Pentamic Assembly of Phospholamban Facilitates Inhibition of Cardiac Function in Vivo*

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Phospholamban has been proposed to coexist as pentamers and monomers in native sarcoplasmic reticulum membranes. To determine its functional unit in vivo, we reintroduced wild-type (pentameric) or monomeric mutant (C41F) phospholamban in the hearts of phospholamban knockout mice. Transgenic lines, expressing similar levels of mutant or wild-type phospholamban, were identified, and their cardiac phenotypes were characterized in parallel. Sarcoplasmic reticulum Ca\(^{2+}\) transport assays indicated similar decreases in SERCA2 Ca\(^{2+}\) affinity by mutant or wild-type phospholamban. However, the time constants of relaxation and Ca\(^{2+}\) transient decline in isolated cardiomyocytes were diminished to a greater extent by wild-type than mutant phospholamban, even without significant differences in the amplitudes of myocyte contraction and Ca\(^{2+}\) transients between the two groups. Langendorff perfusion also indicated that mutant phospholamban was not capable of depressing the enhanced relaxation parameters of the phospholamban knockout hearts to the same extent as wild-type phospholamban. Moreover, in vivo assessment of mouse hemodynamics revealed a greater depression of cardiac function in wild-type than mutant phospholamban hearts. Thus, the mutant or monomeric form of phospholamban was not as effective in slowing Ca\(^{2+}\) decline or relaxation in cardiomyocytes, hearts, or intact animals as wild-type or pentameric phospholamban. These findings suggest that pentameric assembly of phospholamban is necessary for optimal regulation of myocardial contractility in vivo.

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better clarify the physiological effects of monomeric PLB. Our results indicate that although PLB monomers (C14F) inhibit the apparent affinity of the SR Ca\(^{2+}\) uptake system to the same extent as PLB pentamers, they are not as effective in increasing the rate of myocardial relaxation assessed in isolated cardiomyocytes, perfused hearts, or intact animals.

**EXPERIMENTAL PROCEDURES**

**Generation of Transgenic Mice Expressing Mutant PLB in the Knockout Background**—The site-specific mutation C14F was introduced into the PLB cDNA by polymerase chain reaction methodology as described previously (17). The entire expression construct was composed of the cardiac-specific a-myosin heavy chain (a-MHC) promoter (5.5 kb), the PLB coding region with the C14F mutation (0.65 kb), and the SV40 poly(A) signal sequence (0.25 kb) (Fig. 1A). The KpnI-HindIII fragment of the vector pBl31 containing the entire expression construct was released and purified for pronuclear microinjection of fertilized eggs from FVB/N mice. The founder mice, who were PLB heterozygous, were mated back with PLB knockout mice to generate transgenic mice expressing mutant PLB in the PLB knockout background (KO + MU). Transgenic mice with the desired mutation in the knockout background were identified using polymerase chain reaction methodology and Southern and Northern blot analyses in the transgenic lines (data not shown) as described previously (16).

The cardiac homogenates were incubated with equal volumes of loading buffer (20% glycerol, 2% beta-mercaptoethanol, 4% SDS, 0.01% bromphenol blue, and 130 mM Tris.Cl, pH 6.8), the PLB coding region with the C14F mutation (0.65 kb), and the SV40 poly(A) signal sequence (0.25 kb) (Fig. 1A). The KpnI-HindIII fragment of the vector pBl31 containing the entire expression construct was released and purified for pronuclear microinjection of fertilized eggs from FVB/N mice. The founder mice, who were PLB heterozygous, were mated back with PLB knockout mice to generate transgenic mice expressing mutant PLB in the PLB knockout background (KO + MU). Transgenic mice with the desired mutation in the knockout background were identified using polymerase chain reaction methodology and Southern and Northern blot analyses in the transgenic lines (data not shown) as described previously (16), and these KO + WT mice were used as controls for the KO + MU mice in this study.

