Targeted Ablation of the Phospholamban Gene Is Associated With Markedly Enhanced Myocardial Contractility and Loss of β-Agonist Stimulation

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Abstract

Phospholamban is the regulator of the Ca\(^{2+}\)-ATPase in cardiac sarcoplasmic reticulum (SR), and it has been suggested to be an important determinant in the inotropic responses of the heart to β-adrenergic stimulation. To determine the role of phospholamban in vivo, the gene coding for this protein was targeted in murine embryonic stem cells, and mice deficient in phospholamban were generated. The phospholamban-deficient mice showed no gross developmental abnormalities but exhibited enhanced myocardial performance without changes in heart rate. The time to peak pressure and the time to half-relaxation were significantly shorter in phospholamban-deficient mice compared with their wild-type homozygous littermates as assessed in work-performing mouse heart preparations under identical venous returns, afterloads, and heart rates. The first derivatives of intraventricular pressure (±dP/dt) were also significantly elevated, and this was associated with an increase in the affinity of the SR Ca\(^{2+}\)-ATPase for Ca\(^{2+}\) in the phospholamban-deficient hearts. Baseline levels of these parameters in the phospholamban-deficient hearts were equal to those observed in hearts of wild-type littermates maximally stimulated with the β-agonist isoproterenol. These findings indicate that phospholamban acts as a critical repressor of basal myocardial contractility and may be the key phosphoprotein in mediating the heart’s contractile responses to β-adrenergic agonists. (Circ Res. 1994; 75:401-409.)

Key Words: phospholamban • gene targeting • sarcoplasmic reticulum • cardiac contractility • β-agonists

Cardiac β-adrenergic stimulation is associated with increases in the force of contraction and in the rates of rise and fall of force. These changes are mediated by increases in cAMP levels, which lead to phosphorylation of key regulatory proteins that may act as effectors of the adrenergic stimulation. One of these phosphoproteins is phospholamban, the regulator of the Ca\(^{2+}\)-ATPase in cardiac sarcoplasmic reticulum (SR). Dephosphorylated phospholamban is an inhibitor of the Ca\(^{2+}\)-ATPase activity, and phosphorylation relieves this inhibition. The inhibition has been suggested to involve physical direct interaction between the two proteins, followed by conformational changes in the SR Ca\(^{2+}\)-ATPase. In vitro studies have shown that phospholamban is phosphorylated at distinct amino acids by three different protein kinases, namely, serine 10 by protein kinase C, serine 16 by cAMP-dependent protein kinase, and threonine 17 by Ca\(^{2+}\)-calmodulin-dependent protein kinase. Phosphorylation of phospholamban is associated with increases in the affinity of the SR Ca\(^{2+}\) pump for Ca\(^{2+}\) and in some studies, an increase in the V\(_{\text{max}}\) of the Ca\(^{2+}\)-ATPase has also been observed. Phosphorylation of phospholamban in situ and the accompanying increases in SR Ca\(^{2+}\)-uptake rates have been postulated to be partially responsible for the enhanced rate of myocardial relaxation (−dP/dt) observed during adrenergic stimulation of the heart. However, the exact role of phospholamban in the contractile responses of the heart to adrenergic agonists is not presently clear, especially since other key cardiac regulatory proteins have also been shown to be phosphorylated in vivo. These phosphoproteins include troponin I (TnI) and C protein in the myofibrils and a 15-kD (8.5-kD as revealed by molecular cloning) sarcomemal protein. The physiological significance of C-protein phosphorylation is not presently known, but phosphorylation of TnI has been shown to decrease the Ca\(^{2+}\) sensitivity of the myofibrillar Mg\(^{2+}\)-ATPase activity, contributing to the relaxant effects of catecholamines. Phosphorylation of the 15-kD sarcomemal protein has been suggested to regulate transsarcomemal Ca\(^{2+}\) flux contributing to the increases in the maximal rate of developed tension. The relative contribution of phospholamban and TnI phosphorylation to the relaxant effects of β-agonists has been examined by several laboratories. Phosphorylation and dephosphorylation of phospholamban appeared to occur at a faster rate than phosphorylation/dephosphorylation of the 15-kD sarcomemal protein and TnI in vivo. Thus, it was suggested that phosphorylation of phospholamban may play the most prominent role in medi-
ating the relaxant effects of β-adrenergic agonists. However, in such intact tissue studies, it is difficult to assess definitively the relative contribution of each phosphoprotein in the physiological responses observed, and it is even more difficult to assign a specific phosphoprotein responsibility for the observed physiological effect.

