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 conditions. 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) sarcometal 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 myofilibril Mg\(^{2+}\)-ATPase activity, contributing to the relaxant effects of catecholamines. Phosphorylation of the 15-kD sarcolemmal protein has been suggested to regulate transsarcolemmal 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 sarcolemmal 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 phospho-
protein 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 phospholab-
man gene in mouse embryonic stem (ES) cells and generated mice deficient in phospholamban. Phenotypi-
cally, the phospholamban-deficient mice appeared nor-
mal. 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 con-
tractile 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 Ca\(^{2+}\) pump for Ca\(^{2+}\) 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 ΔDash (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–EcoRI 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 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\(^{3}\)) were electroporated with 5 nmol/L of purified targeting fragment by using an IBI Gene Zapper at 800 V · cm\(^{-1}\) 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 G418 was added. One day later, G418 was reduced to 200 μg/mL 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 wa-
tering (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 analy-
sis was performed by standard procedures.\(^{33}\) A random-
primed labeled phospholamban cDNA\(^{34}\) was used as a probe for hybridization.

**Quantitative Western Blots**

Mouse hearts from wild-type, heterozygous, and phospho-
lamban-deficient mice were homogenized in (mmol/L) imid-
azole 10 (pH 7.0), sucrose 300, dithiothreitol 1, sodium meta-
bisulfite 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 trans-
ferred 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\(^{-6}\) cm²/mL \(^{35}\)-labeled anti-mouse IgG (Amersham). The degree of labeling was determined by PHORSIMAGER 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, CaCl\(_2\) 2.5, KCl 4.7, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, sodium EDTA 0.5, NaHCO\(_3\) 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 intraventric-
ular pressure (IVP) measurements a 5-cm polyethylene cath-
ter (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 at 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 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 (in milliseconds), and relaxation (in millimeters of mercury), 35.4 mmHg MAP.

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 dithiotreitol. Ca²⁺ uptake in whole-heart homogenates (0.1 mg/mL) was measured by a modification of the Millipore filtration technique. The reaction contained 40 mmol/L imidazole (pH 7.0), 100 mmol/L KCl, 5 mmol/L MgCl₂, 5 mmol/L NaNO₃, 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. This was done to ensure that strain differences between the ES cells and targeted 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. 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
The fragment shown is the targeted phospholamban allele, yielding the wild-type allele. +/+ indicates homozygous wild-type mice; +/-, heterozygous mice; and -/-, homozygous targeted mice.

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 x 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
Effects of Isoproterenol on Isolated Work-Performing Heart Preparations

Phospholamban has been implicated as one of the phosphoproteins mediating the cardiac responses to β-adrenergic agonists. 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 β-agonist are compared in Fig 4. Two parameters each were measured for contractility (+dP/dt and TTP per millimeter of mercury) and relaxation (~dP/dt and RT½ 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. The ED₅₀ values for isoproterenol stimulation were 10.4 nmol/L for +dP/dt (Fig 4a), 9 nmol/L for TTP per millimeter of mercury (Fig 4b), 11 nmol/L for ~dP/dt (Fig 4c), and 9 nmol/L for RT½ 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 ED₅₀ 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 ED₅₀ values for the isoproterenol effects on heart rate were 31 and 38 nmol/L in the wild-type and phospholamban-deficient hearts, respectively.

SR Ca²⁺ Uptake

The stimulatory effects of isoproterenol in the intact heart have been suggested to be partially mediated through increases in the Ca²⁺-uptake rates by SR vesicles that were due to phospholamban phosphorylation. 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 Ca²⁺ uptake. The incubation conditions under which Ca²⁺ uptake is restricted to SR vesicles in the homogenates have been previously defined, and the validity and advantages of this approach have been discussed. The initial rates of SR Ca²⁺ uptake by cardiac homogenates were assayed over a wide range of [Ca²⁺] similar to the range of [Ca²⁺] occurring intracellularly during relaxation and contraction (Fig 5). Ca²⁺-
uptake rates by the homogenates of the phospholamban-deficient hearts were higher than those by the wild-type hearts, especially at low \([\text{Ca}^{2+}]\), whereas there was no effect on \(V_{\text{max}}\) \((92\pm6.6\text{ versus } 92\pm3.5\text{ mmol Ca}^{2+}\cdot\text{mg}^{-1}\cdot\text{min}^{-1})\) for phospholamban-deficient versus wild-type hearts, \(n=6\). Analysis of these data indicates that the \(E_C^{50}\) of the SR \(\text{Ca}^{2+}\) pump for \(\text{Ca}^{2+}\) was \(110\pm10\text{ nmol/L (n=6)}\) for phospholamban-deficient hearts and \(240\pm20\text{ nmol/L (n=6)}\) for wild-type hearts. The increased sensitivity of the transport system for \(\text{Ca}^{2+}\) in the phospholamban-deficient mice was similar to our previous observations in wild-type hearts, which were stimulated by isoproterenol \((140\pm10\text{ nmol/L (Ca}^{2+})\). Thus, phospholamban deficiency is associated with stimulation of the affinity of the SR \(\text{Ca}^{2+}\) pump for \(\text{Ca}^{2+}\) to levels observed in isoproterenol-stimulated wild-type hearts, where phosphorylation of phospholamban relieves its inhibitory effects on the SR \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\)-ATPase for \(\text{Ca}^{2+}\). 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 \(\beta\)-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 littersmates. 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\(1/2\). The increases in contraction and relaxation parameters were similar to the maximal values produced in wild-type mouse hearts by various means, including \(\text{Ca}^{2+}\) 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 or hearts from the BALB/c strain, which express relatively high levels of the \(\alpha\)-skeletal actin isoform in the ventricle. The increases in contractile and relaxation parameters of the phospholamban-deficient mouse hearts were not asso-
associated 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 we 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-
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 isoform 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 Fabiato\(^{44}\) 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-kDa 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 relationship 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 \(\beta\)-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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References


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