Transgenic Approaches to Define the Functional Role of Dual Site Phospholamban Phosphorylation*

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Phospholamban is a critical regulator of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase activity and myocardial contractility. Phosphorylation of phospholamban occurs on both Ser\(^{16}\) and Thr\(^{17}\) during isoproterenol stimulation. To determine the physiological significance of dual site phospholamban phosphorylation, we generated transgenic models expressing either wild-type or the Ser\(^{16}\) → Ala mutant phospholamban in the cardiac compartment of the phospholamban knockout mice. Transgenic lines with similar levels of mutant or wild-type phospholamban were studied in parallel. Langendorff perfusion indicated that the basal hyperdynamic cardiac function of the knockout mouse was reversed to the same extent by reinsertion of either wild-type or mutant phospholamban. However, isoproterenol stimulation was associated with much lower responses in the contractile parameters of mutant phospholamban compared with wild-type hearts. These attenuated responses were due to lack of phosphorylation of mutant phospholamban, assessed in \(^{32}\)P labeling perfusion experiments. The lack of phospholamban phosphorylation \textit{in vivo} was not due to conversion of Ser\(^{16}\) to Ala, since the mutated phospholamban form could serve as substrate for the calcium-calmodulin-dependent protein kinase \textit{in vitro}. These findings indicate that phosphorylation of Ser\(^{16}\) is a prerequisite for Thr\(^{17}\) phosphorylation in phospholamban, and prevention of phosphoserine formation results in attenuation of the \(\beta\)-agonist stimulatory responses in the mammalian heart.

Phospholamban (PLB)\(^1\) is a regulator of the affinity of the cardiac sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase for Ca\(^{2+}\). Dephosphorylation of PLB is an inhibitor, and phosphorylation of PLB removes its inhibitory effects on the SR Ca\(^{2+}\)-ATPase. Recently, the critical role of PLB in the regulation of cardiac contractility has been defined through gene transfer (1) and knockout (2) technology in the mouse. Cardiac-specific overexpression of PLB was associated with decreases in the affinity of the SR Ca\(^{2+}\)-ATPase and enhanced myocardial performance. Furthermore, the stimulatory effects to \(\beta\)-adrenergic agonists were more pronounced in the PLB-overexpressing hearts, whereas these effects were attenuated in the PLB-knockout hearts compared with wild types (1, 2). These studies suggested that PLB plays a prominent role in the heart’s responses to \(\beta\)-agonists. However, PLB is phosphorylated on both Ser\(^{16}\) and Thr\(^{17}\) during isoproterenol stimulation (3) and the relative contribution of each site in the altered contractile responses of the heart is not presently well known. \textit{In vitro} studies have shown that Ser\(^{16}\) is phosphorylated by cAMP-dependent protein kinase, whereas Thr\(^{17}\) is phosphorylated by Ca\(^{2+}\)-calmodulin-dependent protein kinase (4). Phosphorylation of each site occurs in an independent manner, although it is not presently clear whether the stimulatory effects of the two phosphorylations on SR Ca\(^{2+}\) transport are additive (5–9).

\textit{In vivo} studies have shown that phosphorylation/dephosphorylation of PLB by Ca\(^{2+}\)-calmodulin-dependent protein kinase has as a prerequisite the phosphorylation/dephosphorylation by cAMP-dependent protein kinase (10–15). Furthermore, elevation of intracellular [Ca\(^{2+}\)] to higher levels than those attained by isoproterenol, which resulted in higher peak tension than that elicited during \(\beta\)-adrenergic stimulation, failed to phosphorylate PLB (3, 16). However, a recent study, using phosphorylation site-specific antibodies for PLB, indicated that cAMP-dependent and Ca\(^{2+}\)-calmodulin-dependent phosphorylation of PLB can occur in an independent manner and their effects may be additive \textit{in vivo} (17). Thus, the functional role of dual site phosphorylation of PLB is not clear.

