Replacement of the Muscle-Specific Sarcoplasmic Reticulum Ca$_2^+$-ATPase Isoform SERCA2a by the Nonmuscle SERCA2b Homologue Causes Mild Concentric Hypertrophy and Impairs Contraction-Relaxation of the Heart

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Abstract—The cardiac sarco(endo)plasmic reticulum Ca$_2^+$-ATPase gene (ATP2A2) encodes the following two different protein isoforms: SERCA2a (muscle-specific) and SERCA2b (ubiquitous). We have investigated whether this isoform specificity is required for normal cardiac function. Gene targeting in mice successfully disrupted the splicing mechanism responsible for generating the SERCA2a isoform. Homozygous SERCA2a$^{-/-}$ mice displayed a complete loss of SERCA2a mRNA and protein resulting in a switch to the SERCA2b isoform. The expression of SERCA2b mRNA and protein in hearts of SERCA2a$^{-/-}$ mice corresponded to only 50% of wild-type SERCA2 levels. Cardiac phospholamban mRNA levels were unaltered in SERCA2a$^{-/-}$ mice, but total phospholamban protein levels increased 2-fold. The transgenic phenotype was characterized by a $\approx 20\%$ increase in embryonic and neonatal mortality (early phenotype), with histopathologic evidence of major cardiac malformations. Adult SERCA2a$^{-/-}$ animals (adult phenotype) showed a reduced spontaneous nocturnal activity and developed a mild compensatory concentric cardiac hypertrophy with impaired cardiac contractility and relaxation, but preserved $\beta$-adrenergic response. Ca$_2^+$ uptake levels in SERCA2a$^{-/-}$ cardiac homogenates were reduced by $\approx 50\%$. In isolated cells, relaxation and Ca$_2^+$ removal by the SR were significantly reduced. Comparison of our data with those obtained in mice expressing similar cardiac levels of SERCA2a instead of SERCA2b indicate the importance of the muscle-specific SERCA2a isoform for normal cardiac development and for the cardiac contraction-relaxation cycle. (Circ Res. 2001;89:838-846.)

Key Words: Ca$_2^+$-transporting ATPase ■ alternative splicing ■ gene targeting ■ cardiovascular abnormalities ■ cardiomegaly

Resequestration of Ca$_2^+$ into the cardiac sarcoplasmic reticulum (SR) via the sarco(endo)plasmic reticulum Ca$_2^+$-ATPase (SERCA2a) largely determines the rate of muscle relaxation. This Ca$_2^+$ pump is encoded by the ATP2A2 gene in humans and by Atp2a2 in mice.¹

Tissue-specific alternative processing of the ATP2A2 transcripts results in the expression of the muscle-specific SERCA2a (sarcomeric) isoform in cardiac and slow-twitch skeletal muscle and of the ubiquitous (nonsarcomeric) SERCA2b isoform in virtually every other cell type.² Both isoforms share a common stretch of 993 amino acids, which includes 10 transmembrane segments and the catalytic site, but the C terminus of SERCA2a consists of a tetrapeptide tail located in the cytoplasm, whereas the SERCA2b tail comprises 49 residues, protruding into the endoplasmic reticulum lumen.³ In vitro studies using the COS cell expression systems revealed for SERCA2b a higher apparent Ca$_2^+$ affinity, but its catalytic turnover rate is about half that of SERCA2a.⁴ Both isoforms, however, presented the same sensitivity toward phospholamban (PLB).

