Chronic SR Ca\textsuperscript{2+}-ATPase Inhibition Causes Adaptive Changes in Cellular Ca\textsuperscript{2+} Transport

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Abstract—Phospholamban, the critical regulator of the cardiac SERCA2a Ca\textsuperscript{2+} affinity, is phosphorylated at Ser\textsuperscript{16} and Thr\textsuperscript{17} during β-adrenergic stimulation (eg, isoproterenol). To assess the impact of nonphosphorylatable phospholamban, a S16A, T17A double-mutant (DM) was introduced into phospholamban knockout mouse hearts. Transgenic lines expressing DM phospholamban at levels similar to wild types (WT) were identified. In vitro phosphorylation confirmed that DM phospholamban could not be phosphorylated, but produced the same shift in EC\textsubscript{50} of SERCA2a for Ca\textsuperscript{2+} as unphosphorylated WT phospholamban. Rates of basal twitch [Ca\textsuperscript{2+}], decline were not different in DM versus WT cardiomyocytes. Isoproterenol increased the rates of twitch [Ca\textsuperscript{2+}], decline in WT, but not DM myocytes, confirming the prominent role of phospholamban phosphorylation in this response. Increased L-type Ca\textsuperscript{2+} current (I\textsubscript{Ca}) density, with unaltered characteristics, was the major compensation in DM myocytes. Consequently, the normal β-adrenergic-induced increase in I\textsubscript{Ca} caused larger dynamic changes in absolute I\textsubscript{Ca} density. Isoproterenol increased Ca\textsuperscript{2+} transients to a comparable amplitude in DM and WT. There were no changes in myofilament Ca\textsuperscript{2+} sensitivity, or the expression levels and Ca\textsuperscript{2+} removal activities of other Ca\textsuperscript{2+}-handling proteins. Nor was there evidence of cardiac remodeling up to 10 months of age. Thus, chronic inhibition of SERCA2a by ablation of phospholamban phosphorylation (abolishing its adrenergic regulation) results in a unique cellular adaptation involving greater dynamic I\textsubscript{Ca} modulation. This I\textsubscript{Ca} modulation may partly compensate for the loss in SERCA2a responsiveness and thereby partially normalize β-adrenergic inotropy in DM phospholamban mice. (Circ Res. 2003;92:769-776.)

Key Words: sarcoplasmic reticulum • phospholamban • SR Ca\textsuperscript{2+}-ATPase • cardiomyocytes • transgenic mice

Contractile and relaxation in cardiac muscle are highly regulated by the sympathetic nervous system. Catecholamine-dependent activation of myocardial β-adrenergoreceptors (β-AR) increases cAMP, which activates PKA and phosphorylation of key regulatory proteins. The main regulatory phosphoproteins include the following: (1) the L-type Ca\textsuperscript{2+} channel in the sarcolemmal membrane; (2) phospholamban (PLB)\textsuperscript{2} and theryanodine receptor (RyR)\textsuperscript{3} in the sarcoplasmic reticulum (SR); and (3) troponin I (TnI)\textsuperscript{2} and C-protein in the myofibrils.\textsuperscript{4}Phosphorylation of L-type Ca\textsuperscript{2+} channels increases Ca\textsuperscript{2+} current (I\textsubscript{Ca}) enhancing the Ca\textsuperscript{2+} trigger for SR Ca\textsuperscript{2+} release, as well as increasing cellular and SR Ca\textsuperscript{2+} content. PLB phosphorylation, in response to β-AR stimulation, relieves its tonic inhibition on the Ca\textsuperscript{2+} affinity of the cardiac SR Ca\textsuperscript{2+}-ATPase (SERCA2a), resulting in increases in SR Ca\textsuperscript{2+}-uptake rates, enhanced relaxation, and increased SR Ca\textsuperscript{2+} content. PKA-dependent phosphorylation of the RyR has also been reported to increase the opening probability of this channel in lipid bilayers.\textsuperscript{5,6} On the other hand, TnI phosphorylation by PKA enhances the off-rate for Ca\textsuperscript{2+} from troponin C and may enhance relaxation rate.\textsuperscript{7}

Ex vivo and in vivo studies have suggested that PLB, I\textsubscript{Ca} and TnI are the most critical functional substrates of the β-AR pathway in the heart.\textsuperscript{8-10} Analysis of relative roles of these PKA targets has been aided by mouse models that are PLB-deficient,\textsuperscript{8} expressing phosphorylation site-specific PLB mutants,\textsuperscript{11,12} as well as expressing cardiac TnI phosphorylation site mutants\textsuperscript{13} or slow skeletal TnI in the PLB-KO.\textsuperscript{14} These studies indicated that PLB phosphorylation is a dominant factor in both the inotropic and lusitropic effect of β-AR, and that TnI phosphorylation plays a minor but significant role in the lusitropic effect.\textsuperscript{9,13,14} Although PLB cannot be phosphorylated in the PLB-KO, interpretations can be complicated by an unphysiologically activated SR Ca\textsuperscript{2+}-ATPase activity. Comparisons could be better made using endogenous TnI and the normal level but completely nonphosphorylatable PLB.
PLB phosphorylation is reduced in heart failure (human and animal), and this may be a contributing factor to the deteriorated function. However, long-term effects of dephosphorylated PLB on SR Ca\(^{2+}\) cycling and cardiac function are unknown. Thus, we developed a novel mouse model with a nonphosphorylatable double mutant form of PLB (PLB-DM, where both Ser\(^{16}\) and Thr\(^{17}\) are replaced by Ala) into the PLB-KO background (ie, creating tonic SERCA2a inhibition). This PLB-DM should chronically inhibit SERCA2a and limit the heart’s normal ability to increase SR Ca\(^{2+}\)-ATPase activity in response to sympathetic activation.

