis of variance (ANOVA). The Bonferroni method was used for comparisons between individual pairs. A two sample t test was used to compare sham and LVH groups. A p value of <0.05 was considered significant. Pearson’s r value of ≥0.95 was required for all slopes in Fig. 1.

**RESULTS**

**Validation of Immunoblot Method**—The assay was applied to five mixtures of synthetic standards containing known concentrations of each of the four PLB phosphorylation states (Table 1). Two identical gels, with the same concentrations of each pure synthetic PLB standard and equal volumes of the same five mixtures (Table 1) were run and blotted with four different antibodies (two antibodies on each gel). The mixtures were run in duplicate. The use of fluorescent secondary antibodies made it possible to visualize two blots on the same membrane (Fig. 1A), provided that the primary antibodies were produced in different animals. For the top blot in Fig. 1A, mouse primary antibody (AbU) was paired with rabbit primary antibody (Ab17). For the bottom blot in Fig. 1A, goat primary antibody

| TABLE 1 |
| Known \( X_j \) values (Eq. 2) in mixtures of PLB standards |
| Mixture | \( U \) | \( P16 \) | \( P17 \) | 2P | \( n \) |
|--------|------|------|------|---|---|
| a      | 0.25 | 0.25 | 0.25 | 0.25 | 6  |
| b      | 0.10 | 0.20 | 0.30 | 0.40 | 6  |
| c      | 0.20 | 0.30 | 0.40 | 0.10 | 6  |
| d      | 0.30 | 0.40 | 0.10 | 0.20 | 6  |
| e      | 0.40 | 0.10 | 0.20 | 0.30 | 6  |
(Ab16) was paired with rabbit primary antibody (Ab2P). Visualizing two different epitopes on one membrane required that the secondary antibodies emitted at resolved wavelengths. LI-COR-800CW is conjugated to a dye detected at 800 nm, using a green pseudo-color for display. In contrast, LI-COR680LT is conjugated to a dye detected at 680 nm, using a red pseudo-color for display. In Fig. 1A (top), the green signal corresponds to goat anti-mouse LI-COR-800CW secondary antibody bound to primary mouse antibody AbU, and the red signal corresponds to goat anti-rabbit LI-COR-680LT secondary antibody bound to primary rabbit antibody Ab17. In Fig. 1A (bottom), the green signal corresponds to donkey anti-goat LI-COR-800CW secondary antibody bound to primary goat antibody Ab16, and the red signal corresponds to goat anti-rabbit LI-COR-680LT secondary antibody bound to primary rabbit antibody Ab2P. Fig. 1B shows the red and green signals resolved in separate images. The same accuracy and precision were obtained when four blots, as in Fig. 1B, each stained with one antibody (1, 2, 3, or 4), were performed and analyzed (data not shown).

Fig. 1C shows standard curves obtained from the 2.5, 5.0, and 11-ng PLB standards in Fig. 1B. In C, “I_i” indicates the primary antibody that was used to stain the corresponding blot in B (Table 2), and the slopes of these standard curves are the \( \epsilon_{ij} \) values in Equation 1. The error \( \Delta X_{\text{PLB}} \pm \text{S.E.} \), obtained from known mixtures (Table 1), plotted against the actual \( X_{\text{PLB}} \) for all four PLB phosphorylation states. E, ratio of apparent T-PLB to actual, ± S.E.
was calculated for each mixture from \( n = 5 \) immunoblot experiments. This was also quite accurate (close to 1), ranging from 0.88 to 1.09.

**Application to a Model of LVH**—Left ventricular tissue was harvested from five LVH pigs and five sham controls, as described under “Experimental Procedures.” Left ventricular hypertrophy was quite evident, based on an increase of left ventricular mass by 65\% (S.E.). There was no significant effect of LVH on left ventricular ejection fraction, indicating that the hypertrophy was compensatory and that end-stage heart failure was not present.