The present study was designed to address two major questions. First, does phospholamban play a role in the regulation of basal myocardial contractility? Second, what is the role of phospholamban in mediating the responses of the heart to β-adrenergic stimulation? To address these questions, we disrupted the phospholamban gene in mouse embryonic stem (ES) cells and generated mice deficient in phospholamban. Phenotypically, the phospholamban-deficient mice appeared normal. Their heart rate and blood pressure were similar to those of wild-type homozygous littermates. However, cardiac function, assessed in isolated working-heart preparations, revealed significant increases in all contractile parameters of the phospholamban-deficient mice compared with their wild-type littermates. The increases in cardiac contractile and relaxation function of the phospholamban-deficient mice reflected increases in the affinity of the SR Ca2+ pump for Ca2+ that were due to removal of phospholamban inhibition. Isoproterenol perfusion was not associated with further increases in the cardiac contractile parameters of the phospholamban-deficient mice, whereas it stimulated these parameters in the hearts of wild-type littermates to levels exhibited by the phospholamban-deficient mice. These findings illustrate that phospholamban is an important determinant of basal contractility in the mammalian heart and that phospholamban can play a prominent role in mediating the heart’s contractile responses to β-adrenergic stimulation.

Materials and Methods

Targeting Construct

The mouse phospholamban gene was cloned from a ADash (Stratagene) genomic library derived from a strain 129/SvJ mouse.29 The internal 1.8-kb HindIII fragment of a 5.3-kb Sat 1–EcoR1 genomic fragment was replaced by the Xho I–Sat 1 fragment of the polyadenylation signal–deficient neo gene from pMCneo (Stratagene), resulting in the loss of the HindIII, Xho I, and Sat I sites. A copy of the tk gene (a gift from Dr Roger Askew, University of Cincinnati) was attached to the 3’ end of the targeting construct.

Electroporation, Selection, and Screening of ES Cells

D3 ES cells (5×10^6) were electroporated with 5 nmol/L of purified targeting fragment by using an IBI Gene Zapper at 800 V·cm−2 and 200 μF. Cells were plated on mitomycin C–treated neo primary mouse embryonic fibroblast feeder cells prepared by standard procedures.30 After 24 hours, 500 μg·ml−1 G418 was added. One day later, G418 was reduced to 200 μg·ml−1 and maintained at that level throughout the whole selection period of 10 to 14 days. Ganciclovir (final concentration, 2 nmol/L) was added the third day after electroporation and maintained at that level for the entire selection period. G418– and ganciclovir-resistant colonies were picked and expanded in individual wells of 24-well tissue culture plates (Falcon). Screening for targeted transfectants was done by Southern blot analysis from genomic DNA extracted from the colonies. DNA was digested with HindIII, electrophoretically separated through 0.8% agarose, transferred to nylon membranes, and hybridized with the probe.

The probe was a 1.8-kb phospholamban EcoRI–Ssr I genomic fragment that is positioned immediately 3’ to the homologous region of the targeting sequences and does not hybridize to the targeting construct.

Generation of Targeted Mice

Blastocyst injection and generation of germ-line chimeric mice were performed by the University of Cincinnati Knockout Mouse Service Facility. Phospholamban-targeted ES cells were injected into C57Bl/6 blastocysts and transferred to F1(C3H/HeN×C57Bl/6J) pseudopregnant females by using standard procedures.31 Coat-color offspring were mated to albino nonagouti CF-1 mice (Charles River), resulting in large litter sizes. Agouti offspring were genotyped by Southern blot analysis as described above by using DNA isolated from tail biopsies. Mating of heterozygous animals carrying the targeted phospholamban allele resulted in homozygous mutant mice completely deficient in phospholamban. All animals were housed in ventilated microisolator cages with automatic watering (Cage Rack Model 60236, Lab Products) to prevent the introduction of known mouse pathogens into the colony.

Mouse Heart RNA Analysis

Total RNA from mouse heart was isolated by an acidic guanidinium isothiocyanate procedure.32 Northern blot analysis was performed by standard procedures.33 A random-primed labeled phospholamban cDNA34 was used as a probe for hybridization.

