Targeted Overexpression of the Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase Increases Cardiac Contractility in Transgenic Mouse Hearts

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Abstract—Cardiac hypertrophy and heart failure are known to be associated with a reduction in Ca\(^{2+}\)-ATPase pump levels of the sarcoplasmic reticulum (SR). To determine whether, and to what extent, alterations in Ca\(^{2+}\) pump numbers can affect contraction and relaxation parameters of the heart, we have overexpressed the cardiac SR Ca\(^{2+}\)-ATPase specifically in the mouse heart using the α-myosin heavy chain promoter. Analysis of 2 independent transgenic lines demonstrated that sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase isoform (SERCA2a) mRNA levels were increased 3.88±0.4-fold and 7.90±0.2-fold over those of the control mice. SERCA2a protein levels were increased by 1.31±0.05-fold and 1.54±0.05-fold in these lines despite high levels of mRNA, suggesting that complex regulatory mechanisms may determine the SERCA2a pump levels. The maximum velocity of Ca\(^{2+}\) uptake (\(V_{\text{max}}\)) was increased by 37%, demonstrating that increased pump levels result in increased SR Ca\(^{2+}\) uptake function. However, the apparent affinity of the SR Ca\(^{2+}\)-ATPase for Ca\(^{2+}\) remains unchanged in transgenic hearts. To evaluate the effects of overexpression of the SR Ca\(^{2+}\) pump on cardiac contractility, we used the isolated perfused work-performing heart model. The transgenic hearts showed significantly higher myocardial contractile function, as indicated by increased maximal rates of pressure development for contraction (+dP/dt) and relaxation (–dP/dt), together with shortening of the normalized time to peak pressure and time to half relaxation. Measurements of intracellular free calcium concentration during muscle development and in response to a variety of pathophysiological conditions (reviewed in Reference 13). Previous studies from our laboratory and others have shown that SERCA2a pump expression levels are regulated during muscle development and in response to a variety of pathophysiological conditions (reviewed in Reference 13). Several studies have reported that thyroid hormone markedly

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The sarcoplasmic reticulum (SR) plays a central role in the contraction-and-relaxation cycle of the heart by regulating intracellular calcium (Ca\(^{2+}\)) concentrations (reviewed in Reference 1). Ca\(^{2+}\) release from the SR via the ryanodine receptor initiates muscle contraction, whereas Ca\(^{2+}\) reuptake into the lumen of the SR leads to muscle relaxation. The Ca\(^{2+}\) uptake function of the SR is driven by an ATP-dependent Ca\(^{2+}\) transport pump, the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). Molecular cloning analyses have identified a family of SERCA pumps encoded by 3 highly homologous genes (SERCA1, SERCA2, and SERCA3).2–8

The SERCA2 gene encodes 2 isoforms, SERCA2a and SERCA2b, which differ at the COOH terminus as a result of alternative splicing (SERCA2a comprises 4 amino acids, and SERCA2b comprises 49 amino acids).5–7 SERCA2a is the primary SR isoform expressed in the heart and is also present in slow-twitch skeletal muscle, smooth muscle, and fetal fast-twitch muscle.9,10 In the rat heart, SERCA2a expression can be detected as early as 10 days postcoitum, and its expression levels continue to increase postnatally.10,11 SERCA2b is expressed in most cell types but is present at high levels in cardiac and smooth muscle of the rat and rabbit.7,8 SERCA2a and SERCA2b isoforms not only possess different patterns of expression but also differ in vitro functional characteristics. The SERCA2a isoform exhibits a lower affinity for Ca\(^{2+}\) and a higher rate of turnover for Ca\(^{2+}\) transport compared with the SERCA2b isoform. These differences in pump function have been attributed to the terminal 12 amino acids of SERCA2b.12

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Overexpression of SR Ca\textsuperscript{2+}-ATPase

increases SR Ca\textsuperscript{2+}-ATPase levels and produces enhanced myocardial function.\textsuperscript{14–19} In contrast, decreased SR calcium transport is observed in chronic pressure overload hypertrophy and was shown to be due to decreased expression of the SERCA pump.\textsuperscript{14,20–22} Studies on human hearts also suggest that SR Ca\textsuperscript{2+} transport function is altered in end-stage human heart failure.\textsuperscript{23,24} Intracellular Ca\textsuperscript{2+} measurements using aequorin showed that Ca\textsuperscript{2+} transients in muscle samples from failing human hearts are markedly prolonged in both Ca\textsuperscript{2+} release and uptake phases.\textsuperscript{24} Using tissue samples from failing human hearts, we and others have found that the expression levels of SERCA were decreased both at the mRNA\textsuperscript{25–28} and the protein levels\textsuperscript{29} in end-stage heart failure. The decrease in the levels of SERCA can be closely correlated with a decreased myocardial function.\textsuperscript{29,30} These studies provide correlative evidence to suggest that a decrease in SERCA levels may contribute to contractile dysfunction, but they fail to establish a cause-effect relationship.

