

# Enhanced Myocardial Relaxation In Vivo in Transgenic Mice Overexpressing the $\beta_2$ -Adrenergic Receptor Is Associated with Reduced Phospholamban Protein

Howard A. Rockman, RaeAnn Hamilton, Larry R. Jones,<sup>§</sup> Carmelo A. Milano,<sup>‡</sup> Lan Mao, and Robert J. Lefkowitz\*

Department of Medicine, University of California, San Diego, School of Medicine, La Jolla, California 92093; Departments of \*Medicine and <sup>‡</sup>Surgery, Howard Hughes Medical Institute, Duke University, Durham, North Carolina 27706; and <sup>§</sup>Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, Indiana 46202

## Abstract

To assess the effect of targeted myocardial  $\beta$ -adrenergic receptor (AR) stimulation on relaxation and phospholamban regulation, we studied the physiological and biochemical alterations associated with overexpression of the human  $\beta_2$ -AR gene in transgenic mice. These mice have an  $\sim$  200-fold increase in  $\beta$ -AR density and a 2-fold increase in basal adenylyl cyclase activity relative to negative littermate controls. Mice were catheterized with a high fidelity micromanometer and hemodynamic recordings were obtained in vivo. Overexpression of the  $\beta_2$ -AR altered parameters of relaxation. At baseline, LV dP/dt<sub>min</sub> and the time constant of LV pressure isovolumic decay (Tau) in the transgenic mice were significantly shorter compared with controls, indicating markedly enhanced myocardial relaxation. Isoproterenol stimulation resulted in shortening of relaxation velocity in control mice but not in the transgenic mice, indicating maximal relaxation in these animals. Immunoblotting analysis revealed a selective decrease in the amount of phospholamban protein, without a significant change in the content for either sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase or calsequestrin, in the transgenic hearts compared with controls. This study indicates that myocardial relaxation is both markedly enhanced and maximal in these mice and that conditions associated with chronic  $\beta$ -AR stimulation can result in a selective reduction of phospholamban protein. (*J. Clin. Invest.* 1996; 97:1618–1623.) Key words:  $\beta$ -adrenergic receptor • transgenic mice • phospholamban • myocardial relaxation • sarcoplasmic reticulum

## Introduction

Myocardial  $\beta$ -adrenergic receptor (AR)<sup>1</sup> stimulation activates membrane-bound adenylyl cyclase to generate cAMP. Subsequent activation of cAMP-dependent protein kinases (PKA) leads to phosphorylation of critical regulatory proteins which act in concert to enhance transsarcolemmal  $\text{Ca}^{2+}$  influx (1), decrease sensitivity of the contractile system to  $\text{Ca}^{2+}$  (2), and

Address correspondence to Howard A. Rockman, M.D., Department of Medicine, Basic Science Building, 0613B, UCSD School of Medicine, La Jolla, CA 92093. Phone: 619-534-8988; FAX: 619-534-1626.

Received for publication 19 January 1995 and accepted in revised form 11 January 1996.

1. Abbreviations used in this paper: AR, adrenergic receptor; LV, left ventricle; PKA, protein kinase A; SR, sarcoplasmic reticulum.

The Journal of Clinical Investigation  
Volume 97, Number 7, April, 1996, 1618–1623

most importantly increase  $\text{Ca}^{2+}$  uptake into the sarcoplasmic reticulum (SR) (3). Myocardial relaxation is enhanced by the increase in SR  $\text{Ca}^{2+}$  uptake, which is mediated through the pentameric phosphoprotein phospholamban. Phosphorylation of phospholamban in response to  $\beta$ -AR stimulation regulates SR  $\text{Ca}^{2+}$  ATPase activity through disinhibition of the  $\text{Ca}^{2+}$  pump (3–5). The overall physiological effect after  $\beta$ -AR stimulation is the enhancement of both the inotropic and lusitropic states of the myocardium.

Recent in vitro studies, in isolated rat ventricular cells, have suggested that  $\beta_2$ -AR stimulation results in a pattern of cellular responses that is distinct from  $\beta_1$ -AR stimulation (6). Despite similar increases in intracellular cAMP generation with both  $\beta$ -AR subtypes, marked differences in the kinetics and amplitude of the cytosolic  $\text{Ca}^{2+}$  transient have been shown with  $\beta_2$ -AR stimulation compared with  $\beta_1$ -AR (6, 7). This suggests, in part, that the two  $\beta$ -AR signaling pathways may differ in the mechanism of SR  $\text{Ca}^{2+}$  uptake and release and possibly  $\beta$ -AR coupling to L-type  $\text{Ca}^{2+}$  channels (6). A possible mechanism to account for many of the above findings is a reduced level of phospholamban phosphorylation by PKA in response to  $\beta_2$ -AR agonists (7).

To determine whether these in vitro findings are important in vivo, we studied the physiological and biochemical effects of marked overexpression of the human  $\beta_2$ -AR gene in transgenic mice (8). These mice have an  $\sim$  200-fold increase in  $\beta$ -AR density and a 2-fold increase in basal adenylyl cyclase activity relative to negative littermate controls (8). Associated with biochemical evidence for enhanced  $\beta$ -AR stimulation, in vivo contractility is markedly elevated and is unresponsive to isoproterenol infusion suggesting a maximal activation of the  $\beta$ -AR signaling cascade in these genetically engineered animals (8). Therefore, these transgenic mice provide a unique and powerful model to study the effect of cardiac-specific overexpression of the  $\beta_2$ -AR on in vivo myocardial relaxation and phospholamban regulation.