**Western Blot Analysis**—Quantitative immunoblotting was performed to determine the protein levels of PLB and SR Ca\(^{2+}\)-ATPase in the heart as described previously (16). The cardiac homogenates were incubated with equal volumes of loading buffer (20% glycerol, 2% beta-mercaptoethanol, 4% SDS, 0.01% bromphenol blue, and 130 mM Tris.Cl, pH 6.8), subjected to 13% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto nitrocellulose membranes (Schleicher & Schuell). The membranes were then reacted with a mouse monoclonal antibody to PLB or SR Ca\(^{2+}\)-ATPase (Affinity BioReagents Inc.) at a dilution of 1:1000. After washing out the unbound antibody with Tris-buffered saline (10 mM Tris-HCl and 150 mM NaCl, pH 7.8), the blots were incubated with an alkaline phosphatase-conjugated anti-mouse secondary antibody (1:1000; Cappel Division of Organon Teknika). The PLB and SR Ca\(^{2+}\)-ATPase protein bands were visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates for the alkaline phosphatase reaction.

**Sarcoplasmic Reticulum Ca\(^{2+}\) Uptake Assays**—Mouse hearts were excised, frozen in liquid nitrogen, and stored at −80 °C until processed for SR Ca\(^{2+}\) uptake experiments. The frozen hearts were powdered in liquid nitrogen and homogenized in 50 mM KH2PO4, pH 7.0, 10 mM NaF, 1 mM EDTA, 0.3 mM succrose, 0.3 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. The initial rates of calcium uptake in cardiac homogenates were obtained as described previously (1). Cardiomyocyte Preparations and Contraction Measurements—Isolation of mouse left ventricular myocytes was carried out as described previously (18). Briefly, mouse hearts were perfused in a Langendorff mode with nominally Ca\(^{2+}\)-free Tyrode's solution for 6 min at 37 °C. Perfusion was then switched to the same solution containing 0.8 mg/ml collagenase (type B; Boehringer Mannheim) and 0.03 mg/ml Pronase, with perfusion continuing until the heart became flaccid (~7–12 min). The isolated cells were perfused with normal Tyrode's solution at room temperature (22–23 °C) and field-stimulated at 0.5 Hz. Cell shortening was measured using a video-edge detection system (Crescent Electronics).

**Measurements of Ca\(^{2+}\) Transients**—Cells were incubated with the acetoxymethyl ester form of indo-1 (10 μM; Molecular Probes, Inc.) for 20 min at room temperature. After loading, the cells were superfused with normal Tyrode's solution for 30 min to wash out excess indicator and to allow de-esterification (19). Fluorescence was measured as described by Li et al. (18), with excitation at 355 ± 5 nm restricted to a circular spot of 100 μm in diameter and with emission measured at 405 and 485 nm (F\(_{405}\) and F\(_{485}\) (40-nm bandwidth; Chroma Technology Corp.) by two photomultiplier tubes (Hamamatsu Photonics Technology Corp.).

**Langendorff Perfusion**—Mouse hearts were rapidly excised and cannulated for retrograde aortic perfusion with modified Krebs-Henseleit buffer as described previously (20). The left ventricular pressure was monitored through a PE-50 polyethylene catheter, which was connected to a pressure transducer (041-500-803, COBE Cardiovascular, Inc.). The heart rate, left ventricular systolic pressure (LVSP) and its first derivatives (dP/dt), end diastolic pressure, time-to-peak pressure, and time for half-relaxation were continuously obtained on-line with a heart performance analyzer (HPA-1, Micro-Med, Inc.).

**In Vivo Phosphorylation**—Mouse hearts were perfused in a Langendorff mode as described above. After 30 min of perfusion to stabilize the conditions, the perfusion system was switched to a recirculating mode with modified Krebs-Henseleit buffer containing 2 μCi of [\(^{32}\)P]orthophosphate for 30 min at 37 °C. At the end of this labeling period, isoproterenol (0.1 μM) was administered into the perfusion system (20). After a 2-min stimulation, the hearts were freeze-clamped, stored at −80 °C, and processed for cardiac SR membrane and myofibrillar preparations (21), which were subsequently subjected to SDS-PAGE and autoradiography.

