JCI The Journal of Clinical Investigation

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J Clin Invest. 1997;100(2):380-389. https://doi.org/10.1172/JCI119544.

Research Article

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Overexpression of the Rat Sarcoplasmic Reticulum Ca^{2+} ATPase Gene in the Heart of Transgenic Mice Accelerates Calcium Transients and Cardiac Relaxation

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Abstract

The Ca^{2+} ATPase of the sarcoplasmic reticulum (SERCA2) plays a dominant role in lowering cytoplasmic calcium levels during cardiac relaxation and reduction of its activity has been linked to delayed diastolic relaxation in hypothyroid and failing hearts. To determine the contractile alterations resulting from increased SERCA2 expression, we generated transgenic mice overexpressing a rat SERCA2 transgene. Characterization of a heterozygous transgenic mouse line (CJ5) showed that the amount of SERCA2 mRNA and protein increased 2.6-fold and 1.2-fold, respectively, relative to control mice. Determination of the relative synthesis rate of SERCA2 protein showed an 82% increase. The mRNA levels of some of the other genes involved in calcium handling, such as the ryanodine receptor and calsequestrin, remained unchanged, but the mRNA levels of phospholamban and Na^+/Ca^{2+} exchanger increased 1.4-fold and 1.8-fold, respectively. The increase in phospholamban or Na^+/Ca^{2+} exchanger mRNAs did not, however, result in changes in protein levels. Functional analysis of calcium handling and contractile parameters in isolated cardiac myocytes indicated that the intracellular calcium decline ($t_{1/2}$) and myocyte relengthening ($t_{1/2}$) were accelerated by 23 and 22%, respectively. In addition, the rate of myocyte shortening was also significantly faster. In isolated papillary muscle from SERCA2 transgenic mice, the time to half maximum postrest potentiation was significantly shorter than in negative littermates. Furthermore, cardiac function measured in vivo, demonstrated significantly accelerated contraction and relaxation in SERCA2 transgenic mice that were further augmented in both groups with isoproterenol administration. Similar results were obtained for the contractile performance of myocytes isolated from a separate line (CJ2) of homozygous SERCA2 transgenic mice. Our findings suggest, for the first time, that increased SERCA2 expression is feasible in vivo and results in enhanced calcium transients, myocardial contractility, and relaxation that may have further therapeutic implications. (*J. Clin. Invest.* 1997. 100: 380–389.)

Key words: sarcoplasmic reticulum $Ca2^+$ ATPase • transgenic mice • Ca^{2+} transient measurement • edge detection • contractility

Introduction

Cardiac contraction and relaxation are mediated by the rapidly changing calcium concentration around the myofibrils (1). The major contribution to diastolic calcium lowering is made by the Ca^{2+} ATPase of the sarcoplasmic reticulum (SERCA2)[1] pumping cytosolic calcium into the lumen of the sarcoplasmic reticulum (SR) (2). The calcium pump is a 110,000-D single peptide that moves two molecules of calcium from the cytoplasm of the myocyte into the sequestered space of the SR per molecule of hydrolyzed ATP. Additional contributions to calcium lowering are made by the Na^+/Ca^{2+} exchanger and the calcium pump of the sarcolemma (1). SERCA2 is a member of the P-type ion-motive ATPases that form a phosphoprotein intermediate during ion translocation. Five distinct SERCA isoforms that are encoded by three separate genes have been described (3–5). The SERCA1 gene encodes two alternatively spliced variants, SERCA1a and SERCA1b, which are expressed in adult fast skeletal muscle and neonatal fast skeletal muscle, respectively (3). The SERCA2 gene also encodes two alternatively spliced variants, SERCA2a and SERCA2b (4). SERCA2a is expressed in cardiac and slow-twitch muscles, whereas SERCA2b is mainly expressed in smooth muscle and nonmuscle tissues (6, 7). A third gene, SERCA3, is expressed in a variety of nonmuscle tissues including endothelial and some epithelial cells (5). Detailed information on functional differences and specific physiological roles of these isoforms are not available although it has been shown in vitro that SERCA1 and 2a are similar in sensitivity to calcium and the translocation rate of calcium, whereas, SERCA2b or SERCA3 have either higher or lower affinities for calcium (7, 8).

The level of SERCA2 gene expression in the heart is modified by several physiological and pathophysiological conditions. SERCA2 mRNA is at low levels in fetal rats, but increases markedly after birth to reach a peak in young animals and subsequently decreases in aging rats (9–11). SERCA2 activity is also subjected to regulation by hormonal factors, such as thyroid hormones and by β-adrenergic stimulation. Thyroid hormones stimulate SERCA2 activity through upregulation of its transcriptional activity (12, 13), whereas β-agonists enhance SERCA2 activity through phosphorylation of its inhibitor phospholamban (PLB) (14, 15).

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Received for publication 30 August 1996 and accepted in revised form 11 April 1997.

J. Clin. Invest.

0021-9738/97/07/0380/10 $2.00
Volume 100, Number 2, July 1997, 380–389

1. *Abbreviations used in this paper:* cβA, chicken β-actin; hCMV-enhncr, human cytomegalovirus immediate early enhancer; LV, left ventricle; PLB, phospholamban; SERCA2, sarcoplasmic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum.

Pathophysiological influences on SERCA2 gene expression have been demonstrated in animals undergoing pressure overload induced cardiac hypertrophy (16–19) and in human beings with end-stage heart failure (20–25). In each of these conditions, SERCA2 mRNA and protein levels are markedly decreased. In human-dilated cardiomyopathy, SERCA2 protein, enzyme activity, and calcium transients are decreased according to findings of several groups of investigators (25, 26), however, some investigators were not able to confirm a decrease of SERCA protein levels in human heart failure (27). In addition, in the failing heart, the excitation contraction process is altered and this may be at least, in part, caused by a decrease in SERCA2 activity and resultant abnormalities in calcium transients (28).

To obtain additional insights into the relationship between cardiac SERCA2 level, calcium handling in the heart, and contractile consequences, we constructed transgenic mice expressing a rat SERCA2 transgene in the heart. These animals will allow us to determine if an increased amount of SERCA2 pump produced from the transgene results in a commensurate change in contractile function. Transgenic mice that express the transgene showed increased SERCA2 mRNA and protein levels that resulted in increased contractile performance, showing for the first time that SERCA2 activity can be markedly increased using a transgenic approach.

Methods

Construction of SERCA2 transgenic mice. For the construction of transgenic mice, a rat SERCA2a transgene was cloned into the pBluescript SK plasmid (Stratagene Inc., La Jolla, CA). The rat SERCA2a transcript consists of the first exon except for the first 200 nucleotides of the transcribed but untranslated 5′-end, first intron, second exon, second intron, and part of the third exon obtained from the rat SERCA2 genomic clone described by our laboratory (12, 13). The rest of the exon and the remainder of SERCA2 cDNA was previously obtained by our laboratory from a rat heart cDNA library (12). Expression of the transgene was driven by a human cytomegalovirus immediate early enhancer (hCMV-enhncr) linked to the chicken β-actin (cBA) promoter followed by the cDNA. This region was isolated from pCAGGS (29) and cloned upstream of the rat SERCA2 transgene (Fig. 1 *A*). The mRNA transcript of the transgene consists, therefore, of the first exon of the chicken β-actin, which is transcribed but untranslated, and the rat SERCA2 exons described above. The chicken β-actin intron and the two SERCA2 introns were included because it has been reported that intron inclusion in transgenes enhance their expression in transgenic animals (30, 31). To isolate the expression unit of the SERCA2 gene, the plasmid was cleaved with SalI and BamHI. Standard techniques were used to generate transgenic mice (32). In brief, the pronucleus of eggs from superovulated BalbC mice crossed with C57/Black 6 male mice were injected with 1–2 pl of the purified DNA fragment at a concentration of 2 μg/ml. The injected eggs were then transferred into the oviduct of pseudopregnant BalbC mice. Litters were delivered after ~ 20 d of gestation.