The availability of the PLB knockout mouse in conjunction with site-specific mutagenesis technology have provided us with an excellent opportunity to further examine the interaction between the cAMP-dependent and the Ca\(^{2+}\)-calmodulin-dependent pathways of PLB phosphorylation in the regulation of basal and \(\beta\)-agonist stimulated cardiac contractility \textit{in vivo}. The aims of the present study were to: 1) determine whether reintroduction of PLB in the null background is feasible, and whether it is able to reverse the hyperdynamic cardiac phenotype of the PLB knockout mouse; and 2) elucidate the physiological role of Thr\(^{17}\) phosphorylation in PLB in the absence of Ser\(^{16}\) (Ser\(^{16}\) → Ala) by directing cardiac-specific expression of mutant PLB in the knockout background.

**Experimental Procedures**

**Site-directed Mutagenesis**—The site-specific mutation of Ser\(^{16}\) → Ala (TCC → GCC) was introduced into PLB cDNA by polymerase chain reaction (PCR) methodology. Briefly, a 0.9-kb SalI fragment containing PLB cDNA and the SV40 polyadenylation signal sequence (PLB cDNA-SV40-poly(A)) was released from the α-MHCp-PLB-SV40 fusion gene (1). This SalI PLB cDNA-SV40-poly(A) fragment was then subcloned into a pBluescript SKII(−) vector (Stratagene), which has T3 and T7 primer sites flanking the insert. Polymerase chain reaction mutagenesis was performed by two consecutive PCR amplifications using

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\(^1\) The abbreviations used are: PLB, phospholamban; SR, sarcoplasmic reticulum; PCR, polymerase chain reaction; kb, kilobase(s); MHC, myosin heavy chain; PAGE, polyacrylamide gel electrophoresis; PKA, cAMP-dependent protein kinase.

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two different sets of primers. For the first PCR amplification, 100 pg of the subcloned plasmid DNA containing the 0.9-kb SalI fragment was used as template, along with a 5’ end mutant primer (5’-CT ATG AGA GCC GEC ACT ATT GAA ATG CC-3’) corresponding to nucleotides 92–62 of the PLB coding sequence, and a 3’ end T7 primer, to generate a desired mutant PLB cDNA minor product. Subsequently, an aliquot of the first PCR product as well as the T3 and T7 primers was used for the second PCR. The final amplified product was excised and subcloned into the SalI site of a second pBluescript SK II(—) vector, which was then transformed into XLI-Blue-competent cells. The mutated PLB cDNA-SV40-poly(A) sequence was subcloned into the SalI site of the parent PLB overexpression vector for pBII 31 (1).

Generation and Identification of the Transgenic Mice—The expression fragment containing the cardiac-specific α-myosin heavy chain (α-MHC) promoter, the mutated PLB cDNA, and the SV40 poly(A) signal sequence was used for pronuclear microinjection of fertilized eggs derived from the intercrossing of male PLB knockout (PLB-KO) and female FVB/N mice. The first generation of transgenic mice was PLB-heterozygous. Mice harboring the mutant PLB transgene were identified using PCR methodology and Southern blot analysis (1). Breeding the transgene-positive mice with PLB-KO mice generated offspring expressing the mutant PLB transgene in the PLB-KO background. The α-MHC-PLB-SV40 fusion gene was also used to generate wild-type PLB (PLB-WT) transgenic mice in a similar manner as the PLB Ser16→Ala (PLBKO) transgenic mice.

Western Blot Analysis—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and quantification of PLB and the SR Ca2+-ATPase were performed as described previously (1, 18). For immunodetection of PLB phosphorylation sites, polyclonal antibodies raised against a PLB peptide (residues 9–19) phosphorylated at Ser residues 9–19 phosphorylated at Ser at Ser16 (1:10,000) or at Thr17 (1:5000) (PhosphoProtein Research) were used.

Langendorff Perfusion—Hearts from wild-type and transgenic mice were subjected to retrograde aortic perfusion with modified Krebs-Henseleit buffer as described previously (19). After a 30–40-min stabilization period, cumulative concentrations of isoproterenol (0.1 nM to 1 μM) were administered into the buffer flow line at intervals of 7 min.