The specific questions addressed were as follows: (1) What are the physiological and pathological consequences of chronic SERCA2a inhibition by PLB-DM? (2) How are maximal isoproterenol effects on myocyte Ca\(^{2+}\) transients and contractility changed in PLB-DM mice? (3) What is the relative contribution of PLB, versus other phosphoproteins, in cardiac myocytes during \(\beta\)-AR stimulation? (4) What compensations in myocyte Ca\(^{2+}\) regulation occur when phosphorylation of PLB is unavailable? Our data indicate that there were no cardiac histological alterations up to 10 months of age, although the \(\beta\)-AR–induced acceleration of myocyte relaxation and [Ca\(^{2+}\)]\(_{i}\) decline were completely absent in the PLB-DM myocytes. On the other hand, \(I_{\text{Ca}}\) density was enhanced, which may provide an alternative (or surrogate) pathway for \(\beta\)-AR to exert dynamic control over cardiac function.

Materials and Methods

In Vitro Coexpression Studies

Rabbit PLB wild-type (WT) or PLB-DM and SERCA2a cDNAs were cotransfected into human embryonic kidney cell line 293 (HEK-293), and microsomal Ca\(^{2+}\)–transport activity was assayed.

Generation of Transgenic Mice

PLB-KO 129SvJCF-1 mice\(^{6}\) were mated with transgenic FVB/N mice expressing the S16A, T17A mutant murine PLB cDNA, specifically in cardiac muscle.\(^{17}\) F\(_{1}\) heterozygous PLB offspring carrying the PLB mutant transgene were bred with PLB-KO mice to obtain F\(_{2}\) pups. The PLB-KO offspring carrying the PLB mutant transgene were selected to backcross with PLB-KO mice to F\(_{6}\) generation (see expanded Materials and Methods section in the online data supplement available at http://www.circresaha.org). Handling and maintenance of animals was approved by the ethics committee of the University of Cincinnati. Eight- to 13-week-old mice of either sex were used for the studies.

Quantitative Western Blot Analysis

Quantitative immunoblotting of cardiac homogenates and SR-enriched microsomes\(^{17}\) was used to assess the levels of PLB, SERCA2a, ryanodine receptor, \(\alpha\)- and \(\beta\)-myosin heavy chain isoforms, calsequestrin, calreticulin, and TnI in WT and PLB-DM mouse hearts (see online data supplement).

SR Ca\(^{2+}\) Uptake

Mouse hearts were frozen in liquid nitrogen and later assayed for initial rates of oxalate-supported Ca\(^{2+}\) uptake\(^{6}\) (see on-line supplement).

In Vitro Phosphorylation

PKA or CaMK phosphorylation was performed\(^{11,12}\) using 45 \(\mu\)g of protein via 15% SDS-PAGE and autoradiography. Labeling with \(^{32}\)P-

was assessed using a PhosphorImager and ImageQuant software from Molecular Dynamics.

Left Ventricular Myocyte Measurements

Cell contraction was measured by video edge detection and intracellular-free [Ca\(^{2+}\)]\(_{i}\) ([Ca\(^{2+}\)]\(_{i}\)). This was measured using fluo-3AM (see online data supplement). Cells were perfused with normal Tyrode’s (NT) solution (in mmol/L): NaCl 140, KCl 4, MgCl\(_{2}\) 1, CaCl\(_{2}\) 1, and HEPES 10, with pH 7.4, at 23°C. Twitches (steady state at 0.5 Hz) were field stimulated. To assess cytosolic Ca\(^{2+}\) removal by non-SR pathways and SR Ca\(^{2+}\) content, 10 mmol/L caffeine was applied for 10 seconds in NT. Isoproterenol (ISO; 100 nmol/L) was used to activate \(\beta\)-AR. Under basal conditions, PLB phosphorylation was less than 5% at either P-Ser16 or P-Thr17, compared with the levels obtained on ISO stimulation (100%), assessed by the PLB phosphorylation site specific antibodies.

Twitch and caffeine-induced Ca\(^{2+}\) transient decline were fit with single exponential declines, described by time constants (\(\tau\)) or rate constants (\(\lambda\)). SR Ca\(^{2+}\) content was calculated from caffeine-induced Ca\(^{2+}\) transient amplitude (after converting [Ca\(^{2+}\)]\(_{i}\), values to total cytosolic [Ca\(^{2+}\)] using buffering constants\(^{18}\)).

\(^{32}\)P-Labeling of Isolated Ventricular Myocyte Proteins

Isolated myocytes were labeled with \(^{32}\)P and stimulated with ISO.\(^{9}\) After boiling and electrophoresis, the degree of \(^{32}\)P-incorporation was assessed (see online data supplement).

Electrophysiology of Isolated Left Ventricular Myocytes

Ventricular myocytes were studied using whole-cell patch clamp.\(^{19}\) \(I_{\text{Ca}}\) was recorded using Na\(^{+}\) and K\(^{+}\)-free external solution (to prevent contaminating Na and K channel currents) containing (in mmol/L): CaCl\(_{2}\) 2, MgCl\(_{2}\) 1, TEA-Cl 135, 4-aminopyridine 5, glucose 10, and HEPES 10 (pH 7.3). The pipette solution contained (in mmol/L): Cs-aspartate 100, CsCl 20, MgCl\(_{2}\) 1, MgATP 2, GTP 0.5, EGTA 5, and HEPES 5 (pH 7.3). \(I_{\text{Ca}}\) was activated by depolarization pulses (0.1 Hz) from a holding potential of \(-50\) mV. To measure ISO-induced \(I_{\text{Ca}}\) response, 10 mmol/L BAPTA replaced pipette EGTA to minimize Ca\(^{2+}\)-dependent inactivation. Outward Na\(^{+}\)–Ca\(^{2+}\) exchanger current was activated (with membrane potential held at \(-40\) mV) by rapidly switching the external solution to one in which equimolar LiCl was substituted for NaCl.\(^{20}\)

Force Measurements of Skinned Fiber Bundles

Skinned fiber bundles were isolated and the [Ca\(^{2+}\)]-force relations (at sarcomere length of 2.0 \(\mu\)m) were measured\(^{21}\) and fit to the Hill equation to derive the \(-\log [\text{Ca}^{2+}]\) at half-maximal activation (pCa\(_{50}\)) and Hill coefficient.

