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Accurate quantitation of phospholamban phosphorylation by immunoblot

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Abstract

We have developed a quantitative immunoblot method to measure the mole fraction of phospholamban (PLB) phosphorylated at Ser16 (X_p) in biological samples. In cardiomyocytes, PLB phosphorylation activates the sarcoplasmic reticulum calcium ATPase (SERCA), which reduces cytoplasmic Ca^{++} to relax the heart during diastole. Unphosphorylated PLB (uPLB) inhibits SERCA at low $[Ca^{++}]$ and phosphorylated PLB (pPLB) is less inhibitory, so myocardial physiology and pathology depend critically on X_p . Current methods of X_p determination by immunoblot provide moderate precision but poor accuracy. We have solved this problem using purified uPLB and pPLB standards, produced by solid-phase peptide synthesis. In each assay, a pair of blots is performed with identical standards and unknowns, using antibodies partially selective for uPLB and pPLB, respectively. When performed on mixtures of uPLB and pPLB, the assay measures both total PLB (tPLB) and X_p with accuracy of 96% or better. We assayed pig cardiac SR and found that X_p varied widely among four animals, from 0.08 to 0.38, but there was remarkably little variation in the ratios of X_p /tPLB and uPLB/SERCA, suggesting that PLB phosphorylation is tuned to maintain homeostasis in SERCA regulation.

Keywords

Phospholamban; phosphorylation; SERCA; heart failure; western blot; X_p Phospholamban phosphorylation and heart disease

Heart disease is the number one cause of mortality in the United States across all demographics, and the incidence is rising [1]. In cardiac sarcoplasmic reticulum (CSR), phospholamban (PLB) phosphorylation acts as a molecular switch that activates SERCA (SR Ca-ATPase). Active SERCA increases the calcium gradient across the SR during diastole [2], accelerating relaxation and leading to a more forceful systolic contraction [3]. In the presence of unphosphorylated PLB, SERCA is inhibited at submicromolar Ca^{2+} , but beta adrenergic phosphorylation of PLB relieves this inhibition [4–7]. Because SERCA activity is reduced in many instances of heart failure [8–10], SERCA activation is a widely pursued goal for development of new therapies [11]. SERCA inhibition can be relieved by PLB phosphorylation, either at S16 by β -adrenergic activation of PKA [5, 12–14] or at T17

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by Ca-dependent CaMII-Kinase [5], but S16 phosphorylation is more important physiologically [15–18]. In heart failure, phosphorylation of PLB may be reduced [9, 19], thus it is desirable to increase the fraction of PLB that is phosphorylated (X_p) so that SERCA remains in its active state. PLB phosphorylation at S16 has been examined [2], mimicked [20–23], and altered [10, 24], with the goal of developing therapies for heart failure, but the rational development and testing of these therapies is limited because PLB phosphorylation has not been measured accurately in biological systems. In the present study, we have solved this problem.

Quantitation of X_p in healthy versus failing myocardium is needed to establish correlations between PLB phosphorylation and cardiac function. These correlations will aid in development of prognostic indicators for differential diagnosis and in treatment of heart failure. For example, it has been shown that the R9C mutation in PLB leads to dilated cardiomyopathy [25]. This mutation hinders binding to PKA, thus decreasing phosphorylation [26], but this effect has not been defined in a quantitative measurement of X_p , so its role in the disease mechanism remains unclear. Thus X_p measured from biopsied heart tissue (from animal models or patients) would provide crucial insight into the etiology and stages of heart failure, and into the efficacies of therapeutic interventions.

There is one method that has been shown to measure X_p accurately – quantitation of radioactivity after reaction of PLB with γ - ^{32}P -ATP in the presence of PKA [27]. However, this method requires an accurate measurement of total PLB (tPLB), and it is limited in accuracy by the unknown basal phosphorylation state of PLB in most tissue samples. Thus it has usually been used accurately only to analyze simple systems containing purified proteins [28]. It seems clear that a method involving quantitative immunoblots is the method of choice.