In Vivo Phosphorylation—Mouse hearts were perfused in a recirculating system containing 2 mM of [32P]ATP (30 μCi) at the end of this labeling period, isoproterenol (0.1 μM) was administered into the perfusion system and hearts were stimulated for 2 min (19). After stimulation, preparations were freeze-clamped and homogenized in phosphate buffer (50 mM KH2PO4, 10 mM NaF, 1 mM EDTA, 0.3 mM sucrose, and 0.3 mM phenylmethylsulfonyl fluoride, pH 7.0) containing 0.5 mM dithiothreitol and 1 μM o-acidic acid. Microsomal fractions enriched in SR membranes and myofibrillar proteins were prepared as described previously (20).

In Vitro Phosphorylation—Cyclic AMP-dependent protein kinase (PKA) phosphorylation of the cardiac homogenates (60 μg) was carried out at 30 °C in 30 μl of reaction mixture containing 50 mM K+ phosphate buffer (pH 7.0), 10 mM MgCl2, 5 mM NaF, 0.5 mM EGTA, 0.1 mM ATP, 20 μCi of [γ-32P]ATP, and 45 units of the PKA-catalytic subunit. For endogenous Ca2+-calmodulin-dependent protein kinase (Ca2+/CAM) phosphorylation of the cardiac homogenates, 0.5 mM CaCl2, 2 μM calmodulin, and 1 μM protein kinase inhibitor peptide (5–24) amide were added to the above reaction mixture. Reactions were terminated with 30 μl of SDS sample buffer after 2 min (PKA) or 5 min (Ca2+/CAM) incubation, which was associated with optimal phosphate incorporation in PLB. Thirty μg of protein was subjected to 15% SDS-PAGE and autoradiography.

Statistical Analysis—Data are expressed as mean ± S.E. Statistical analysis was performed using Student’s t test for unpaired observations and analysis of variance followed by Bonferroni’s t test for multiple comparisons. Values of p < 0.05 were considered statistically significant.

RESULTS

Reintroduction of Wild-type PLB into the PLB Knockout Mouse Hearts—To determine whether the hyperdynamic cardiac function of the PLB-KO mice can be reversed by reintroduction of the missing PLB gene, we used the α-MHC promoter to direct expression of mouse PLB cDNA in the cardiac compartment of the PLB knockout mouse. Four PLB-WT transgenic lines were identified by PCR and Southern blot analyses of mouse genomic DNA. Northern blot analysis of total RNA isolated from the hearts of the PLB-WT transgenic mice revealed expression of the transgene, which migrated at 1.0 kb

Mouse hearts were perfused in a Langendorff mode, and data were collected and analyzed using the Digi-Med data acquisition system. WT, wild-type controls; PLB-KO, PLB knockout; PLB-WT, transgenic mice with wild-type PLB reintroduced in the knockout background, heart rate; +dP/dt, maximal rate of left ventricular pressure increase; −dP/dt, maximal rate of left ventricular pressure decrease; TTP, time to peak pressure; RT1/2, time for half-relaxation. Values are mean ± S.E., n, number of hearts.

This 1.0-kb message was not present in either control or PLB-KO mouse hearts. However, the control hearts showed the presence of the endogenous PLB messages migrating at 2.8 and 0.7 kb (data not shown).

To assess the protein levels of PLB in the transgenic hearts, we performed quantitative Western blot analysis of cardiac homogenates from PLB-WT in parallel with wild-type control mice. The levels of PLB were 0.7-fold in one line and 2.0-fold in three transgenic lines compared with control hearts. The line, which expressed PLB levels (0.7-fold) closer to those present in control hearts, was chosen for evaluation of the physiological effects of PLB reintroduction in the knockout background. Sarcolpasmic reticulum membranes isolated from the transgenic hearts indicated that the reintroduced PLB was inserted in the SR and there were no alterations in the SR Ca2+-ATPase levels compared with control hearts (data not shown).