The goal of this study was to demonstrate whether alterations in SERCA levels can affect cardiac contractility. In vitro studies have shown that adenovirus-mediated gene transfer of SERCA into fetal/neonatal myocytes can increase Ca\textsuperscript{2+} transport function and enhance contractility.\textsuperscript{31,32} In this study, we chose a transgenic (TG) approach to address this question. The TG approach allows us to specifically alter SERCA pump levels within the intact heart. The TG mouse model also provides us with an in vivo system to test the functional consequences of altered SERCA expression on cardiac muscle physiology.

In a recent study, He et al\textsuperscript{33} used the chicken \beta-actin promoter linked to the viral (cytomegalovirus [CMV]) enhancer to overexpress SERCA2a in a TG mouse model. In this model the amounts of SERCA2a mRNA and protein were increased 2.6-fold and 1.2-fold, respectively. Functional analysis of calcium handling and contractile parameters in isolated cardiac myocytes indicated that the intracellular calcium decline (t\textsubscript{1/2}) and myocyte relengthening (t\textsubscript{1/2}) were accelerated by 23% and 22%, respectively. In addition, the rate of myocyte shortening was also significantly faster, and cardiac function measured in vivo demonstrated significantly accelerated contraction and relaxation in SERCA2 TG mice.

However, in the above study, SERCA2a was overexpressed using the chicken \beta-actin promoter (driven by the viral CMV enhancer), which is not tissue specific. This transgene could drive SERCA2a expression in multiple tissues (including muscle and nonmuscle tissues). In particular, overexpression of SERCA2a in other tissues, including vascular smooth muscle, may complicate the interpretation of the results by altering Ca\textsuperscript{2+} homeostasis in multiple tissues. To avoid these complications, we chose to use the cardiac tissue–specific mouse \alpha-myosin heavy chain (\alpha-MHC) promoter\textsuperscript{34,35} to target pump overexpression specifically to the heart. This promoter has been shown by many investigators to consistently overexpress high levels of the desired protein to the cardiac compartment.

In this study, we report the generation and characterization of 2 independent SERCA2a TG lines that vary in transgene copy number and levels of SERCA2a mRNA and protein. Overexpression of the SERCA2a mRNA resulted in an increase in the total amount of SERCA pumps in a copy number-dependent manner. In response to increased protein expression, SERCA2a TG hearts showed increased SR Ca\textsuperscript{2+} uptake (V\textsubscript{max}). However, the apparent affinity of the SERCA for Ca\textsuperscript{2+} remained unchanged in TG hearts. Functional analysis revealed that TG hearts were hyperdynamic, exhibiting increased rates of contraction and relaxation. These findings suggest that the increased expression of SERCA pumps directly enhances cardiac muscle contractility.

Materials and Methods

SERCA2a Transgene

The SERCA2a transgene was constructed by cloning the rat SERCA2a cDNA\textsuperscript{6} downstream of the 5.5-kb \alpha-MHC gene promoter\textsuperscript{36} at the SalI and HindIII sites. The SERCA2a cDNA contained the entire coding sequence (starting at the AUG codon, NcoI site) and the complete 3' untranslated region. Linkers were added to the 5' and 3' ends of the cDNA to facilitate cloning into 5.5-kb \alpha-MHC promoter vector. An additional human growth hormone polyadenylation- ation site (Hgh poly A) was included to ensure that the transcript would be polyadenylated. The transgene was removed from the vector through restriction digestion (with BamHI), isolated from an agarose gel, and purified in a cesium chloride gradient. The resulting DNA was resuspended in 5 mmol/L Tris-HCl, pH 7.4, and 0.1 mmol/L EDTA at a concentration of 5 \mu g/mL for microinjection into mouse oocytes.

Production of SERCA2a TG Mice

TG mice were generated by the Transgenic Core Facility, University of Cincinnati, following established procedures.\textsuperscript{36} Transgene DNA was microinjected into the pronuclei of fertilized mouse oocytes derived from superovulated FVB/N females. Injected embryos were implanted into the oviducts of pseudopregnant foster females. TG mice were identified using polymerase chain reaction (PCR)\textsuperscript{37} and Southern blot analysis\textsuperscript{38} of genomic DNA from tail biopsies. PCR primers derived from the second intron of the \alpha-MHC promoter (5'-GCCCAACCAGAAATGACAGA-3') and SERCA2a cDNA exon 4 (5'-TCACTTTCTTGACCGAGG-3') produced a 390-bp fragment in TG samples. Six positive founder mice were obtained and bred with non-TG (NTG) cohorts to establish stable TG lines. The transgene copy number was determined using Southern blot analysis in comparison with endogenous \alpha-MHC gene. In brief, 10 \mu g of genomic DNA prepared from tail biopsies was digested with EcoRI and electrophoresed. The blots were hybridized with the \textit{NdeI}-to-SalI fragment of the mouse \alpha-MHC promoter (NTG samples gave a 3.0-kb endogenous band, whereas TG samples gave 3.0-kb and 2.7-kb bands). Intensities of the TG band were compared with the endogenous band to determine copy number.