Existing methods lack accuracy

However, the properties of PLB have prevented accurate measurement of endogenous X_p by previous immunoblot/densitometry techniques. Phosphorylation of PLB causes only a very slight shift in SDS-PAGE, and this shift is complicated by the multiple oligomeric forms of PLB that are observed [16, 27–29]. The ideal solution would be a pair of antibodies specific for pPLB and uPLB, respectively. Fortunately, there is a commercially available antibody, designated Ab285, that is almost completely specific for pPLB [30]. Unfortunately, an antibody completely specific for uPLB has not been reported. Instead, commercial antibodies that bind uPLB cross-react with pPLB to varying degrees [16, 30–32]. For example, Ab1d11 [16, 30, 33], AbA1 [34] [35], Ab8A3 [31] [36], and Ab2D12 [32] all bind both uPLB and pPLB, with different degrees of selectivity [32].

As we show below, antibodies that are completely specific for uPLB and pPLB are not strictly required for an accurate assay of X_p . The most important requirement is a source of reliable standards, of known purity and concentration, for both uPLB and pPLB, so that the selectivity of antibodies can be quantitated on immunoblot. Primarily as a result of this lack of reliable standards, previous X_p measurements have not been accurate.

The present study

We describe here a quantitative immunoblot method, involving the use of extremely pure synthetic standards for pPLB and uPLB, that measures both X_p and tPLB with high accuracy and precision. In our approach, western blots with identical standards and samples were performed, one labeled with Ab285 (almost completely specific for pPLB) and the other with an antibody that is partially selective for uPLB. Following quantitative densitometry of the two immunoblots, a system of simultaneous equations is used to solve

accurately for X_p . Using mixtures of standard samples of uPLB and pPLB, the method is shown to be accurate (less than 5% error), and the method is then used to determine X_p in samples from pig cardiac sarcoplasmic reticulum.

Materials and methods

PLB standards

Solid-phase peptide synthesis and HPLC purification were used to prepare porcine uPLB and pPLB standards with > 95% purity, as previously reported [37–40]. PLB phosphorylation was accomplished by incorporation of p-Ser at position S16 as Fmoc-Ser(PO(OBzl)OH)-OH (EMD Chemicals) during peptide synthesis. Characterization was accomplished by mass spectrometry (MALDI-TOF) and Edman protein sequencing. [37–40]. PLB concentrations were measured with the BCA assay (Pierce) [2, 38] and by amino acid analysis [38].

Antibodies

Primary antibodies included Ab8A3 [31] [36] (gift from Diana Bigelow, University of Kansas), Ab2D12 [32] (Abcam, Cambridge, MA), AbA1 [34] [35] (Millipore, Billerica, MA), and Ab285 [41] (Merck, Westpoint, PA). The first three were monoclonal mouse antibodies. Ab285 is a polyclonal rabbit antibody raised against a cytoplasmic fragment of pSer16-PLB. Fluorescent dye-conjugated secondary antibodies, used to visualize and quantitate immunoblots, were goat anti-mouse IR-800CW and goat anti-rabbit IR-680LT (LICOR Biosciences).

Electrophoresis and Western Blot

PLB was dissolved in Laemmli buffer (Biorad) with 5% β -mercaptoethanol, separated by SDS-PAGE on 10–20% Tris-Tricine gels (Biorad) at constant voltage (120V) for 90 minutes at 25 °C, and transferred to 0.45 micron Immobilon-FL PVDF membranes (Millipore) in Towbin transfer buffer [42] for 50 minutes at constant current (300mA), blocked overnight in pure Odyssey blocking buffer (LI-COR Biosciences), and rinsed for 1 minute in ddH₂O. Membranes were then incubated in primary antibody for 1 or 2 hours (at concentrations recommended by the manufacturer) in blocking buffer consisting of 50% Odyssey blocking buffer and 50% TBS containing 0.1% Tween 20 (TBST). The primary antibody was washed from each blot 3 times for 15 minutes with TBST, before adding secondary antibody at a dilution of 1:15,000 for 35 minutes. The membrane was then washed 3 times for 15 minutes with TBST, and the blots were stored in TBS. Proteins were detected and analyzed using LI-COR Odyssey, using the 700nm channel for the IR-680LT and the 800nm channel for the 800CW (LI-COR Biosciences). Analysis by densitometry was accomplished with Odyssey software. The resolution was 169 μ m and the focus offset was 0.0 mm. Boxes were drawn around an area that encompassed all PLB bands (monomeric and oligomeric). The background was local, determined as the average or median of the intensities at the top and the bottom of the box. The greatest linearity was obtained at a border width of 1 pixel.