Quantitative Western Blots

Mouse hearts from wild-type, heterozygous, and phospholamban-deficient mice were homogenized in (mmol/L) imidazole 10 (pH 7.0), sucrose 300, dithiothreitol 1, sodium metabisulfite 1, and phenylmethylsulfonyl fluoride 0.3. Protein concentration was determined by the Bio-Rad method, with bovine serum albumin used for the standard curve. Total heart homogenates (25 μg) were electrophoresed through 10% to 20% sodium dodecyl sulfate–polyacrylamide gels and transferred to 0.22-μm nitrocellulose membranes. The membranes were incubated overnight with a monoclonal antibody specific for phospholamban (UBI). The antibody-antigen complex was detected by using 2×10^6 cpm/ml 35S-labeled anti-mouse IgG (Amersham). The degree of labeling was determined by PhosphORImager and ImageQUANT software (Molecular Dynamics).

Mouse Heart Perfusion

Mice of either sex were anesthetized intraperitoneally with 1 mg/g body wt sodium pentobarbital and protected with 1.5 U heparin to prevent intracoronary microthrombi.35–38 After thoracotomy, the hearts were attached by the aorta to a 20-gauge cannula and temporarily retrogradely perfused with oxygenized Krebs-Henseleit solution (KHS) containing (mmol/L) NaCl 118, CaCl2 2.5, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, sodium EDTA 0.5, NaHCO3 25, and glucose 11. The KHS cylinder was located 70 cm above the heart with an overflow to produce a hydrostatic pressure of 50 mm Hg. For intraventricular pressure (IVP) measurements a 5-cm polyethylene catheter (PE-50) was inserted into the left atrium through the pulmonary vein, guided through the mitral valve during opening, advanced into the left ventricle, and forced through the ventricular apex. The proximal end of the catheter was flanged over heat to prevent it from slipping out of the ventricle. The distal end was cut to a point to allow easy penetration through the ventricular wall. This end was connected through a larger-diameter catheter to a pressure transducer. The frequency response of this fluid-filled transducer-catheter tubing system was flat to 15 Hz. The opening of the pulmonary vein in the left atrium was connected to the venous return cannula, and antegrade work-performing perfusion was initiated. Venous return could be adjusted between 0 and 12 mL/min. During
baseline observations, venous return was set to 5 mL/min. Atrial pressure was monitored through a side arm. The aortic outflow passed through a bubble chamber (Windkessel) containing 1.0-mL air that served as elastic recoil. The elastic recoil is necessary to maintain coronary flow during diastole. A variable resistance determined the aortic pressure (afterload), which was initially set to 50 mm Hg to produce a comparable baseline. The second component in the aortic pressure recording (Fig 3) was caused by the elastic recoil produced by a 5-mL air chamber in the aorta outflow line. Since our emphasis was on quantification of afterload, we used only the computer-calculated mean aortic pressure (MAP), which was not influenced by the pressure oscillations. Temperature of the KHS was continuously monitored and kept at 37.4°C. Coronary and aortic flow were separately measured, and together they measure the cardiac output (CO). All recordings were monitored on a six-channel P7 Grass polygraph. The signals were digitized via a TL-1 DNA interface board (Axon Instrument) and analyzed by computer software custom-designed for an IBM-compatible computer. The amplified and digitized signal from the IVP transducer was continuously differentiated/displayed and analyzed and to provide the first derivative of IVP (+dP/dt and −dP/dt, in millimeters of mercury per second) and the duration of contraction and relaxation. The output from the data-analysis program indicated the most negative and most positive peak pressure and the end-diastolic pressure. From these data, time to peak pressure (TPP, in milliseconds) during contraction, peak pressure (in millimeters of mercury), and the absolute contraction time (TPP per millimeter of mercury) were continuously computed as were relaxation (in millimeters of mercury), half-relaxation time (RT½, in milliseconds), and RT½ per millimeter of mercury.35

Drug Infusion
Work-performing mouse heart preparations were subjected to continuous infusions of increasing concentrations of isoproterenol from 0.8 to 800 nmol/L. Each isoproterenol concentration was infused for 3 minutes into the venous return line before the volume control in order not to alter venous return. The maximum volume added by the infusion was 400 μL/min. In all hearts, the infusions were initiated at similar venous returns (≈5 mL/min), afterloads (≈50 mm Hg), and heart rates (≈380 beats per minute). From the beginning to the end of the isoproterenol infusions, venous return did not change, although coronary flow increased somewhat and aortic flow decreased to the same extent. Afterload increased slightly from the initial 50.8±0.3 to 54.3±2.2 mm Hg MAP.