Morphological Studies

Histological evaluation was performed on the hearts from 10- to 12-month-old SERCA2a TG and wild-type mice. The tissues were fixed in 10% formalin, dehydrated, and embedded in paraffin.\textsuperscript{39} Longitudinal sections (5 \mu m) of the heart (cut at 50-\mu m intervals) were stained with hematoxylin and eosin. Longitudinal sections were made to demonstrate all 4 heart chambers and valves. Hearts were examined for hypertrophy, hyperplasia, inflammation, septal defects, necrosis, mineralization, fibrosis, cytoplasmic vacuolization, and altered cell orientation.

Northern Blot Analysis

Total RNA from cardiac tissue was isolated, using Ultra Spec II (RNA isolation kit; Biotec Laboratories, Inc), from 11- to 14-week old mice. SERCA2a mRNA levels were estimated using Northern blot analysis of 10 \mu g of total cardiac RNA following established procedures.\textsuperscript{40} For dot blots, serial dilutions (1.25, 2.5, and 5 \mu g) of total RNA were blotted onto nitrocellulose membranes. Both North-
em blots and dot blots were hybridized with an Ncol fragment from the rat SERCA2 cDNA. Dot blots were also hybridized with a mouse GAPDH.

**Western Blot Analysis**

Hearts from adult TG mice and littermates (11 to 14 weeks old) were homogenized in the following (in mmol/L): imidazole 10, pH 7.0, sucrose 300, DTT 1, and sodium bisulfite 1.41 Protein concentrations were determined by Bio-Rad protein assay. The relative SERCA2, phospholamban, and cardiac actin protein levels in TG versus NTG samples were determined by quantitative immunoblotting as described below. Equal amounts of protein extract from both TG and NTG littermates (1, 2, and 4 mg) were separated by SDS-PAGE on a 13% polyacrylamide gel and then transferred to nitrocellulose using a Bio-Rad transblot apparatus. Filters were incubated with a polyclonal antibody for SERCA2 (1:500 dilution), a cardiac actin monoclonal antibody (1:2500; Sigma), or phospholamban monoclonal antibody (1:1000; Affinity Bioreagents) overnight. Secondary antibodies were peroxidase-labeled anti-rabbit or anti-mouse IgG (Kirkegaard & Perry Laboratories). Antibody signals were detected through an enhanced chemiluminescence kit (Kirkegaard & Perry Laboratories).

**Ca**\textsuperscript{2+} Uptake Assays

Heart tissue from TG and NTG mice was homogenized in the following (in mmol/L): potassium phosphate 50, pH 7.0, NaF 10, EDTA 1, sucrose 300, phenylmethylsulfonyl fluoride 0.3, and DTT 0.5. The SR Ca\textsuperscript{2+} uptake activity was measured using a modified Millipore filtration method.43,44 Cardiac homogenates (100 μg/mL) were incubated at 37°C in 1.5 mL of reaction mixture containing 40 mmol/L imidazole, pH 7.0, 100 mmol/L KCl, 5 mmol/L MgCl\textsubscript{2}, 5 mmol/L Na\textsubscript{2}S, 5 mmol/L potassium oxalate, 1 μmol/L Ruthenium red, and 0.5 mmol/L EGTA to yield free Ca\textsuperscript{2+} concentration in the range of 0.03 to 3 μmol/L (containing 1 μmol/L Ca\textsuperscript{2+}) as determined by the computer program (Calcium Titration Program).45 The uptake reaction was started by adding 5 mmol/L ATP to the reaction mixture. At serial time intervals, 300-μL samples were vacuum filtered through Millipore (0.45 μm HAWP) nitrocellulose membrane, and the vesicles remaining on the filters were washed by grinding using 1.5 mL of reaction mixture containing 200 mmol/L imidazole, pH 7.0, 100 mmol/L KCl, 5 mmol/L MgCl\textsubscript{2}, 5 mmol/L Na\textsubscript{2}S, 5 mmol/L potassium oxalate, 1 μmol/L Ruthenium red, and 0.5 mmol/L EGTA to yield free Ca\textsuperscript{2+} concentration in the range of 0.03 to 3 μmol/L (containing 1 μmol/L Ca\textsuperscript{2+}) as determined by the computer program (Calcium Titration Program).45 The uptake reaction was started by adding 5 mmol/L ATP to the reaction mixture. At serial time intervals, 300-μL samples were vacuum filtered through Millipore (0.45 μm HAWP) nitrocellulose membrane, and the vesicles remaining on the filters were washed, dissolved, and then processed for liquid scintillation counting. The rates of Ca\textsuperscript{2+} uptake were calculated by the least-squares linear regression analysis of uptake values at 30, 60, and 90 s. The calcium concentration required for half of the maximum velocity for Ca\textsuperscript{2+} uptake (K\textsubscript{Ca}) was determined by nonlinear curve fitting using Origin 3.5 (MicroCal Software Inc.).