SERCA quantitation

10 mg of pig CSR was dissolved in 1mL of Laemmli sample buffer (Biorad) containing 4% β -mercaptoethanol, loaded on a 4–20% Tris-HCl gel and electrophoresed at 120V for 90 minutes at 25 °C. The gel was stained overnight with a solution containing 0.1% coomassie brilliant blue, 40% methanol, 40% H₂O, and 16% acetic acid, then destained for 1 hour in a solution containing 20% methanol, 5% isopropanol, 10% glacial acetic acid and 65% H₂O. The mass % of SERCA was calculated by densitometry with USCANIT software.

Analysis of pig cardiac SR

Pig cardiac SR was prepared from the ventricle of a fresh pig heart, placed on ice within 10 seconds of sacrifice, obtained from Lindenfelser's Meats, Inc (Monticello, MN). One ventricle was dissected and homogenized in a blender with 250 mL of homogenization buffer containing 10 mM NaHCO₃, 10 mM Tris-HCl (pH to 7.2 with KOH), 0.8 M Benzamidine, 0.5 g/L Aprotinin, 0.5 g/L Leupeptin, 1 M PMSF and 1 g/L pepstatin. The homogenate was centrifuged at 4° C for 20 minutes at 11,000 × g (8,500 rpm in a JA-14). The pellets were discarded and the supernatant was filtered with cheese cloth. The volume of the supernatant was measured, and enough KCl was added to make a 0.6 M KCl solution, which was incubated at 4° C with gentle stirring for 1 hour, then centrifuged at 4 °C, for 45 minutes at 100,000 × g (36,000 rpm in Beckman 45Ti rotor). The pellet was resuspended using a hand homogenizer, in 15 mL of sucrose buffer (10% sucrose, 20 mM MOPS and 1 mM NaN₃, pH to 7.0 with KOH, 25 g/L aprotinin, 25 g/L leupeptin, 50 g/L benzamidine, 0.1 M PMSF, and 0.1 g/mL pepstatin A) and centrifuged again for 45 minutes at 100 000 × g. The pellet was resuspended in 1 mL sucrose solution per 40 g of wet weight of tissue with a hand homogenizer. The homogenate was then aliquoted into microfuge tubes (~0.5 mL/tube) flash frozen; and stored at -80 °C. Electrophoresis and immunoblots were carried out as described above.

Derivation of the fraction of PLB phosphorylated

The measured intensity for one band in a western blot using antibody i is given by

$$I_i = \sum \epsilon_{ij} c_k \quad \text{Eq. 1}$$

where ϵ_{ij} is a proportionality constant (fluorescence units per ng PLB) that describes the slope of the standard curve for uPLB ($j = u$) or pPLB ($j = p$). The variable c_k is the concentration of uPLB ($k = u$) or pPLB ($k = p$) in the unknown sample. As shown below, calculating c_u and c_p requires that one antibody ($i = a$) preferentially binds uPLB, and the other antibody ($i = b$) preferentially binds pPLB, satisfying the condition $\epsilon_{au} > \epsilon_{ap}$ and $\epsilon_{bu} < \epsilon_{bp}$. Thus, for our system involving two species (uPLB and pPLB) and two antibodies (a partially selective for uPLB and b partially selective for pPLB), Eq. 1 expands to

$$\begin{aligned} I_a &= \epsilon_{au} c_u + \epsilon_{ap} c_p \\ I_b &= \epsilon_{bu} c_u + \epsilon_{bp} c_p \end{aligned} \quad \text{Eq. 2}$$