To determine transgene integration, genomic DNA was extracted from tails of 3-wk-old mice and subject to Southern blot analysis. The tail DNA was digested with ApaI endonuclease, resolved on an agarose gel, transferred on to a nylon membrane, and hybridized to a [^{32}P]-labeled transgene-specific probe, which corresponded to the first chicken β-actin intron (Fig. 1 *A*). In SERCA2 transgene positive animals, ApaI digestion would generate a 1.2-kb fragment that specifically hybridized to the probe. Two transgenic lines were obtained, CJ5 and CJ2. To determine the copy number of the SERCA2 trans-

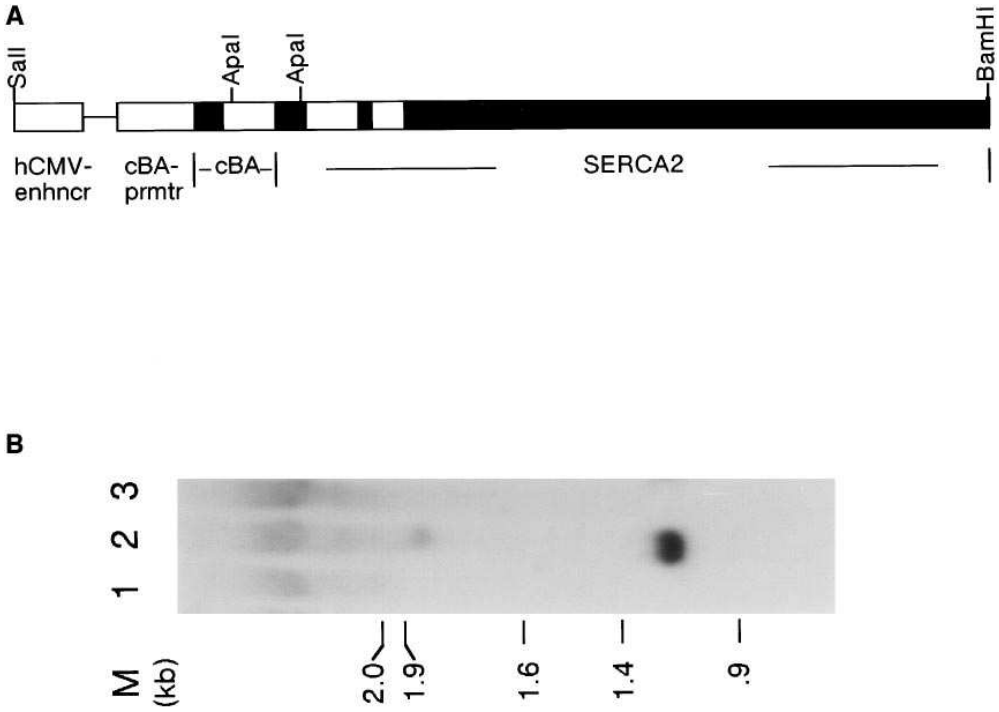

Figure 1. (*A*) Schematic depiction of the SERCA2 transgene fragment (SalI–BamHI) used to generate SERCA2 transgenic mice. hCMV-enhncr, human cytomegalovirus immediately–early enhancer; cBA prmtr, chicken β-actin promoter. cBA between vertical border lines: first exon (*filled box*; no coding region) and first intron (*open box*) of chicken β-actin gene. SERCA2 between vertical border lines: first exon, first intron, second exon, second intron followed by the rest of the SERCA2 cDNA (*filled boxes for exons and open boxes for introns*). An intron fragment of chicken β-actin (~ 900 bp) was isolated and used as a probe for Southern blot analysis. (*B*) Mouse tail DNA was digested with ApaI and hybridized to the transgene-specific probe, [^{32}P]-labeled ApaI–ApaI fragment (see above). Lanes *1*, *2*, and *3* represent DNA isolated from three littermates of CJ5 line. The animals in lane *2* are transgene positive and lanes *1* and *3* represent transgene negative littermates.

gene in the CJ2 and CJ5 lines, the approach previously described (32) was used using mouse tail DNA digested with ApaI subject to Southern blots as described above. A probe corresponding to the first exon of SERCA2 was used for hybridization and quantitation. Signals resulting from SERCA2 transgenes and the endogenous gene were quantitated densitometrically on Southern blots.

Northern blot analysis. Isolation of tissue RNA was as described by Chomczynski et al. (33). Isolation of polyA RNA afterwards was done by use of an Oligo(dT) kit (Qiagen Inc., Chatsworth, CA). For electrophoresis, Northern transfer and hybridization standard protocols were used (34). To characterize the SERCA2 transgene expression, a 1.8-kb EcoRI restriction fragment corresponding to the 5′-end of the rat SERCA2 cDNA was used. The restriction fragment of mouse phospholamban, rabbit calsequestrin, rabbit ryanodine receptor, and dog Na^+/Ca^{2+} exchanger cDNA were isolated and used to characterize the expression of these calcium handling genes. Rabbit calsequestrin and ryanodine receptor constructs were generous gifts from Dr. A. Zarain-Herzberg and the cDNA for the Na^+/Ca^{2+} exchanger was a generous gift from Dr. K. Philipson.

To assure that the rat SERCA2 cDNA probe showed similar hybridization for the rat SERCA2 transgene and the mouse SERCA2 gene, we used the following approach. The membrane was washed first at a lower stringency (0.5 × SSC/0.1% SDS, 50°C), then at a higher stringency (0.2 × SSC/0.1% SDS, 55°C), and it was found that the specific hybridization signal was reduced by the same proportion between control and transgenic samples that suggests that the probe has a similar specificity for SERCA2 of both species when membranes were washed at 0.2 × SSC/0.1% SDS, 55°C.

Western blot analysis. Antibodies used in the study are as follows. A rabbit polyclonal antibody against rat SERCA2 (SERCA2 Ab) was used as previously described (35). In addition to the SERCA2 Ab that was raised against the rat SERCA2, a commercial SERCA2 Ab (Affinity BioReagents, Inc., Neshanic Station, NJ) raised against the mouse SERCA2 was also used for comparison. It was found that the two antibodies produced essentially identical results in Western blot analysis. A mouse monoclonal antibody against canine phospholamban, referred to as PLB Ab and Na^+/Ca^{2+} exchanger antibody were purchased from Affinity BioReagents, Inc. A mouse monoclonal antibody against rabbit sarcomeric α-actin, referred to as α-actin Ab was purchased from Sigma Chemical Co. (St. Louis, MO).

Immunoblotting. 100 mg of mouse ventricle was homogenized as described by Meyer et al. (25). The crude homogenate was quantitated for protein content with a Bradford Reagent (BioRad Laboratories, Richmond, CA). 40 μg of protein was resolved on a 4–20% gradient Tris-glycine polyacrylamide gel (Novex Experimental Technology, San Diego, CA). After electrophoresis, protein was transferred on to a nitrocellulose membrane in a BioRad semi-dry transfer apparatus according to the instructions by the manufacturer (BioRad Laboratories). The membrane was cut between 70- and 97-kD prestained markers (Gibco-BRL, Grand Island, NY). The upper portion was used for immunoblotting with SERCA2 Ab and the lower portion was used for simultaneous immunoblotting with α-actin Ab and PLB Ab. An ECL kit (Amersham Corp., Arlington Heights, IL) was used for signal detection.