**Functional Analysis of TG Hearts Using Isolated Work-Performing Heart Preparations**

The anterograde, work-performing heart preparation has been previously described.46 In brief, 12- to 16-week-old, age-matched mice of either sex were anesthetized with 30 mg/kg pentobarbital sodium IP and injected with 500 U/kg heparin sodium (Elkins Sinn) to prevent intracardiac blood coagulation. A 12-lead ECG and heart rate were recorded for each mouse before opening the chest and removing the heart. Hearts were removed via midsternal incision, immediately suspended on a 20-gauge stainless steel cannula, and then connected to the perfusion apparatus. Retrograde perfusion with heated (37.4°C) and oxygenated Krebs-Henseleit (KH) buffer (containing in mmol/L) NaCl 118, KCl 4.7, CaCl\textsubscript{2} 2.5, MgSO\textsubscript{4} 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.2, sodium EDTA 0.5, NaHCO\textsubscript{3} 25, and glucose 11) was started to clear the coronary vasculature of the remaining blood and to insert the intraventricular catheter for pressure measurements. After a short period of stabilization on retrograde perfusion, the pulmonary vein was cannulated, and perfusion of the heart was then switched from retrograde to anterograde. Venous return (preload) was adjusted and regulated with a micrometer and continuously measured using a dual-channel Transonic flowmeter. Aortic pressure (afterload) was adjusted with a second micrometer. To set basal loading conditions, flow was adjusted to deliver an initial “venous return” (preload) of 5 mL/min cardiac output and an afterload of 50 mm Hg aortic pressure, providing a basal workload of 250 mL/min×mm Hg/s. Cardiac function curves were performed after stabilization of the heart at basal loading conditions by increasing and decreasing the aortic pressure (pressure loading) stepwise, while keeping the venous return constant. Following pressure loading, volume loading was performed by stepwise increasing and decreasing of the venous return at a constant aortic resistance. Because ventricular end-diastolic and left atrial pressures were monitored continuously, any developing cardiac failure was recognized and corrected immediately. All hearts were compared under identical loading conditions ranging from 250 mm Hg×mL/min (50 mm Hg mean aortic pressure) to 400 mm Hg×mL/min (80 mm Hg mean aortic pressure). The perfusion fluid, oxygenation, and temperature were constant throughout the experiment. Heart rate, aortic pressure, left intraventricular (systolic, diastolic, and end-diastolic) pressure, and atrial pressure were continuously monitored, and the first derivative of left intraventricular pressure, +dP/dt and –dP/dt, time to peak pressure (TPP)/mm Hg, and time to half relaxation (RT\textsubscript{1/2})/mm Hg were calculated with a custom-designed computer program. Cardiac output and coronary flow were computer calculated.

**Oxygen Consumption in Work-Performing Hearts**

Coronary arterial perfusion buffer and venous effluent samples were collected anaerobically, and the PO\textsubscript{2} and PCO\textsubscript{2} values of these samples were measured using an automated blood gas analyzer (model 248, CIBA Corning Diagnostics Corp). Oxygen consumption (MV\textsubscript{O2}) by the perfused hearts was computed by multiplying the coronary flow by the arteriovenous difference in oxygen content and normalized per gram of tissue mass as follows:

\[
\text{MV}_{\text{O}_2} (\mu\text{L O}_2/\text{min} \times \text{g}^{-1}) = \frac{(P_{\text{O}_2} - P_{\text{V O}_2}) \times \text{coronary flow (mL/min)} \times (C/760) \times 1000}{\text{heart wet weight (g)}}
\]

where \(P_{\text{O}_2}\) and \(P_{\text{V O}_2}\) represent the perfusate and venous partial pressures of \(\text{O}_2\) (mm Hg), respectively, and \(C = 0.0239 \text{ (Bunsen solubility coefficient of oxygen dissolved in perfusate at 37°C, in mL O}_2/\text{atm} \cdot \text{g}^{-1} \cdot \text{mL}^{-1}\)).

**Unanesthetized Blood Pressure and Heart Rate Measurements**

The blood pressures were measured in the unanesthetized mice via tail cuff (Natsume model KN-210 Photosensor unit, modified for mice; cuff size 7 mm) and recorded on a Grass P7 polygraph. Heart rate was computed from the amplitude of the pulse signal. The mice were acclimated to the chamber before the actual measurements of the pressures were taken. A set of 30 measurements at 3 different time periods was averaged for each mouse.