**Closed-chest Preparations**—Mouse hemodynamics were obtained using closed-chest preparations as described previously (22). Briefly, mice were anesthetized with intraperitoneal injection of a mixture of ketamine (100 mg/kg), xylazine (5 mg/kg), and morphine (2.5 mg/kg). The trachea was intubated with a 24-gauge Jelco intravenous catheter. A custom-made 1-French bipolar pacemaker (Numed) was positioned in the right atrium via the right internal jugular vein. A 1.4-French Millar high fidelity catheter was introduced into the right carotid artery and advanced into the left ventricle. Analog signals for left ventricular systolic pressure and rates of left ventricular pressure increase and decline as well as electrocardiogram were obtained on-line, recorded on a Gould WindowGraf four-channel recorder, and digitized via an A-D board at 1000 Hz.

**Statistical Analysis**—Data are expressed as means ± S.E. Statistical analysis was performed using analysis of variance for multiple comparisons and Student's t test for unpaired observations. Values of p < 0.05 were considered statistically significant. For the SR Ca\(^{2+}\) uptake assays, the data were analyzed by nonlinear regression analysis using MicroCal Origin software. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's test for multiple comparisons and the one-tailed Student's t test for comparison of means.

**RESULTS**

**Cardiac-specific Expression of Mutant PLB in the Knockout Background**—Cardiac specific expression of wild-type or mutant PLB was driven using the a-MHC promoter (Fig. 1A) as described previously (2). The codon TGT (Cys\(^{11}\)) in the coding region of PLB was mutated to TTT (Phe) by site-directed polymerase chain reaction mutagenesis. The expression construct sequence and mutation were confirmed by restriction digestion and DNA sequencing and subsequently used for pronuclear microinjection to generate transgenic mice. Eleven founder mice harboring the mutated PLB transgene in the knockout background were identified using polymerase chain reaction analysis of genomic DNA isolated from tail biopsies. Furthermore, the transgene and its expression were detected using Southern and Northern blot analyses in the transgenic lines (data not shown) as described previously (16).

To examine the PLB protein expression levels, cardiac homogenates from transgenic mice expressing either wild-type or mutant PLB in the knockout background were processed in parallel with nontransgenic wild-type mice for Western blot analysis. Quantitative immunoblotting indicated that the reintroduced mutant PLB migrated as monomers, whereas the reintroduced wild-type PLB migrated mainly as pentamers, similar to endogenous PLB in wild-type hearts (Fig. 1B). In 11 transgenic lines, the cardiac PLB protein expression levels varied between 20 and 70% of those present in nontransgenic wild-type hearts. Transgenic mice with 70% PLB expression levels (Fig. 1C) were selected to breed and propagate for further biochemical and physiological studies. Transgenic mice expressing similar levels of wild-type PLB in the knockout hearts (17) were used as controls. Assessment of the SR Ca\(^{2+}\)-ATPase protein levels showed no significant alterations upon expression of either wild-type or mutant PLB in the knockout mouse hearts (data not shown).

**Sarcoplasmic Reticulum Ca\(^{2+}\) Uptake Assays**—To examine the effects of mutant PLB expression on SR Ca\(^{2+}\) pump func-
cardiac homogenates from transgenic mice expressing wild-type (KO+WT; ○; n = 12) or mutant (KO+MU; ●; n = 12) PLB in the knockout background were prepared in parallel with those of PLB knockout mice (KO; □; n = 7), and the initial rates of SR Ca\(^{2+}\) uptake were assessed as a function of increasing [Ca\(^{2+}\)]. Values are means ± S.E., and n indicates the number of hearts used for analysis.

type PLB or mutant PLB reintroduced in the knockout background. Myocytes expressing wild-type PLB relaxed more slowly than those expressing mutant PLB (Fig. 3, A and B). The time constant for cell relaxation during the steady-state twitch was significantly faster in the mutant PLB myocytes (τ = 45.1 ± 2.5 ms in mutant versus 83.3 ± 5.2 ms in wild-type). The amplitude of cell shortening was slightly but not significantly higher in mutant PLB myocytes (Fig. 3B). Consistent with cell mechanics, the decline of the Ca\(^{2+}\)-transient was faster in myocytes with mutant PLB compared with wild-type PLB (τ = 162 ± 7.5 ms in mutant versus 196 ± 11 ms in wild-type). Peak [Ca\(^{2+}\)], tended to be higher with mutant PLB, but again this effect was not significant (Fig. 3, C and D).