## Methods

The animals in this study were handled according to the animal welfare regulations of the University of California, San Diego, and the protocol was approved by the Animal Subjects Committee of this institution.

**Microsurgical techniques.** The generation and characteristics of transgenic mice which overexpress the human  $\beta_2$ -AR have been previously described in detail elsewhere (8). Briefly, the transgene construct was created using the murine alpha myosin heavy chain promoter ligated to the cDNA coding for the human  $\beta_2$ -AR terminated with a portion of the SV-40 intron (8). Three founder lines were identified and propagated (TG4, TG33, TG35) all of which have significantly elevated  $\beta$ -AR density; however, only mice from line TG4 were used in this study. Adult TG4 mice contain a 195-fold increase

in  $\beta$ -AR density and display approximately a 2-fold increase in basal adenylyl cyclase activity relative to negative littermate controls. Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (90–110 mg/kg) and xylazine (4–6 mg/kg). Using microsurgical procedures as described previously (8–10), under a dissecting microscope animals were placed in a supine position and a midline cervical incision made to expose the trachea and carotid arteries. Endotracheal intubation was performed using a blunt 20-gauge needle which then was connected to a volume-cycled rodent ventilator with a tidal volume of 0.2 ml and respiratory rate of 105/min. A midline incision was made and either carotid artery was cannulated with a flame stretched PE 50 catheter. The catheter was connected to modified P50 transducers (Statham, Oxnard, CA) for measurement of carotid artery pressure. The external jugular vein was cannulated with a PE 50 catheter which was used for isoproterenol infusion (0.005–1 ng). After bilateral vagotomy, the chest was opened and a high fidelity 2F micromanometer catheter was inserted into the left atrium, advanced into the left ventricle (LV) just in front of the mitral valve, and secured into position. The micromanometer-recorded systolic pressure was then matched to that in the aorta. Continuous high fidelity LV and fluid-filled aortic pressure were recorded simultaneously at baseline and 45–60 s after each dose of isoproterenol on an 8-channel chart recorder and in digitized form at 1,000 Hz for later analysis. Experiments were then terminated with an overdose of pentobarbital. Hearts were rapidly excised, and individual chambers were separated, weighed, and then frozen in liquid N<sub>2</sub> for later Northern analysis.

Parameters measured were heart rate, aortic pressure, LV systolic and diastolic pressure, and the maximal and minimal first derivative of LV pressure (dP/dt<sub>max, min</sub>). 10 sequential beats were averaged for each measurement. The time constant of isovolumic pressure decay (Tau) was calculated by fitting the decay of the LV pressure from dP/dt<sub>min</sub> to EDP + 3 mmHg with a monoexponential equation assuming a zero asymptote. The equation simplifies to  $P = P_0 \exp(-t/T)$ , where  $P_0$  is the pressure at time 0 (dP/dt<sub>min</sub>) and T is Tau. The validity of the zero-asymptote model has been confirmed previously in other species (11). T was determined from the inverse slope of the linear ln P vs. t plot which was generated using  $\sim$  22–27 points in the control mice and 14–15 points for the TG4 mice (12, 13). Correlation coefficient for all studies were  $> 0.98$ .

**Immunoblotting.** In separate experiments, 19 (10 control and 9 TG4) mouse hearts were removed, and left ventricles were dissected free and quickly frozen in liquid N<sub>2</sub>. Hearts used for immunoblotting were not studied physiologically or exposed to isoproterenol. Left ventricular tissue was homogenized in 300  $\mu$ l of 10 mM NaHCO<sub>3</sub> buffer at 4°C, to which 600  $\mu$ l of 20% SDS was added and then pelleted at room temperature at 12,000 g for 5 min. The supernatant was aliquoted and frozen at  $-70^{\circ}$ C for later use. Samples were thawed and diluted 5 $\times$  with 10 mM NaHCO<sub>3</sub> and resuspended at 100,000 rpm in a Sorvall RC M100 (Sorvall Instruments, Newton, CT) for 5 min. The pellet containing the contractile proteins was discarded and the protein concentration on the supernatant of the SDS extract was determined by the method of Lowry (14). Purified phospholamban (15) (range 1–6 ng) and 3  $\mu$ g of protein from each sample were electrophoresed in a 10% SDS-polyacrylamide gel until the tracking dye reached the bottom of the gel. Samples were transferred electrophoretically to Hybond-ECL nitrocellulose membrane in 39 mM glycine, 48 mM Tris, 20% methanol at 600 mA for 2.5 h. After blocking with 5% nonfat dried milk, filters were incubated with the phospholamban antibody (1:5,000 dilution of phospholamban monoclonal antibody 2D12 generated to phospholamban peptide 2–25 [16]) and then with a horseradish peroxidase-coupled second antibody by use of chemiluminescent substrate (ECL; Amersham Corp., Arlington Heights, IL). Filters were developed on Kodak BioMax MR film suitable for ECL. Exposure times were chosen to obtain densitometric scans within the linear response range of the radiographic film. To reduce variability between blots, known concentrations of purified phospholamban were run on each gel and as a standard to quantify the amount of phospholamban in each sample (17). Quantitative

evaluation of autoradiograms was performed using an IS-1000 digital imaging system (Alpha Innotech Corp., San Leandro, CA). Protein extracts from each sample were run on multiple gels to confirm reproducibility of results.