Echocardiographic Measurements

Echocardiography studies were performed for noninvasive assessment of left ventricular function and dimensions, as described previously.\(^{22}\)

Statistics

Data are presented as mean\(\pm\)SEM. Statistical analysis was performed using linear regression and ANOVA. Mean data were compared using \(t\) tests, Newman-Keuls rank test, or Mann-Whitney test, as appropriate, and differences were considered significant if \(P<0.05\).

Results

In Vitro Studies on PLB Double Mutant

Previous work in expression systems showed that single site-mutagenesis of Ser\(^{16}\) or Thr\(^{17}\) to Ala in PLB did not alter the interaction between PLB and SERCA2a. In the present
study, we measured the in vitro effect of mutating both sites to Ala (PLB-DM).

Figure 1 shows the [Ca\(^{2+}\)]-dependence of Ca\(^{2+}\) transport in microsomes isolated from HEK-293 cells transfected with SERCA2a alone or in combination with wild-type PLB (PLB-WT) or PLB-DM. Western blot analysis indicated similar SERCA2a expression in each case, and the two PLB forms were expressed equally. Therefore, the PLB/SERCA2a ratio was the same in cells expressing WT or mutant PLB. The apparent Ca\(^{2+}\) affinity of SERCA2a was reduced significantly and similarly when SERCA2a was coexpressed with either WT PLB or mutant PLB (SERCA2a alone, \(K_m = 0.26 \pm 0.02 \ \text{mmol/L}\); +WT-PLB, \(0.69 \pm 0.04 \ \text{mmol/L}\); +PLB-DM, \(0.76 \pm 0.04 \ \text{mmol/L}\)). Thus, mutating both Ser\(^{16}\) and Thr\(^{17}\) to Ala do not alter SERCA2a inhibition by PLB in vitro.

Transgenic Mice With Cardiac Expression of PLB Double Mutant

To assess the functional significance of dual site PLB phosphorylation in vivo, nonphosphorylatable PLB was introduced into PLB-KO mouse hearts. Genomic tail DNA from transgenic mice (F6 generation) was extracted, and PLB mutant cDNA was amplified by PCR and sequenced. Sequence analysis confirmed the presence of the expected mutant PLB transgene. WT mice, with the same genetic background as PLB-KO mice, were used as controls in the following studies.

Western blots of cardiac homogenates processed in parallel were used to analyze PLB expression levels. Figure 2A shows that PLB expression in lines 72 and 78 was 132% and 139% of that in WT. To assess SR localization of the mutant PLB, SR-enriched microsomal fractions (isolated by differential centrifugation) were analyzed by Western blot. Levels of PLB were similar between cardiac homogenates and microsomal preparations in both WT and transgenics (Figure 2B), indicating that mutant PLB targeted correctly to the SR.

We also found that the levels of SERCA2a protein expression in both cardiac homogenates (Figure 2C) and SR-enriched microsomes (Figure 2D) were similar between transgenic and WT controls.

In Vitro Phosphorylation of PLB

To determine whether the expressed mutant PLB could be phosphorylated in vitro, cardiac homogenates were incubated with [\(\gamma\)-32P] ATP and PKA catalytic subunit, or Ca\(^{2+}\) plus calmodulin. Autoradiographs (Figure 3) show that 32P-incorporation into PLB was only detected in WT hearts. Thus, mutant PLB could not be phosphorylated in vitro by either PKA or CaMK.

Sarcoplasmic Reticulum Ca\(^{2+}\) Uptake

Initial rates of SR Ca\(^{2+}\) uptake were measured in cardiac homogenates at different [Ca\(^{2+}\)]. The EC\(_{50}\) values in PLB-DM hearts were slightly higher (0.34 \pm 0.01 mmol/L in line 72; 0.32 \pm 0.02 mmol/L in line 78) than those in WT control hearts (0.25 \pm 0.01 mmol/L), consistent with the higher levels of PLB in PLB-DM hearts. There were no differences in the maximal Ca\(^{2+}\)-uptake velocity between WT (58.8 \pm 9.6 nmol/mg per min) and PLB-DM hearts (49.1 \pm 2.9 and 51.1 \pm 3.7 nmol/mg per min in lines 72 and 78, respectively). Thus, mutant PLB functionally interacts with SERCA2a in vivo. Because both transgenic lines expressed similar levels of PLB-DM protein and exhibited no significant differences in the EC\(_{50}\) values for Ca\(^{2+}\) uptake, line 78 was used for further functional studies.

Cardiomyocyte Ca\(^{2+}\) Transients and Contractions

Figures 4A and 4B show that 100 nmol/L ISO increased Ca\(^{2+}\) transient amplitude in both WT and PLB-DM myocytes under steady state conditions (0.5 Hz). Normalized traces (Figures 4C and 4D) emphasize the prominent acceleration of [Ca\(^{2+}\)], decline in WT, but not PLB-DM. In pooled data, ISO
reduced $\tau$ by $\approx 50\%$ in WT, but did not change $\tau$ in PLB-DM (Figure 4E). These results show that PLB is essential in the enhanced rate of $[Ca^{2+}]$, decline and SR $Ca^{2+}$ uptake with ISO. However, $Ca^{2+}$ transient amplitudes were increased even in PLB-DM myocytes. Thus, tonic SERCA2a inhibition by nonphosphorylatable PLB does not prevent the inotropic effect of ISO.