ATP2A2 expression is tightly regulated by developmental, cell-specific, and hormonal cues.⁵⁻⁷ Reductions in the expression and/or activity of SERCA2 contribute to the altered Ca$_2^+$ homeostasis and diastolic dysfunction of failing hearts.⁸ Recent advances in transgenic mouse technology have made it possible to experimentally address the physiological relevance of increases or decreases in SERCA expression. Over-expression resulted in enhanced myocardial function,⁹⁻¹¹ whereas total Atp2a2 inactivation (SERCA2$^{-/-}$) was found lethal, with heterozygous mice (SERCA2$^{+/-}$) showing im-
paired cardiac contractility and relaxation. The physiological meaning of the SERCA2a/2b diversity, however, remains unknown. We hypothesized that expression of sarcomeric SERCA2a rather than the nonmuscle SERCA2b variant is important for the normal structure and function of the cardiomyocyte. To investigate this hypothesis, we have by gene targeting specifically disrupted the SERCA2a splicing mechanism. This resulted in a complete loss of SERCA2a expression in the heart and its replacement by SERCA2b. We show that the nonsarcomeric SERCA2b can be targeted to the myocardial SR where, it can functionally substitute remarkably well for SERCA2a. However, transgenic mice suffered from an increased incidence of early embryonic and neonatal lethality with pronounced cardiac malformations. The surviving mice all developed a mild compensatory concentric hypertrophy with impaired contractility, but preserved β-adrenergic response. These phenotypes were not seen in heterozygotes lacking one functional Atp2a2 allele with reduced cardiac levels of SERCA2a as a consequence. Our transgenic animals express the SERCA2b variant. Hence, we must ascribe the above phenotypes specifically to the switch in isoform.

Materials and Methods
A targeting vector was constructed to mutate the endogenous Atp2a2 gene of R1 embryonic stem cells by homologous recombination (Figure 1). Genomic clones were derived from a mouse 129/SvJ genomic library. The 5′ Atp2a2 flank, consisting of a 4.5-kb fragment reaching from exons 15 through 22, was subcloned 5′ to the neomycin resistance (neo) cassette of Pnd49, which was kindly provided by E.D. Rosen. This fragment contained the inactivated (silent mutation) muscle-specific 5′ donor splice site (5′D1) and a synthetic strong polyadenylation site (SPA), which replaces the weak upstream polyadenylation site (pAu) used to produce the class 2 donor splice site (5′(silent mutation) muscle-specific 5′ splice site (5′M1)) muscle-specific 5′ splice site (5′M2)). The neo gene replaced the untranslated exons 23 and 24 and the muscle-specific exon 25 of Atp2a2.

Embryonic stem cells heterozygous for targeted recombination at the Atp2a2 locus were aggregated with Swiss morula-stage embryos to generate chimera offspring. Chimeric germline-transmitting males were testbred with Swiss females to obtain heterozygous mice (SERCA2a+/−), which were further intercrossed to generate homozygous (SERCA2a−/−) progeny. Among 159 offspring genotyped at weaning age, 44 were WT (28%), 85 heterozygous (54%), and 30 homozygous (18%) for the mutated Atp2a2 allele. The percentage of SERCA2a−/− mice was significantly lower than the expected 1:2:1 Mendelian ratio, which indicates a loss of homozygous mutants during embryonic and/or neonatal development. The average number of viable implanted embryos at days E12.5 and E14.5 of embryonic development was 11±0.4 in WT (n=7) but only 8.2±0.9 in −/− litters (n=5, P<0.05). These analyses demonstrated an increased number of intrauterine resorptions in SERCA2a−/− mice; the resorptions amounted to 21±4.6% of total implants in −/− compared with 1.2±1.2% in WT (P<0.05). The average litter size obtained at birth, 7.8±2.2 (n=84 litters) for SERCA2a−/− mice, was 20% lower as compared with WT mice, 10±1.3 (n=51 litters, P<0.05).

Histological analysis of embryonic hearts (E12.5 to E14.5) revealed that the −/− hearts were hypoplastic with a thinner compact layer of the ventricular wall, which suggested an impaired cardiac function (Figure 2A). Furthermore, whole-mount immunostaining on E9.5 embryos with SERCA2a- and SERCA2b-specific antisera documented the expression of SERCA2a in this early stage of heart development in WT mice and a switch to SERCA2b in −/− hearts (data not shown).