Preparation of adult cardiac myocytes. Ventricular myocytes from adult mouse hearts were prepared by a modified collagenase-dissociation method used for rat hearts (36). In brief, the mouse heart was excised and the aorta was cannulated and connected to the perfusion rig. The heart was perfused for 4 min in calcium-free minimal essential medium (MEM) consisting of, in mmol/liter, 113 NaCl, 4.7 KCl, 0.6 KH_2PO_4, 0.6 Na_2HPO_4, 1.2 $MgSO_4$, 12 $NaHCO_3$, 20 D-glucose, 10 Na^+-Hepes, 30 taurine, 2 creatine, 2 carnitine, and 1 × basal medium eagle amino acids (Gibco-BRL), pH 7.4. Perfusion was then switched to MEM plus, in wt/vol, 0.08% collagenase B, 0.1% trypsin, 0.1% BSA, and 25 μmol/liter $CaCl_2$ under continuous equilibrium with 95% O_2/5% CO_2. After 15 min of enzymatic digestion, the atria were then removed, ventricles were cut into several pieces and the cells were dispersed by gentle agitation through a wide bore serologic pipette. Myocytes were filtered and washed and 1 mmol/liter calcium was reintroduced gradually to the cells.

Determination of relative protein synthesis rate. Relative protein synthesis rate was determined as described (37). In brief, adult cardiac myocytes were prepared as described above. 30 min after the preparation, myocytes were washed once with Met-deficient, high glucose-containing DME (Gibco-BRL). 1 ml of the same medium containing 100 μCi of [^{35}S]-amino acid mixture consisting of labeled methionine and cysteine (Trans ^{35}S-Label, ICN Biomedicals Inc., Irvine, CA) was added and incubated at 37°C for 4 h. To harvest cells, cells were washed twice with cold 1 × PBS, lysed by incubation for 10 min with 200 μl single detergent lysis buffer (1% Triton X-100, 50 mM Tris, pH 8.0, 150 mM NaCl, 100 μg/ml PMSF, 1 μg/ml aprotinin) (34) and subsequently scraped and collected. The lysates were vortexed for 30 s. The quantitation of protein was performed as described above.

Immunoprecipitation. The immunoprecipitation protocol as described by Kessler (37) was used. In brief, 300 μg of protein in crude lysate after protein labeling was mixed with 6 μl of SERCA2 Ab in a total volume of 300 μl and incubated at 4°C for 1 h on a rotating platform. 100 μl of 10% fixed *S. aureus* Cowan I (SAC) was added and was further incubated as above for 1 h. To purify the immunoprecipitated proteins, the mixture was spun at 10,000 *g* for 1 min at room temperature and the pellet was washed with the cold lysis buffer. This was repeated three more times. The pellet was finally resuspended in 50 μl of 1 × Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris, pH 6.8) and heated at 85°C for 10 min. In the supernatant, radioactive counts were determined and equal counts of radioactivity were loaded on SDS-PAGE for each sample.

Measurement of Ca^{2+} transient. Myocytes were incubated with 10 μmol/liter Indo-1/AM for 15–30 min at 37°C in an atmosphere of 5% CO_2/95% air in cell culture medium (DME/F12) supplemented with 1 mg/ml BSA plus 0.01% (wt/vol) Pluronic (Molecular Probes, Eugene, OR). The cells were then rinsed and the media replaced with Tyrode's solution (136 mmol/liter NaCl, 5 mmol/liter KCl, 0.5 mmol/liter $MgCl_2$, 2 mM $CaCl_2$, pH 7.4) and allowed to sit at room temperature for ~ 20 min before fluorescence measurements were begun to allow deesterification of the Indo-1/AM. Fluorescence measurements were performed at room temperature using the methods and apparatus (Photon Technologies Inc., South Brunswick, NJ) previously described (38). Cells were stimulated to contract (0.3 Hz) using platinum electrodes in 1 ml Tyrode's solution in a Biophysica (Sparks, MD) coverslip chamber. The data were collected at 20 Hz. The experiment was performed by investigators blinded to the identity of animals.

Myocytic shortening measurement (edge detection). Contractility studies of cultured myocytes were performed with a video edge motion detector (Crescent Electronics, Sandy, UT) interfaced to a standard CCTV video camera (Panasonic Corp., Tokyo, Japan), which was attached to a Nikon Diaphot microscope (Melville, NY). A 40× Zeiss phase two objective was used (Carl Zeiss, Inc, Thornwood, NY). Output from the edge detector was interfaced to the RCQC reference channel of the Photon Technologies photometry system. Calibration was achieved by focusing the microscope on 26-μm microspheres (Dupont, Wilmington, DE); voltage output from the video system was linear with distance (0.037 V/μm). Cells were stimulated to contract at 0.3 Hz or 0.5 Hz. Edge detection records were obtained at 60 or at 240 Hz. Edge detection measurements were performed on myocytes at room temperature in Tyrode's solution on cells that were not loaded with Indo-1. This experiment was performed by investigators blinded to the identity of the animals from which the cells were isolated.

Isolated papillary muscle contraction. Left ventricular (LV) papillary muscles from eight SERCA2 transgenic mice and eight negative littermates of either sex (body weight 27.6±1.3 grams) were excised under oxygenated Tyrode's solution containing 30 mM 2,3-butanedione monoxime (BDM). They were inserted into Ω-shaped clamps made from strips of platinum foil, tied with 6.0 braided silk suture,

and mounted on hooks of platinum wire in a 0.5-ml muscle chamber. Muscles were perfused with 2.5 mM Ca^{2+} Tyrode's solution at 37°C and stimulated at 2 Hz through the platinum clamps (1.5 V, 0.25 ms duration). Force was measured with an isometric force transducer (OPT1L; Scientific Instruments, Heidelberg, Germany) and recorded on a strip chart recorder. Muscles were stretched over 30–60 min to the length at which active force development was maximal (L_{max}). Forces (mN) were normalized by the muscle cross-sectional areas to yield stresses (mN/mm^2). The cross-sectional area was calculated for each muscle as the ratio of muscle volume (determined by weighing) and muscle length at L_{max}.

The relaxation time (RT_{50}) was determined as the time from the peak of contraction to 50% of maximum developed stress during relaxation. Postrest potentiation was studied by stopping stimulation for intervals ranging from 0.5 to 15 s and resuming regular stimulation. The stress of the first postrest contraction reached a maximum after rest intervals of 5 to 15 s. The time to half maximum postrest potentiation (half-time) was determined for each muscle as the rest interval after which the stress of the first postrest contraction was closest to the average of the stresses of the steady-state contraction and the maximum postrest contraction. Data are expressed as mean±SEM. Statistical comparisons were made by unpaired Student's *t* test.