Using an additional method of immunoblotting (18), the level of SR Ca<sup>2+</sup> ATPase, calsequestrin, and phospholamban protein was determined in five control and five TG4 hearts. For these experiments hearts were extracted and homogenized as above. 50  $\mu$ g of protein from each sample was electrophoresed in an 8% SDS-polyacrylamide gel by the method of Porzio and Pearson (19). Samples were transferred to 0.2  $\mu$ m pore size nitrocellulose membranes by electrophoresis at 3 A for 60 min in 50 mmol/liter NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.5). The nitrocellulose membrane was cut into horizontal strips according to the mobility regions of SR Ca<sup>2+</sup> ATPase, phospholamban, and calsequestrin. Immunoblotting was performed using a 1:500 dilution of monoclonal antibody 2A7-A1 (generated to the cardiac isoform of the canine SR Ca<sup>2+</sup> pump), 1:500 dilution of phospholamban monoclonal antibody 2D12 (16), and 1:100 dilution of the polyclonal rabbit antibody to canine cardiac calsequestrin (10, 18). After blocking with 2% bovine serum albumin, strips were incubated with the appropriate antibodies and then with <sup>125</sup>I-labeled protein A (Du Pont-New England Nuclear, Boston, MA) and exposed to radiographic film. In addition, nitrocellulose sheets were analyzed with the use of a GS-250 Molecular Imaging System (Bio-Rad Laboratories, Richmond, CA) for quantification of radioactivity associated with antibody reacting bands. For phospholamban the entire lane was selected to include all oligomeric forms of the molecule. After background subtraction, density was integrated within each box which is linearly related to the amount of protein in each band. Each sample was run on multiple gels to confirm reproducibility of results. Densitometric values were normalized to a control sample to correct for some variability in loading and efficiency of transfer.

**Statistical analysis.** Data are expressed as mean value  $\pm$  SE. To examine the effect of isoproterenol on changes in dP/dt between the control and TG4 groups during isoproterenol infusion, a two-way repeated measures ANOVA was used. Post-hoc analysis with regard to differences in mean values between the groups at a specific dose was conducted with the use of Scheffé analysis. In other data sets, statistical significance of differences in mean values of physiological parameters and densitometric data from control and transgenic (TG4) mice was assessed with a Student's *t* test for unpaired data. Comparisons of Tau between groups was assessed by a paired and unpaired *t* test with Bonferroni correction for multiple comparisons. For all analyses, *P* < 0.05 was considered significant.

## Results

**Phenotypic and hemodynamic effects of targeted  $\beta_2$ -AR overexpression.** Targeted  $\beta$ -AR stimulation in the TG4 mice did not result in an increase in heart weight, either absolute or normalized to body weight compared with negative littermate control animals (Table I). As described previously basal contractility was greatly enhanced in the TG4 mice as shown by a 92% increase in dP/dt<sub>max</sub> compared with control animals (Table I) (8). This was associated with a 33% increase in basal heart rate without a significant difference in basal LV systolic or end diastolic pressures.

Overexpression of the  $\beta_2$ -AR significantly altered parameters of relaxation. At baseline, LV dP/dt<sub>min</sub> in the TG4 mice was significantly more negative compared with control animals, indicating markedly enhanced myocardial relaxation (Fig. 1 A). Isoproterenol stimulation resulted in shortening of relaxation velocity, in control mice as demonstrated by the 86% fall in dP/dt<sub>min</sub> at peak stimulation (Fig. 1 A). In contrast,  $\beta$ -AR stimulation had a small but nonsignificant effect on dP/dt<sub>min</sub> in the TG4 animals under the study conditions.

Table I. Baseline In Vivo Physiologic Parameters in Control and Transgenic Mice Overexpressing the Human  $\beta_2$ -AR

	Control <i>n</i> = 8	Transgenic <i>n</i> = 9
Body weight (grams)	23.67 $\pm$ 3.28	25.76 $\pm$ 3.99
Heart weight (mg)	120.2 $\pm$ 20.2	129.6 $\pm$ 17.3
LV/body weight (mg/gram)	3.42 $\pm$ 0.32	3.41 $\pm$ 0.47
LV/tibial length (mg/mm)	4.49 $\pm$ 1.01	4.85 $\pm$ 0.64
Heart rate (beats/min)	352 $\pm$ 8.6	468 $\pm$ 25*
dP/dt <sub>max</sub> (mmHg/s)	4186 $\pm$ 189	8027 $\pm$ 954*
dP/dt <sub>min</sub> (mmHg/s)	-3456 $\pm$ 212	-5763 $\pm$ 500*
Peak LV systolic pressure (mmHg)	68 $\pm$ 2.5	74 $\pm$ 4.1
LV end diastolic pressure (mmHg)	3.4 $\pm$ 1.0	4.2 $\pm$ 1.0
Mean aortic pressure (mmHg)	50 $\pm$ 3.6	51 $\pm$ 2.2

dP/dt<sub>max,min</sub>, maximal and minimal first derivative of LV pressure. Data are mean  $\pm$  SE, \**P* < 0.005 vs. control negative littermates.

Since dP/dt<sub>min</sub> may be affected by loading conditions, the preload independent measure of isovolumic relaxation, Tau (20), was calculated for both the control and TG4 mice. During basal conditions, Tau was significantly shorter in the TG4 mice compared with the negative controls, indicating faster relaxation (Tau shortened by 43%, control 13.6 ms vs. TG4 7.8 ms). Isoproterenol stimulation resulted in shortening of the relaxation time constant, Tau, in control mice by 17%, confirming the results obtained with dP/dt<sub>min</sub> (Fig. 1 B). In contrast, isoproterenol stimulation had no effect on Tau in the TG4 mice, suggesting that relaxation velocity during basal conditions was already maximal (Fig. 1 B). Importantly, maximal relaxation velocity in the TG4 was equal to that obtained after maximal stimulation with  $\beta$ -AR agonist in control animals (Fig. 1).