Reversal of the PLB Knockout Hyperdynamic Cardiac Function by Reinsertion of Wild-type PLB—To determine whether the reintroduced PLB was capable of reversing the hyperdynamic cardiac function associated with PLB deficiency, hearts from PLB-WT and PLB-KO mice were subjected to Langendorff perfusion in parallel with control hearts. The PLB-KO hearts exhibited significantly enhanced myocardial performance compared with wild-type controls, as characterized by significant increases in the maximal rates of cardiac contraction (+dP/dt) and relaxation (−dP/dt) (Table I). Reinsertion of PLB in the knockout background (PLB-WT) was associated with significant depression of the contractile parameters (Table I). However, since the levels of reintroduced PLB were 0.7-fold of those present in control hearts, reversal of the enhanced cardiac contractile parameters was not complete, or to the levels observed in control hearts (Table I). It is interesting to note that when the relative levels of PLB or PLB/SR Ca2+-pump in the three animal models were plotted against 1.2

| Parameters | WT (n = 7) | PLB-KO (n = 7) | PLB-WT (n = 7) |
|------------|-----------|---------------|---------------|
| HR (beats/min) | 376 ± 12 | 377 ± 9 | 389 ± 13 |
| LVDP (mmHg) | 55 ± 0.9 | 68 ± 0.6 | 59 ± 0.9 |
| EDP (mmHg) | 4.5 ± 0.2 | 1.0 ± 0.3 | 2.8 ± 0.6 |
| −dP/dt (mmHg/s) | 2756 ± 104 | 5590 ± 142 | 3610 ± 140 |
| +dP/dt (mmHg/s) | 1712 ± 98 | 3232 ± 124 | 2136 ± 93 |
| VTt (ms) | 39 ± 1.2 | 27.1 ± 0.3 | 32.1 ± 0.5 |
| RT1/2 (ms) | 27.3 ± 1.3 | 15.4 ± 0.4 | 25.3 ± 1.2 |

a p < 0.05 vs. WT.

b p < 0.05 vs. PLB-KO.
FIG. 1. Correlation between basal contractile parameters and PLB expression levels. The relative ratios of PLB to the SR Ca$^{2+}$-ATPase (SERCA) were plotted against the basal maximal rates of contraction (○, +dP/dt) and relaxation (●, -dP/dt) in the perfused hearts from various mouse models: wild-type control (Control), PLB knockout (PLB-KO), and transgenic mice expressing the wild-type PLB in the knockout background (PLB-WT). The close linear correlations between the PLB/SR Ca$^{2+}$-ATPase levels and the contractile parameters are depicted by regression lines. Values are mean ± S.E. of four to five determinations.

FIG. 2. Western blot analysis of PLB and SR Ca$^{2+}$-ATPase. A, Western blots of PLB and SR Ca$^{2+}$-ATPase using cardiac SR-enriched preparations from transgenic mice with reintroduced wild-type PLB (PLB-WT) or mutant PLB (PLB-MU) in the PLB knockout background. SR-enriched fractions (5, 10, and 15 μg) were subjected to SDS-PAGE and immunoblotting as described previously (1). PLB$_H$ designates the high molecular weight form of PLB. B, the relative ratio of PLB to SR Ca$^{2+}$-ATPase protein levels in cardiac homogenates and cardiac microsomal fractions from PLB-WT and PLB-MU mice. Values are mean ± S.E. of four to five determinations.

Three lines of transgenic mice were generated, which were identified by PCR and Southern blot analyses. The levels of PLB were 0.7-fold in two lines and 2.0-fold in one line, compared with control hearts. One of the lines expressing 0.7-fold PLB, which was similar to the PLB levels expressed in PLB-WT hearts (Fig. 2), was selected for breeding and further studies. Analysis of the SR Ca$^{2+}$-ATPase levels showed that there was no alteration upon introduction of mutant PLB in the mouse heart (Fig. 2A).