Myocyte contraction results agree with the $[Ca^{2+}]$ results. Twitch contraction amplitude of WT and PLB-DM myocytes was similar under control conditions and both increased comparably with ISO (Figure 5A). The maximum rate of control twitch contraction was slightly higher in PLB-DM than WT (not shown), consistent with the tendency toward higher $\Delta[Ca^{2+}]$, in PLB-DM (Figure 4F). With ISO, relaxation $t_{1/2}$ decreased (Figure 5B) and maximal relaxation rate (not shown) increased only in WT myocytes, while no change was observed in PLB-DM myocytes. These data indicate that PLB-DM myocytes have adapted mechanisms to increase contractility on ISO stimulation, presumably to compensate for the inability of PLB to be phosphorylated during sympathetic stimulation.

SR $Ca^{2+}$ content was also assessed by caffeine-induced $Ca^{2+}$ transients on termination of steady state stimulation at 0.5 Hz. Without ISO, mean SR $Ca^{2+}$ content was higher in PLB-DM versus WT myocytes (100.1 ± 15.2 versus 64.3 ± 13.0 μmol/L cytosol; n=12, 18), but the difference was not significant ($P=0.08$), presumably due to high cell-to-cell variation in this unpaired comparison. ISO increased SR $Ca^{2+}$ content significantly in both PLB-DM and WT myocytes (by 22% and 24%, respectively; n=12, 9). Thus, ISO can still increase SR $Ca^{2+}$ load, even when PLB cannot be phosphorylated.

Altered SR $Ca^{2+}$ leak could also alter SR $Ca^{2+}$ load. Resting $Ca^{2+}$ sparks are indicative of this leak rate. We measured $Ca^{2+}$ spark frequency and characteristics by confocal microscopy in both PLB-DM and WT myocytes. There was no major difference in $Ca^{2+}$ spark frequency or duration, although there was a slight increase in $Ca^{2+}$ spark amplitude and a counterbalancing reduced spatial spread. Thus, there was little alteration in SR $Ca^{2+}$ leak rate between PLB-DM and WT myocytes.

**Ca$^{2+}$ Removal Systems**

The rate constant of twitch $[Ca^{2+}]$, decline ($\lambda_{Tw}$) was faster after ISO in WT, but unchanged in PLB-DM (Figure 5C). The rate constant of $[Ca^{2+}]$, decline during caffeine exposure ($\lambda_{Ca}$) reflects primarily $Ca^{2+}$ removal via Na$^{+}$-$Ca^{2+}$ exchange, and this was $\approx 10$ times slower than $\lambda_{Tw}$ but comparable between WT and PLB-DM (Figure 5D). We conclude that Na$^{+}$-$Ca^{2+}$ exchange function is unaltered in PLB-DM myocytes and unaffected by ISO. The rate constant of $[Ca^{2+}]$, decline attributed to SERCA2a ($\lambda_{SA}$) is inferred as $\lambda_{SA} = \lambda_{Tw} - \lambda_{Ca}$. In WT, $\lambda_{SR}$ was significantly increased by ISO, but no difference was seen in PLB-DM. Thus, SERCA2a function could not be increased without PLB phosphorylation. Finally, the fraction of $Ca^{2+}$ removal due to the SR ($\lambda_{SA}/\lambda_{Tw}$) was $\approx 88\%$ in both wild-type and PLB-DM myocytes without ISO (Figure 5E). With ISO, this fraction increased (to 94%) only in WT.
Phosphorylation of PLB in Cardiac Myocytes

Possible compensatory phosphorylation of the mutant PLB on Ser16, the protein kinase C site, was examined in myocytes labeled with [32P]-orthophosphate. Autoradiographs from WT myocytes exhibited low levels of basal PLB phosphorylation, which increased 2.3-fold upon ISO exposure (Figure 6A). However, the PLB-DM myocytes exhibited no PLB phosphorylation before or after ISO. TnI phosphorylation increased similarly in WT and PLB-DM.

Compensatory Mechanisms

Quantitative immunoblots were used to assess possible compensatory changes in Ca2+ handling proteins in PLB-DM mice. The protein expression levels in PLB-DM (versus WT) were unaltered for SERCA2a (109 ± 8%), calsequestrin (101 ± 8%), calreticulin (101 ± 6%), and ryanodine receptor (106 ± 5%). Also, no alterations were seen in Tnl or α-myosin heavy chain in PLB-DM hearts versus WT. Expression of β-myosin heavy chain was barely detectable in either WT or PLB-DM hearts.

Myofilament Ca2+ sensitivity was assessed in skinned fiber preparations. Figure 6B shows that [Ca2+]-force curves for PLB-DM and WT preparations were identical (pCa50 was 5.70 ± 0.01 for mutant and 5.69 ± 0.01 for WT preparations). Thus, myofilament Ca2+ sensitivity was unaltered in PLB-DM.

Whole-cell ICa was measured using patch-clamp. Membrane capacitance was not different in PLB-DM (110 ± 3 pF, n = 65) versus WT myocytes (115 ± 4 pF, n = 35). The Eca-dependence of ICa was comparable in WT and PLB-DM (Figures 7A through 7C), but peak ICa density was 25% higher in PLB-DM versus WT (PLB-DM: 9.5 ± 0.3 A/F, n = 56; WT: 7.6 ± 0.4 A/F, n = 35; P < 0.05). Figures 7B, inset, and 7F showed that ICa decline during the pulse was significantly faster (at +10 mV) in PLB-DM (15.5 ± 0.8 ms, n = 60) versus WT (20.9 ± 0.9 ms, n = 18) myocytes. Despite the faster ICa inactivation in PLB-DM, integrated ICa was still significantly higher in PLB-DM versus WT (Figure 7E). The more rapid inactivation could have been secondary to higher ICa in PLB-DM (and consequent SR Ca2+ loading and release). Thus, we measured ICa inactivation in the presence of 10 μmol/L ryanodine. Ryanodine significantly slowed ICa inactivation in both PLB-DM (33.1 ± 2.5 ms, n = 7) and WT (35.7 ± 1.9 ms, n = 5) cells, such that these were no longer different. ISO produced almost identical percent increases in ICa in both cell types (Figure 7D). Thus, there is no intrinsic difference in ICa characteristics, there is simply more ICa in PLB-DM. The expression level of L-type Ca2+ channel α1C subunit was also increased by 20 ± 3% (n = 4; data not shown), consistent with the increased ICa being due to more Ca2+.