Neonatal mortality was also significantly higher in SERCA2a−/− animals, as follows: 18±1.1% of newborn −/− mice died within the first 4 days after birth (most of them at P0 or P1) compared with 3.5±0.8% in WT litters (P<0.05). These preterminal mice (P0 to P4, referred to as early phenotype) showed a lower body weight, lack of milk consumption, signs of fatigue, striking sluggishness of their movements, and impairment in regaining an upright position when turned on the back. Histological analyses (Figure 2B) revealed severe cardiac malformations such as dilated right ventricle with hypertrophy of the left ventricle (LV), ventricular and atrial septum defects, fibrosis in the atrium, coarctation, and/or hypoplasia of the ascending aorta suggesting a hypoplastic left heart syndrome, leading to malfunctioning of

Ventricular myocytes were enzymatically isolated from 3-month-old mice. Single-cell shortening was measured with a video-edge detector (Crescent Electronics) during field stimulation at 1 Hz. [Ca2+], transients were recorded with fluo-3 during whole-cell voltage clamp. To obtain diastolic [Ca2+], values, fluorescence signals were calibrated as described by Trafford et al. Cells were stimulated at a rate of 1 Hz with 25-ms depolarizing steps from a holding voltage of −70 to +20 mV. All experiments were performed at 37°C. Statistical significance was determined by unpaired Student t test or ANOVA. Values are mean±SEM; n indicates the number of mice. Values of P<0.05 were considered as statistically significant. An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results
SERCA2a-Isomorph Deficiency Leads to Increased Incidence of Fetal/Neonatal Mortality and Cardiac Structural Malformations
The mutation strategy illustrated in Figure 1A was followed to disrupt muscle-specific splicing of Atp2a2 transcripts. Heterozygous offspring (SERCA2a+/−) were intercrossed to generate homozygous (SERCA2a−/−) progeny. Among 159 offspring genotyped at weaning age, 44 were WT (28%), 85 heterozygous (54%), and 30 homozygous (18%) for the mutated Atp2a2 allele. The percentage of SERCA2a−/− mice was significantly lower than the expected 1:2:1 Mendelian ratio, which indicates a loss of homozygous mutants during embryonic and/or neonatal development. The average number of viable implanted embryos at days E12.5 and E14.5 of embryonic development was 11±0.4 in WT (n=7) but only 8.2±0.9 in −/− litters (n=5, P<0.05). These analyses demonstrated an increased number of intrauterine resorptions in SERCA2a−/− mice; the resorptions amounted to 21±4.6% of total implants in −/− compared with 1.2±1.2% in WT (P<0.05). The average litter size obtained at birth, 7.8±2.2 (n=84 litters) for SERCA2a−/− mice, was 20% lower as compared with WT mice, 10±1.3 (n=51 litters, P<0.05).

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Figure 1. Atp2a2 gene targeting strategy to disrupt splicing of the muscle-specific SERCA2a isoform and verification. A, Top, Schematic organization of 3′ end of WT Atp2a2. Tissue-specific alternative processing results in expression of 4 different classes of mRNA (classes 1 through 4). Wide boxes indicate open reading frames; narrow boxes, untranslated regions. Processing signals are indicated as follows: 5′D1 is a donor splice site used in muscle; 5′D2, donor splice site used exclusively in brain; 3′A, 3′ acceptor splice site common to muscle and neurons; and pAu and pAd, upstream (u) and downstream (d) polyadenylation sites, respectively. j1 and j4 indicate polyadenylation sites used in class 2 (pAu) and class 3 (pAd) mRNA, respectively. j2 and j3 indicate optional muscle- and neuron-specific splicing (classes 1 and 4 mRNA, respectively). Class 1 mRNA encodes the SERCA2a isoform, whereas classes 2 through 4 all encode SERCA2b. Middle, Targeting vector containing the corrupted 5′ donor splice site 5′D1 (silent mutation creating a novel SmaI restriction site), and the synthetic polyadenylation site (SPA). The neor cassette used for positive selection replaced exons 23 through 25, whereas a cda (cytosine deaminase) cassette was used for negative selection against random integrants. Bottom, Targeted Atp2a2 allele that allows only the expression of the nonmuscle SERCA2b isoform (class 2 mRNA). E1 and E2 are the 3′ and 5′ external probes used for Southern blot analysis. Diagnostic fragments discriminating between WT and the correctly targeted allele are indicated by thin lines above or below the genes. B, PCR + SmaI digest. C, RNase Protection. D, Western Blot.
the neonatal heart and to premature death. SERCA2a−/− mice that survived the first postnatal days developed to adulthood (adult phenotype). These adult −/− mice were fertile but showed a markedly reduced spontaneous nocturnal activity as measured by the total distance covered in a treadmill test (2.1 ± 0.5 × 10³ m for −/− versus 8.0 ± 0.8 × 10³ m for WT, n = 10, P < 0.05). Changes in SERCA expression in slow-twitch skeletal muscle fibers, normally expressing SERCA2a in the WT but SERCA2b in −/− mice, may be at least partially responsible for the decreased activity in the treadmill test.