**Molecular alterations in response to targeted  $\beta_2$ -AR overexpression.** Associated with the above hemodynamic changes in the TG4 mice exposed to chronic cardiac-specific  $\beta_2$ -AR stimulation were selective molecular alterations in the level of the  $\text{Ca}^{2+}$  transport proteins. Phospholamban protein levels in the TG4 mice were quantified by immunoreactivity. The amount of phospholamban was determined by Western analysis after electrophoresing graded concentrations of purified phospholamban protein in each gel along with samples of cardiac extract from each group. A linear relationship between the scanned density and the amount of purified phospholamban loaded is shown in Fig. 2, which was reproducible on multiple blots.

Analysis of samples from the transgenic mouse hearts exposed to chronic  $\beta_2$ -AR stimulation revealed a significant reduction in total phospholamban in the LV compared with con-

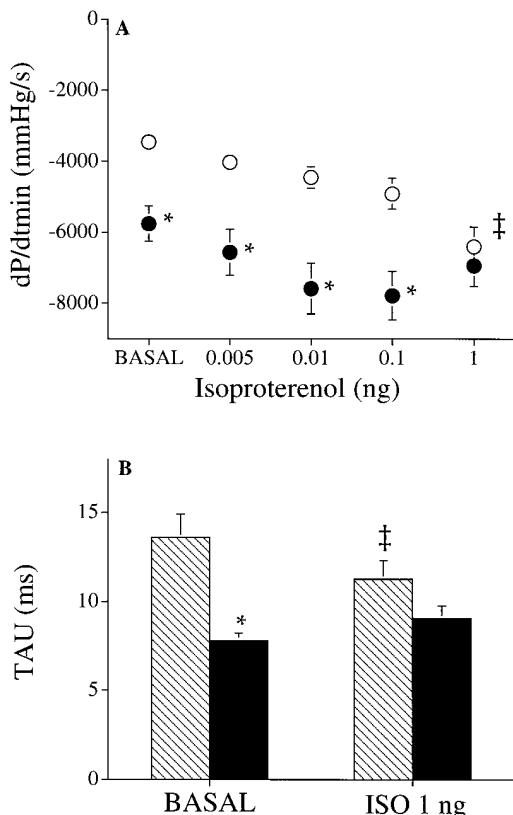
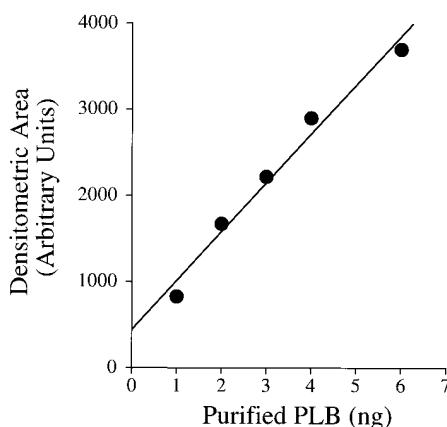


Figure 1. Myocardial relaxation is markedly enhanced in transgenic mice which overexpress  $\beta_2$ -AR. (A) The minimal first derivative of LV pressure (dP/dt<sub>min</sub>) is significantly shortened in the transgenic group (*n* = 9, filled circles) compared with control animals (*n* = 8, open circles). Control mice showed a further enhancement of relaxation velocity in response to isoproterenol stimulation. In contrast,  $\beta$ -AR stimulation in transgenic mice showed a trend toward enhanced relaxation velocity but this did not reach statistical significance (ANOVA). The pattern of change in dP/dt<sub>min</sub> in response to isoproterenol was statistically different between the two groups overall (ANOVA, *P* < 0.005). Post-hoc Scheffé analysis revealed \**P* < 0.005 transgenic vs. control at the indicated times points, ‡*P* < 0.005 control ISO 1 ng vs. control basal. (B) Time constant of isovolumic LV pressure decline (Tau). The load-independent measure of relaxation demonstrates marked enhancement of relaxation in the transgenic animals. Lack of response to isoproterenol (ISO) in the transgenic animals indicates that myocardial relaxation at baseline was maximal. Transgenic mice (*n* = 9), filled bars; control mice (*n* = 8), hatched bars. \**P* < 0.001 transgenic vs. control at basal, ‡*P* < 0.05 ISO 1 ng vs. basal for control animals, *t* test with Bonferroni correction for multiple comparisons.

trol. Fig. 3 depicts a representative Western blot showing reduced levels of phospholamban in the transgenic group. Quantification of phospholamban protein levels by densitometric scanning indicates a significant reduction of phospholamban in TG4 mice (*n* = 9), compared with negative littermate controls (*n* = 10), and represents an  $\sim$  44% reduction in phospholamban protein in the transgenic group (Fig. 4).