Figure 5. Myocyte contraction and Ca2+ transient analysis. Twitch contractions at 0.5 Hz steady state were assessed for amplitude (A) and relaxation half-time, t1/2 (B; n = 33, 13, 29, and 17 for the 4 bars). Rate constants for [Ca2+]i decline during twitches (λinj) and the inferred fractional contribution of SR to twitch [Ca2+]i decline (λSR/λinj) are shown (C-E; n = 18, 9, 14, and 15 for the 4 bars; *P < 0.05).

Figure 6. Phosphorylation in intact myocytes and myofilament Ca2+ sensitivity. A, Representative autoradiogram showing 32P incorporation into myocytes (~ISO) in the presence of 1 mmol/L CaCl2 and 0.15 mCi [32P]-orthophosphate. Samples were boiled, run on SDS-PAGE (4% to 20% gradient gels), and autoradiographed. PLB indicates PLB monomer (on boiling); MLC, myosin light chain; cTnl, cardiac TnI. B, Myofilament Ca2+ sensitivity (in the absence of cAMP/PKA) at sarcomere length = 2 μm. Force was normalized to value at 3 μmol/L Ca2+ (mean ± SEM, n = 9 each, from 6 and 5 hearts).
Concentration-dependent to basal WT (A) and PLB-DM (B) myocytes. Holding potential was I
(Inset, normalized superimposed traces at -10 mV and pulses were to the indicated test potentials at 0.1 Hz
with Li
. When the peak outward current shift was normalized to cell capacitance, Na
-Ca
 exchange current density was comparable for PLB-DM and WT mice, consistent with unaltered [Ca 2+
] i decline rates are unaffected by
affinity of the myofilaments (due to phosphorylation of PLB and TnI, respectively). 10,13,14 Studies
in PLB-KO mice have demonstrated that PLB phosphorylation is critical for the vast majority of the β-AR lusitropic effect. 9 Our results here with PLB-DM confirm this. Indeed, twitch relaxation and [Ca 2+
] decline rates are unaffected by ISO in either PLB-KO or PLB-DM myocytes. On the other hand, the role of TnI is more apparent when force (or pressure) is developed, 9,14 and a somewhat larger fractional contribution by TnI phosphorylation was inferred using mice with mutant, nonphosphorylatable cardiac TnI. 13 A unique advantage of the PLB-DM is that the SR Ca 2+
-ATPase is tonically inhibited by PLB, as in the normal basal WT state (in contrast to the tonic maximally active state in the PLB-KO). In balance, PLB phosphorylation is critical for the β-AR–induced lusitropic effect, with TnI playing a complementary role.

Compensatory Changes in PLB-DM Mice
The PLB-DM heart cannot enhance the Ca 2+
-ATPase during normal sympathetic tone and modulation. We explored compensations that may allow dynamic modulation of cardiac myocyte contractility (and perhaps explain the lack of pathophysiology). Interestingly, there was a small increase in the rates of contraction and a trend toward higher basal twitch Ca 2+
 transient amplitude and SR Ca 2+
 load in the PLB-DM myocytes. However, there were no alterations in the protein expression levels of SERCA2a, ryanodine receptor, calsequestrin, or calreticulin. Moreover, there was no increase in V max of SR Ca 2+
 uptake in the PLB-DM, ruling out any contribution of CaMK-dependent channels (rather than altered regulation). The enhanced I ca
 could be an important compensatory mechanism, in the PLB-DM, allowing a greater absolute increase in I ca
 density with β-AR activation. This could cause the increased SR Ca 2+
 content and enhanced SR Ca 2+
 release during twitches with ISO (even without phosphorylatable PLB).

Outward Na
-Ca
 exchange current was induced by replacing external Na
 with Li
. When the peak outward current shift was normalized to cell capacitance, Na
-Ca
 exchange current density was comparable for PLB-DM and WT (1.03 ± 0.05 versus 0.93 ± 0.07 A/F, respectively, n = 40, 20). Thus, Na
-Ca
 exchange function was not altered in PLB-DM myocytes, consistent with unaltered [Ca 2+
] decline during caffeine exposure (Figure 5D).

In Vivo Assessment of Cardiac Function
Echocardiographic studies revealed no significant differences in fractional shortening or left ventricular dimensions between PLB-DM and WT mice at 10 to 12 weeks of age. Furthermore, there were no apparent differences during aging of PLB-DM mice to 10 months (Table).

Discussion

PLB phosphorylation is a major determinant of β-AR contractile response in the heart, 8,9,11,12 and basal phosphorylation can be compensatory during depressed cardiac function. Thus, we expected that the absence of phosphorylatable PLB in the novel PLB-DM mouse would cause a pathological cardiac phenotype. Surprisingly, this tonic SERCA2a inhibition, by comparable levels of PLB-DM (versus WT), caused no pathophysiological phenotype up to 10 months of age. Rather, the inability to regulate PLB by phosphorylation seemed to be partially compensated by greater I ca
 density (which can be modulated by β-AR). The enhanced I ca
 may allow animals to maintain a dynamic β-AR–mediated lusitropic response, even though the lusitropic effect is greatly diminished. Notably, the adaptation to nonphosphorylatable PLB does not involve altered expression or function of SERCA2a or Na
-Ca
 exchange. This unique animal model is also a valuable tool for studying regulation of other Ca 2+
 transport systems without the complication of the prominent PLB effects (see following sections).