**Total SERCA2 mRNA and Protein Levels Are Decreased in SERCA2a−/− Hearts**

The absence of SERCA2a mRNA and switching to SERCA2b mRNA in SERCA2a−/− mice were confirmed by ribonuclease-protection assays (Figure 1C). Remarkably, the total SERCA2 mRNA expression levels in −/− hearts were reduced to 40 ± 2.1% of WT levels (Figure 3A, P < 0.05). Western blot analysis (Figure 1D) and immunostaining of cardiac tissue sections (data not shown) with isoform-specific antisera further confirmed the isoform switch to SERCA2b protein. By consequence the SERCA2a mRNA and protein are completely absent in −/− hearts. Although SERCA2b successfully replaced SERCA2a, the overall expression of SERCA2 in −/− hearts was reduced to 49 ± 3% of WT levels (Figure 3B; n = 5 hearts, P < 0.05). No compensatory ectopic expression of SERCA1 or of SERCA3 in −/− hearts was observed by Western blot analysis. Although PLB mRNA levels were unchanged in SERCA2a−/− hearts (Figure 3A), the PLB protein levels were increased 2.2 ± 0.14-fold (Figure 3B; n = 5 hearts, P < 0.05). As a result of the inverse changes of PLB and SERCA2 protein expression in SERCA2a−/− mice, the overall PLB/SERCA2 ratio in −/− hearts was ~4.5 times higher than in WT hearts.

**Cardiac Hypertrophy in SERCA2a−/− Hearts**

The hearts of adult −/− mice showed a marked reduction in their long axes and a loss of the normal ellipsoid form in favor of a more spherical appearance (Figure 4A). Heart weight/body weight ratios increased from 4.5 ± 0.1 mg/g in WT to 5.2 ± 0.1 mg/g in −/− mice (P < 0.05; n = 8). Ventricular mRNA levels of atrial natriuretic factor, α-skeletal actin, and β-myosin heavy chains, markers of cardiac hypertrophy of diverse etiologies, were increased respectively 3-, 1.6-, and 1.6-fold in ventricles of 18-day-old and adult −/− hearts (Figure 4D). Sections of −/− hearts showed differences in gross morphology (Figures 4B and 4C) with changes in chamber size, ventricular wall thickness, and overall myocyte organization consistent with concentric hypertrophy (Table 1). The increases in heart size and mass were largely accounted for by increases in the myocyte dimensions. Staining of cross sections of adult hearts with Sirius red excluded excessive interstitial fibrosis as the cause of hypertrophy.
Echocardiographic measurements (Table 1) showed an increase in the LV posterior wall and septal wall thickness of −/+ hearts relative to age-matched controls. In −/− hearts, both LV internal end-diastolic diameter (−22%) and LV end-systolic diameter (−21%) were decreased. The relative wall thickness (an index of concentric hypertrophy) was increased compared with age-matched control groups. However, the percentage of fractional shortening remained unchanged, indicating a functional compensated balance.