Solve for c_u and c_p :

$$\begin{aligned} c_u &= [I_b \epsilon_{ap} - I_a \epsilon_{bp}] / [\epsilon_{ap} \epsilon_{bu} - \epsilon_{au} \epsilon_{bp}] \\ c_p &= [I_a \epsilon_{bu} - I_b \epsilon_{au}] / [\epsilon_{ap} \epsilon_{bu} - \epsilon_{au} \epsilon_{bp}] \end{aligned} \quad \text{Eq. 3}$$

Calculate tPLB and X_p :

$$\begin{aligned} \text{tPLB} &= c_p + c_u \\ X_p &= c_p / \text{tPLB} \end{aligned} \quad \text{Eq. 4}$$

Statistical Analysis

Statistical analysis was performed using Origin 8.1. For validation of the method, the accuracies of X_p and tPLB values, performed on known mixtures of standards, were calculated as $X_p(\text{app})/X_p$ and $\text{tPLB}(\text{app})/\text{tPLB}$, so that 100% accuracy is given by a ratio of 1.00. Precision is expressed as the coefficient of variation (CV = standard deviation/mean).

For analysis of true unknowns (pig CSR), values are reported as mean $\pm$ SEM, and one-way ANOVA was used to compare mean values, with a P-value of < 0.01 considered significant. Exact values are reported for $P = 0.01$. Slope correlation coefficients (from linear regression) are expressed as Pearson's r .

Results

Densitometry for oligomeric forms of PLB

PLB has been shown to exist as a mixture of monomers and oligomers (mainly pentamers), in both native lipid bilayers and on SDS-gels [16, 28, 41, 43, 44], and oligomerization is enhanced by phosphorylation [28, 43]. Therefore, it is essential to determine the effect of the PLB oligomeric state on quantitation by western blot, since it is entirely possible that an antibody would bind less effectively to the five protomers in a PLB pentamer than to the isolated monomer. Fortunately, it has been shown that heating PLB samples to 100°C in SDS solution, just before electrophoresis, greatly decreases the extent of oligomerization observed on the gel [29]. Therefore, PLB standard curves were analyzed by western blot with and without this pre-heating procedure Fig. 1. Heating substantially decreased the extent of PLB oligomerization, but there was no significant effect on the observed immunoblot intensity from any of the antibodies (Fig. 1), demonstrating clearly that these immunoblot intensity values are independent of PLB's oligomeric state.

Validation of Methodology

Fig. 2 documents the accuracy and precision of our method. Each immunoblot (e.g., Fig. 2A) contains a complete set of synthetic standards (both uPLB and pPLB) and "unknown" mixtures. Fig. 2B shows how the proportionality constants (e_{ij} in Eq. 1) are determined from standard curves, quantifying each antibody's sensitivity to uPLB and pPLB. For antibody *a* (Ab8A3, Ab2D12, and AbA1), $e_{au} > e_{ap}$, confirming that antibody *a* prefers to bind uPLB over pPLB. Conversely, antibody *b* (Ab285) greatly prefers to bind pPLB ($e_{bu} < e_{bp}$) (Fig. 2B). Fig. 2C and Fig. 2D show that the values of X_p and tPLB, determined for the "unknown" mixtures from these blots using Eq. 4 are extremely accurate -- no more than 10% error in all cases. One-way ANOVA showed no significant variation among the mean values for X_p (app) or tPLB, for any of the antibody pairs (Table 1). This high degree of both accuracy and precision is particularly remarkable in light of the large variability observed for the relative sensitivities of antibodies in separate blots (Table 2). Thus a key requirement of our method is that standards and unknowns are all run on each individual blot *a* or *b*, with identical standards and unknowns run on both blots *a* and *b*.