β-Adrenergic Lusitropic Effect
Ventricular twitch relaxation and [Ca 2+
], decline are greatly accelerated by β-AR stimulation (lusitropic effect; Figures 4E and 5B). The mechanism involves acceleration of SR Ca 2+
 uptake and reduced Ca 2+
 affinity of the myofilaments (due to phosphorylation of PLB and TnI, respectively). 10,13,14 Studies in PLB-KO mice have demonstrated that PLB phosphorylation is responsible for the vast majority of the β-AR lusitropic effect. 9 Our results here with PLB-DM confirm this. Indeed, twitch relaxation and [Ca 2+
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phosphorylation of SERCA2a. Myofilament Ca\textsuperscript{2+} sensitivity was also unaltered in the PLB-DM (although it can be modulated by Tnl phosphorylation).\textsuperscript{4,7,24} Thus, with slightly reduced SR Ca\textsuperscript{2+}-ATPase function in the PLB-DM (see previous section), some other compensatory change in Ca\textsuperscript{2+} transport may have indirectly prevented a decline in SR Ca\textsuperscript{2+}.

The Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger normally competes with the SR Ca\textsuperscript{2+}-ATPase for Ca\textsuperscript{2+} during relaxation, and reduced Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange function could increase SR Ca\textsuperscript{2+} load (all other things being equal). The converse is observed in heart failure, where upregulated Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange can lower SR Ca\textsuperscript{2+} load and cause contractile dysfunction.\textsuperscript{25} However, in the PLB-DM there was no change in either Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange current or its ability to cause relaxation and [Ca\textsuperscript{2+}], decline during caffeine exposure.

On the other hand, $I_{Ca}$ density was significantly increased in the PLB-DM versus WT, similar to previous observations in mice overexpressing a superinhibitory PLB-mutant (V49G).\textsuperscript{26} Such an increase would enhance both the trigger for SR Ca\textsuperscript{2+} release as well as the SR Ca\textsuperscript{2+} load (by increasing Ca\textsuperscript{2+} influx per beat). $I_{Ca}$ is normally the other major functional target for PKA (other than PLB), with respect to enhancing Ca\textsuperscript{2+} transients in the β-AR inotropic response. Uregulated $I_{Ca}$ may be a unique compensatory mechanism, in that it partially replaces the β-AR–induced modulation of PLB with an alternative PKA target (enhanced $I_{Ca}$). Thus, the enhanced $I_{Ca}$ can produce the higher SR Ca\textsuperscript{2+} content and release in PLB-DM compared with WT myocytes. Moreover, the greater absolute increase in $I_{Ca}$ with ISO may directly offset (in part) the loss of PLB phosphorylation in the dynamic β-AR inotropic response. Indeed, there was still a very substantial inotropic effect of β-AR stimulation (Figures 4F and 5A), although the lusitropic effect was abolished (Figures 4E and 5B) in the PLB-DM. Notably, the relative ISO stimulation of Ca\textsuperscript{2+} transients in PLB-DM (versus WT) is much better maintained than we have seen for the PLB-KO mouse where $I_{Ca}$ was not increased.\textsuperscript{9,19} Although the increase in $I_{Ca}$ seems clearly to be a compensatory factor in the PLB-DM, we cannot yet rule out other prospects (eg, ryanodine receptor or sorcin properties).

The larger $I_{Ca}$ and SR Ca\textsuperscript{2+} load in the PLB-DM may work synergistically to enhance the fraction of SR Ca\textsuperscript{2+} release, which may also accelerate $I_{Ca}$ inactivation.\textsuperscript{27} Consistent with this notion, when ryanodine inhibited SR Ca\textsuperscript{2+} release, the difference in $I_{Ca}$ inactivation (PLB-DM versus WT) was abolished. Thus, increased $I_{Ca}$ may be the crucial compensatory mechanism that keeps twitch and Ca\textsuperscript{2+} transient amplitudes from declining in the face of chronically inhibited SR Ca\textsuperscript{2+}-ATPase.

Reduced levels of PLB phosphorylation (and higher levels of type-1 phosphatase expression) have been implicated in contractile dysfunction in failing human hearts.\textsuperscript{15,28} In this context, we expected that PLB-DM mice might exhibit depressed Ca\textsuperscript{2+} transients and cardiac function. However, in vivo cardiac function, assessed by echocardiography, revealed no significant differences between PLB-DM and age-matched WT controls even up to 10 months of age. Furthermore, no signs of cardiac hypertrophy were detected at the organ and cellular levels. We conclude that the detrimental effect of chronic SERCA2a inhibition by non-phosphorylatable PLB is ameliorated by compensatory mechanisms (eg, enhanced $I_{Ca}$) during development. These results in our PLB-DM model are different from recent findings in another transgenic model with cardiac overexpression of the PLB-Arg9Cys human mutant, which traps protein kinase A and prevents PLB phosphorylation.\textsuperscript{29} Chronic inhibition of SERCA2a in the PLB-R9C hearts was associated with dilated cardiomyopathy and terminal heart failure at 4 to 8 months of age.\textsuperscript{29} The reason for these apparent different phenotypes between our PLB-DM and the PLB-R9C models may include differences in the following: (1) compensatory mechanisms; (2) the degree of PLB expression levels (overexpression of PLB-R9C versus normal levels of PLB-DM); and (3) the mouse genetic background. Alternatively, lack of PLB phosphorylation may not be the only mechanism underlying the phenotype of the PLB-R9C mutant, which sequesters PKA and may prevent phosphorylation of other key phosphoproteins.