In separate experiments, we also quantified the levels of SR  $\text{Ca}^{2+}$  ATPase and calsequestrin in extracts from five control and five TG4 hearts. No difference in the level of SR  $\text{Ca}^{2+}$  ATPase or calsequestrin protein was observed in the transgenic hearts compared with littermate controls (Fig. 5) (control vs. TG4: SR  $\text{Ca}^{2+}$  ATPase 0.97  $\pm$  0.26 vs. 1.23  $\pm$  0.34, *P* = NS; calse-



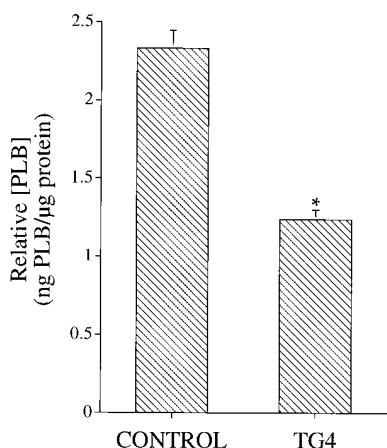
**Figure 2.** Quantitation of phospholamban (PLB) immunoreactivity in murine heart. Different amounts of purified PLB (1–6 ng) were loaded onto a 10–12.5% SDS gel and PLB immunoreactivity was detected by Western blotting. Linear relationship between various amounts of PLB and scanned area is demonstrated. Purified PLB served as a standard to minimize variability between blots. Regression equation  $y = 567.4x + 448$ ;  $R = 0.99$ .

questrin  $0.95 \pm 0.08$  vs.  $0.78 \pm 0.06$ ,  $P = \text{NS}$ ; arbitrary units). In contrast, phospholamban levels in the same samples were significantly reduced in the transgenic heart, confirming earlier data ( $1.11 \pm 0.06$  vs.  $0.85 \pm 0.08$ ,  $P = 0.025$ , Fig. 5).

## Discussion

The findings of this study indicate that targeted overexpression of the human  $\beta_2$ -AR is associated with marked enhancement of myocardial relaxation and reduced phospholamban protein levels.

In this study we used both the peak rate of LV pressure decline ( $dP/dt_{\text{min}}$ ) and the time constant of LV isovolumic pressure decline (Tau) to assess and quantitate myocardial relaxation *in vivo* in mice. Previous studies in larger species have shown that loading conditions and  $\beta$ -adrenergic tone are important determinants of relaxation velocity (13, 20). The effect of increasing membrane  $\beta_2$ -AR in myocytes is to markedly enhance relaxation processes as shown by both the load dependent ( $dP/dt_{\text{min}}$ ) and independent (Tau) measures. LV relax-

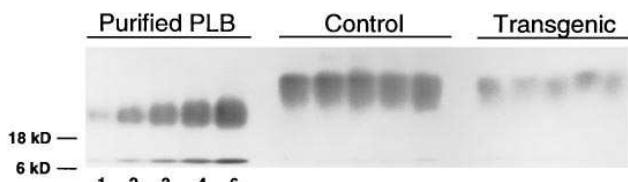


**Figure 4.** Quantification of relative concentration of phospholamban (PLB) in mouse heart. The amount of PLB in each sample was determined from a standard of purified PLB (see Fig. 2) and normalized for the amount of protein loaded. Control  $n = 10$ , transgenic  $n = 9$ .  $^*P < 0.00001$  vs. control samples.

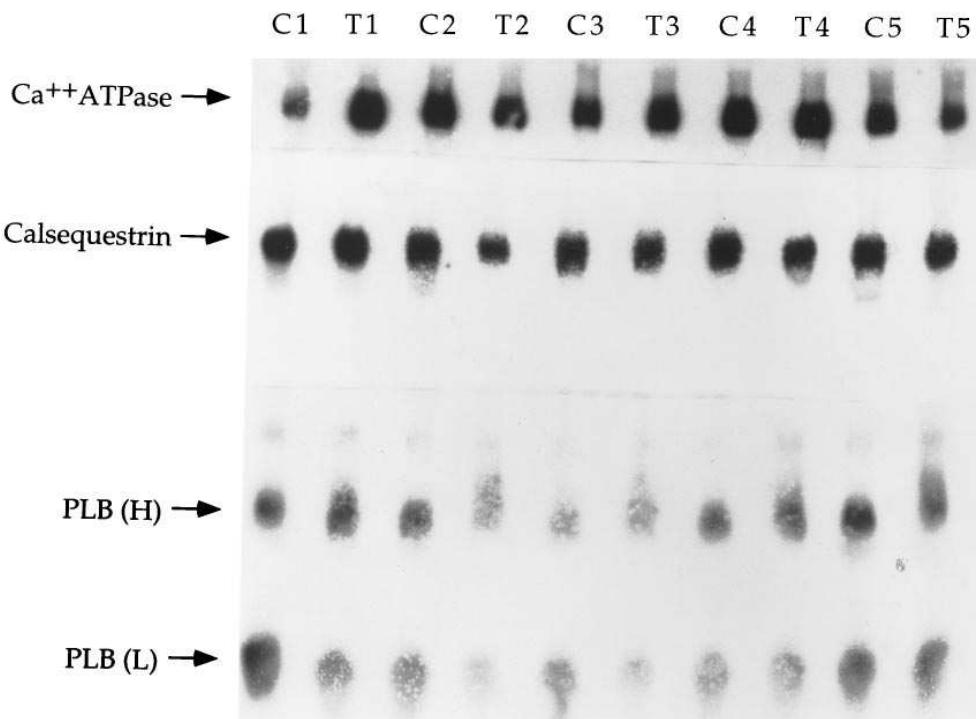
ation velocity is mostly determined by the rate of  $\text{Ca}^{2+}$  sequestration by the SR. It is interesting to note that Tau is considerably shorter in normal mice compared with larger species (range of Tau in the dog 23–40 ms) (11–13). Given the high heart rates of these small animals it follows that active processes involving myocardial relaxation would be considerably faster to allow time for adequate ventricular filling.