Ca²⁺ Uptake
Hearts from 8- to 9-week-old wild-type and phospholamban-deficient littermate mice were frozen under liquid nitrogen and stored at −80°C until processed. Frozen hearts were homogenized in 50 mmol/L KH₂PO₄ (pH 7.0), 10 mmol/L NaF, 1 mmol/L EDTA, 0.3 mol/L sucrose, 0.3 mmol/L phenylmethylsulfonyl fluoride, and 0.5 mmol/L dithiothreitol. Ca²⁺ uptake in whole-heart homogenates (0.1 mg/mL) was measured by a modification of the Millipore filtration technique.39 The reaction contained 40 mmol/L imidazole (pH 7.0), 100 mmol/L KCl, 5 mmol/L MgCl₂, 5 mmol/L NaCl, 0.5 mmol/L potassium oxalate, 0.5 mmol/L EDTA, 1 μmol/L ruthenium red, and various concentrations of CaCl₂ to yield 0.02 to 5 μmol/L free Ca²⁺. Homogenates were incubated at 37°C for 2 minutes in the above buffer, and the reaction was initiated by the addition of ATP (final concentration, 5 mmol/L). The rates of Ca²⁺ uptake were calculated by least-squares linear regression analysis of 30-, 60-, and 90-second values of Ca²⁺ uptake. The data were analyzed by nonlinear regression using ORIGIN (version 6.0) software.

Statistics
Data are presented as mean±SEM. The number (n) of mice used is indicated. Statistical analysis was performed by t test for paired comparisons between wild-type and phospholamban-deficient mice. Values were also tested by ANOVA using SUPER ANOVA software from Abacus.

Results
Targeted Deletion of Phospholamban-Coding Region
The murine phospholamban gene was isolated from a genomic library constructed with DNA from a 129/SvJ mouse, which is isogenic to the 129/Sv strain from which the D3 ES cell line was established.29 This was done to ensure that strain differences between the ES cell and targeting DNA did not negatively affect targeting efficiency. The targeting strategy (Fig 1a through 1c) was designed to eliminate the entire phospholamban-coding region. The targeting vector consisted of 5.3 kb of genomic sequence in which an internal 1.8-kb HindIII fragment containing the protein-coding region was replaced with a neomycin (neo) gene, which expresses resistance to the antibiotic geneticin (G418). A copy of the herpes simplex thymidine kinase gene (tk) was attached to the 3' end of the phospholamban-neo fragment for negative selection purposes.40 The purified phospholamban-neo-tk construct was introduced into D3 ES cells by electroporation. ES cell lines containing a disrupted phospholamban gene allele were identified by Southern blot analysis of HindIII-digested genomic DNA, which restricts the targeted allele outside both regions of homology. A phospholamban genomic fragment that flanks the 3' region of homology was used as a probe (Fig 1d). The combined use of probe and restriction sites lying outside of the region of homology ruled out both a random integration event and large deletions at either end of homology. Further analysis of positive clones using three additional restriction endonucleases and additional probes specific for the 5' region of homology and for the neo gene confirmed that homologous integration had occurred without gross rearrangements, deletions, or secondary random integrations of the targeting sequences (data not shown). Seven clones from a total of 520 G418-resistant colonies contained the desired targeted phospholamban allele. Three of these lines (227, 380, and 472) were karyotyped to ensure that no obvious chromosomal abnormalities had occurred.