Concentrations of the four PLB phosphorylation states were determined from immunoblots obtained from tissue homogenates (Fig. 2A), using the method described in Fig. 1. As described in Fig. 1, the accuracy and precision of the assay was verified by including known mixtures of synthetic standards in each immunoblot (left side of Fig. 2A). Two controls containing known values of \( X \) (\( X = 0.25 \) for each phosphorylation state as in mixture A in Table 1) gave apparent \( X \) values of \( X_U = 0.31 \pm 0.01 \), \( X_{P16} = 0.24 \pm 0.01 \), \( X_{P17} = 0.20 \pm 0.01 \), and \( X_{2P} = 0.25 \pm 0.01 \). The apparent/actual total PLB value of the control was 1.05 \pm 0.03.

Mole fractions of the four PLB species, determined from the right half of Fig. 2A, are plotted in Fig. 2B. In sham (control) hearts (open bars in Fig. 2B), the greatest mole fraction was observed for P16-PLB (\( X_{P16} = 0.47 \pm 0.006 \)), followed by U-PLB (\( X_U = 0.30 \pm 0.014 \)), doubly phosphorylated 2P-PLB (\( X_{2P} = 0.16 \pm 0.007 \)), and P17-PLB (\( X_{P17} = 0.07 \pm 0.007 \)). ANOVA showed significant differences among all the mole fractions of all four PLB phosphorylation states, and the Bonferroni test for multiple comparisons showed significant differences between all pairs (\( p < 0.05 \)). In summary, all four states are significantly populated, and the order of mole fractions is P16-PLB \( \approx \) U-PLB \( \approx \) 2P-PLB \( \approx \) P17-PLB. The mole fraction of TP-PLB, the combined mole fraction of all phosphorylated PLB (P16-PLB \( \approx \) P17-PLB \( \approx \) 2P-PLB) was 0.70 \pm 0.005.

A similar pattern was seen in LVH hearts (closed bars in Fig. 2B). Again, ANOVA showed significant differences among all
the mole fractions of all four PLB phosphorylation states, and the Bonferroni test showed significant differences between all pairs (p < 0.05). All four states were significantly populated, and the order of mole fractions is P16-PLB > U-PLB > 2P-PLB > P17-PLB. The mole fraction TP-PLB was 0.74 + 0.008.

LVH caused a significant decrease in the mole fraction of U-PLB (Fig. 2B, p = 0.005), and a corresponding increase in both P16-PLB and TP-PLB, when normalized to the U-PLB mole fraction (Fig. 2C, p < 0.05). LVH did not cause a significant change in total PLB for the two groups but did cause a significant increase in the variability (S.E.) of this measure (Fig. 2D).

Effects of Specific Phosphorylation States of PLB on SERCA Activity—Synthetic human U-PLB, P16-PLB, P17-PLB, and 2P-PLB were evaluated for their potency to inhibit SERCA (Fig. 3). By fitting the data with Equation 3, the inhibition of Ca-ATPase activity was quantified by the decrease in pKCa (the pCa value corresponding to 50% activation) in Fig. 3A. Each PLB phosphorylation state displayed a significantly different value of pKCa (Fig. 3B), according to both ANOVA and the Bonferroni comparisons, showing that they are functionally distinct. Inhibitory potency, expressed as −ΔpKCa, the decrease in pKCa compared with SERCA alone, is plotted in Fig. 3C. As expected, U-PLB was the most inhibitory species. P16-PLB was the least inhibitory, followed by 2P-PLB and P17-PLB (Fig. 3C). Thus, phosphorylation at Ser-16 provides the greatest reversal of SERCA-PLB inhibition, followed by phosphorylation at both Ser-16 and Thr-17, followed by phosphorylation at Thr-17 alone.

DISCUSSION

This study demonstrates the potential of synthetic peptide chemistry for solving important problems in cardiac pathophysiology. The availability of accurate synthetic standards of all four phosphorylation states of PLB, we were able to provide new accuracy and precision in determining (a) the concentrations of these proteins in normal and diseased cardiac tissue and (b) their functional properties.