Langendorff perfusion indicated that the basal contractile parameters, end-diastolic pressure, left ventricular systolic pressure, and heart rate of the PLB-mutant (PLB-MU) hearts were similar to those of the PLB-WT hearts. To determine the functional importance of Ser$^{16}$ phosphorylation in the β-adrenergic responses, hearts from PLB-KO, PLB-WT, and PLB-MU mice were perfused in a Langendorff mode and subjected to increasing concentrations of isoproterenol. Isoproterenol stimulated +dP/dt, -dP/dt, and heart rate in PLB-WT, PLB-MU, and PLB-KO hearts in a dose-dependent manner (Fig. 3). Under maximal isoproterenol stimulation, the contractile parameters were similar between the PLB-KO and PLB-WT groups, whereas those of the PLB-MU hearts were much lower (Fig. 3, A and B). It is interesting to note that, when the stimulatory responses were considered as absolute increments (mmHg/s) over the values observed in non-stimulated hearts, the degrees of stimulation in either the +dP/dt or -dP/dt values were similar between PLB-KO and PLB-MU hearts. The rate of contraction (+dP/dt) increased ~1000 mmHg/s in either PLB-MU (3357 ± 193 to 4600 ± 195 mmHg/s, n = 7) or PLB-KO (5616 ± 165 to 6497 ± 143 mmHg/s, n = 6) hearts (Fig. 3A). However, this increase was ~3000 mmHg/s in PLB-WT (2752 ± 120 to 5958 ± 206 mmHg/s, n = 7) and in non-transgenic control (3610 ± 140 to 6587 ± 298 mmHg/s, n = 6) hearts. Similarly, the rate of relaxation (-dP/dt) increased ~2000 mmHg/s in PLB-MU (2334 ± 133 to 4928 ± 191 mmHg/s, n = 7) and PLB-KO (3297 ± 118 to 5439 ± 88 mmHg/s, n = 6) hearts (Fig. 3B), whereas an increase of ~3000 mmHg/s was observed in PLB-WT hearts (2136 ± 90 to 5396 ± 322 mmHg/s, n = 7) and in non-transgenic control hearts (1711 ± 116 to 4641 ± 170 mmHg/s, n = 6).

In Vivo $^{32}$P Incorporation in PLB—The observation that the degree of changes in contractile parameters was similar between PLB-MU and PLB-KO hearts during isoproterenol stimulation, suggested that mutation of Ser$^{16}$ to Ala in PLB attenuated or abolished its regulatory role in the responses to β-agonists. This might be due to lack of phosphorylation of Thr$^{17}$ in the mutated PLB form during β-adrenergic stimulation. To determine whether Thr$^{17}$ was phosphorylated in vivo, PLB-WT and PLB-MU hearts were perfused in parallel with buffer containing [$^{32}$P]orthophosphate. The hearts were stimulated with 0.1 μM isoproterenol as described previously (19). Cardiac myofibrillar and SR-enriched membrane preparations were isolated and subjected to SDS-gel electrophoresis and autoradiography (Fig. 4). Examination of the degree of [$^{32}$P]labeling of the proteins in the SR-enriched membrane fraction indicated that phosphorylation of PLB was pronounced only in the PLB-WT hearts and incorporation of [$^{32}$P]phosphate in this protein was barely detectable in the PLB-MU hearts (Fig. 4A). However, the degree of [$^{32}$P]-incorporation in troponin I and C-protein in the myofibrillar fraction, isolated from the same hearts, was similar between PLB-MU and PLB-WT mice (Fig. 4B). Furthermore, PLB-WT and PLB-MU hearts were perfused in parallel with non-radioactive buffer and stimulated with isoproterenol, as described above. The SR-enriched membrane preparations were subjected to SDS-gel electrophoresis and immunoblotting using antibodies specific to Ser$^{16}$ or Thr$^{17}$ phosphorylated PLB peptides. Both phosphoserine (Fig. 5A) and phosphothreonine (Fig. 5B) were detected in isoproterenol-stimulated PLB-WT hearts. However, in PLB-MU hearts, there was a very low degree of phosphothreonine formation (Fig. 5B) but no phosphoserine detection (Fig. 5A), consistent with mutation of this site to Ala.