| M-Mode Echocardiography Measurements of Basal Left Ventricular Systolic Function |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
|                   | 3 Months          |                   | 10 Months         |                   |                   |
|                   | WT (n=5)          | PLB-DM (n=4)      | WT (n=5)          | PLB-DM (n=4)      |                   |
| HR, bpm           | 385±17            | 389±32            | 456±5             | 447±18            |                   |
| EDD, mm           | 3.7±0.2           | 3.8±0.2           | 3.9±0.2           | 4.0±0.2           |                   |
| ESD, mm           | 2.4±0.2           | 2.4±0.1           | 2.4±0.01          | 2.3±0.1           |                   |
| FS, %             | 35.7±1.5          | 36.3±1.9          | 39.5±1.0          | 41.6±2.5          |                   |
| EDbWT, mm         | 0.59±0.03         | 0.53±0.01         | 0.70±0.03         | 0.72±0.03         |                   |
| EDPWT, mm         | 0.70±0.02         | 0.64±0.03         | 0.71±0.04         | 0.78±0.05         |                   |
| h/r               | 0.35±0.02         | 0.31±0.01         | 0.36±0.03         | 0.38±0.01         |                   |

$n$ indicates the number of mice; HR, heart rate; EDD, end-diastolic dimension; ESD, end-systolic dimension; FS, fractional shortening; EDbWT, end-diastolic septal wall thickness; EDPWT, end-diastolic posterior wall thickness; and h/r, wall thickness to cavity radius ratio. Values represent mean±SEM with $P$ value assessed by the Student $t$ test between 3 months and 10 months and wild-type and PLB mutant mice. $^*P<0.05$ vs age-matched wild-type.

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PLB-DM as a New Investigative Tool

Because PLB phosphorylation is so important in cardiac β-AR effects, preventing phosphorylation, while retaining the normal inhibitory role of PLB on SR Ca\(^{2+}\)-ATPase, has distinct advantages. For example, we have already used these PLB-DM myocytes to more clearly evaluate the effects of ryanodine receptor versus PLB phosphorylation in the cellular environment. These studies suggest that the main effects of PKA on SR were mediated by PLB and not ryanodine receptor phosphorylation.

In summary, this is the first study to replace native cardiac PLB with a nonphosphorylatable form in vivo. This dual site PLB phosphorylation mutant abolished the lusitropic effect of β-AR stimulation, without greatly altering basal contraction, relaxation, or Ca\(^{2+}\) transients. A key compensatory mechanism may be enhanced \(I_{ca}\) density. This could both offset the reduced SR Ca\(^{2+}\)-uptake rate in the PLB-DM (retaining higher SR Ca\(^{2+}\) load) and increase the fraction of SR Ca\(^{2+}\) released. Since \(I_{ca}\) is strongly enhanced by β-AR activation, this compensation may also help the heart retain the dynamic β-AR modulation necessary during sympathetic activation.

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Chronic SR Ca\textsuperscript{2+}-ATPase Inhibition Causes Adaptive Changes in Cellular Ca\textsuperscript{2+} Transport

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Methods

*Generation of Transgenic Mice Expressing a PLB Mutant in the Null Background*

To identify different genotypes, PCR analysis of genomic DNA from biopsied tails was performed. Identification of mice expressing the PLB-DM required the use of two specific S16A,T17A transgene oligonucleotide primers: 5’CACATAGAAGCCTAGCCCACAC3’ and 5’GATTCTGACGTGCTTGAGG3’, which produced a 150 bp band on 1.2% agarose gels. Mice harboring one or both PLB-targeted alleles were identified, using a set of oligonucleotide primers complementary to the neo cassette (5’TCTCTTGCTTTACGGTATC3’) and the 3’ untranslated region of PLB (5’TGTGGGTGCAAAGTTAGGC3’). This primer set produced a 450 bp band, which was visualized on 1.2% agarose gels. Identification of mice expressing the endogenous PLB required a specific set of oligonucleotide primers that were complementary with a portion of the 3’ untranslated region of PLB (5’GTGGTCACTACTGTCAGAAC3’ and 5’TGTGGGTTGCAAAGTTAGGC3’). This set of primers produced a 450 bp band, which was visualized on 1.2% agarose gels. For each PCR reaction performed, a specific set of oligonucleotide primers were used as an internal control (5’TCCTCAAAGATGCTCAATTAG3’ and 5’GTAACTCACTCATGCAAAGT3’), which recognized the endogenous single copy mouse TSHγ gene (thyrotropin stimulating hormone γ subunit) and produced a 380 bp band on 1.2% agarose gels. WT 129SvJ/CF-1 mice were used as controls. The handling and maintenance of animals was approved by the ethics committee of the University of Cincinnati. Eight to 13-week-old mice of either sex were used for the following studies.

*Quantitative Western Blot Analysis*

Quantitative immunoblotting of cardiac homogenates and microsomes enriched in SR membranes was carried out as follows. A pool of three to six hearts was prepared from either WT or PLB-DM mice and homogenized at 4°C in a buffer, composed of 10 mM imidazole (pH 7.0), 300 mM sucrose, 1 mM dithiothreitol (DTT), 1 mM sodium metabisulphite and 0.3 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates and microsomes were incubated with equal volumes of loading buffer (20% glycerol, 2% β-mercaptoethanol, 4% SDS, 0.001% bromophenol blue, and 130 mmol/L Tris-HCl (pH 6.8)) for 1 hr at room temperature. For all proteins assessed, preliminary experiments were performed to obtain the correct linear range for quantitation in Western blot analyses. Electrophoretic separation and transfer of proteins were performed according to a previous study. The blots were subsequently washed 3 times with Tris-buffered saline and incubated with their respective mouse monoclonal or rabbit polyclonal secondary-labeled antibodies for 3 hr at room temperature. For peroxidase-labeled secondary antibodies (Amersham), 0.5% milk buffer was used and for 35S-labeled secondary antibodies (0.09 Ci, Amersham) 2% milk buffer was used. The degree of labeling was visualized and quantified using the PhosphorImager and the ImageQuant analysis software.
SR Ca$^{2+}$-Uptake

The frozen hearts were powdered and homogenized in (in mM) 50 KH$_2$PO$_4$, 10 NaF, 1 EDTA, 300 sucrose, 0.3 PMSF and 0.5 DTT (pH 7.0). The initial rates of Ca$^{2+}$-uptake in whole-heart homogenates were obtained by regression analysis.3