$\beta$ -AR stimulation mediated through activation of PKA can importantly increase the amplitude and rate of the intracellular  $\text{Ca}^{2+}$  transient. Although decreased myofilament  $\text{Ca}^{2+}$  sensitivity might contribute to the more rapid relaxation with  $\beta$ -AR stimulation due to the increased off-rate of  $\text{Ca}^{2+}$  from troponin C, evidence suggests that enhancement of relaxation is mainly attributed to stimulation of the SR  $\text{Ca}^{2+}$  pump rather than a decrease in myofilament sensitivity (3, 21). Phosphorylation of phospholamban by PKA acts to regulate SR  $\text{Ca}^{2+}$  ATPase activity to enhance  $\text{Ca}^{2+}$  uptake through disinhibition of the SR  $\text{Ca}^{2+}$  pump (3–5, 10). It is of interest that a recent *in vitro* study in rat ventricular myocytes suggested that  $\beta_2$ -AR signaling pathways may produce changes in the cytosolic  $\text{Ca}^{2+}$  transient via different mechanisms than  $\beta_1$ -AR signal transduction (7). In support of this hypothesis was a reduced extent of phospholamban phosphorylation in response to  $\beta_2$ -AR stimulation compared with  $\beta_1$ -AR stimulation, suggesting that  $\beta_2$ -AR effects may rely on mechanisms other than PKA-dependent pathways (7).

In this study there did not appear to be differences in mobility shift for phospholamban to suggest differential phosphorylation in the transgenic group compared with negative littermates. A possible explanation is that we studied the effect of chronic  $\beta_2$ -AR stimulation in the intact heart as opposed to the *in vitro* cell culture system with a shorter exposure to  $\beta_2$ -AR agonists. Furthermore, elevated sympathetic nervous activity associated with the brief duration of anesthesia required to exercise the heart may have increased the level of phospholamban phosphorylation in the control hearts to that of the TG4's. To adequately test for differences in phospholamban phosphorylation in the intact heart in these animals, metabolic labeling experiments will be required. However, we do demonstrate that in response to chronic  $\beta_2$ -AR stimulation, there is a selective reduction in phospholamban protein content that may operate in part to enhance SR  $\text{Ca}^{2+}$  uptake and relaxation processes *in vivo*. Although the physiological significance of this finding is not certain, a reduction in phospholamban content would not only result in enhanced myocardial relaxation but



**Figure 3.** Western blot showing that overexpression of  $\beta_2$ -AR is associated with downregulation of myocardial phospholamban protein levels. 3  $\mu$ g of unboiled protein extract from the LV chamber of control and transgenic hearts was loaded onto a 10% SDS polyacrylamide gel. Quantification of phospholamban (PLB) was derived from a standard of purified phospholamban (1, 2, 3, 4, and 6 ng) run with each gel. Phosphorylation-induced mobility shift is only noted between purified phospholamban and the heart samples lanes. No apparent mobility shift is observed between control and transgenic hearts. Migration of molecular mass standards (kD) is indicated at the left.



**Figure 5.** SR  $\text{Ca}^{2+}$  ATPase, calsequestrin, and phospholamban (PLB) immunoreactivity in hearts from control (C,  $n = 5$ ) and transgenic (T,  $n = 5$ ) mice showing the selective reduction in phospholamban with targeted  $\beta_2$ -AR stimulation. The autoradiograph of the nitrocellulose sheet is shown. Radioactivity was quantified with use of a GS-250 Molecular Imaging System (Bio-Rad Laboratories). Densitometric quantification of the various autoradiograms, control vs. transgenic (arbitrary units): SR  $\text{Ca}^{2+}$  ATPase  $0.97 \pm 0.26$  vs.  $1.23 \pm 0.34$ ,  $P = \text{NS}$ ; calsequestrin  $0.95 \pm 0.08$  vs.  $0.78 \pm 0.06$ ,  $P = \text{NS}$ ; PLB  $1.11 \pm 0.06$  vs.  $0.85 \pm 0.08$ ,  $P = 0.025$ . Both high (H) and low (L) molecular weight forms of PLB were used in the quantification (see text).

would also limit the ability for  $\beta$ -AR stimulation to modulate SR  $\text{Ca}^{2+}$  uptake under conditions of varying sympathetic activity. Consistent with this hypothesis is a recent study in which the phospholamban gene has been disrupted. Knockout mice lacking phospholamban have increased  $\text{Ca}^{2+}$  uptake into the SR associated with enhanced myocardial contraction and relaxation which lack isoproterenol responsiveness as demonstrated in both the isolated working heart preparation and in vivo (22, 23). Normal control of cardiac contractility mediated through catecholamine stimulation of  $\beta$ -AR is an important mechanism for sustaining the circulation particularly under conditions of rest and exercise.