Chimeric mice were generated by blastocyst injection of ES cells from the three karyotyped lines. Thirty coat color–chimeric mice were obtained. Seventeen were male chimeras, and mating of these animals to strain CF-1 females allowed identification of nine animals that were fertile. Five of the nine male mice transmitted the ES cell–derived agouti coat color to their offspring. One of these males has produced a total of 25 offspring and transmitted the modified phospholamban allele to 64% (16 of 25) of its agouti offspring. Heterozygous mice carrying one wild-type and one disrupted phospholamban allele appeared normal and were fertile. Heterozygous animals have been intercrossed to generate homozygous mice for the altered phospholamban allele. Genotypes of the progeny were determined by polymerase chain reaction (PCR) of tail DNA biopsies. Genotypes of all of the animals used for cardiac functional
studies were also confirmed by Southern blot analysis of cardiac DNA. A representative example of such a litter is shown in Fig 2. Of 100 offspring from 10 heterozygous crossings, 23 were homozygous wild-type, 50 were heterozygous, and 27 were homozygous for the disrupted allele, yielding the expected 1:2:1 distribution of progeny. Consistent with the targeting scheme designed to completely remove the coding region of the phospholamban gene, PCR-based amplification of the genomic coding region of the gene yielded no product from the progeny homozygous for the disrupted phospholamban allele. No cardiac muscle phospholamban mRNA or protein was detected in homozygotes. Consequently, mice homozygous for the targeted allele were completely deficient in phospholamban. The phospholamban-deficient mice were phenotypically indistinguishable from wild-type littermates at the gross level. Furthermore, the ECG recordings and systolic blood pressure by tail-cuff measurements in unanesthetized phospholamban-deficient mice and wild-type littermates did not reveal significant differences.

**Cardiovascular and Contractile Parameters in Work-Performing Heart Preparations**

To assess quantitatively the effects of the absence of phospholamban on myocardial contractility, isolated work-performing heart preparations from phospholamban-deficient mice were perfused in parallel with hearts from wild-type littermates under identical load conditions. The MAPs (afterload) were adjusted by a micrometer (to 50 mm Hg) acting as a Starling resistance. The venous returns (CO values) were also adjusted with a micrometer to 5 mL/min. Fig 3 shows representative recordings of the contractile parameters of wild-type and phospholamban-deficient hearts. Although the spontaneous heart rate and cardiac power (MAP×CO) were similar between the phospholamban-deficient and wild-type hearts, IVP and the maximal rates of pressure development were significantly different. The systolic IVP was increased from 80 to 115 mm Hg, and the diastolic and end-diastolic IVPs were decreased from −6 to −25 mm Hg and from +10 to +2 mm Hg, respectively, in the phospholamban-deficient heart compared with the wild-type heart. Since the stroke volumes (CO/heart rate) in both hearts were similar (see above), the changes in end-diastolic pressure were most likely caused by changes in left ventricular rest volumes. However, the methodology to measure left ventricular volumes has not been developed yet. The maximal rates of pressure development (dP/dt) were increased from +2800 to +5625 mm Hg/s for contraction and from −2500 to −5125 mm Hg/s for relaxation in the phospholamban-deficient heart compared with the wild-type heart.

A comparison of the contractile and relaxation function of several hearts from 8- to 9-week-old phospholamban-deficient (n=8) and wild-type (n=14) mice is shown in Fig 3.
shown in the Table. The conditions of the comparison were equal in terms of heart rate and heart and body weights. In addition, the perfusion fluid, oxygenation, and temperature were constant. Since cardiac contractility is strongly affected by venous return and afterload, we have, in previous experiments, \textsuperscript{35,37-38} determined the minimum venous return (\textasciitilde 5 mL/min CO) and afterload (\textasciitilde 50 mm Hg MAP) required to maintain continuous function of the hearts without any changes in end-diastolic and left atrial pressure. Consequently, the comparison of contractile and relaxation function of the hearts of either group was done at similar afterload, venous return, stroke volume, cardiac power, heart rate, and perfusion conditions. The only differences observed between the two groups were in the left IVP and the derivatives of IVP. The differences in systolic, diastolic, and end-diastolic IVP and in the rates of pressure change (\textpm dP/dt) were large and significant to the highest level of probability (Table). In addition, the computed contraction time (TPP per millimeter of mercury) and relaxation time (RT\textfrac{1}{2} per millimeter of mercury) were significantly shorter in the phospholamban-deficient hearts (Table).