In Vitro Phosphorylation of PLB—The lack of phosphorylation of Thr$^{17}$ in the perfused PLB-MU hearts could be due to structural alterations in PLB upon mutation of Ser$^{16}$ to Ala, rendering the adjacent Thr$^{17}$ residue inaccessible to protein kinase. To determine whether this mutation in PLB alters its ability to become phosphorylated by the Ca$^{2+}$-calmodulin-dependent protein kinase, cardiac homogenates from PLB-WT
and PLB-MU mice were incubated with Ca\(^{2+}\) and calmodulin in the phosphorylation assay buffer and processed for SDS-polyacrylamide gel electrophoresis and autoradiography. The degree of \(^{32}\)P incorporation in PLB was similar between PLB-WT and PLB-MU hearts (Fig. 6A), suggesting that Thr\(^{17}\) in PLB-MU hearts could be phosphorylated in vitro. Formation of phosphothreonine in PLB-MU hearts was also verified by immunodetection, using a polyclonal antibody raised to a PLB peptide phosphorylated at Thr\(^{17}\) (data not shown). These data indicate that mutation of Ser\(^{16}\) did not prevent phosphorylation of the adjacent Thr\(^{17}\) residue in PLB by the Ca\(^{2+}\)-calmodulin-dependent protein kinase. However, incubation of the same cardiac homogenates with the protein kinase A catalytic subunit under optimal phosphorylation conditions indicated that only the PLB in PLB-WT hearts could be phosphorylated, consistent with the lack of the Ser\(^{16}\) (Ser\(^{16}\) → Ala) site in PLB-MU hearts (Fig. 6B).

**DISCUSSION**

Our results are the first to demonstrate that a cardiac phenotype, generated by gene targeting, can be reversed by reininsertion of the missing gene in the null background. In previous studies, we showed that ablation of PLB resulted in enhanced basal cardiac contractile parameters assessed at the cellular, organ, and intact animal levels (2, 18, 22, 23). In this study, we used the \(\alpha\)-myosin heavy chain promoter to direct cardiac-specific expression of wild-type PLB in the knockout background and observed reversal of the hyperdynamic function of the PLB-deficient hearts. The degree of inhibition of the contractile parameters was proportional to the expression levels of PLB, in agreement with our previous studies in PLB-heterozygous and PLB-homozygous mice (2, 21). The success of PLB transgenesis in the genetically altered background, accompanied by reversal of the knockout phenotype, indicated that the PLB-deficient mouse provides an attractive model system for expression of various PLB mutants in the heart and elucidation of their functional properties.
of structure-function relationships in vivo. With the first mutant we studied, we sought to elucidate the functional significance of dual site PLB phosphorylation during β-adrenergic stimulation. A site-specific mutation was introduced into the PLB coding region, converting Ser16 to Ala, and mutant PLB expression was directed in the knockout background. Quantitative immunoblotting of cardiac homogenates and SR-enriched microsomal preparations showed that all the mutant PLB was inserted in the SR membranes. Transgenic mice, expressing similar levels of wild-type or mutant PLB in the heart, exhibited similar contractile parameters under basal conditions, indicating that the mutant PLB was capable of modulating contractility in a manner similar to that for wild-type PLB. However, isoproterenol stimulation was associated with much lower enhancement of the rates of contraction and relaxation in the PLB-mutant hearts compared with PLB-wild-type hearts, whereas the heart rate responses were similar between these groups. It is interesting to note that the maximal increases in contractile parameters of the PLB-mutant hearts were similar to those of PLB-knockout hearts under β-adrenergic stimulation. These findings suggest that mutation of Ser16 in PLB compromised the contribution of this phosphoprotein in the stimulatory responses of the heart to isoproterenol. Actually, when [32P]orthophosphate was included in the perfusate buffer, there was no 32P labeling of mutant PLB observed, even under maximal isoproterenol stimulation. However, in the same hearts, the degree of phosphorylation of troponin I and C-protein in the myofibrils was similar between hearts expressing mutant or wild-type PLB. Thus, cardiac phosphoproteins other than PLB were responsible for mediating the attenuated responses of the PLB-mutant hearts. To exclude the possibility that the lack of phosphothreonine in vivo was due to mutation of the adjacent Ser16 to Ala, cardiac homogenates from PLB-wild-type and PLB-mutant mice were incubated in vitro under optimal phosphorylation conditions for the Ca2+-calmodulin-dependent protein kinase. The degree of mutant PLB phosphorylation was similar to that of wild-type PLB and phosphothreonine formation was verified using the phospholamban phosphorylation site-specific antibody, indicating that the mutant PLB form was capable of being phosphorylated on Thr17.