Left Ventricular Myocyte Preparation and Measurements

Mouse cardiomyocytes were prepared as described previously.4 Cells were plated on laminin coated perfusion chambers for recording. Intracellular free Ca$^{2+}$ ([Ca$^{2+}]_i$) was measured using fluo-3 AM where: $[\text{Ca}^{2+}]_i = K_d \cdot R/((K_d/([\text{Ca}^{2+}]_{rest})+1–R)$ where $[\text{Ca}^{2+}]_{rest} = 100$ nM, $K_d = 1100$ nM, and $R$ is emitted fluorescence divided by the resting fluorescence (after background subtraction). Cell contraction as % of resting cell length (RCL) was recorded using a video edge detector. Cells were superfused with a normal Tyrode solution (in mM): NaCl 140, KCl 4, MgCl$_2$ 1, CaCl$_2$ 1, and HEPES 10, with pH 7.4). Twitches (steady state at 0.5 Hz) were field stimulated. To assess cytosolic Ca$^{2+}$ removal by non-SR pathways and SR Ca$^{2+}$ content, 10 mM caffeine was applied for 10 sec in normal Tyrode solution. Isoproterenol (ISO; 100 nM) was used to activate ?-AR. All measurements were done at 23°C, to minimize run-down of cell condition and I$_{Ca}$, and also to allow comparison of the data with other studies in the field.

Ca$^{2+}$ transient amplitude ($[\text{Ca}^{2+}]_i$) was calculated and the post-peak decay time constant was obtained by fit to $[\text{Ca}^{2+}]_i(t) = A \cdot \exp((t-t_{peak})/?)$, which in mice is dominated by SR Ca$^{2+}$-uptake. However, Na$^+$/Ca$^{2+}$ exchange and slow processes also contribute to twitch [Ca]$^{2+}$ decline.5 To better isolate SR Ca$^{2+}$-uptake, we used rate constants (=1/?) to describe the rate of removal of Ca$^{2+}$ by all processes (?_T_w) and then subtract the rate due to the non-SR processes (?_Caff), to obtain the rate constant for SR Ca$^{2+}$-uptake (?_SR). These rate constants can be used to estimate the relative contributions of the SR Ca$^{2+}$-ATPase or Na/Ca exchange to twitch [Ca$^{2+}]_i$ decline (as ?_SR/ ?_T_w and ?_Caff/ ?_T_w respectively).6 This analysis is less rigorous than one we have used before involving numerical reconstruction of Ca$^{2+}$ fluxes via each system during a twitch Ca$^{2+}$ transient.5,7 However, we generally find very comparable results.6

32P-Labeling of Isolated Ventricular Myocyte Proteins

Isolated mouse ventricular myocytes were labeled with $^{32}$P and stimulated with isoproterenol (ISO).8 Half of the $^{32}$P-labeled myocytes from each group were treated with ISO for 5 min and the other half were processed in parallel. All samples were boiled in microcentrifuge tubes for 5 min to dissociate oligomeric PLB to its monomeric form, before SDS–PAGE (using a 4-20% polyacrylamide gradient gel). An aliquot of samples containing 50 ?g myocyte protein, as determined by the Bio–Rad protein assay, was applied to each well. The degree of labeling in SDS–PAGE gels was assessed using a PhosphorImager and the ImageQuant software from Molecular Dynamics.

Skinned Fibers

Measurements of the Ca$^{2+}$-force relation were carried out on fiber bundles from left ventricular papillary muscles of 4-6 month old mice.9 Mice were anesthetized with pentobarbital sodium (50 mg/kg b.w. ip), hearts were quickly removed, and put into cold high relaxing (HR) solution (in mmol/L): KCl 53, EGTA 10, MOPS 20, free Mg$^{2+}$ 1, MgATP$^2-$ 5, creatine phosphate 12, and 10 IU/ml creatine phosphokinase (pH 7.0). The
Ionic strength of all solutions was 150 mmol/L. Fiber bundles approximately 150-200 μm in width and 4-5 mm long were mounted between a micromanipulator and a force transducer with cellulose-acetate glue. Fibers were skinned in HR solution containing 1% Triton X-100 for 30 min. A resting sarcomere length of 2.0 μm was established from laser diffraction patterns. Isometric tension was recorded on a chart recorder. After skinning, the fibers were initially washed in HR solution followed by solutions of varying Ca\(^{2+}\) concentrations (Ca\(^{2+}\) range from 10\(^{-7}\) to 10\(^{-4.5}\)M). All solutions also contained the protease inhibitors pepstatin A (2.5  g/ml), leupeptin (1  g/ml) and phenylmethylsulfonyl fluoride (50  M).

**In Vivo Echocardiographic Measurements of Cardiac Dimensions and Function**

2D guided M-mode echocardiography (9 MHz) and color-flow directed Doppler (5-7 MHz) were performed using an Interspec Apogee CX-200 ultrasound (Interspec-ATL, Ambler, PA). Mice were lightly anesthetized with 2.5% tribromoethanol (0.01 ml/gi.p) and were allowed to breathe spontaneously. 2D-guided M-mode echoes were obtained from short-axis views at the level of the largest LV-diameter. LV end-diastolic (EDD) and end-systolic (ESD) dimensions were measured from original tracings by using the leading edge convention of the American Society Echocardiography. LV percent fractional shortening (LVFS), velocity of circumferential shortening corrected for heart rate (Vcf\(_{c}\)), and end-diastolic wall-thickness/cavity ratio were calculated. There were no differences between any parameters of WT and PLB-DM mice (Fig. 1).

**References**


**Figure Legend**

**Online Figure 1:** Representative M-mode echoes of PLB-WT and mutant PLB-DM.

*EDD:* end-diastolic dimension; *ESD:* end-systolic dimension; *Sw Th:* LV septal wall thickness; *Pw Th:* LV posterior wall thickness.