SERCA2b Hearts Show Significant In Vivo Decreases in Cardiac Contractility and Relaxation

Under baseline conditions, heart rate, left ventricular systolic pressure, and end-diastolic pressure were similar in both SERCA2a+/− and WT mice. Several physiological parameters, however, showed impairment in the LV contractility of SERCA2a−/− hearts (Table 2). Both rate of contraction (+dP/dt) and rate of relaxation (−dP/dt) were significantly depressed in −/− hearts. The time to peak pressure (TTP) and half relaxation time were prolonged in −/− hearts compared with WT hearts. During dobutamine infusion for β-adrenergic stimulation, the rates of contraction and relaxation were increased in a dose-dependent manner in −/− and WT hearts (Figure 4E). The absolute values of +dP/dt and −dP/dt were significantly lower in SERCA2a−/− mice under basal conditions and at all levels of β-adrenergic stimulation. The fractional dobutamine-dependent increase in these parameters was similar in −/− and WT hearts.

Reduced SR Ca2+-Uptake Activity in SERCA2a−/− Hearts

The maximum rate of ATP-dependent oxalate-facilitated Ca2+ uptake was significantly reduced (P<0.05) in cardiac homogenates of SERCA2a−/− mice (to 56% of WT levels),
with a $V_{\text{max}}$ of 51 ± 1.9 and 29 ± 0.9 nmol Ca\textsuperscript{2+}/mg protein/min in WT and −/− hearts, respectively (Figure 3C). This decrease in SR Ca\textsuperscript{2+} uptake is consistent with the decreased overall SERCA2 protein levels in −/− samples, with a $K_{0.5}$ of 0.19 ± 0.01 m\textsuperscript{mol/L} in −/− hearts versus 0.28 ± 0.02 m\textsuperscript{mol/L} in WT hearts ($P<0.05$).

### Decreased Rate of Ca\textsuperscript{2+} Removal by the SR in Isolated Ventricular Myocytes

The amplitude of shortening in field-stimulated unloaded cells was 6 ± 0.5% of the resting cell length $L_0$ in SERCA2a\textsuperscript{−/−} ($n_{\text{cells}}=18$) versus 5 ± 0.7% of $L_0$ in control ($n_{\text{cells}}=19$, $P=NS$); TtP was 89 ± 2 ms in SERCA2a\textsuperscript{−/−} versus 89 ± 1 ms in control ($P=NS$). The rate of relaxation was slower in SERCA2a\textsuperscript{−/−}; $\tau$ was 48 ± 3 ms in SERCA2a\textsuperscript{−/−} versus 39 ± 2 ms in control. Ca\textsuperscript{2+} uptake in the SR was further studied in cells under whole-cell voltage clamp. Representative examples of [Ca\textsuperscript{2+}]\textsubscript{i} transients in a control myocyte and a SERCA2a\textsuperscript{−/−} cell are shown in Figure 5Aa. The amplitude of [Ca\textsuperscript{2+}]\textsubscript{i} transients during steady-state stimulation at 1 Hz tended to be smaller in SERCA2a\textsuperscript{−/−} cells; $\Delta F/F_0$ was 3.0 ± 0.37, for WT ($n=14$ cells) and 2.3 ± 0.29 for −/− ($n=13$, $P=0.2$). The TtP of the [Ca\textsuperscript{2+}] transient was not significantly different (31 ± 1 ms for WT versus 34 ± 1 ms for −/−), but the $\tau$ value for the decline of the [Ca\textsuperscript{2+}] transient was significantly higher in −/− cells (55 ± 4 ms in WT versus 73 ± 5 ms in −/−; Figure 5Ba). Resting [Ca\textsuperscript{2+}], of cells in which the fluorescence records were calibrated (see Materials and Methods) was comparable in both cell types, as follows: 64 ± 9 nmol/L for WT cells and 57 ± 9 nmol/L for −/− cells ($n=7$ and 12 respectively, $P=NS$). The amplitude of the transient inward current on repolarization, reflecting Ca\textsuperscript{2+}...
TABLE 1. Morphometric Measurements and Echocardiographic Analysis