**Cardiomyocyte Mechanics and Ca\(^{2+}\) Transients**—To determine whether the observations at the subcellular level were associated with similar findings at the cellular level, left ventricular myocytes were isolated, paced at 0.5 Hz, and used for measurements of contractile parameters and Ca\(^{2+}\) transients. Reintroduction of wild-type PLB in the knockout background was associated with significant depression of the cell shortening fraction (7.7 ± 0.7%, n = 28) and prolongation of the time constant (τ) of relaxation (83.3 ± 5.2 ms, n = 28) compared with PLB knockout cardiomyocytes (cell shortening: 10.6 ± 0.8%, n = 33; τ = 34.0 ± 2.1 ms, n = 33).

Fig. 3 shows representative traces of steady-state cell shortening and Ca\(^{2+}\) transients of transgenic myocytes with wild-
Functional Unit of Phospholamban in Vivo

In Vivo Phosphorylation of Mutant PLB—The stimulatory effects of isoproterenol suggested that both mutant and wild-type PLB can become phosphorylated and that this relieves their inhibitory effects. Thus, it was of special interest to determine the degree of mutant PLB phosphorylation and to compare it to that of wild-type PLB. Transgenic mouse hearts were perfused with buffer containing [32P]orthophosphate in the presence of optimal isoproterenol concentration (0.1 μM), and at the peak of the inotropic response, the hearts were freeze-clamped. Cardiac SR membranes and myofibrillar fractions were then prepared and subjected to SDS-PAGE. Autoradiography indicated that wild-type PLB migrated mainly as pentamers, whereas mutant PLB migrated as monomers (Fig. 5A), consistent with Western blotting analysis (Fig. 1B). Furthermore, the degree of phosphorylation ([32P] incorporation) was similar between mutant and wild-type PLB, suggesting that mutant PLB was phosphorylated to the same extent as wild-type PLB. There was no alteration in the degree of phosphorylation of troponin I, which served as an internal control in these experiments, between the same wild-type and mutant PLB hearts (Fig. 5A). In addition, pentameric wild-type PLB could be dissociated to monomers upon boiling, prior to loading onto SDS-polyacrylamide gel (data not shown).

In Vivo Assessment of Cardiac Function—To determine whether these cardiac alterations in ex vivo preparations reflected similar findings in vivo, anesthetized closed-chest preparations of transgenic mice were atrially paced at 300 beats/min and studied in parallel with PLB knockout mice. Reintroduction of wild-type PLB into the knockout background significantly repressed cardiac contractile parameters, whereas reintroduction of monomeric mutant PLB only slightly inhibited mouse hemodynamics (Table II). The maximal rates of left ventricular systolic pressure development (+dP/dt) and decline (−dP/dt) and τ were significantly different between wild-type and mutant transgenic mice (Table II). These data

![Image]

FIG. 3. Myocyte twitch contraction and Ca^{2+} transients. Left ventricular myocytes were isolated and stimulated at 0.5 Hz. All measurements were performed during steady-state (SS) twitches at 1 mM Ca^{2+} and room temperature. A, representative raw traces of myocyte contractions in unloaded isolated cardiomyocytes; B, pooled data of the amplitude of cell shortening; C, representative raw traces of Ca^{2+} transients in isolated cardiomyocytes loaded with 10 μM indo-1/AM; D, pooled data of the time constant (τ) of Ca^{2+} decline. RCL, resting cell length; KO+WT, transgenic mouse with wild-type PLB reintroduced in the knockout background; KO+MU, transgenic mouse with C41F mutant PLB reintroduced in the knockout background; *, p < 0.05, ***, p < 0.001 versus KO+WT mice.