In vivo hemodynamic assessment. The procedure was as described previously (39). In brief, age-matched transgenic and control littermates were used. Animals were anesthetized with a mixture of ketamine (90–110 mg/kg) and xylazine (4–6 mg/kg) and placed in a supine position under a dissecting microscope. A midline incision was made and the carotid artery was cannulated with a PE 50 catheter, which was connected to a modified P50 transducer (Statham, Oxnard, CA) for measurement of carotid artery pressure. The external jugular vein was cannulated with a PE 50 catheter that was used for isoproterenol infusion (0.005–1 ng). Isoproterenol doses $>$ 1 ng led to a marked increase in heart rate and were, therefore, not used. After bilateral vagotomy, the chest was opened and a high fidelity 1.8 or 2 F micromanometer catheter was inserted into the left atrium and advanced into the LV. Continuous LV and 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 2,000 Hz for later analysis. Parameters measured were heart rate, aortic pressure, LV systolic and diastolic pressure (dP/dt_{max} and dP/dt_{min}). 10 sequential beats were averaged for each measurement. The time constant of isovolumic pressure decay (Tau) was determined assuming the fall in LV pressure (P_{LV}) during isovolumic relaxation to be monoexponential with variable asymptote. The equation $P_{LV} = P_o e^{-t/T} + P_B$ allows calculation of extrapolated LV pressure assuming diastole to be infinite in duration (40, 41), where P_O is the pressure at peak negative dP/dt, t is the time from peak negative dP/dt, P_B is the asymptote and T is Tau. Calculation of Tau was performed using $\sim$ 20–22 points from the decay of LV pressure for both control and transgenic animals. Correlation coefficient for all studies were $>$ 0.98 (42). The experiments were performed by investigators blinded to the identity of the animals.

Statistical analysis. Data are expressed as mean value±SEM. To examine the effect of isoproterenol on changes in hemodynamic parameters between control and SERCA2 overexpressing transgenic mice, a two-way repeated measure ANOVA was used. Post-hoc analysis with regard to differences in mean values between the groups at a specific dose was performed with a Newman-Keuls test. A students *t* test was used to test for statistical difference in Tau, body, and chamber weights.

Results

Characterization of transgenic mice. Southern blot analysis of mouse tail DNA with a transgene-specific probe identified three separate founder mice. One male founder was sterile and two female founders passed on the transgene to their offspring. One of the lines, CJ5, was characterized in further detail. A second line, CJ2, also showed increased SERCA2 expression and was used in a more limited number of studies to assure that the functional effects of SERCA2 overexpression in the CJ5 line were not due to positional effects of SERCA2 transgene integration. Physiological measurements such as heart weight/body weight ratio, heart rate, and left ventricular pressure are summarized in Table I. No significant difference occurred in these parameters between control and SERCA2 transgenic animals (Table I). The transgene used to produce the mice and a representative Southern blot is shown in Fig. 1. In the heterozygous CJ5 line, the transgene copy number was two and it was six in heterozygous CJ2 mice. In heterozygous CJ5 mice, the total amount of SERCA2 mRNA was 2.6-fold higher than in transgene negative littermates. In contrast, in heterozygous CJ2 mice, SERCA2 mRNA levels were increased by 1.5-fold indicating that expression from the transgene incorporation site in CJ5 mice is higher than from the integration site in CJ2 mice. Because of the lower level of overexpression of SERCA2 mRNA in the CJ2 line, and the difficulty in breeding the CJ2 homozygotes, most of the studies were performed in CJ5 heterozygous mice. In Fig. 2, a representative Northern blot with mRNA from CJ5 mice coding for SERCA2 and other calcium-related genes the ryanodine receptor, calsequestrin, phospholamban, and the Na^+/Ca^{2+} exchanger is shown. Quantitation of the total amount of SERCA2 message indicated that this was increased by 2.6±0.20 fold ($P < 0.01$). When some of the Northerns were hybridized with a probe (ApaI fragment) corresponding to the chicken β-actin/SERCA2 fusion area, a signal runs at the same position as SERCA2, making it highly likely that the SERCA2 transgene–derived message contributed to the increase in total SERCA2 mRNA levels (Swanson, E., M.R. Sayen, and W.H. Dillmann, unpublished observation). In addition, we determined the level of the mRNAs coding for other calcium handling related genes. mRNAs for the ryanodine receptor and calsequestrin showed no significant change in their message level. In contrast, the mRNA for phospholamban was increased by 43±13% ($P < 0.01$) and the Na^+/Ca^{2+} exchanger mRNA was increased by 83±8% ($P < 0.01$). As shown in a Western blot (Fig. 2 *B*), the increase in phospholamban and

Table I. Cardiovascular and Basal Contractile Parameters in SERCA2 Transgenic and Wild-Type Mice

	Wild-type mice $n = 13$	SERCA2 transgene $n = 13$	*P* value*
Body weight (grams)	26.44±1.24	24.51±1.26	NS
Heart weight (mg)	138.22±6.79	123±5.7	= 0.09
Heart weight/body weight (mg/gram)	5.25±0.17	5.041±0.11	NS
Heart rate (bpm)	365±10.4	388±14	NS
LVSP (mmHg)	77±2.1	78±2.1	NS
LVEDP (mmHg)	2.69±0.37	3.14±0.44	NS
LV dP/dt_{max} (mmHg/s)	5428±387	7006±331	< 0.001
LV dP/dt_{min} (mmHg/s)	−4032±256	−4674±323	= 0.01
Tau (ms) ($n = 10$)	16.67±1.7	13.43±0.68	= 0.01

*Compared by paired *t* test or ANOVA. LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure.