In an animal model of heart failure, decreased levels of SR  $\text{Ca}^{2+}$  ATPase (24) have been associated with LV dysfunction. Results obtained with human heart samples are more controversial, some investigators noting decreases in these two proteins (25, 26) and others no changes (18, 27). In a recently developed model of right ventricle heart failure in the mouse, we observed downregulation of both phospholamban mRNA and protein levels with a small but significant decrease in the steady state levels of the SR  $\text{Ca}^{2+}$  ATPase protein. In this regard, we have hypothesized previously that the observed downregulation of phospholamban may serve as an adaptive mechanism in the early phase of heart failure by disinhibition of the  $\text{Ca}^{2+}$  pump (10). The results of this study are consistent with this hypothesis since catecholamine levels can be significantly elevated in the early phases of heart failure (28). Sustained sympathetic activity may both phosphorylate and downregulate phospholamban and may act to enhance  $\text{Ca}^{2+}$  pump activity and increase SR loading in the attempt to maintain contractile and relaxation processes. However, associated with decreased levels of phospholamban would be the loss of the normal regulatory control mechanisms for SR  $\text{Ca}^{2+}$  uptake.

Previous studies with isoproterenol administration have led to a cardiac hypertrophic phenotype. Whether these findings

are related to the activation of  $\beta$ -AR signaling pathways or to isoproterenol-induced myocardial fibrosis is not clear (29). In vitro, the hypertrophic response from  $\beta$ -AR stimulation is likely due to stimulation of nonmyocyte cells releasing a variety of growth factors (30) and possibly induction of myocyte beating (31). Our data are consistent with this hypothesis, since we are unable to demonstrate the presence of cardiac hypertrophy (as assessed by LV weight) in the TG4 mice which have myocyte-targeted overexpression of  $\beta_2$ -AR and thus myocyte cell-specific  $\beta$ -adrenergic stimulation. It is likely that isoproterenol administration to the whole animal stimulates a variety of cell types in the heart, which may indirectly promote myocyte growth.

In summary, using a unique transgenic model of cardiac tissue targeted  $\beta_2$ -AR overexpression we assessed the physiological and biochemical events associated with in vivo myocardial relaxation. The findings of this study indicate that myocardial relaxation is both markedly enhanced and maximal in these mice and that chronic  $\beta$ -AR stimulation can lead to decreased phospholamban protein content without changes in SR  $\text{Ca}^{2+}$  ATPase or calsequestrin.

## Acknowledgments

We gratefully acknowledge Drs. John Ross, Jr. and Kirk U. Knowlton for their careful review of the manuscript and Carol Kent for her expert technical assistance.

This work was supported in part by the American Heart Association (grant 94-286 to H.A. Rockman).

## References

1. Tsien, R.W., B.P. Bean, P. Hess, J.B. Lansman, B. Nilius, and M.C. No wycky. 1986. Mechanisms of calcium channel modulation by  $\beta$ -adrenergic

agents and dihydropyridine calcium agonists. *J. Mol. Cell. Cardiol.* 18:691–710.