Effects of Isoproterenol on Isolated Work-Performing Heart Preparations

Phospholamban has been implicated as one of the phosphoproteins mediating the cardiac responses to \(\beta\)-adrenergic agonists, \textsuperscript{16-19,21,25-28} Thus, hearts from phospholamban-deficient and wild-type littermates were subjected to perfusion with increasing isoproterenol concentrations under similar load conditions, and the effects of this \(\beta\)-agonist are compared in Fig 4. Two parameters each were measured for contractility (\(+dP/dt\) and TPP per millimeter of mercury) and relaxation (\(-dP/dt\) and RT\textfrac{1}{2} per millimeter of mercury) (Fig 4a through 4d). The results obtained with the wild-type littermates were quantitatively and qualitatively similar to those we reported with different mouse strains in earlier studies.\textsuperscript{34,35} The ED\textsubscript{50} values for isoproterenol stimulation were 10.4 nmol/L for \(+dP/dt\) (Fig 4a), 9 nmol/L for TPP per millimeter of mercury (Fig 4b), 11 nmol/L for \(-dP/dt\) (Fig 4c), and 9 nmol/L for RT\textfrac{1}{2} per millimeter of mercury (Fig 4d). In contrast, the isoproterenol dose-response curves for contraction and relaxation in the phospholamban-deficient mice began at a maximum level and were not further increased by isoproterenol. Thus, no ED\textsubscript{50} values could be obtained. The values, including the maximal and minimal values, in the phospholamban-deficient hearts were not statistically different from each other in Fig 4a through 4d. Furthermore, these values were not statistically different from the values of the maximally stimulated contractile parameters in the hearts of wild-type littermates, obtained at 400 and 800 nmol/L isoproterenol. Despite the lack of contractile responses to isoproterenol by the phospholamban-deficient hearts, the response of heart rate to this agonist was similar between the phospholamban-deficient and wild-type littermate hearts (Fig 4e). The two dose-response curves were not statistically different from each other, and the ED\textsubscript{50} values for the isoproterenol effects on heart rate were 31 and 38 nmol/L in the wild-type and phospholamban-deficient hearts, respectively.

SR Ca\textsuperscript{2+} Uptake

The stimulatory effects of isoproterenol in the intact heart have been suggested to be partially mediated through increases in the Ca\textsuperscript{2+}-uptake rates by SR vesicles that are due to phospholamban phosphorylation.\textsuperscript{34,35,37-38} To determine whether the lack of isoproterenol-stimulatory effects in the phospholamban-deficient mouse hearts was due to lack of phospholamban in SR, unfractionated SR vesicles in homogenates of phospholamban-deficient and wild-type littermate hearts were used to determine the initial rates of Ca\textsuperscript{2+} uptake. The incubation conditions under which Ca\textsuperscript{2+} uptake is restricted to SR vesicles in the homogenates have been previously defined,\textsuperscript{41} and the validity and advantages of this approach have been discussed.\textsuperscript{42,43} The initial rates of SR Ca\textsuperscript{2+} uptake by cardiac homogenates were assayed over a wide range of [Ca\textsuperscript{2+}]\textsubscript{0} similar to the range of [Ca\textsuperscript{2+}] occurring intracellularly during relaxation and contraction (Fig 5). Ca\textsuperscript{2+}-
uptake rates by the homogenates of the phospholamban-deficient hearts were higher than those by the wild-type hearts, especially at low [Ca\textsuperscript{2+}], whereas there was no effect on V_{max} (92±6.6 versus 92±3.5 nmol Ca\textsuperscript{2+}·mg\textsuperscript{-1}·min\textsuperscript{-1} for phospholamban-deficient versus wild-type hearts, n=6). Analysis of these data indicates that the EC\textsubscript{50} of the SR Ca\textsuperscript{2+} pump for Ca\textsuperscript{2+} was 110±10 nmol/L (n=6) for phospholamban-deficient hearts and 240±29 nmol/L (n=6) for wild-type hearts. The increased sensitivity of the transport system for Ca\textsuperscript{2+} in the phospholamban-deficient mice was similar to our previous observations in wild-type hearts, which were stimulated by isoproterenol (140±10 nmol/L Ca\textsuperscript{2+}).\textsuperscript{34} Thus, phospholamban deficiency is associated with stimulation of the affinity of the SR Ca\textsuperscript{2+} pump for Ca\textsuperscript{2+} to levels observed in isoproterenol-stimulated wild-type hearts, where phosphorylation of phospholamban relieves its inhibitory effects on the SR Ca\textsuperscript{2+} pump.