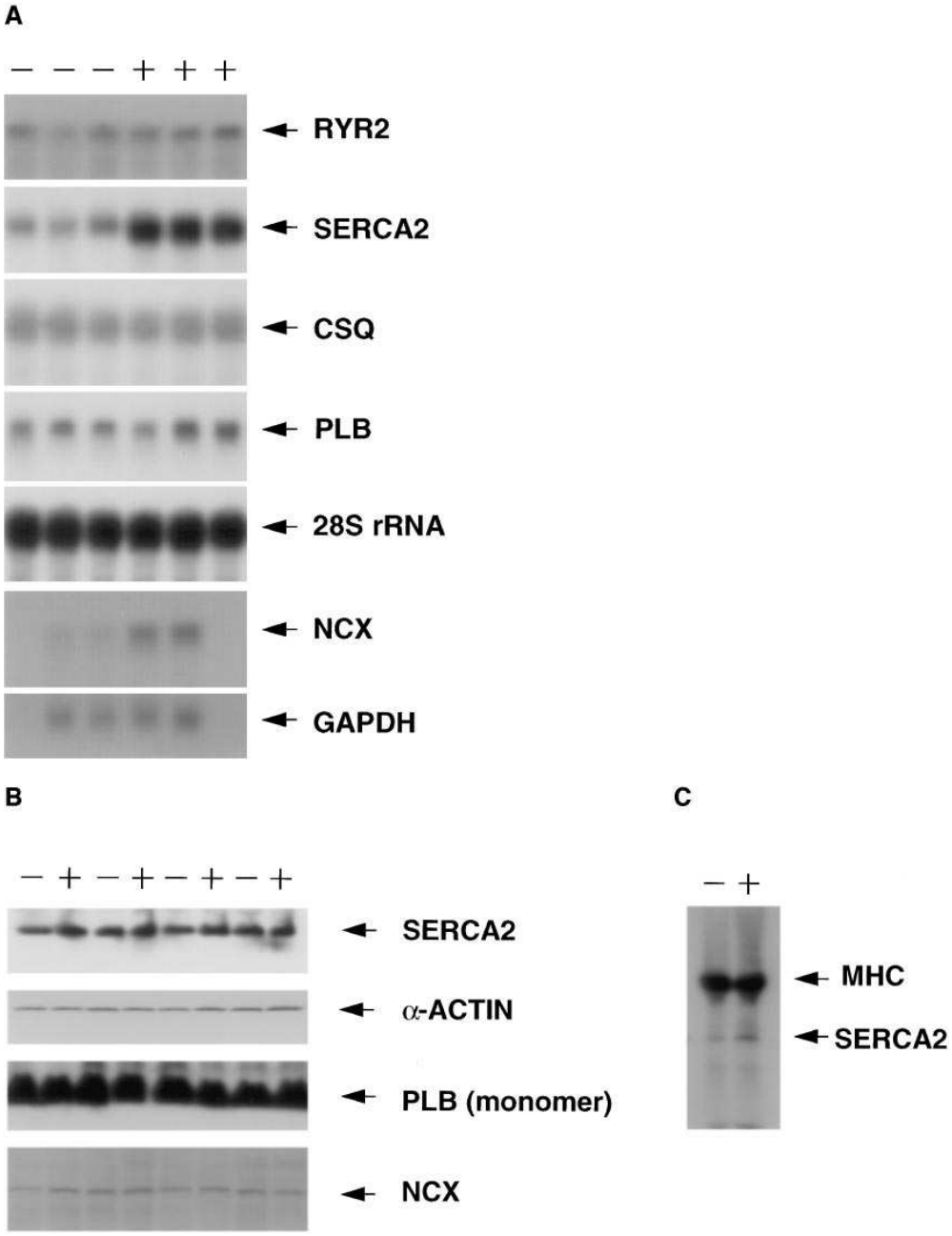

Figure 2. (*A*) Northern blot analysis: RNA samples isolated from mouse heart ventricles were sequentially hybridized to [^{32}P]-labeled probes corresponding to ryanodine receptor (*RYR*), SR Ca^{2+} ATPase (*SERCA2*), Calsequestrin (*CSQ*), phospholamban (*PLB*), and 28S rRNA which was used as a loading control. In addition, PolyA RNA was isolated and hybridized to Na^{+}/Ca^{2+} exchanger (*NCX*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNAs. − and + denote for control and transgenic littermates, respectively. (*B*) Western blot analysis: Homogenates of the mouse hearts were electrophoresed, transferred, and probed with antibodies against SERCA2, PLB, and α-actin which served as a loading control. −, transgenic negative; +, SERCA2 transgene positive. (*C*) Relative synthesis rate of SERCA2: Isolated myocytes of control (−) or transgenic (+) animals were incubated in the presence of [^{35}S]-labeled amino acids for 4 h, lysed, precipitated with SERCA2 Ab and purified with 10% fixed *S. aureus* Cowan I (SAC). The purified proteins were boiled in 1 × Laemmli buffer and resolved on an 8% SDS-PAGE. *MHC*, myosin heavy chain.

Na^{+}/Ca^{2+} exchanger mRNA levels did not lead to an increase in the corresponding protein level. In contrast, the increase in SERCA2 mRNA levels led to a 20±11% ($P < 0.05$) increase in SERCA2 protein level, when normalized to either phospholamban or α-actin proteins.

To gain more insight into the apparent discrepancy between the 160% increase in SERCA2 mRNA levels in transgenic mice versus a moderate 20% increase in SERCA2 protein levels, we determined the relative synthesis rate of SERCA2 protein. Isolated cardiac myocytes from both transgenic and control mice were plated and incubated with [^{35}S]-labeled amino acids for 4 h and subsequently lysed with 1% Triton X-100 lysis buffer. The lysates were then incubated with SERCA2 Ab followed by affinity purification. The purified proteins were then resolved on SDS-PAGE as shown in Fig. 2 *C*. MHCα (~ 190 kD) was copurified together with SERCA2 and used as a loading standard. When normalized to MHCα, radiolabeled SERCA2 exhibited an 82±24% ($P < 0.01$) increase in transgenic mice relative to control mice indicating a much higher synthesis rate for SERCA2 protein.

Calcium transients and contractile behavior in isolated myocytes. To determine if the increase in SERCA2 expression resulted in corresponding functional alterations, calcium transients and myocyte relengthening was determined in adult cardiomyocytes isolated from SERCA2 transgenic and control mice. For the determination of calcium transients $[Ca]_i$, myocytes were first isolated from adult mice and then incubated with Indo-1 fluorescence and paced to contract (0.3 Hz) at room temperature. Considering the uncertainties regarding the calcium binding affinity of Indo-1 in cardiac myocytes (43) and

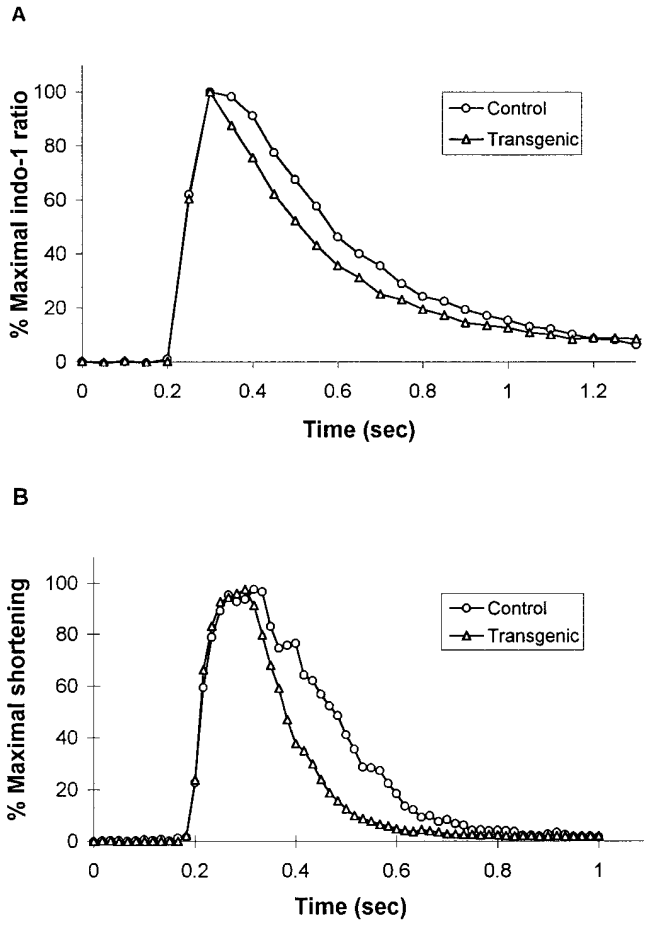

Figure 3. (*A*) Intracellular $[Ca]_i$ measurement: Calcium transients were determined using Indo-1. The data were collected with 50-ms intervals. The peak point of each trace relative to the baseline was set to 100% maximum, the data at each time point were represented as the percentage of the maximum value. The graph is based on the average of 35 and 33 cells, respectively, for transgenic and control animals from three independent experiments ($n = 3$). Student's two tailed t test yielded a $P < 0.01$ for the difference at 50% decline (*downslope*) ($t_{1/2}$) between transgenic cells (*triangle*) and control cells (*circle*). (*B*) Myocytic shortening measurement (edge detection): The contraction is represented as the percentage of shortening of cells relative to the maximum length (cells at rest), which is set to 100%. The data were collected with 17-ms interval. The graph is based on the average of 33 control cells and 38 transgenic cells from three independent experiments ($n = 3$). Student's two tailed t test yielded a $P < 0.05$ for the difference at 50% relaxation (*downslope*) between transgenic and control animals.

the potential for intracellular compartmentalization of Indo-1 (44, 45), data were presented as the ratio of the Indo-1 fluorescence obtained at 405 and 485 nm rather than as a calculated intracellular calcium concentration. Furthermore, the transients were normalized to the respective basal diastolic and peak systolic ratios (to yield the percent maximum for each trace). As seen in Fig. 3 *A*, the kinetics of the upstroke of calcium transients were identical for control and transgenic myocytes with both transients reaching peak Indo-1 $\sim$ 100 ms after stimulation. After the peak values, however, the transients declined more rapidly for the SERCA2 transgenic myocytes than for control cells. The times to reach 50% of decline ($t_{1/2}$) for SERCA2 transgenic and control myocytes were 200 and 261 ms, respectively. This represented a 23±7% faster $t_{1/2}$ in transgenic relative to control myocytes ($P < 0.01$).