Quantification of PLB Phosphorylation States in Cardiac Tissue Homogenates—We have extended our previous quantitative immunoblot assays (30), which measured mole fractions of two PLB phosphorylation states, to measure mole fractions of all four PLB phosphorylation states (Fig. 1). Our assay allows accurate quantification even though AbU, Ab16, and Ab17 are not completely specific (Fig. 1C). The use of the synthetic peptides and two-color immunoblots provides accurate concentrations of all four species in known mixtures (Fig. 1D). We used this assay to determine the unknown concentrations of these phosphorylation states of PLB in tissue homogenates from sham and LVH hearts, showing that all four states are present in both groups (Fig. 2B), with the concentration of P16-PLB nearly twice that of U-PLB, four times that of 2P-PLB, and six times that of P17-PLB. 2P-PLB is significantly populated even though it requires phosphorylation at adjacent sites. Including 2P-PLB increases the mole fraction of Thr-17 phosphorylated PLB (2P-PLB + P17-PLB) from 0.07 to 0.23, which suggests that CamKII has substantial activity in the resting heart, even in the absence of β-adrenergic stimulation. LVH causes a small but significant decrease in U-PLB (Fig. 2B), a corresponding increase in the ratios of P16-PLB and total P-PLB to U-PLB (Fig. 2C), and an increase in the variability of total PLB (Fig. 2D).

The effect of cardiac hypertrophy on PLB phosphorylation is controversial. Some authors have reported a decrease in phosphorylation at Ser-16 (53, 54), Thr-17 (55, 56), or both (57), whereas other reports show unaltered PLB phosphorylation (56) or an increase in total PLB phosphorylation (58) (59). Some of this variability may be due to different models of cardiac hypertrophy being used, but the present method offers unprecedented accuracy and precision, so its future application to diverse forms of heart disease should provide invaluable insight into cardiac pathology. For example, the present study suggests that measuring a ratio of mole fractions (e.g. X16/X11) provides the most reliable biomarkers for LVH (Fig. 2C). The observed increase in PLB phosphorylation with LVH may be due to the need to compensate for the decreased in SERCA activity that occurs in heart failure (60). Indeed, the hypertrophic pig hearts used in this study did not exhibit significant loss of cardiac function; future studies on more advanced hypertrophy, including end-stage heart failure, will be informative.

In the future, this assay can be used to further investigate correlations between the mole fractions of PLB phosphoryla-
tion states and cardiac physiology and pathology, as affected by CaMKII, PKA, and protein phosphatase-1. This assay requires a small sample such that precious cardiac tissues from biopsies or donors can be assayed. Biopsy is routinely performed for diagnosis of the presence and/or etiology of cardiomyopathy (61–63). It has a <1% complication rate (62). When this assay is applied to biopsy tissue, the effects of age, gender, the etiology of heart failure, and the stage of heart failure on PLB phosphorylation can be determined quantitatively.

**Direct Measurement of Inhibitory Potencies of All Four PLB Phosphorylation States**—We used four phosphorylation state-specific co-reconstitutions of PLB with SERCA to determine the inhibitory potencies of PLB phosphorylation states (Fig. 3). Our *in vitro* system has facilitated the simultaneous control of PLB/SERCA stoichiometry and the purity of each PLB phosphorylation state, which is not feasible in cardiac tissue samples (8). Although our assay utilizes SERCA1a from fast-twitch skeletal muscle, our measured potencies apply as well to the heart, where SERCA2a predominates, as these two isoforms have been shown to have identical regulatory interactions with PLB (44, 64).

Each PLB phosphorylation state has a distinct effect on SERCA activity (Fig. 3 and Table 3). These differences are not due to differences in affinity for SERCA, as doubling the PLB/SERCA ratio had no effect on SERCA activity. This is consistent with previous studies showing that PLB, phosphorylated PLB, and phosphomimetic PLB mutants saturate SERCA, both physically and functionally, at protein/lipid concentrations comparable with those in this study, which are lower than physiological concentrations (11–14, 27). Thus, PLB is effectively a subunit of SERCA, and phosphorylation relieves SERCA inhibition by changing the structure of the SERCA-PLB complex (11–14, 27).