### Table 1

Basal contractile parameters of Langendorff perfused mouse hearts

| Parameters                  | KO+MU | KO+WT | KO+WT |
|-----------------------------|-------|-------|-------|
| n                           | 6     | 6     | 6     |
| Body weight (g)             | 34 ± 3.0 | 28.5 ± 1.6 | 29.4 ± 3.0 |
| Heart rate (beats/min)      | 383 ± 20 | 382 ± 14 | 382 ± 11 |
| LVSP (mm Hg)                | 73.9 ± 2.1 | 59.9 ± 1.8 | 63.6 ± 1.6 |
| EDP (mm Hg)                 | 1.1 ± 0.3 | 2.8 ± 0.8 | 1.8 ± 0.2 |
| +dP/dt (mm Hg/s)            | 5896 ± 84 | 3718 ± 105 | 3940 ± 224 |
| −dP/dt (mm Hg/s)            | 3715 ± 169 | 2153 ± 105 | 2666 ± 158 |
| TTP (ms)                    | 26.0 ± 0.5 | 31.7 ± 0.3 | 31.7 ± 0.7 |
| RT1/2 (ms)                  | 14.7 ± 0.5 | 25.3 ± 1.4 | 19.3 ± 1.1 |

*a* KO, PLB knockout mice; KO+WT, transgenic hearts with wild-type PLB reintroduced in the knockout background; KO+MU, transgenic hearts with C41F mutant PLB reintroduced in the knockout background; EDP, end diastolic pressure; τ, time to peak pressure; RT1/2, time to half-relaxation.

*b* p < 0.01 versus KO.

*c* p < 0.05 versus KO+WT.

*d* p < 0.01 versus KO+WT (statistical analysis was performed using one-way analysis of variance followed by Bonferroni’s t test for multiple comparisons).
corroborated our ex vivo findings and suggested that pentameric PLB was a stronger repressor of cardiac function compared with monomeric PLB, associated with the C41F mutation.

**DISCUSSION**

This study was designed to determine the functional unit of PLB in vivo by reintroducing wild-type (pentameric) or monomeric mutant (C41F) PLB into the cardiac compartment of PLB knockout mice. Although expression of either PLB form was capable of inhibiting the SR Ca$^{2+}$-ATPase Ca$^{2+}$ affinity to the same extent, our findings indicate that monomeric PLB was less effective than the pentameric form in reversing the enhanced relaxation parameters of the PLB knockout hearts.

The availability of the PLB knockout mouse coupled with a strong cardiac-specific α-MHC promoter allowed us for the first time to assess the functional significance of pentameric or monomeric PLB in a background free of the endogenous protein in vivo. Transgenic lines expressing similar levels of mutant or wild-type PLB were chosen to assess the effects of each PLB form in a physiologically relevant manner. Calcium transport studies showed that wild-type or mutant PLB inhibited the apparent affinity of SR Ca$^{2+}$-ATPase for Ca$^{2+}$ to the same extent. Furthermore, there were no alterations in the maximal velocity of the enzyme, consistent with the finding that the levels of SERCA2 were similar between these transgenic lines. However, the pentameric form of PLB depressed the relaxation rate during the steady-state twitch and prolonged the Ca$^{2+}$ transient decline to a greater extent than the mutant or mono-

**Fig. 4. Effects of isoproterenol on cardiac contractile parameters.** Mouse hearts were perfused in a Langendorff mode with increasing concentrations of isoproterenol. A, +dP/dt (maximal rate of left ventricular pressure increase); B, −dP/dt (maximal rate of left ventricular pressure decline); C, time-to-peak pressure (TTP); D, time for half-relaxation (RT1/2). □, PLB knockout mice; ○, transgenic mice with wild-type PLB reintroduced in the knockout background; ●, transgenic mice with C41F mutant PLB reintroduced in the knockout background. Values are means ± S.E. (n = 6).

**Fig. 5. Autoradiogram of SDS-polyacrylamide gels of microsomal membrane proteins (A) and myofibrillar proteins (B) isolated from $^{32}$P-perfused hearts.** Hearts were perfused in a Langendorff mode with buffer containing $^{32}$P-labeled orthophosphate (2 mCi) and stimulated with isoproterenol (0.1 μM). KO+WT, transgenic mice with wild-type PLB reintroduced in the knockout background; KO+MU, transgenic mice with C41F mutant PLB reintroduced in the knockout background; PLBp and PLBm, pentameric and monomeric forms of PLB, respectively; TnT, troponin T; TnI, troponin I; LC2, myosin light chain.