**Intracellular Ca\textsuperscript{2+} and Twitch Force Measurements in Intact Trabeculae**

The intact mouse cardiac muscle preparation has been previously described.47 In brief, 12- to 19-week-old mice of either sex were anesthetized with 10 to 20 mg of pentobarbital sodium (IP) and injected with 20 to 30 mg of heparin sodium. The hearts were retrogradely perfused with modified KH buffer with high K\textsuperscript{+} (20 mmol/L) and gassed with a 95% O\textsubscript{2}–5% CO\textsubscript{2} gas mixture in a dissection dish at room temperature. Trabeculae suitable for force measurements and fura-2 microinjection were quickly dissected from the right ventricle and mounted between a force transducer and a micromanipulator in a perfusion bath. Geometrically suitable (long, thin, nonbranched) trabeculae were found in a minority of the hearts, so that the overall success rate of these experiments was ~20%. The trabeculae were superfused with KH buffer (containing the following [in mmol/L]: NaCl 112, KCl 5, CaCl\textsubscript{2} 0.5, MgSO\textsubscript{4} 1.2, NaH\textsubscript{2}PO\textsubscript{4} 2, NaHCO\textsubscript{3} 25, and glucose 10) equilibrated with 95% O\textsubscript{2}–5% CO\textsubscript{2}. The perfusion rate was 5 to 10 mL/min, and the preparations were stimulated at 0.5 Hz. All experiments were performed at room temperature (~22°C). The preparations were field stimulated with
Figure 1. Structure of the SERCA2a transgene construct and determination of copy number. A, The SERCA2a transgene consists of the rat SERCA2a cDNA entire coding sequence and the complete 3' untranslated region cloned downstream of the 5.5 α-MHC gene promoter at the SalI and HindIII sites. An additional polyadenylation site (Hgh poly A) was included to ensure that the transcript would be polyadenylated. B, Southern blot analysis of genomic DNA isolated from mouse tail biopsies. Genomic DNA (10 μg) was digested with EcoRI and electrophoresed. The blots were hybridized with the NdeI-to-SalI fragment of the mouse α-MHC promoter (NTG samples gave a 2.6-kb endogeneous band, whereas TG samples gave 3.0-kb and 2.6-kb bands).

5-ms pulses, with a Grass SD9 stimulator. After stabilization of the preparations, fura-2 potassium salt was microinjected iontophoretically into one cell and allowed to spread throughout the muscle via gap junctions. [Ca2+] was determined by measuring the epifluorescence of fura-2 excited at 380 and 340 nm. The fluorescent light was collected at 510 nm by a photomultiplier tube (model R2693; Hamamatsu). The output of the photomultiplier tube was filtered at 100 Hz, collected by an analog-to-digital converter, and stored in the computer for later analysis. Intracellular [Ca2+] was given by the following equation (after subtraction of the autofluorescence of the muscle):

\[
[Ca^{2+}]_i = K_d \frac{(R - R_{min})/(R_{max} - R)}
\]

where \( R \) is the observed ratio of fluorescence (340 nm/380 nm), \( K_d \) is the apparent dissociation constant, \( R_{min} \) is the ratio of 340 nm/380 nm at saturating \([Ca^{2+}]\), and \( R_{max} \) is the ratio of 340 nm/380 nm at 0 \([Ca^{2+}]\). The values for \( K_d \), \( R_{min} \), and \( R_{max} \) were 3.5, 7.20, and 0.47 μmol/L, respectively. After equilibration of the loaded fura-2, intracellular free Ca2+ concentration and contractile force were measured.

Statistical Analyses
TG and NTG parameters were compared using either one-way ANOVA followed by a Dunnett's/Student-Newman-Keuls post hoc test or a 2-way ANOVA as appropriate using SigmaStat software. Results are expressed as the mean±SEM. The level of statistical significance was \( P\leq0.05 \).

Results
Characterization of SERCA2a TG Mouse Lines
In this study the cardiac α-MHC gene promoter was used to direct the rat SERCA2a cDNA expression specifically to the heart (Figure 1A). PCR and Southern blot analysis of founder mice identified 6 TG lines containing 2 to 13 copies of the SERCA2a transgene. Of these 6 founder lines, 4 mice (founder mice 100, 19, 16, and 3) showed germ-line trans-
analysis indicated that SERCA2a mRNA levels were significantly increased in TG hearts (Figure 2A). The rat SERCA2a message migrated at the same level as the endogenous mouse (4-kb) transcript; however, overexpression of the transgene can be recognized by the abundance of SERCA2a mRNA levels. Furthermore, the SERCA2a overexpression was limited to the heart (data not shown). Precise quantification by dot-blot analysis revealed that the SERCA2a mRNA level was increased 3.88\(\pm\)0.4-fold in line 100 and 7.9\(\pm\)0.2-fold in line 3 (Figure 2B). The SERCA2a mRNA levels were normalized for loading variations by a GAPDH cDNA probe. Our data show that line 3, with the highest transgene copy number (13 copies), expressed the highest SERCA2a mRNA level (8-fold).

**SERCA Protein Levels Are Increased in TG Hearts**

To determine whether SERCA2a protein levels were increased in TG hearts, Western blot analysis was performed using cardiac homogenates from age-matched TG and NTG littermates.\(^41\) SERCA2a protein was quantitated using a polyclonal antibody raised against SERCA2a protein.\(^42\) A representative Western blot is shown in Figure 3. Our protein analysis revealed that total SERCA2a protein was increased 1.31\(\pm\)0.05-fold in TG 100 and 1.54\(\pm\)0.05-fold in TG 3. These data suggest that there is no strict correlation between SERCA2a mRNA and protein levels in the TG mice. However, the line that produced the highest SERCA2a mRNA levels (TG 3) also shows the greatest increase in SERCA2a protein levels. To determine whether increased expression of SERCA2a has affected the expression of other proteins, we performed quantitative immunoblotting on phospholamban (a regulator of the SERCA pump) and actin (a myofilament protein) levels using specific antibodies. The levels of phospholamban and actin were not altered in TG hearts (Figure 3).