U-PLB decreased $pK_{Ca}$ by 0.39, corresponding to an increase of $K_{Ca}$ by a factor of 2.4. P16-PLB was the least inhibitory PLB species, decreasing $pK_{Ca}$ by only 0.14, corresponding to an increase in $K_{Ca}$ by a factor of 1.4. Thus phosphorylation at P16 has the greatest effect in relieving SERCA inhibition. Intermediate results were observed for P17-PLB ($\Delta pK_{Ca} = 0.33$) and for 2P-PLB ($\Delta pK_{Ca} = 0.22$) (Fig. 3 and Table 3), showing that the effects of phosphorylation at the two sites are not additive because double phosphorylation produces an effect intermediate between the two singly phosphorylated states of Ser-16 phosphorylation when both Ser-16 and Thr-17 are phosphorylated on the same PLB (Fig. 3 and Table 3).

Determination of the contribution of any given phosphorylation state to the activation of SERCA requires knowledge of both its mole fraction and its potency ($pK_{Ca}$ shift). We now know that P16-PLB makes the largest contribution, both in terms of its concentration and $pK_{Ca}$ shift, followed by 2P-PLB, which has the next largest concentration and $pK_{Ca}$ shift, and finally P17-PLB, which has the smallest concentration and the smallest $pK_{Ca}$ shift from the reference point of the $pK_{Ca}$ of SERCA bound to U-PLB (Fig. 3C). Thus, increases in P16-PLB provide coarse adjustments to SERCA inhibition, whereas 2P-PLB and P17-PLB finely tune the PLB/SERCA regulatome.

**Implications for Cardiac Physiology and Pathology**—SERCA activation is an established therapeutic goal in treatment of heart failure (65). Therefore, the high concentration of P16-PLB in cardiac tissue homogenates (Fig. 2) and its low inhibitory potency (Fig. 3 and Table 3) establishes it (not P17-PLB or 2P-PLB) as the best template for loss-of-inhibition PLB mutants in gene therapy (25, 27, 66, 67). Similarly, these results indicate that activation of PKA should be therapeutically more effective than activation of CaMKII. In fact, elevation of CaMKII activity could reduce the effects of PKA activation because both P17-PLB and 2P-PLB are more inhibitory than P16-PLB (Fig. 3 and Table 3). This may explain why P17-PLB elevation at the onset of ischemia fails to improve SERCA activity and cardiac function (21). This small effect of Thr-17 phosphorylation on SERCA activity could be therapeutically useful in acidosis and ischemia, where elevated CaMKII leads to arrhythmia, necrosis and apoptosis (21), so treatment with small-molecule CaMKII inhibitors (68, 69) can occur without appreciable decreases in SERCA activity.

**Conclusions**—We have used solid-phase peptide synthesis to produce pure samples of each of the four PLB phosphorylation states, to permit the accurate quantification of both the concentration and potency of each PLB phosphorylation state. These four states are all present in pig cardiac tissue in different concentrations, with small but significant perturbations caused by induced cardiac hypertrophy, and each has a distinct inhibitory effect on SERCA activity. This assay is applicable to extremely small tissue samples. Thus, it is now feasible to determine these concentrations in human tissue samples and to relate them quantitatively to cardiac physiology and pathology, providing essential information for future therapeutic developments. These tools are particularly important for the fight against heart failure, the leading health problem for aging Americans.

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**TABLE 3**

| Sample | $K_{Ca}^{\mu M}$ | $pK_{Ca}$ | $-\Delta pK_{Ca}$ | $n$ |
|--------|-----------------|----------|-------------------|-----|
| No PLB | 0.32 ± 0.01 | 6.50 ± 0.01 |         | 4   |
| U-PLB  | 0.78 ± 0.02 | 6.11 ± 0.01 | 0.39 ± 0.02 | 4   |
| P16-PLB| 0.44 ± 0.03 | 6.36 ± 0.03 | 0.14 ± 0.03 | 4   |
| P17-PLB| 0.67 ± 0.03 | 6.17 ± 0.02 | 0.33 ± 0.03 | 4   |
| 2P-PLB | 0.53 ± 0.05 | 6.28 ± 0.04 | 0.22 ± 0.04 | 4   |
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