**Table II**

**Mouse hemodynamics**

In vivo mouse cardiac function was assessed using closed-chest preparations, which were atrially paced at 300 beats/min. Values are means ± S.E. (n = number of mice).

| Parameters | KOa | KO+WT | KO+MU |
|------------|-----|-------|-------|
| n          | 5   | 6     | 6     |
| LVSP (mm Hg) | 122 ± 12 | 95 ± 10b | 98 ± 13 |
| +dP/dt (mm Hg/s) | 15,724 ± 1191 | 8107 ± 767b | 12,300 ± 800c |
| −dP/dt (mm Hg/s) | 12,200 ± 800 | 7200 ± 650b | 10,125 ± 525c |
| T (ms)     | 3.9 ± 0.6 | 5.8 ± 0.8b | 4.5 ± 0.4c |
| RT1/2 (ms) | | | |

a KO, PLB knockout mice; KO+WT, transgenic mice with wild-type PLB reintroduced in the knockout background; KO+MU, transgenic mice with C41F mutant PLB reintroduced in the knockout cardiac background; ±dP/dt, maximal rates of LVSP development and decline; τ, time constant of LVSP decline.

b p < 0.05 versus KO.

c p < 0.05 versus KO+WT.
meric PLB in isolated cardiomyocytes. Consistent with the findings at the cellular level, this differential effect was also observed in Langendorff perfused hearts. Furthermore, assessment of cardiac contractile parameters in the intact mouse revealed a greater depression of cardiac relaxation rates by wild-type PLB than by mutant PLB. These findings on cardiac contractile parameters were similar to previous results in models overexpressing similar levels of wild-type or mutant PLB in the presence of the endogenous protein (16). However, the findings on SR Ca\(^{2+}\) transport measurements were different from those in the overexpression models (16). Overexpression of pentameric PLB was more effective than that of monomeric PLB in inhibiting the initial rates of SR Ca\(^{2+}\) transport, whereas reintroduction of either form of PLB in the null background inhibited this SR parameter to the same extent. The reason for this apparent discrepancy could be due to the following: 1) the stoichiometric ratio of PLB to SERCA2 (0.7 in the transgenic mice in this study versus 2.0 in the overexpression models compared with 1.0 in the wild-type mice), which may affect the nature of interaction between these proteins; 2) the coexistence of PLB pentamers and monomers in the overexpression models, which may mask the maximal inhibitory effects by monomers; 3) compensatory mechanisms, associated with altered PLB expression levels, which may be different between the knockout and overexpression models; and 4) the genetic background of the two models (129/SvJ and CF-1 for the knockout models versus FVB/N for the overexpression models), which may contain different modifier alleles.