Maximal Velocity (V\(_{\text{max}}\)) of SR Ca\(^{2+}\) Uptake Is Increased in SERCA2a TG Hearts

To study the effects of SERCA2a overexpression on SR Ca\(^{2+}\) transport function, the initial rates of ATP-dependent, oxalate-facilitated SR Ca\(^{2+}\) uptake were assessed using cardiac homogenates under conditions that restrict Ca\(^{2+}\) uptake to the SR.\(^{43,44}\) Ca\(^{2+}\) uptake rates for TG mice were higher than for their NTG littermates (Figure 4A). The maximum velocity of Ca\(^{2+}\) uptake (V\(_{\text{max}}\)) was significantly higher (37%; n=5) in TG mice (line 100) than in NTG (age- and sex-matched) littermates (n=5). This increase in SR Ca\(^{2+}\) uptake is consistent with increases in SERCA2a protein levels in TG hearts.

**SERCA2a TG Hearts Show a Significant Increase in the Rates of Contraction and Relaxation**

To determine whether an increase in SERCA2a pump levels affects the overall cardiac function, we used the isolated work-performing heart preparation to measure contractile and relaxation function. This model allows us to compare myo-
cardiac contractile parameters in individual mouse hearts under identical afterload conditions (50 mm Hg mean aortic pressure) and preload conditions (5 mL/min venous return, an approximation of preload), producing a cardiac minute work (mean aortic pressure×cardiac output) of 250 mm Hg×mL/min. Although isolated TG hearts showed similar heart rates (360 to 390 bpm) and end diastolic pressures compared with age-matched NTG hearts, both systolic and diastolic intraventricular pressures were increased under identical load conditions (Table). Furthermore, the maximal rate of pressure development (360 to 390 bpm) and end diastolic pressures compared with control values for all pressure-loading conditions examined. Our results also demonstrate that both control and TG hearts respond to increased cardiac minute work with increased contractility, as evidenced by increased +dP/dt. However, the TG and NTG hearts did not respond to increased pressure loading with increased rates of relaxation. The hearts from TG 3 rather showed a decline in the rate of relaxation under high-pressure loading conditions.

**Effect of Pressure and Volume Loading on Contractile Parameters of TG Hearts**

To evaluate the capacity of SERCA2a TG mouse hearts to respond to increased work, we performed pressure loading on 7 TG 100, 4 TG 3, and 6 NTG hearts. We determined left ventricular functional response to increased pressure-loading conditions by plotting cardiac minute work (from 250 to 400 mm Hg×mL/min) versus the rate of pressure development and relaxation (+dP/dt and –dP/dt in mm Hg/s; Figure 6). The TG hearts showed significantly higher rates of contraction and relaxation compared with control values for all pressure-loading conditions examined. Our results also demonstrate that both control and TG hearts respond to increased cardiac minute work with increased contractility, as evidenced by increased +dP/dt. However, the TG and NTG hearts did not respond to increased pressure loading with increased rates of relaxation. The hearts from TG 3 rather showed a decline in the rate of relaxation under high-pressure loading conditions.

**SERCA2a TG Hearts Show a Sizable Increase in Ca2+ Transient Amplitude, With a Concomitant Boost in Contractility**

An increase in SERCA2a expression might logically be predicted to increase the ability of the SR to store calcium, such that more calcium is available to be released during each heartbeat. If so, the increased contractility in SERCA2a TG hearts would be attributable to an increase in activator Ca2+ availability. To determine whether this was the case, we measured intracellular Ca2+ and contractile force simultaneously in fura-2-loaded isometrically contracting trabeculae. The experiments were performed in 2 mmol/L [Ca2+]c, at a stimulation rate of 2 Hz (22°C). Figure 7 shows typical records of Ca2+ transients (top) and the corresponding twitch force (bottom) in muscles from hearts of normal (left) and SERCA2a TG (right) mice. The TG hearts exhibit a sizable increase in Ca2+ transient amplitude relative to the normal

### Cardiac Parameters of SERCA2a Overexpression Versus Controls (FVB/N)

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<th></th>
<th>Control (n=6)</th>
<th>Transgenic Line 100 (n=7)</th>
<th>Transgenic Line 3 (n=4)</th>
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<td>3986.8±25</td>
<td>4448±42.6‡</td>
<td>5380±43.8‡</td>
</tr>
<tr>
<td>Maximal rate of relaxation, mm Hg/s (–dP/dt)</td>
<td>–3245±42.1</td>
<td>–3697±38.8‡</td>
<td>–4589±41.6‡</td>
</tr>
<tr>
<td>TPP, ms/mm Hg</td>
<td>0.425±0.003</td>
<td>0.386±0.004‡</td>
<td>0.314±0.003§</td>
</tr>
<tr>
<td>RT1/2, ms/mm Hg</td>
<td>0.488±0.007</td>
<td>0.432±0.006‡</td>
<td>0.331±0.004‡</td>
</tr>
<tr>
<td>PaO2, mm Hg</td>
<td>674.4±2.6</td>
<td>657±1.7†</td>
<td>660±1.55†</td>
</tr>
<tr>
<td>PVaO2, mm Hg</td>
<td>275.28±9</td>
<td>263.1±9.1</td>
<td>241.4±9.71*</td>
</tr>
<tr>
<td>MVaO2 (µL O2 · min⁻¹ · g⁻¹)</td>
<td>159.48±9.4</td>
<td>180.5±19.9</td>
<td>173.0±8.9</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P<0.05; †P<0.01; ‡P<0.001.
Figure 5. SERCA2a TG hearts show a significant increase in the rates of contraction and relaxation. Isolated work-performing heart preparations were used to measure contractile and relaxation function of SERCA2a-overexpressing mouse hearts. Contractile parameters of TG and NTG hearts were measured under identical afterload (50 mm Hg mean aortic pressure) and preload conditions (5 mL/min venous return, approximation of preload). The maximal rates of pressure development for contraction (+dP/dt) (A) and relaxation (−dP/dt) (B), TPP (C), and RT1/2 (D) are indicated for wild type (NTG), TG line 3, and TG line 100. Data are mean ± SEM.

