Accurate quantitation of phospholamban phosphorylation by immunoblot

Naa-Adjeley Ablorh, Tyler Miller, Florentin Nitu, Simon J. Gruber, Christine Karim, David D. Thomas*

Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN 55455, USA

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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$^{2+}$ to relax the heart during diastole. Unphosphorylated PLB (uPLB) inhibits SERCA at low [Ca$^{2+}$] but 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 sarcoplasmic reticulum (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.

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techniques. Phosphorylation of PLB causes only a very slight shift in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (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 to use a pair of antibodies specific for phosphorylated PLB (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 immunoblots. Primarily as a result of this lack of reliable standards, previous $X_p$ measurements have not been accurate.

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 labeled 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 (<5% error), and the method is then used to determine $X_p$ in samples from pig CSR.

**Materials and methods**

**PLB standards**

Solid-phase peptide synthesis and high-performance liquid chromatography (HPLC) purification were used to prepare porcine uPLB and pPLB standards with greater than 95% purity, as reported previously [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 (matrix-assisted laser desorption/ionization time-of-flight, MALDI–TOF) and Edman protein sequencing. [37–40]. PLB concentrations were measured with the BCA (bis-chloroimino acid) assay (Pierce) [2,38] and by amino acid analysis [38].

**Antibodies**

Primary antibodies included Ab8A3 [31,36] (a gift from Diana Bigelow, University of Kansas), Ab2D12 [32] (Abcam), AbA1 [34,35] (Millipore), and Ab285 [41] (Merck). 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 (LI-COR Biosciences).

**Electrophoresis and Western blot**

PLB was dissolved in Laemmli buffer (Bio-Rad) with 5% β-mercaptoethanol, separated by SDS–PAGE on 10–20% Tris–tricine gels (Bio-Rad) at constant voltage (120 V) for 90 min at 25 °C, and transferred to 0.45-μm Immobilon-FL polyvinylidene difluoride (PVDF) membranes (Millipore) in Towbin transfer buffer [42] for 50 min at constant current (300 mA), blocked overnight in pure Odyssey blocking buffer (LI-COR Biosciences), and rinsed for 1 min in doubly distilled H2O (ddH2O). Membranes were then incubated in primary antibody for 1 or 2 h (at concentrations recommended by the manufacturer) in blocking buffer consisting of 50% Odyssey blocking buffer and 50% Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST). The primary antibody was washed from each blot three times for 15 min with TBST before adding secondary antibody at a dilution of 1:15,000 for 35 min. The membrane was then washed three times for 15 min with TBST, and the blots were stored in TBS. Proteins were detected and analyzed using LI-COR Odyssey with the 700-nm channel for the IR-680LT and the 800-nm channel for the 800CW (LI-COR Biosciences). Analysis by densitometry was accomplished with Odyssey software. The resolution was 169 μm, 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, better (10%) than the average or median of the Intensities at the top and bottom of the box. The greatest linearity was obtained at a border width of 1 pixel.

**SERCA quantitation**

Pig CSR (10 mg) was dissolved in 1 ml of Laemmli sample buffer (Bio-Rad) containing 4% β-mercaptoethanol, loaded on a 4–20% Tris–HCl gel, and electrophoresed at 120 V for 90 min 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 and then was destained for 1 h in a solution containing 20% methanol, 5% isopropanol, 10% glacial acetic acid, and 65% H2O. The mass percentage of SERCA was calculated by densitometry with U SCANIT software.