The C41F mutation, chosen in this study, was based on previous in vitro (13) and in vivo (16) studies, which indicated that this mutation resulted in pentamer destabilization and formation of PLB monomers as revealed by SDS-PAGE. However, it is not entirely clear whether the migration pattern of C41F versus wild-type PLB on SDS-polyacrylamide gels reflects the in vivo situation. Future studies using chemical cross-linking (24), EPR spectroscopy (10), or low-angle laser light scattering measurements in conjunction with high-performance gel chromatography (25) may provide further insights into the oligomeric/monomeric state of PLB in vivo and its molecular dynamics upon mutagenesis or phosphorylation. Furthermore, it could be argued that replacement of cysteine with phenylalanine, which has a bulky side chain, may diminish the inhibitory effects of monomeric (C41F) PLB on cardiac relaxation parameters due to its inefficient interaction with the SR Ca\(^{2+}\)-ATPase. However, biochemical reactivity studies of the three Cys residues (Cys\(^{36}\), Cys\(^{41}\), and Cys\(^{46}\)) in the transmembrane domain of wild-type (pentameric) PLB indicated that Cys\(^{36}\) and Cys\(^{46}\) reacted with 5,5'-dithiobis(2-nitrobenzoic acid), whereas Cys\(^{41}\) remained unreactive (26). When PLB was denatured or a monomeric (L37A) form of PLB was used, all three Cys residues including Cys\(^{41}\) reacted with 5,5'-dithiobis(2-nitrobenzoic acid) (26). These studies suggest that Cys\(^{41}\) in wild-type PLB may be located within the pore of the PLB pentamer, which is protected and most insensitive to conformational modification. Furthermore, it has been hypothesized that the PLB transmembrane helix is oriented with Cys\(^{36}\) and Cys\(^{46}\) projecting into the lipid hydrocarbon, thus allowing interaction with the SR Ca\(^{2+}\)-ATPase, and Cys\(^{41}\) on the helix interface within the pentameric structure of PLB away from the binding region of SR Ca\(^{2+}\)-ATPase. Thus, mutation of Cys\(^{41}\) to Phe should not alter the interface of the inhibitory domain of PLB with the SR Ca\(^{2+}\)-ATPase and their physical interaction. This assumption is also supported by previous in vitro expression studies of SR Ca\(^{2+}\)-ATPase and PLB (13) and by this study, in which C41F mutant or monomeric PLB was capable of inhibiting the SR Ca\(^{2+}\)-ATPase Ca\(^{2+}\) affinity to the same extent as wild-type or pentameric PLB.

Langendorff perfusion in the presence of \(^{32}\)P-containing buffer revealed that mutant PLB could be phosphorylated by isoproterenol. The degree of phosphorylation of monomeric PLB appeared to be similar to that of pentameric PLB, suggesting that the functional differences in cardiac preparations expressing wild-type or mutant PLB were not due to alterations in the phosphorylation capability of the mutant form of PLB. Under maximal stimulation by isoproterenol, the functional differences between the two forms of PLB, observed under basal conditions, were abolished, and the contractile parameters were similar between the two groups. It is interesting to note that both the non-phosphorylated and phosphorylated forms of mutant PLB migrated with similar apparent monomeric molecular weight, indicating that phosphorylation did not promote oligomerization of PLB in vivo. These findings using in vivo phosphorylation conditions and SDS-PAGE are different from previous results based on EPR studies, which suggested that in vitro phosphorylation of wild-type or monomeric (L37A) PLB resulted in oligomer formation in lipid bilayers (10).

In summary, our findings indicate that cardiac-specific expression of the monomeric form of PLB (C41F) is associated with inhibition of the SR Ca\(^{2+}\)-ATPase Ca\(^{2+}\) affinity, similar to wild-type or pentameric PLB, but the mutant form is not as effective as wild-type PLB in suppressing cardiac relaxation parameters, assessed at the cellular, organ, and intact animal levels. This apparent discrepancy between biochemical and physiological findings may be explained by the following possibilities. 1) The assay used to measure initial rates of SR Ca\(^{2+}\) uptake is not sufficiently sensitive to detect small differences in Ca\(^{2+}\) affinity. 2) The Ca\(^{2+}\) uptake assay conditions in vitro do not adequately reflect the dynamic situation in vivo. 3) The pentameric form of PLB may induce a Ca\(^{2+}\) leak in SR membranes via PLB pentamers (7, 8, 27), ryanodine receptors, or uncoupled Ca\(^{2+}\)-ATPase, which slows down net Ca\(^{2+}\) uptake in the intact cell, but not the apparent initial SR Ca\(^{2+}\) uptake rates under in vitro conditions. 4) There could be an increased SR Ca\(^{2+}\) load in the myocytes expressing pentameric PLB that slows down net SR Ca\(^{2+}\) uptake during relaxation in vivo. These possibilities may be addressed in future studies designed to elucidate the mechanisms underlying the differences between the regulatory effects of the C41F PLB mutant in SR Ca\(^{2+}\) transport assays in vitro and in functional measurements in vivo.

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Functional Unit of Phospholamban in Vivo
Pentameric Assembly of Phospholamban Facilitates Inhibition of Cardiac Function

*in Vivo*

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