To characterize the contractile properties of cardiac myocytes isolated from control or SERCA2 transgenic mice, contractile motion was monitored with a standard video camera interfaced to a video edge detector. Myocytes used for the contractility studies were not loaded with Indo-1. Myocytes were isolated from the heterozygous CJ5 line as well as the homozygous CJ2 line for these studies to assure that alteration in contractile function resulted from SERCA2 transgene expression but not from positional effects related to SERCA2 transgene integration. For myocytes isolated from CJ5 mice, we analyzed 33 cells isolated from three control mice and 38 cells obtained from three transgenic mice. Cardiac myocytes isolated from both transgenic positive and negative littermates were found to be nearly identical in their length, averaging 123.3 and 123.8 μm, respectively. Contractile motion was normalized to resting cell length. For control and transgenic myocytes, electrical stimulations resulted in contractions to 80.6 and 76.6% of the resting length. While this difference suggested a greater degree of contractility for the transgenic myocytes, it was not statistically significant. As with Indo-1 measurements, however, edge detection records from the transgenic myocytes exhibited a more rapid relaxation with the data for the transgenic cells being statistically different from the control myocytes ($P < 0.05$). To better illustrate this phenomenon, data from control and transgenic cells were normalized to the maximum shortening for each data set (Fig. 3 *B*). The $t_{1/2}$ for 50% of maximal relaxation for control and transgenic myocytes averaged 128 and 100 ms, respectively. This represented a 22±8% ($P < 0.05$) faster relaxation in transgenic myocytes. In subsequent studies, we analyzed the contractile behavior of cardiac myocytes from CJ2 and CJ5 SERCA2 transgenic mice using a stimulation frequency of 0.5 Hz and a sampling rate of 240 Hz. The results for CJ2 myocytes are summarized in Table II and show that expression of SERCA2 resulted in a significantly greater rate of cell shortening and relengthening. Using a higher stimulation frequency of 0.5 Hz versus 0.3 Hz and a higher sampling rate of 240 Hz versus 60 Hz than in the first set of experiments, we also found a greater rate of cell shortening for CJ5 myocytes. These data, therefore, suggest that the increased SERCA2 expression leads to a functional enhancement in SR uptake of intracellular calcium. This results in a greater rate of shortening,

Table II. Contractile Measurement in Isolated Myocytes by Edge Detection

Contractile parameters	Wild-type ($n = 6$)	SERCA2 transgenic ($n = 4$)
L_{max} (μm)	122.6±1.6	124.6±0.9
L_{min} (μm)	100.2±1.0	97.8±1.0
−dL/dt (μm/s)	−276.6±10.4	−377.0±16.3*
+dL/dt (μm/s)	190.1±8.4	264.9±5.5*
%CS	18.3±0.5	21.5±0.2*

Mean±SEM; *$P < 0.05$ vs control; t test with Bonferroni correction for five comparisons. %CS, %cell shortening; L_{max}, maximal myocyte length; L_{min}, minimum myocyte length; −dL/dt, rate of shortening; +dL/dt, rate of relengthening; n, number of animals; From each heart, 11–17 myocytes were studied.

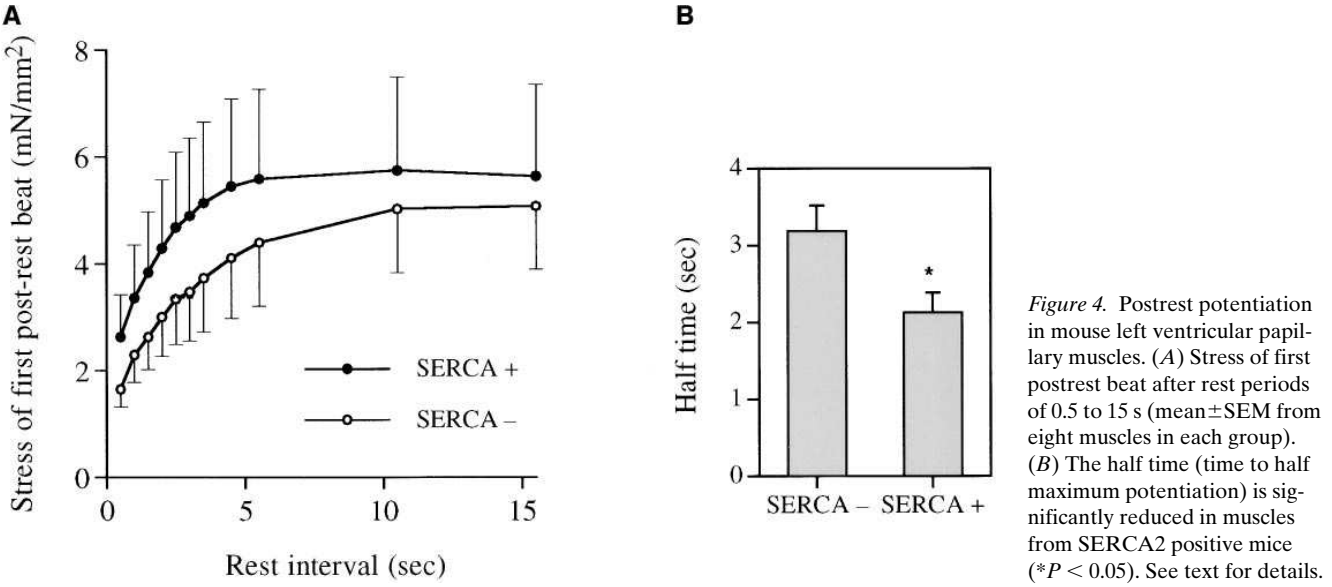

Figure 4. Postrest potentiation in mouse left ventricular papillary muscles. (*A*) Stress of first postrest beat after rest periods of 0.5 to 15 s (mean±SEM from eight muscles in each group). (*B*) The half time (time to half maximum potentiation) is significantly reduced in muscles from SERCA2 positive mice (*$P < 0.05$). See text for details.

as well as relaxation, of myocytes from SERCA2 transgenic mice. The results also demonstrate that the same changes occur in CJ2 and CJ5 myocytes and that this effect is, therefore, related to SERCA2 transgene expression.

Contractile behavior of isolated papillary muscles. The 16 mouse left ventricular papillary muscles had a length of 2.2±0.1 mm and a cross-sectional area of 0.41±0.04 mm^2 with no significant differences between the muscles from SERCA2 transgenic mice and the negative littermates. The muscles from the SERCA2 transgenic mice had a significantly shorter relaxation time RT_{50} of 33.8±1.3 ms compared to 39.8±0.8 ms in the muscles from the negative littermates ($P = 0.002$).

To investigate potential changes of intracellular calcium handling during periods of rest, we studied postrest potentiation in isolated left ventricular papillary muscles. When regular stimulation was interrupted by a short rest interval (0.5–15 s), the resumption of regular stimulation resulted in a larger potentiated contraction (Fig. 4 *A*). The stress of the postrest contraction increased with the duration of the rest interval for muscles from the transgene negative littermates and from the SERCA2 transgene animals. The time to half maximum potentiation (half-time) was 3.19±0.33 s in the negative littermates (Fig. 4 *B*). In muscles from SERCA2 transgenic mice, the half-time was significantly reduced to 2.13±0.27 s ($P = 0.024$).