**Analysis of pig CSR**

Pig CSR was prepared from the ventricle of a fresh pig heart (obtained from Lindenfelder’s Meats, Monticello, MN, USA) and placed on ice within 10 s of sacrifice. 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, 0.1 μg/mL aprotinin, 0.1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 μg/mL pepstatin. The homogenate was centrifuged at 4°C for 20 min at 11,000g (8500 rpm in a JA-14 rotor). The pellets were discarded, and the supernatant was filtered with cheesecloth. 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 h and then centrifuged at 4°C for 45 min at 100,000g (36,000 rpm in a 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, 2.5 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 min at 100,000g. The pellet was resuspended in 1 ml of sucrose solution per 40 g of wet weight 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 fraction of PLB phosphorylated**

The measured intensity for one band in a Western blot using antibody i is given by...
where $e_{ij}$ is a proportionality constant (fluorescence units/ng PLB) that describes the slope of the standard curve for uPLB ($j = u$) or pPLB ($j = p$). The variable $c_i$ 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 $e_{aa} > e_{ap}$ and $e_{au} < e_{ap}$. 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

\[ I_i = \sum e_{ij}c_k, \quad \text{(1)} \]

Solve for $c_u$ and $c_p$:

\[ c_u = [I_u/e_{up} - I_u/e_{up}]/[e_{up}e_{au} - e_{au}e_{up}], \]

\[ c_p = [I_p/e_{up} - I_p/e_{up}]/[e_{up}e_{au} - e_{au}e_{up}]. \quad \text{(3)} \]

Calculate tPLB and $X_p$:

\[ \text{tPLB} = c_p + c_u, \]

\[ X_p = c_p/tPLB. \quad \text{(4)} \]

### Statistical analysis

Statistical analysis was performed using Origin 8.1. For validation of the method, the accuracies of $X_u$ and tPLB values, performed on known mixtures of standards, were calculated as $X_u(app)/X_u$ and tPLB(app)/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 means ± standard errors of the mean (SEM), and one-way analysis of variance (ANOVA) was used to compare mean values, with a P value of less than 0.01 considered as significant. Exact values are reported for $P \geq 0.01$. 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]. It is essential to determine the proportion of the PLB oligomeric state on quantitation by Western blot because it is entirely possible that an antibody would bind less effectively to the five pentomers 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 an SDS–PAGE gel and its subsequent Western blot [29]. Therefore, PLB standard curves were analyzed by Western blot with and without this preheating 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), clearly demonstrating 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 ($c_{ij}$ in Eq. (1)) are determined from standard curves, quantifying each antibody’s sensitivity to uPLB and pPLB. For antibody $a$ (Ab8A3, Ab2D12, or AbA1), $e_{aa} > e_{ap}$, confirming that antibody $a$ prefers to bind uPLB over pPLB. Conversely, antibody $b$ (Ab285) greatly prefers to bind pPLB ($e_{ap} < e_{ap}$) (Fig. 2B). Fig. 2C and D show that the values of $X_u$ 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 $X_u(app)$ 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 all standards and unknowns are run on each individual blot $a$ and $b$.

#### Potential sources of systematic error

Lack of uPLB and pPLB standards and assumptions made about antibodies create systematic errors in tPLB and $X_u$ 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 ($e_{aa} = 0$), tPLB is systematically underestimated in the presence of any pPLB, which leads to overestimation of $X_u$ (Fig. 3, method 1). If it is assumed that antibody $a$ is completely specific for uPLB ($e_{ap} = 0$), pPLB is systematically overestimated ($X_u$ and $X_u$ 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 $X_u$ and tPLB accurately, with all three choices of antibody $a$, whereas 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 CSR

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

We quantitated the SERCA content in the pig CSR samples using Coomassie stain (data not shown). Thus, we obtained the molar concentrations of SERCA, as well as those of iPLB, 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 and 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. 1. PLB oligomeric state does not affect immunoblot intensity. Typical immunoblots and standard curves are shown for samples preheated to 100 °C (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 \( X_p \) (0.00–1.00, as indicated). (B) Typical standard curves from panel A for uPLB (squares) and pPLB (circles) from blots incubated with the indicated primary antibody. Resulting slopes (proportionality constants \( \epsilon_{ij} \) in Eqs. (1) and (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 (squares), Ab2D12 (circles), or AbA1 (triangles). (D) Accuracy and precision of tPLB values: tPLB(\text{app})/tPLB \pm \text{SEM} for the same mixtures as in panel C.
was also found to be essentially invariant among the four pigs (Table 3). The regulation of SERCA by PLB, as indicated by calcium dependence of ATPase activity, was also invariant among the four pigs (Fig. 4 and Table 3). 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 2), standards and samples must be run on the same blot. 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.