**Discussion**

The findings of the present study indicate that ablation of phospholamban expression in the mammalian heart is associated with significant increases in the heart's contractile parameters and in the affinity of the SR Ca\textsuperscript{2+}-ATPase for Ca\textsuperscript{2+}. This is the first evidence presented indicating the role of phospholamban in the regulation of basal contractility in the mammalian heart. The generation of an animal model deficient in phospholamban expression has allowed us to perform physiological and biochemical studies to elucidate the role of phospholamban in the intact heart and in the cardiac responses to β-adrenergic agonists. The working-heart preparation used in the present study enabled us to quantify myocardial contractility in the phospholamban-deficient mice and to make definitive functional comparisons between these mutant mice and their wild-type littermates. The phospholamban-deficient and wild-type mice had similar heart rates, body weights, and heart weights, but they differed significantly in their cardiac IVPs and their contraction and relaxation parameters, expressed as rates of pressure development and TPP or RT\textsubscript{½}. The increases in contraction and relaxation parameters were similar to the maximal values produced in wild-type mouse hearts by various means, including Ca\textsuperscript{2+} loading, force-frequency response, and pressure and/or volume loading (data not shown). Furthermore, the increased contractile parameters in phospholamban-deficient hearts exceeded the hyperdynamic values we previously observed when using either hyperthyroid hearts\textsuperscript{35} or hearts from the BALB/c strain, which express relatively high levels of the α-skeletal actin isoform in the ventricle.\textsuperscript{37} The increases in contractile and relaxation parameters of the phospholamban-deficient mouse hearts were not asso-

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type Mice</th>
<th>Phospholamban-Deficient Mice</th>
<th>P Value*</th>
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<tr>
<td>n</td>
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<tr>
<td>Body wt, g</td>
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<td>Heart wt (both ventricles), mg</td>
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<td>Heart wt/body wt, mg/g</td>
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<td>HR, bpm</td>
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<td>MAP (afterload), mm Hg</td>
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<td>Left IVP, mm Hg</td>
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<td>End-diastolic</td>
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<td>Stroke volume, μL</td>
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<td>Cardiac power (left ventricle), mm Hg · mL · min\textsuperscript{-1}</td>
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<td>Contraction properties</td>
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<td>+dP/dt, mm Hg/s</td>
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</tbody>
</table>

*n indicates number of mice; HR, heart rate; bpm, beats per minute; MAP, mean aortic pressure; IVP, intraventricular pressure; TPP, time to peak pressure; and RT\textsubscript{½}, half-relaxation time. Values are mean±SEM.

*Assessed by t test for paired comparisons between control and mutant mice. Values were also tested by ANOVA using SUPER ANOVA software from Abacus.
ciliated with any increases in cardiac work in the isolated preparations, since venous return and afterload in both groups were set to identical values. However, it is not presently known whether stroke volume, stroke work, or CO are altered after phospholamban ablation in the intact animal, but tail blood pressure measurements and heart rate were similar to those in wild-type littermates. Thus, since heart rate was not altered and venous return (CO) and afterload (MAP) were set in the isolated work-performing preparations, the stroke volume (CO/heart rate) and stroke work (MAP×CO/heart rate) were similar between phospholamban-deficient and wild-type littermates. Although preload was not measured directly, it must be that left ventricular end-diastolic pressure, one indirect measure of preload, was reduced in the phospholamban-deficient mice.

Evidence from in vitro and in vivo studies indicates that dephosphorylated phospholamban is an inhibitor of the SR Ca^{2+}-ATPase and that phosphorylation relieves this inhibition. The mechanism by which phospholamban exerts its inhibitory effects has been generally agreed to involve a decrease in the affinity of the SR Ca^{2+}-ATPase for Ca^{2+}. However, it is currently under debate whether phospholamban has an effect on the V_{max} of the SR Ca^{2+}-ATPase. The phospholamban-deficient mice provided an attractive system for further elucidation of the phospholamban-regulatory effects on the SR Ca^{2+}-ATPase. Biochemical analysis of the SR Ca^{2+} transport system indicated that the affinity of the Ca^{2+}-ATPase for Ca^{2+} was significantly increased in the phospholamban-deficient hearts compared with wild-type hearts. However, the maximal velocity of Ca^{2+} transport was similar between phospholamban-deficient and wild-type littermate hearts. These findings indicate that phospholamban deficiency leads to an enhancement of SR Ca^{2+}-uptake rates in the intact heart, thereby enhancing the rate of myocardial relaxation. These increases in SR Ca^{2+}-uptake rates are expected to lead to higher amounts of Ca^{2+} sequestered by the SR per unit time, which would be available for release, resulting in higher force and rate of contraction, as observed in the phospholamban-deficient hearts.