The absolute stresses of the postrest contractions were not significantly different between the two groups because of large muscle to muscle variations. To compare the magnitude of postrest potentiation, the stress of the largest postrest contraction was normalized to the steady-state stress for each muscle. This postrest ratio was significantly different between muscles from SERCA2 transgenic mice (2.26±0.14) and negative littermates (3.04±0.16) ($P = 0.003$). At steady-state, muscles from SERCA2 transgenic mice operated, therefore, at a larger percentage of the maximum stress achieved during postrest potentiation.

In vivo phenotype in SERCA2 overexpressing mice. Cardiac function under in vivo conditions was evaluated by cardiac catheterization. We found that the left ventricular systolic pressure and heart rate were not significantly different between SERCA2 transgenic and control littermates with or without infusion of isoproterenol. In contrast at baseline, LV dP/dt_{max} was significantly more positive and LV dP/dt_{min} was significantly more negative (Fig. 5, *C* and *D*). The administration of isoproterenol, however, reduced the difference in both dP/dt_{max} and dP/dt_{min} between transgenic positive and negative littermates in a dose-dependent manner and completely abolished the difference at the maximum dose of 1 ng (Fig. 5, *C* and *D*). Since dP/dt_{min} may be affected by loading conditions, the preload independent measure of isovolumic relaxation, Tau (40, 41), was calculated for both control and SERCA2 mice under basal conditions. Tau was significantly shorter in SERCA2 mice compared with the negative controls indicating faster relaxation (Tau shortened by 20%, control at 16.6 ms versus in SERCA2 13.4 ms) ($P < 0.01$). Isoproterenol stimulation resulted in significant shortening of the relaxation time constant, Tau, in control mice. In contrast, isoproterenol stimulation had no effect on Tau in the SERCA2 mice suggesting that relaxation velocity during basal conditions was already close to its maximal value. In line with these observations, Tau in SERCA2 mice under basal conditions was similar to isopro-

Table III. Relaxation as Measured by Tau

Mean	Basal	ISO 1000
Wild-type ($n = 8$)		
Tau ms	16.67	12.79‡
SEM	1.71	0.44
SERCA2 transgenic ($n = 10$)		
Tau	13.43*	11.36
SEM	0.68	0.32

Isovolumic relaxation was determined by Tau. Tau was calculated as described in Methods. *Tau was significantly shorter ($P < 0.01$) in SERCA2 mice. ‡Isoproterenol stimulation resulted in significant shortening of Tau in wild-type mice, but not in SERCA2 mice. ISO 1000 = a dose of 1,000 pg of isoproterenol was administered.

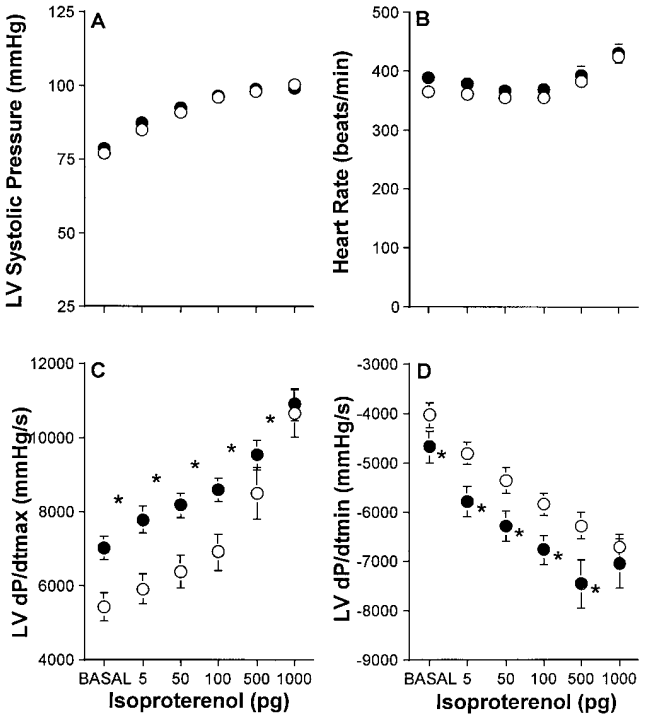

Figure 5. In vivo hemodynamic assessment: In vivo assessment of the left ventricular contractile function in control mice ($n = 13$) (○) and transgenic ($n = 13$) (●) mice. Cardiac catheterization was performed in intact anesthetized animals. Four measured parameters are shown at baseline and after progressive doses of isoproterenol. (*A*) LV systolic pressure; (*B*) Heart rate; (*C*) LV dP/dt_{max}; (*D*) LV dP/dt_{min}. Data were analyzed with a two-way repeated ANOVA, $^*P < 0.015$, control versus transgenic. A difference between group main effect in response to isoproterenol was found for (*C*) LV dP/dt_{max} ($P < 0.02$) and (*D*) LV dP/dt_{min} ($P < 0.05$). The pattern of change between groups was statistically different for (*C*) LV dP/dt_{max} ($P = 0.005$).

terenol stimulated values in control mice. The results related to Tau are summarized in Table III.

Discussion

The gene coding for the cardiac/slow skeletal muscle isoform of the Ca^{2+} ATPase of the sarcoplasmic reticulum (SERCA2) plays an important role in regulating cytoplasmic calcium levels in cardiac myocytes and is the most important contributor to the lowering of calcium levels during cardiac relaxation (2). Decreased expression of the SERCA2 gene may contribute to the diminished contractile function occurring in animal models and in human beings with severe congestive heart failure (25). The detailed functional alterations which result from changes in SERCA2 levels are, however, currently unclear. To obtain more insight into the functional consequences of selective changes in SERCA2 levels, we generated transgenic mice expressing a rat SERCA2 gene in their hearts. Our results provide for the first time direct evidence that increased expression of SERCA2 by itself leads to enhanced cardiac contractility.

In a heterozygous line of SERCA2 transgenic mice (CJ5 line), the level of SERCA2 mRNA increased 2.6-fold over the level in transgene negative littermates. This marked increase in SERCA2 mRNA levels resulted, however, in a much smaller but significant 20% increase in SERCA2 protein as determined by Western blots. The increase in SERCA2 protein resulted from an increase in SERCA2 formation and led to contractile alterations as described below. The relative synthesis rate of SERCA2 protein in transgenic mice was increased by 82% indicating that a significant fraction of SERCA2 mRNA gets translated into protein, however, the increase in relative protein synthesis rate was not fully commensurate with a 160% increase in SERCA2 mRNA. The discrepancy between the increased SERCA2 mRNA and relative protein synthesis rate may result, in part, from the efficiency of translation for the SERCA2 transgene derived mRNA. The SERCA2 transgene was shortened by 200 nt in the 5′ transcribed, but untranslated region. In addition, the transcriptional start of the transgene derived fusion mRNA occurs at the beginning of the first chicken β-actin exon and the resultant mRNA is composed of 150 nt of transcribed, but untranslated chicken β-actin nucleotide sequence fused to the truncated SERCA2 mRNA as described above. SERCA2 protein levels may also be much lower than mRNA levels because of accelerated degradation of SERCA2 protein formed in excess of that derived from the endogenous gene. SERCA2 protein contains an internal signal peptide sequence that provides for incorporation into the membrane of the SR (46). SERCA2 protein that cannot be properly anchored in the SR membrane and remains in the cytoplasm may be prone to rapid degradation. This explanation would require that only a limited number of SR membrane insertion sites are available for newly formed SERCA2 protein. A difference in the amino acid sequence between rat and mouse SERCA2 gene products could also account for increased degradation of the SERCA2 transgene product and less SERCA2 protein accumulation. Assessment of this contributing factor is currently difficult because the mouse SERCA2 sequence is not known.