Potential Sources of Systematic Error

Lack of uPLB and pPLB standards and assumptions made about antibodies create systematic errors in tPLB and X_p measurements. Without standards, accurate measurements cannot be made. When standards are used, Eq. 4 must be used to achieve accuracy, without simplifying assumptions. For example, if it is assumed that antibody *a* reacts equally with uPLB and pPLB ($e_{au} = e_{ap}$) in determination of tPLB, tPLB is underestimated in the presence of any pPLB, which leads to overestimation of X_p (Fig. 3, Method 1). If it is assumed that antibody *a* is completely specific for uPLB ($e_{ap} = 0$), tPLB is systematically overestimated and X_p is systematically underestimated (Fig. 3, Method 2). If it is assumed that antibody *b* (Ab285) is completely specific for pPLB ($e_{bu} = 0$), tPLB and X_p are both overestimated (Fig. 3, Method 3). In the current method (Fig. 3, Method 4), all e_{ij} values were quantitatively included in calculations (Eq. 4). Our method returns X_p and tPLB accurately, with all three choices of antibody *a*, while the systematic errors in Methods 1–3 depend on the choice of antibody *a* (Fig. 3), due to different values of K_{UP} (Table 2).

Application to pig cardiac sarcoplasmic reticulum (CSR)

X_p and tPLB were measured in cardiac sarcoplasmic reticulum prepared from 4 different pigs (Fig. 4). Standard curves were used to calculate proportionality constants ϵ_{ij} , and Eq. 4 was used to solve for X_p and tPLB. Mixtures of synthetic standards were used as controls (“ X_p control”), yielding accurate values for X_p and tPLB (data not shown), verifying the results of Fig. 2. X_p values were determined with high precision (SEM = 0.01 for all four pig samples), but there was considerable variation among the four pigs, ranging from 0.08 ± 0.01 to 0.38 ± 0.01 (Fig. 4, Table 3). Similarly, tPLB values were determined with high precision, but varied widely among the four pigs, from 3.32 ± 0.01 nmol/mg to 13.0 ± 1.1 nmol/mg (Table 3). Concordantly, ANOVA showed that X_p and tPLB both showed statistically significant variation among the four pigs (Table 3). However, a striking linear correlation between X_p and tPLB was observed (Pearson’s $r = 0.99$), such that CSR containing higher levels of tPLB also have a higher X_p (Fig. 4B, Table 3).

We quantitated the SERCA content in the pig CSR samples using coomassie stain (data not shown). We thus obtained the molar concentrations of SERCA, as well as those of tPLB, uPLB, and pPLB in the four CSR samples. Combining these measurements, we determined the molar ratios of tPLB/SERCA, uPLB/SERCA, and pPLB/SERCA (Table 3, Fig. 4C). For all four samples, the tPLB/SERCA ratio was consistently greater than 2 (as reported previously in rabbits and rats [36]), and uPLB/SERCA was consistently greater than pPLB/SERCA. Both tPLB/SERCA and pPLB/SERCA showed statistically significant variation among the pigs ($P < 0.01$), but it is remarkable that uPLB/SERCA was essentially constant, showing no significant variation among the four pigs ($P = 0.230$) (Table 3, Fig. 4C). The Ca-dependence of SERCA enzymatic activity was also found to be essentially invariant among the four pigs (Table 3).

Discussion

We have established an immunoblot method for the measurement of X_p (the fraction of PLB that is phosphorylated at S16) and tPLB (the total PLB level). This method is extremely accurate ($> 96\%$ for X_p , $> 90\%$ for tPLB, as shown in Fig. 2 and Table 1). The key to the accuracy of this method is the use of purified synthetic standards of uPLB and pPLB, along with antibodies that are partially selective for each, and rigorous calculations (Eq. 4). Without these features, substantial systematic errors result (Fig. 3).