Features of our method

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 and >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. Although pPLB/SERCA and tPLB/SERCA varied widely, there were no significant variations in uPLB/SERCA among the four pigs (Fig. 4 and Table 3). The regulation of SERCA by PLB, as indicated by calcium dependence of ATPase activity, was also invariant among the four pigs (Table 3, pKC_a).

Table 1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>K_{UP}</th>
<th>n (blots)</th>
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<tbody>
<tr>
<td>Ab8A3</td>
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<tr>
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<td>47</td>
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<td>AbA1</td>
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<td>36</td>
</tr>
<tr>
<td>ANOVA</td>
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</tbody>
</table>

Note. Ab8A3 was used as the pPLB-selective antibody. The uPLB-selective antibody a is given in the top row. Values in the second, third, and fourth columns indicate accuracy (apparent/actual) ± precision (CV = standard deviation/mean). P > 0.01 indicates no significant variation (n = 8 measurements for each value of X_p for a total of 40).

Table 2

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Note. K_{UP} = \frac{\text{u/CP}}{\text{p/CP}}, where i = a or b. CV, coefficient of variation (=standard deviation/mean). Primary antibody incubation times were varied from 1 to 2 h.

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 and >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. Although pPLB/SERCA and tPLB/SERCA varied widely, there were no significant variations in uPLB/SERCA among the four pigs (Fig. 4 and Table 3). The regulation of SERCA by PLB, as indicated by calcium dependence of ATPase activity, was also invariant among the four pigs (Table 3, pKC_a).
not attempt quantitative calculations such as those in Eq. (4) [16].
Even with standards, Fig. 3 illustrates how assumptions about anti-
body selectivity can lead to systematic errors in determination of
both tPLB and Xp.

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 a negligible effect on cardiac contractility
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from Santa Cruz Biotechnology, Santa Cruz, CA, USA, or from
Badrilla, Leeds, UK) and a threonine-17-phosphorylated pPLB
synthetic standard.

Application to biological membranes

We have used our method to make the first accurate measure-
ments of Xp and tPLB in biological samples. We found that the frac-
tion of pPLB (Xp) in pig CSR is variable, ranging from 0.08 ± 0.01 to
0.38 ± 0.03 (Table 3). Remarkably, even the greatest Xp value was
less than 0.5, leaving considerable reserve for response to β-adren-
ergic stimulation or response to phosphomimetic therapies [53].
The tPLB level was also quite variable, ranging from 3.32 ± 0.05 to
13.0 ± 1.13 nmol/mg total protein (Table 3). Despite the wide
variation in Xp and tPLB among the pigs, the ratio of Xp to tPLB was
essentially constant (Fig. 4B), suggesting that PLB phosphory-
lation increases 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 and then calculated molar ra-
tios tPLB/SERCA, uPLB/SERCA, and pPLB/SERCA (Table 3). Differ-
ences in tPLB/SERCA, pPLB/SERCA, and Xp 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 signifi-
cantly among the four pigs over a wide range of Xp and SERCA mea-
surements (Fig. 4C and Table 3). Similarly, the calcium dependence of
SERCA ATPase activity was essentially invariant (Table 3). These
results suggest that myocytes in nonfailing myocardium maintain
SERCA activity by keeping uPLB/SERCA within a narrow range. Fu-
ture studies are needed to test this hypothesis more rigorously. For
example, freshly harvested tissue should be homogenized and ana-
lized quickly using phosphatase inhibitors to ensure that the phos-
phorylation status of PLB is captured accurately [46].