Discussion

The goal of the present study was to determine whether and to what extent alterations in SERCA2a pump levels affect cardiac contractility, using a TG mouse model. In this study, we used the cardiac tissue–specific α-MHC promoter to direct the cardiac tissue–specific overexpression of the SERCA2a pump. Our analyses of SERCA2a TG hearts revealed that SERCA2a mRNA levels were increased severalfold (3.88 ± 0.4-fold and 7.90 ± 0.2-fold), whereas SERCA2a protein levels were increased by 1.31 ± 0.05-fold and 1.54 ± 0.05-fold over those of controls. The maximum velocity (V_{max}) of SR membrane Ca^{2+} uptake was increased in TG hearts in correspondence with increased protein levels. Our studies using the isolated work-performing heart model demonstrate that TG hearts have increased rates of contraction/relaxation with decreased TPP and RT1/2 under resting conditions and respond to increased cardiac load with increased myocardial contractility. Reduction in RT1/2 indicates enhanced SR Ca^{2+} uptake in TG hearts, whereas a reduction in the TPP is indicative of increased Ca^{2+} availability. Our results suggest that increased Ca^{2+} uptake in the TG hearts results in an increased availability of Ca^{2+} for contraction. Thus, by increasing SERCA2a levels, we have not only increased the rate of relaxation but also the rate of contraction. These data provide convincing evidence that the SERCA2a pump level is an important determinant of myocardial contractility.

Level of SERCA2a Pump Expression Can Directly Affect Cardiac Contractility

In this study, we have analyzed 2 independent SERCA2a TG lines that vary in transgene copy number, SERCA2a mRNA expression, and SERCA protein levels. Data from both lines 3 and 100 independently demonstrate an increase in contractile function in response to SERCA2a overexpression. More importantly, this study demonstrates that different levels of pump expression can affect contractile parameters to different extents. Although there is no strict correlation between SERCA2a mRNA and protein levels, TG 3 mice that produced the highest overexpression of the SERCA2a message (7.9-fold) and SERCA2a protein levels (1.54-fold) also demonstrated the highest level of cardiac performance, whereas TG 100, which produced a 3.88-fold increase in SERCA2a message and protein levels (1.31-fold), exhibited a lesser but significant increase in cardiac contractility. Thus, analysis of these 2 lines allows us to conclude that changes in Ca^{2+}-ATPase levels can directly affect cardiac contractility. Our studies using the intact mouse ventricular trabeculae method revealed directly that muscles from TG hearts exhibit an increased Ca^{2+} transient amplitude, with a concomitant boost in twitch force. This experimental approach, which is the most direct one available for the characterization of calcium cycling and contractile activation in mice, verifies that SERCA2a overexpression is capable of increasing the calcium-sequestering ability of the SR in a physiologically significant manner. Recently, He et al reported that SERCA2a overexpression (1.2-fold) in transgenic mouse (using the CMV enhancer and the chicken β-actin promoter) resulted in increased Ca^{2+} transients in isolated myocytes and increased contractile function. Furthermore, using adenovirus-mediated gene transfer of SERCA into fetal/neonatal myocytes, Hajjar et al and Inesi et al have shown that increased SERCA protein levels produced an increase in peak Ca^{2+} levels, shorter Ca^{2+} transients, and enhanced contractility. These studies together provide strong evidence that SERCA pump level is a critical determinant of myocardial contractility.
Overexpression of SR Ca²⁺-ATPase

SERCA2a Overexpression Results in an Increased Rate of SR Ca²⁺ Uptake Without a Change in the Apparent Ca²⁺ Affinity

Our studies show that Ca²⁺ uptake rates (V_{max}) were significantly higher in TG hearts (37% increase over NTG hearts). However, the Ca²⁺ dependence of SR Ca²⁺ uptake (apparent affinity for Ca²⁺) was not altered in TG hearts, despite an increase in SERCA pump level. This is particularly interesting, since the level of phospholamban is unaltered in the TG hearts. On the basis of the increased expression of the SERCA2a protein, one might expect that the phospholamban/SERCA2a ratio might be altered. Phospholamban/SERCA2a ratios have been shown to influence the Ca²⁺ dependence of Ca²⁺ uptake in studies in which the phospholamban level has been altered. A recent report by Hajjar et al.⁵⁰ has indicated that alterations in phospholamban/SERCA2a ratios by adenoviral gene transfer of phospholamban result in altered Ca²⁺ affinity of the Ca²⁺ pump and Ca²⁺ transients. Furthermore, ablation of the phospholamban gene results in an increase in the SERCA pump affinity for calcium and increased rates of