When the method was applied to pig CSR obtained from different animals (Fig. 4), the X_p values were observed to be quite variable, ranging from 0.08 to 0.38 for four animals. Values of tPLB also varied widely, but there was a remarkably precise linear correlation between tPLB and X_p (Fig. 4B). We also measured SERCA content and used it to calculate PLB/SERCA ratios. While pPLB/SERCA and tPLB/SERCA varied widely, there were no significant variations in uPLB/SERCA among the four pigs (Fig. 4, Table 3). The regulation of SERCA by PLB, as indicated by Ca dependence of ATPase activity, was also invariant among the four pigs (Table 3, pK_{Ca}).

Features of our method

The key features of our method are calibration within each immunoblot and a rigorously accurate calculation of X_p and tPLB with Eq. 4 which combines two immunoblots. Calibration requires that both uPLB and pPLB standards of known concentration and phosphorylation state are available to generate standard curves and determine a unique set of proportionality constants for each blot. Due to variability of K_{UP} among immunoblots (Table), standards and samples must be run on the same blot. In order to use Eq. 4 to calculate X_p and tPLB reliably, the two blots (*a* and *b*), must have identical standards and

unknowns, and blots *a* and *b* must be incubated with two antibodies with opposite selectivity for uPLB and pPLB.

If any of these features are missing, differences in antibody selectivity and sensitivity of isolated experiments introduce systematic errors into X_p (app) and tPLB(app) calculations Fig. 3. Indeed, most measurements of PLB phosphorylation have lacked standards for uPLB and pPLB, so measurements were relative, inherently lacking accuracy [18, 45–50]. For example, a sample that has 4 times more phosphorylation than a control may represent an increase from 0.03 to 0.12 (probably having little physiological effect) [16] [51] or from 0.25 to 1.00 (probably having a substantial physiological effect) [50]. There is only one paper in the literature in which standards of both uPLB and pPLB were used, along with a pair of antibodies with differential sensitivity, but that study did not attempt quantitative calculations such as those in Eq. 4 [16]. Even with standards, Fig. 3 illustrates how assumptions about antibody selectivity can lead to systematic errors in determination of both tPLB and X_p .

This study focused on PLB phosphorylation at S16 because that site is more important physiologically than T17 [18], due mainly to the lower level of phosphorylation at T17 [16]. Phosphorylation of PLB at T17 must be potentiated by S16 phosphorylation ([52]), and T17 phosphorylation has negligible effect after S16 has been phosphorylated [27]. Nevertheless, this method has the capacity to measure PLB phosphorylation at T17, using an antibody that is selective for T17-pPLB (commercially available from Santa Cruz Biotechnology, Inc. CA, USA, or Badrilla Ltd, Leeds, UK.), and threonine-17-phosphorylated pPLB synthetic standard.

Application to biological membranes

We have used our method to make the first accurate measurements of X_p and tPLB in biological samples. We found that the fraction of phosphorylated phospholamban (X_p) in pig CSR is variable, ranging from 0.08 ± 0.01 to 0.38 ± 0.03 (Table 3). Note that even the greatest X_p value was less than 0.5, leaving considerable reserve for response to β -adrenergic stimulation or response to phosphomimetic therapies [53]. The total PLB level, tPLB, was also quite variable, ranging from $(3.32 \pm 0.05 \text{ nmol/mg total protein})$ to $(13.0 \pm 1.13 \text{ nmol/mg total protein})$ (Table 3). Despite the wide variation in X_p and tPLB among the pigs, the ratio of X_p to tPLB was essentially constant (Fig. 4B), suggesting that PLB phosphorylation increases in order to compensate for the inhibitory effects of high PLB expression. To further explore this hypothesis, we measured SERCA content in the same pig CSR samples, then calculated molar ratios tPLB/SERCA, uPLB/SERCA and pPLB/SERCA (Table 3). Differences in tPLB/SERCA, pPLB/SERCA and X_p were all statistically significant among the four pigs ($P < 0.01$), as was the difference in SERCA content ($P < 0.01$), but uPLB/SERCA did not vary significantly among the four pigs, over a wide range of X_p and SERCA measurements (Fig. 4C, Table 3). Similarly, the Ca-dependence of SERCA ATPase activity was essentially invariant (Table 3). These results suggest that myocytes in non-failing myocardium maintain SERCA activity by keeping uPLB/SERCA within a narrow range. Future studies are needed to test this hypothesis more rigorously. For example, freshly harvested tissue should be homogenized and analyzed quickly, using phosphatase inhibitors to ensure that the phosphorylation status of PLB is captured accurately [46].