To determine if increased SERCA2 expression led to changes in mRNA or protein levels of other genes involved in cardiac calcium handling, a number of these mRNAs were quantitated. The level of mRNAs coding for the ryanodine receptor and calsequestrin were similar in control and SERCA2 transgenic mice. In contrast, phospholamban mRNA levels showed a significant 43% increase in SERCA2 transgenic mice and Na^+/Ca^{2+} exchanger mRNA increased by 83%, however, phospholamban or Na^+/Ca^{2+} exchanger protein levels were not altered. It appears, therefore, that the contractile alterations that are described below result primarily from increased SERCA2 levels.

Functional alterations that may result from increased SERCA2 protein levels were first explored by determining calcium transients and contractile behavior in isolated adult cardiac myocytes obtained from heterozygous CJ5 mice. Isolated cardiac myocytes allow for a load independent determination of such parameters. Calcium transients determined with Indo-1 show an upstroke phase of the calcium transient which is not significantly different from that in transgene negative myocytes. In contrast, the downslope of the calcium transients, reflecting the cytoplasmic calcium lowering primarily mediated by calcium uptake into the sarcoplasmic reticulum and to a lesser extent calcium extrusion from the myocytes, was significantly accelerated with a 23±7% faster decline of $t_{1/2}$. The accelerated calcium transient correlated with a significantly 22±8% faster relengthening of myocytes as determined by

edge detection (Fig. 3 *B*). In addition, when myocyte contractility was determined at a stimulation frequency of 0.5 Hz and a sampling rate of 240 Hz, a greater rate of cell shortening occurred in myocytes from SERCA2 transgene positive mice of the CJ5 and CJ2 lines. It should also be noted that cell shortening in the range of 18–20% found for cardiac myocytes is higher than the percent shortening found in myocytes from other species.

To assure that increased SERCA2 expression and the resultant functional alterations were not, in part, due to positional effects where the SERCA2 transgene was integrated, we characterized the contractile behavior of myocytes prepared from a separate line of SERCA2 transgene mice, the homozygous CJ2 animals. The rate of shortening and relengthening was significantly greater than in transgene negative littermates. These findings clearly indicate that the increased SERCA2 expression and the resultant functional changes are due to additional copies of the SERCA2 transgene in the genome of transgenic CJ2 and CJ5 mice. We also found that SERCA2 mRNA expression normalized to transgene copy numbers was 5.2 times higher in CJ5 than in CJ2 lines. The SERCA2 transgenes are probably localized in a more actively transcribed region of the genome in CJ5 than in CJ2 mice.

Postrest contractions have been recognized as an important experimental tool for investigating excitation–contraction coupling. When regular stimulation is resumed after a short period of rest, the first postrest contraction may either be larger (postrest potentiation) or smaller (postrest decay) than the previous steady-state contractions (47, 48). These behaviors appear to reflect a net gain or a net loss, respectively, of SR calcium during rest (47). Previously, postrest contractile behavior has not been reported in the mouse. In our study, mouse left ventricular papillary muscles exhibited postrest potentiation that reached a maximum after rest periods of 5–15 s. These findings agree at least qualitatively with results in the rat (49) and seem to reflect the accumulation of calcium in the SR up to a presumably saturated level. In muscles from the SERCA2 overexpressing mice, the time to half maximum postrest potentiation was significantly shorter than in the negative littermates suggesting that SR calcium loading during a rest interval occurred at a faster rate in muscles from the transgenic mice. The stresses of postrest contractions were not significantly different between groups because of large muscle to muscle variability. However, the ratio of postrest to steady-state beats was significantly smaller in the transgenic mice than in the negative littermates. In other words, under steady-state conditions (2 Hz stimulation), the muscles from SERCA2 overexpressing mice operated at a larger percentage of the force reached in maximum postrest contractions. This result would be consistent with an increase in peak stress developed at steady state (2 Hz stimulation) in transgenic mice. While we did see such an increase, it did not reach statistical significance. In addition, we found that the relaxation time RT_{50} is significantly shorter in isolated left ventricular papillary muscles from SERCA2 transgenic mice. These findings provide further support for the hypothesis that the SERCA2 transgene increased SR calcium-pumping efficiency.

To determine whether the increased SERCA2 protein levels and the resultant enhanced diastolic calcium lowering as measured by calcium transients would lead to augmented in vivo relaxation, dP/dt_{min} was determined by cardiac catheterization in intact mice. Adult SERCA2 transgenic mice demonstrated enhanced basal relaxation which was confirmed by the load independent time constant of LV isovolumic pressure decay (Tau). These results are in accordance with the increased rate of myocyte relengthening found in SERCA2 mice. Interestingly, under in vivo conditions the dP/dt_{max} was also significantly increased in SERCA2 mice suggesting augmented contractile function. Increase in SERCA2 action will result in enhanced calcium import into the sarcoplasmic reticulum resulting in higher amounts of calcium available for discharge through the ryanodine receptor into the cytoplasm during the next contraction cycle (1). The contractile behavior of the heart under in vivo conditions and that of isolated myocytes is similar and both show increased values related to contraction and relaxation. Contractile measurements under in vivo conditions are influenced by loading conditions, especially preload, with an increase in end diastolic pressure positively influencing left ventricular dP/dt_{max} (50). Since end diastolic pressure was not significantly different between SERCA2 transgenic and control mice, loading did not markedly influence our results.

Isoproterenol stimulation resulted in significant further enhancement of LV dP/dt_{max} and dP/dt_{min} in both control and SERCA2 transgenic mice. This may indicate that in SERCA2 transgenic mice phospholamban still exerts an inhibitory influence on SERCA2 function and protein kinase A–induced phosphorylation of phospholamban progressively removes the inhibitory influence on SERCA2 pump function. At the maximal isoproterenol doses, LV dP/dt_{max} and dP/dt_{min} in control and SERCA2 animals were not significantly different from each other. This could be due to material properties of the heart that only allow for certain maximal LV dP/dt_{max} and dP/dt_{min} to occur.

It is interesting to note that the SERCA2 overexpressing transgenic mice described in this report and the phospholamban knock-out mice described earlier (51), present a similar contractile phenotype with significant enhancement of both maximal and minimal first derivative of left ventricular pressure. In both of these animal models, the calcium pumping activity of the SERCA2 is enhanced in the phospholamban knock-out mice due to the removal of the inhibitory effects of phospholamban on SERCA2 (51, 52) and in the SERCA2 mice described in this report due to increased expression of SERCA2. Similar contractile changes observed in both of these models with increased SERCA2 activity further confirm the central role which this calcium pump plays in cardiac function. Enhancing the activity of SERCA2 in the failing heart may, therefore, lead to improved contractile performance.

Acknowledgments

We thank J. Price, Scripps Clinic and Research Foundation, La Jolla, CA for helping with the production of transgenic animals. We are indebted to Gerd Hasenfuss, M.D. (University of Freiburg, Germany) who generously provided us with the setup for the isometric force measurements in isolated muscle preparations and for helpful discussions regarding the postrest potentiation protocol.

This work was supported by National Institutes of Health grants HL52946 (W.H. Dillmann), HL03041 (H.A. Rockman), and a postdoctoral training grant HL0744-14 (H. He).

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