The role of PLB phosphorylation by cAMP-dependent and Ca2+-calmodulin-dependent protein kinases has been the subject of several studies. Reports by Raeymaekers et al. (7), Tada et al. (8), and Kranias (6) indicated that the stimulatory effects of the two protein kinases on sarcoplasmic reticulum Ca2+-transport can be additive, whereas a study by Colyer and Wang (9) suggested that maximal stimulation of the Ca2+-pump occurs by PLB phosphorylation at a single site and that additional phosphorylation of the other site does not further stimulate pump activity. Furthermore, all the in vitro studies agree that phosphorylation of PLB by cAMP-dependent and Ca2+-calmodulin-dependent protein kinases occurs in an independent manner, whereas in vivo findings (3, 13, 24) indicate that phosphorylation/depolyphorylation of Thr17 occurs only subsequent to phosphorylation/depolyphorylation of Ser16 during β-adrenergic stimulation. Our findings in transgenic animals demonstrated that: (a) Thr17 in PLB cannot be phosphorylated in the absence of Ser16 phosphorylation, even under maximal isoproterenol stimulation of intact, beating hearts; and (b) phosphorylation of Thr17 in PLB does not require prior phosphorylation of Ser16 in vitro experiments. Thus, phosphorylation of Thr17 occurs independently of Ser16 phosphorylation in vitro, whereas phosphorylation of the adjacent Ser16 residue appears to be a prerequisite for in vivo phosphothreonine formation during β-agonist stimulation. This apparent discrepancy between in vivo and in vitro findings may be due to differences in the levels of calcium available to activate the SR Ca2+-calmodulin-dependent protein kinase. In vitro conditions generally include optimal calcium concentrations. However, in vivo studies, phosphorylation of Ser16 may be required to occur first and enhance the SR Ca2+ uptake rates and, thus, SR Ca2+ load. This would lead to increased Ca2+ levels released by the SR, activation of the Ca2+-calmodulin-dependent protein kinase, and phosphorylation of Thr17 in PLB. The phosphorylation and activation of the sarcoplasmic Ca2+ channels may also contribute to the increased Ca2+ levels required for in vivo phosphorylation of Thr17 in PLB (17). However, the inhibition of the SR-associated protein phosphatase 1 activity, which was suggested to be an important determinant for Thr17 phosphorylation (17), did not appear to play any role in our transgenic experiments, even under maximal isoproterenol stimulation.

The functional significance of Ca2+-calmodulin-dependent phosphorylation of PLB has been previously examined in intact cardiac myocytes (15, 25). Phosphorylation of PLB by Ca2+-calmodulin-dependent protein kinase II was suggested to increase the Vmax of the SR Ca2+-ATPase, whereas phosphorylation by protein kinase A increased the Ca2+ affinity of the pump (15). Furthermore, inhibition of Ca2+-calmodulin-dependent protein kinase II was shown to slow-down sarcoplasmic reticulum Ca2+ uptake and the decline of [Ca2+] even after inhibition of protein kinase A (25), suggesting that the phosphothreonine in PLB may be important in regulation of diastolic Ca2+ and prevention of cytosolic Ca2+ overload especially under pathophysiological conditions (17, 26, 27).

In summary, our findings indicate that the hyperdynamic PLB knockout phenotype can be reversed by reintroduction of PLB in the null background, and demonstrate the potential power of this technology in performing PLB structure-function studies in vivo. Expression of mutant PLB in which Ser16 was replaced by Ala in the knockout background indicated that the phosphorylation of Thr17 in PLB requires prior phosphorylation of Ser16 during β-adrenergic stimulation. In the absence of Ser16 phosphorylation, the degree of the stimulatory effects by β-agonists was similar to that obtained in PLB knockout hearts, suggesting that cardiac phosphoproteins other than PLB mediate these responses. Future studies using transgenic mice harboring the Thr17→Ala or Ser16-Thr17→Ala-Ala mutations in PLB will further delineate the interrelationship of dual site phosphorylation in PLB and elucidate the functional relevance of each phosphorylation site under physiological and pathophysiological conditions.
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