Potential applications to research in physiology and pathology

Now that we can quantitate c_u , c_p , X_p , tPLB, and SERCA with accuracy and precision, many questions concerning the role of PLB in cardiac function and pathology can be addressed more quantitatively to evaluate their roles in cardiovascular physiology and pathology. This

method should standardize X_p and tPLB measurements across studies, even if the blots are performed in different laboratories, where the choice of primary antibodies and other experimental conditions may vary. The accuracy of our method should normalize X_p and tPLB values across animal systems, sample preparations, disease states, and therapies so that cross-study comparisons can be made. With this method in hand, it is possible to test the hypothesis that the uPLB/SERCA ratio increases in heart failure, resulting in decreased SERCA activity (increased pK_{Ca}) [27]. Thus significant increases in uPLB/SERCA ratio may be a useful biomarker for heart disease and/or response to therapy in animal models of heart failure. The variation in X_p and tPLB (Fig. 4) suggests that it is uPLB/SERCA that should be measured in these models, providing reference values for comparisons during diagnosis and over the course of treatment.

Potential clinical applications

Our method has potential for clinical applications to assess changes in X_p in human cardiac tissue, in response to current treatments such as β -adrenergic agonists, Ca^{++} channel blockers, phosphodiesterases, and future treatments in gene and drug therapy. Since milligram quantities of tissue are sufficient for replicate measurements of X_p , tPLB and uPLB/SERCA, it is plausible that human biopsies (1–2 mm³ of tissue) [54], which are performed routinely under suspicion of myocarditis, cardiomyopathy and amyloidosis, can also be assayed for these values. Myocardial biopsy is a non-surgical procedure with a complication rate of <1% to the patient [54]. Accurate tPLB calculations can also quantitatively determine changes in PLB expression, revealing factors that affect calcium regulation at the level of transcription or translation. Thus our method can play an important role in evaluating the success of gene or drug therapies for heart failure. More generally, our approach can provide a quantitative measure of any post-translational modification where (a) purified standards are available for both the unmodified and modified state of the protein and (b) antibodies are available with differential sensitivity to the posttranslational modification.

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Abbreviations used

tPLB	total phospholamban
uPLB	unphosphorylated phospholamban
pPLB	phosphorylated phospholamban
Ab	antibody
SR	sarcoplasmic reticulum

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Fig. 1. PLB oligomeric state does not affect immunoblot intensity. Typical immunoblots and standard curves are shown for samples pre-heated to 100° (red) and unheated (blue) for uPLB ("u" on blots, circles on graphs) or pPLB ("p" on blots, squares on graphs) are shown for Ab285, Ab8A3, Ab2D12, and AbA1. Corresponding western blots are above each graph.

Fig. 2.

Validation of the method used to determine X_p and tPLB. (A) Western blots of 5, 10, and 15 ng of uPLB or pPLB standards, and 12 ng of uPLB/pPLB mixtures (duplicates) of known X_p (0.00 to 1.00, as indicated). (B) Typical standard curves from a for uPLB (squares) and pPLB (circles), from blots incubated with the indicated primary antibody. Resulting slopes (proportionality constants ϵ_{ij} in Eq. 1 and Eq. 2) are indicated. (C) Accuracy and precision of X_p values: $X_p(\text{app})/X_p \pm \text{SEM}$, for mixtures of known X_p . Antibody *a* is Ab8A3 (square), Ab2D12 (circle), AbA1 (triangle). (D) Accuracy and precision of tPLB values: $t\text{PLB}(\text{app})/t\text{PLB} \pm \text{SEM}$, for the same mixtures as in C.

Fig. 3.