Potential applications to research in physiology and pathology

Now that we can quantitate Cus, Cps, Xp, tPLB, and SERCA with accu-
acy and precision, many questions concerning the role of PLB in
cardiac function and pathology can be addressed more quantita-
tively. This method can standardize Xp and tPLB measurements
across studies even when the blots are performed in different labo-
atories, where the choice of primary antibodies and other exper-
imental conditions may vary. The accuracy of our method will also
normalize Xp and tPLB values across animal systems, sample pre-
parations, disease states, and therapies so that cross-study compar-
sions 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 pKCa) [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 Xp 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, such as the
assessment of changes in Xp in human cardiac tissue in response
to current treatments including β-adrenergic agonists, Ca2+ chan-
nel blockers, phosphodiesterases, and future treatments in gene
and drug therapy. Because milligram quantities of tissue are suffi-
cient for replicate measurements of Xp, tPLB, and uPLB/SERCA, it is
plausible that human biopsies (1–2 mm3 of tissue) [54], which are
performed routinely under suspicion of myocarditis, cardiomyopa-
thy, or amyloidosis, can also be assayed for these values. Myocar-
dial biopsy is a nonsurgical procedure with a complication rate of
less than 1% to the patient [54]. Accurate tPLB calculations can also
quantitatively determine changes in PLB expression, revealing fac-
tors that affect calcium regulation at the level of transcription or
translation. Thus, our method can play an important role in eval-
uating the success of gene or drug therapies for heart failure. More
generally, our approach can provide a quantitative measure of
any protein modified by phosphorylation or with another post-
translational modification where (i) purified standards are avail-
able for both the unmodified and modified state of the protein
and (ii) antibodies are available with differential sensitivity to the
posttranslational modification.

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and the Biophysical Spectroscopy Facility. We acknowledge Octa-
vian Cornea for assistance in the preparation of the manuscript.

Table 3
Application to pig CSR.

<table>
<thead>
<tr>
<th>Pig</th>
<th>Xp (nmol/mg)</th>
<th>tPLB (nmol/mg)</th>
<th>uPLB (nmol/mg)</th>
<th>pPLB (nmol/mg)</th>
<th>SERCA (nmol/mg)</th>
<th>pKCa</th>
<th>pPLB/SERCA (mol/mol)</th>
<th>uPLB/SERCA (mol/mol)</th>
<th>tPLB/SERCA (mol/mol)</th>
<th>p</th>
<th>Mean</th>
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<td>1</td>
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<td>3.32 ± 0.01</td>
<td>3.07 ± 0.08</td>
<td>0.25 ± 0.10</td>
<td>1.48 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>2.25 ± 0.05</td>
<td>2.08 ± 0.05</td>
<td>5.90 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>0.14 ± 0.01</td>
<td>5.79 ± 0.10</td>
<td>4.98 ± 0.08</td>
<td>4.92 ± 0.20</td>
<td>2.22 ± 0.01</td>
<td>0.36 ± 0.01</td>
<td>2.60 ± 0.04</td>
<td>2.24 ± 0.03</td>
<td>5.88 ± 0.04</td>
<td>0.14 ± 0.01</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>3</td>
<td>0.23 ± 0.01</td>
<td>6.70 ± 0.20</td>
<td>5.18 ± 0.18</td>
<td>8.88 ± 0.20</td>
<td>2.03 ± 0.01</td>
<td>0.75 ± 0.02</td>
<td>3.31 ± 0.09</td>
<td>2.56 ± 0.09</td>
<td>5.87 ± 0.06</td>
<td>0.23 ± 0.01</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>0.38 ± 0.01</td>
<td>13.0 ± 1.1</td>
<td>8.09 ± 0.69</td>
<td>30.3 ± 2.6</td>
<td>3.74 ± 0.04</td>
<td>1.32 ± 0.11</td>
<td>3.48 ± 0.14</td>
<td>2.16 ± 0.18</td>
<td>5.88 ± 0.01</td>
<td>0.65 ± 0.13</td>
<td>0.22 ± 0.01</td>
</tr>
</tbody>
</table>

Note. Values are means ± SEM. P < 0.01 indicates significant variation among the four pigs. pKCa is the pCa value giving half-maximal calcium ATPase activity. n = 5 measurements per pig for all values except pKCa, where n = 2.
References