Figure 6. Effect of pressure loading on contractile parameters in TG and NTG hearts. The effects of pressure loading on contractile function were examined in TG 100 (n=7), TG 3 (n=4), and NTG (n=6) hearts. The left ventricular function was plotted as cardiac minute work (mean aortic pressure x cardiac output) vs the rates of contraction and relaxation (+dP/dt and –dP/dt in mm Hg/s). Comparisons were made between and among control and TG groups under different loading conditions. Data are mean±SEM.

Figure 7. Ca²⁺ transients (top) and twitch force (bottom) in representative mouse ventricular trabeculae of normal (left) and TG (right) hearts. The muscles were superfused with modified KH buffer at 2 mmol/L [Ca²⁺] and stimulated at 2 Hz (22°C). SERCA2a TG hearts showed 1.84 μmol/L systolic [Ca²⁺], and 30.4 mN/mm² systolic force, whereas those of NTG hearts were 1.02 μmol/L and 18.0 mN/mm², respectively.

Figure 8. SERCA2a TG hearts showed a sizable increase in Ca²⁺ transients and contractility. Pooled data are shown for systolic and diastolic [Ca²⁺], (A) and force (B) in trabeculae from normal control (n=4) and TG hearts (n=4). Both systolic [Ca²⁺] and force were significantly higher in muscles from TG hearts with no significant changes in diastolic [Ca²⁺] and force. All muscles were superfused with KH buffer containing 2 mmol/L [Ca²⁺], and stimulated at 2 Hz. Data are mean±SEM.
contraction and relaxation. On the other hand, overexpression of phospholamban in TG mice results in decreased SERCA pump affinity for Ca\(^{2+}\) and decreased rates of relaxation and contraction. In this study, we show that the apparent SERCA2a affinity for Ca\(^{2+}\) remains unchanged in TG hearts despite an increase in SERCA2a protein levels. Two possible explanations are the following. (1) Although the total phospholamban level has not been altered, the monomer/pentamer ratio for phospholamban maybe adjusted for increased SERCA2a. (2) Phospholamban exists at saturating levels in the mouse heart, and thus increases in SERCA2a levels do not result in a compensatory change in the affinity of the pump for Ca\(^{2+}\). Our study suggests that increases in the SERCA2a pump level may not necessarily produce a shift in the apparent affinity of the pump for Ca\(^{2+}\). Currently, the in vivo functional ratio between phospholamban and SERCA2a is unknown. It is also not known whether phospholamban is in excess or in a limited quantity in the heart. Studies have shown that phospholamban exists both as a monomer and as a pentamer, and the equilibrium between these 2 states is highly regulated. There are still controversies as to whether the active form of phospholamban is a monomer or a pentamer, although some studies have suggested that the monomer is the more active form (David D. Thomas, oral communication, January 1998).

**Similarities and Differences Between SERCA2a and SERCA1a TG Overexpression Models**

In a parallel study, we have overexpressed the fast skeletal muscle SERCA1a pump in the TG mouse heart to determine whether the SERCA1a pump is functionally distinct from the SERCA2a pump. Ectopic expression of the SERCA1a pump resulted in a 2.5-fold increase in total SERCA pump level but decreased the endogenous SERCA2a level to 50% as compared with NTG hearts. In contrast, SERCA2a overexpression resulted in a severalfold increase in the mRNA level, but only a modest increase (1.3- to 1.5-fold) in protein levels. The different levels of SERCA protein expression observed with these models may be due to differences in the model system or to isoform-specific differences. It is well known that the density of SERCA1a pumps is 3- to 5-fold greater in fast skeletal muscle SR as compared with SERCA2a in slow-twitch or cardiac muscle. Both models demonstrate that increases in Ca\(^{2+}\)-ATPase levels can directly modify cardiac contractility, although the magnitude of the cardiac contractility is model dependent. We also found that the SERCA1a pump can be regulated by phospholamban. However, it remains to be determined whether these models differ in their response to neurohormonal regulation.

In conclusion, we have demonstrated that SERCA2a is a critical determinant of cardiac contractility. Our studies demonstrate that different levels of SERCA2a pump can affect cardiac contractility to different extents. Taken together, the results from our studies and those of He et al., Inesi et al, and Hajjar et al demonstrate that alteration of SR Ca\(^{2+}\)-ATPase levels can affect Ca\(^{2+}\) transport and cardiac function. The SERCA2a TG mice with increased Ca\(^{2+}\)-ATPase levels in the heart provide us with an interesting model to study the role of SERCA in Ca\(^{2+}\) homeostasis and heart failure.

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**References**


Overexpression of SR Ca\textsuperscript{2+}-ATPase

Targeted Overexpression of the Sarcoplasmic Reticulum Ca2+-ATPase Increases Cardiac Contractility in Transgenic Mouse Hearts

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