Comparison of methods for measuring X_p and tPLB for the same sample ($X_p = 0.5$). The line at 1.00 indicates the accurate value. The antibody a (partially selective for uPLB) was Ab8A3 (squares), Ab2D12 (circles), or AbA1 (triangles). Methods 1 through 4 and their assumptions are described in the text. Only Method 4 (ours) returns accurate values for both tPLB and X_p .

Fig. 4. Application to pig cardiac SR. (A) Western blots of standards (2.5, 7.5 and 15ng), control mixtures ($X_p = 0.25, 0.5$ and 0.75), and 0.5μ g of pig CSR from pigs 1–4 from left to right. (B) tPLB vs. X_p (C) PLB/SERCA ratios vs. X_p for uPLB, pPLB, and tPLB.

Table 1Accuracy of X_p and tPLB in known mixtures

	Ab8A3	Ab2D12	AbA1	ANOVA
X_p	0.98 ± 0.06	1.04 ± 0.04	1.05 ± 0.01	$P = 0.98$
tPLB	1.10 ± 0.03	1.07 ± 0.03	1.06 ± 0.05	$P = 0.07$

Note: Ab285 was used as the pPLB-selective antibody *b*. The uPLB-selective antibody *a* is given in the top row. Values in columns 2 through 4 indicate accuracy (apparent/actual) $\pm$ precision (coefficient of variation = SD/mean). $P > 0.01$ indicates no significant variation ($n = 8$ measurements for each value of X_p for a total of 40).

Table 2K_{UP}, antibody specificity

Ab	K _{up}		n (blots)
	mean	CV	
285	0.033	129%	5
8A3	7.47	47%	4
2D12	4.59	36%	4
A1	2.11	26%	4

Note: $K_{UP} = \epsilon_{iu}/\epsilon_{ip}$, where $i = a$ or b . CV = coefficient of variation = $SD/mean$). Primary antibody incubation times were varied from 1 to 2 h.

Table 3

Application to pig CSR

	Pig 1	Pig 2	Pig 3	Pig 4	Mean	ANOVA
X_p	0.08 ± 0.01	0.14 ± 0.01	0.23 ± 0.01	0.38 ± 0.01	0.21 ± 0.03	P < 0.01
tPLB (nmol/mg)	3.32 ± 0.01	5.79 ± 0.10	6.70 ± 0.20	13.0 ± 1.1	7.21 ± 1.04	P < 0.01
uPLB (nmol/mg)	3.07 ± 0.08	4.98 ± 0.08	5.18 ± 0.18	8.09 ± 0.69	5.33 ± 0.51	P < 0.01
pPLB (nmol/mg)	0.25 ± 0.1	4.92 ± 0.2	8.88 ± 0.2	30.3 ± 2.6	1.88 ± 0.52	P < 0.01
SERCA (nmol/mg)	1.48 ± 0.01	2.22 ± 0.01	2.03 ± 0.01	3.74 ± 0.04	2.37 ± 0.24	P < 0.01
pPLB / SERCA (mol/mol)	0.17 ± 0.01	0.36 ± 0.01	0.75 ± 0.02	1.32 ± 0.11	0.65 ± 0.13	P < 0.01
tPLB / SERCA (mol/mol)	2.25 ± 0.05	2.60 ± 0.04	3.31 ± 0.09	3.48 ± 0.14	2.91 ± 0.14	P < 0.01
uPLB / SERCA (mol/mol)	2.08 ± 0.05	2.24 ± 0.03	2.56 ± 0.09	2.16 ± 0.18	2.26 ± 0.05	P = 0.230
pK _{Ca}	5.90 ± 0.01	5.88 ± 0.04	5.87 ± 0.06	5.89 ± 0.01	5.88 ± 0.01	P = 0.97

Note: Values are mean ± SEM. P < 0.01 indicates significant variation among the four pigs. pK_{Ca} is the pCa value giving half-maximal Ca-ATPase activity. n = 5 measurements per pig for all values except pK_{Ca}, where n = 2.