Phospholamban has also been shown to be phosphorylated in vivo during β-adrenergic stimulation of intact hearts, and phosphorylation of phospholamban has been implicated in mediating the relaxant effects of β-agonists in the heart. Thus, it was of special interest to note that the contractile parameters of phospholamban-deficient mice could not be further stimulated by isoproterenol perfusion. In parallel studies, the hearts from wild-type littermates were stimulated by isoproterenol in a dose-dependent manner. The contractile parameters of the wild-type hearts, when maximally stimulated by isoproterenol, were similar to those of the nonstimulated phospholamban-deficient hearts. Consequently, phosphorylation of phospholamban and relief of its inhibitory effects, in-

Fig 5. Graph showing enhancement of the affinity of cardiac sarcoplasmic reticulum (SR) Ca^{2+} transport for Ca^{2+} in phospholamban-deficient hearts. The initial rates of Ca^{2+} uptake by SR vesicles in homogenates prepared from phospholamban-deficient (a) and wild-type (b) littermate mouse hearts were assayed over a wide range of [Ca^{2+}]. Values are mean±SEM of six different hearts, each assayed in triplicate.

![Graph showing enhancement of the affinity of cardiac sarcoplasmic reticulum (SR) Ca^{2+} transport for Ca^{2+} in phospholamban-deficient hearts.](image)
duced by isoproterenol in the wild-type hearts, is associated with increases in the contractile parameters to levels present in the phospholamban-deficient mice. The phospholamban-deficient hearts maintain the SR Ca^{2+}-ATPase activity in its noninhibited form with high affinity for Ca^{2+}, a form that is associated with a hyperdynamic state of the heart even under basal load conditions. The lack of additional isoproterenol effects on the contractile parameters of the phospholamban-deficient mice leads to the intriguing possibility that the inotropic effects of isoproterenol are mainly mediated by increases in the Ca^{2+} sensitivity of the SR transport system, through phosphorylation of phospholamban. Consistent with this hypothesis, early studies by Fabiato and Fabiato44 showed that the relaxant effects of cAMP in skinned myocardial cellular preparations were not associated with any discernible effects on the Ca^{2+} sensitivity of the contractile proteins. However, it remains to be determined whether phosphorylation of TnI and the 15-kD sarcolemmal protein play a role in the inotropic responses of the phospholamban-deficient hearts to isoproterenol.

In summary, our findings indicate that phospholamban plays a critical role in the regulation of the basal contractile parameters in the mammalian heart without affecting heart rate. The close relation between increases in the affinity of the SR Ca^{2+}-ATPase for Ca^{2+} and changes in indexes of myocardial relaxation and contractility provides strong evidence for the functional significance of phospholamban in the heart. Furthermore, the present results provide insight into the mechanisms by which β-adrenergic agonists modulate cardiac contractility. It is interesting to propose that the presence of phospholamban in the mammalian heart keeps the contractile parameters in an “inhibited” state, such that ablation or phosphorylation of phospholamban by physiological stimulants relieves its inhibitory effects and results in increased contractile parameters. However, it remains to be determined whether compensatory mechanisms such as alterations in adrenergic receptors, G proteins, contractile proteins, and Ca^{2+}-cycling proteins have occurred as a result of phospholamban ablation. The generation of an animal model deficient in phospholamban will enable us to distinguish between these possibilities and will allow us to perform additional in vivo studies to elucidate the role of phospholamban in the regulation of smooth muscle tone and slow-twitch skeletal muscle contractility. Furthermore, the phospholamban-deficient mice provide an attractive system for in vivo structure/function studies by directing mutant phospholamban transgenes to specific tissues by use of appropriate promoters. Such studies will provide insight into potential therapeutic strategies targeting phospholamban in cardiac and smooth muscle, which may be beneficial in several diseases such as hypertrophy, congestive heart failure, hypertension, coronary artery disease, and asthma.

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