2. Solaro, R.J., A.J.G. Moir, and S.V. Perry. 1976. Phosphorylation of troponin I and inotropic effect of adrenaline in the perfused rabbit heart. *Nature (Lond.)*. 262:615–617.
3. Sham, J.S.K., L.R. Jones, and M. Morad. 1991. Phospholamban mediates  $\beta$ -adrenergic-enhanced  $\text{Ca}^{2+}$  uptake in mammalian ventricular myocytes. *Am. J. Physiol.* 261:H1344–H1349.
4. Lindemann, J.P., L.R. Jones, D.R. Hathaway, B.G. Henry, and A.M. Watanabe. 1983.  $\beta$ -adrenergic stimulation of phospholamban phosphorylation and  $\text{Ca}^{2+}$ -ATPase activity in guinea pig ventricles. *J. Biol. Chem.* 258:464–471.
5. Wegener, A.D., J.P. Lindemann, H.K.B. Simmerman, and J.R. Jones. 1989. Phospholamban phosphorylation in intact ventricles. *J. Biol. Chem.* 264:11468–11474.
6. Xiano, R.-P., and E.G. Lakatta. 1993.  $\beta_1$ -adrenoceptor stimulation and  $\beta_2$ -adrenoceptor stimulation differ in their effects on contraction, cytosolic  $\text{Ca}^{2+}$ , and  $\text{Ca}^{2+}$  current in single rat ventricular cells. *Circ. Res.* 73:286–300.
7. Xiano, R.-P., C. Hohl, R. Altschuld, L. Jones, B. Livingston, B. Ziman, B. Tantini, and E.G. Lakatta. 1994.  $\beta_2$ -adrenergic receptor-stimulated increase in cAMP in rat heart cells is not coupled to changes in  $\text{Ca}^{2+}$  dynamics, contractility, or phospholamban phosphorylation. *J. Biol. Chem.* 269:19151–19156.
8. Milano, C.A., L.F. Allen, H.A. Rockman, P.C. Dolber, T.R. McMinn, K.R. Chien, T.D. Johnson, R.A. Bond, and R.J. Lefkowitz. 1994. Enhanced myocardial function in transgenic mice overexpressing the  $\beta_2$ -adrenergic receptor. *Science (Wash. DC)*. 264:582–586.
9. Rockman, H.A., S.P. Wachhorst, L. Mao, and J. Ross, Jr. 1994. Angiotensin II receptor blockade prevents ventricular hypertrophy and ANF gene expression with pressure overload in mice. *Am. J. Physiol.* 266:H2468–H2475.
10. Rockman, H.A., S. Ono, R.S. Ross, L.R. Jones, M. Karim, V. Bhargava, and J. Ross, Jr., and K.R. Chien. 1994. Molecular and physiological alterations in murine ventricular dysfunction. *Proc. Natl. Acad. Sci. USA*. 91:2694–2698.
11. Yellin, E.L., M. Hori, C. Yoran, E.H. Sonnenblick, S. Gabbay, and R.W.M. Frater. 1986. Left ventricular relaxation in the filling and nonfilling intact canine heart. *Am. J. Physiol.* 250:H620–H629.
12. Weisfeldt, M.L., J.W. Frederiksen, F.C.P. Yin, and J.L. Weiss. 1978. Evidence of incomplete left ventricular relaxation in the dog. *J. Clin. Invest.* 62:1296–1302.
13. Blaustein, A.S., and W.H. Gaasch. 1983. Myocardial relaxation. VI. Effects of  $\beta$ -adrenergic tone and asynchrony on LV relaxation rate. *Am. J. Physiol.* 244:H417–H422.
14. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
15. Wegener, A.D., and J.R. Jones. 1984. Phosphorylation-induced mobility shift in phospholamban in sodium dodecyl sulfate-polyacrylamide gels. *J. Biol. Chem.* 259:1834–1841.
16. Jones, L.R., and L.J. Field. 1993. Residues 2–25 of phospholamban are insufficient to inhibit the  $\text{Ca}^{2+}$  transport ATPase of cardiac sarcoplasmic reticulum. *J. Biol. Chem.* 268:1–3.
17. Ungerer, M., G. Parruti, M. Bohm, M. Puzicha, A. DeBlasi, E. Erdmann, and M.J. Lohse. 1993. Expression of  $\beta$ -arrestins and  $\beta$ -adrenergic receptor kinases in the failing human heart. *Circ. Res.* 74:206–213.
18. Movsesian, M.A., M. Karimi, K. Green, and L.R. Jones. 1994.  $\text{Ca}^{2+}$ -transporting ATPase, phospholamban, and calsequestrin levels in nonfailing and failing human myocardium. *Circulation*. 90:653–657.
19. Porzio, M.A., and A.M. Pearson. 1977. Improved resolution of myofibrillar proteins with sodium dodecyl sulfate A-polyacrylamide gel electrophoresis. *Biochim. Biophys. Acta*. 490:27–34.
20. Varma, S.K., R.M. Owen, M.L. Smucker, and M.D. Feldman. 1989. Is  $\tau$  a preload-independent measure of isovolumetric relaxation? *Circulation*. 80:1757–1765.
21. Endoh, M., and J.R. Blinks. 1988. Actions of sympathomimetic amines on the  $\text{Ca}^{2+}$  transients and contractions of rabbit myocardium: reciprocal changes in myofibrillar responsiveness to  $\text{Ca}^{2+}$  mediated through  $\alpha$ - and  $\beta$ -adrenoceptors. *Circ. Res.* 62:247–265.
22. Luo, W., I.L. Grupp, J. Harrer, S. Ponniah, G. Grupp, J.J. Duffy, T. Doetschman, and E.G. Kranias. 1994. Targeted ablation of the phospholamban gene is associated with markedly enhanced myocardial contractility and loss of  $\beta$ -agonist stimulation. *Circ. Res.* 75:401–409.
23. Hoit, B.D., S.F. Koury, E.G. Kranias, N. Ball, and R. Walsh. 1995. In vivo echocardiographic detection of enhanced left ventricular function in gene-targeted mice with phospholamban deficiency. *Circ. Res.* 77:632–637.
24. Feldman, A.M., E.O. Weinberg, P.E. Ray, and B.H. Lorell. 1993. Selective changes in cardiac gene expression during compensated hypertrophy and the transition to cardiac decompensation in rats with chronic aortic banding. *Circ. Res.* 73:184–192.
25. Arai, M., N.R. Alpert, D.H. MacLennan, P. Barton, and M. Periasamy. 1993. Alterations in sarcoplasmic reticulum gene expression in human heart failure: a possible mechanism for alterations in systolic and diastolic properties of the failing myocardium. *Circ. Res.* 72:463–469.
26. Feldman, A.M., P.E. Ray, C.M. Silan, J.A. Mercer, W. Minobe, and M.R. Bristow. 1991. Selective gene expression in failing human heart: quantification of steady-state levels of messenger RNA in endomyocardial biopsies using the polymerase chain reaction. *Circulation*. 83:1866–1872.
27. Bohm, M., B. Reiger, R.H.G. Schwinger, and E. Erdmann. 1994. cAMP concentrations, cAMP dependent protein kinase activity, and phospholamban in non-failing and failing myocardium. *Cardiovasc. Res.* 28:1713–1719.
28. Cohn, J.N., B. Levine, M.T. Olivari, V. Garberg, D. Lura, G.S. Francis, A.B. Simon, and T. Rector. 1984. Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. *N. Engl. J. Med.* 311:819–823.
29. Benjamin, I.J., J.E. Jalil, L.B. Tan, K. Cho, K.T. Weber, and W.A. Clark. 1989. Isoproterenol-induced myocardial fibrosis in relation to myocyte necrosis. *Circ. Res.* 65:657–670.
30. Long, C.S., W.E. Hartogensis, and P.C. Simpson. 1993.  $\beta$ -adrenergic stimulation of cardiac non-myocytes augments the growth-promoting activity of non-myocyte conditioned medium. *J. Mol. Cell. Cardiol.* 25:915–925.
31. Long, C.S., K. Kariya, L. Karns, and P.C. Simpson. 1992. Sympathetic modulation of the cardiac myocyte phenotype: studies with a cell-culture model of myocardial hypertrophy. *Basic Res. Cardiol.* 87(Suppl. 12):19–31.