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<th>WT  (n=5)</th>
<th>SERCA2a−/− (n=5)</th>
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<tr>
<td>Heart/body weight ratio, mg/g†</td>
<td>4.5±0.1</td>
<td>5.2±0.1*</td>
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<tr>
<td>Cross area myocyte, μm²</td>
<td>210±4</td>
<td>290±9*</td>
</tr>
<tr>
<td>2D area LV wall, mm²</td>
<td>10.0±0.29</td>
<td>12.0±0.48*</td>
</tr>
<tr>
<td>2D luminal area LV, mm²</td>
<td>2.4±0.2</td>
<td>1.90±0.1*</td>
</tr>
<tr>
<td>Length isolated myocyte, μm†</td>
<td>120±2</td>
<td>150±3*‡</td>
</tr>
<tr>
<td>Width isolated myocyte, μm†</td>
<td>24±0.9†</td>
<td>27±0.5*‡</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>230±38</td>
<td>190±12</td>
</tr>
<tr>
<td>PWth, mm</td>
<td>0.63±0.06</td>
<td>1.30±0.15*</td>
</tr>
<tr>
<td>SepTh, mm</td>
<td>0.81±0.10</td>
<td>1.40±0.10*</td>
</tr>
<tr>
<td>Relative wall thickness (2 · PWth/LVEDD)</td>
<td>0.37±0.06</td>
<td>0.97±0.16*</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.4±0.18</td>
<td>2.6±0.13*</td>
</tr>
<tr>
<td>% FS</td>
<td>47±2.5</td>
<td>45±2.0</td>
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PWh indicates posterior wall thickness; SepTh, intraventricular septal wall thickness; LVEDD, left ventricular end-diastolic dimension; LVEDD, left ventricular end-systolic dimension; and FS, fractional shortening. Values are mean±SEM of n mice. *P<0.05 vs WT (†n=8; and †n=10).

Discussion

Disruption of SERCA2a Splicing Results in a Switch to the SERCA2b Isoform and Decrease in SERCA2 Levels

The replacement of SERCA2a by SERCA2b in the transgenic hearts was accompanied by a nearly 50% decrease in SERCA2 mRNA and protein levels. The observed phenotype (cardiac hypoplasia with cardiac malformations in the embryo and mild compensatory cardiac hypertrophy after birth in −/− mice) appears to relate to the SERCA2a→b isofrom switch and not to the overall reduction of the SERCA2 pump levels. Indeed, heterozygous Atp2a2 knockout mice (SERCA2a+/−), which lack one functional copy of the Atp2a2 allele, showed comparable reductions (~35%) of total SERCA2 protein and activity levels in the heart, yet they do not develop cardiac hypertrophy or cardiac malformations.12,13 The main difference with our transgenic mice is the presence of SERCA2a and not SERCA2b in the hearts.

SERCA2 protein levels were decreased significantly only in muscle and brain tissue of our SERCA2a−/− mice and not in any of the tested nonmuscle organs, including liver, kidney, and testes (data not shown). Thus, only tissues that predominantly synthesize SERCA2 mRNA containing exon 25 (ie, class 1 in muscle, class 4 in neuronal cells) show a decrease in total SERCA2 levels observed in −/− mice. This tissue-specific downregulation might point to a role of the deleted regions (ie, the untranslated exons 23 and 24 and the muscle-specific exon 25) in mRNA processing/stability. Indeed, posttranscriptional mechanisms are partially held responsible for the higher SERCA2a expression in cardiac versus smooth muscle30 and for the changes in cardiac SERCA2 mRNA levels in the perinatal period.6

The upregulation of the PLB protein level in contrast to its mRNA level, in our SERCA2a−/− hearts, suggests a posttranscriptional regulation. Posttranscriptional regulation of PLB expression was also reported earlier in the SERCA2a−/− mouse, but in the latter case PLB levels were decreased.19 We thus confirm that PLB expression posttranscriptionally adapts to a lowered SERCA2 expression, but that the direction of the change differs with the SERCA2 splice variant. Altering the relative expression levels of PLB and SERCA2 has profound effects on cardiac performance. Cardiac relaxation and contractility are depressed in transgenic mice overexpressing PLB.21 In contradistinction, SERCA2-overexpressing9,10 or PLB-ablated mice22 exhibit an accelerated cardiac relaxation and augmented contractility. Thus, it can be expected that the apparent posttranscriptional upregulation of PLB combined with the depressed SERCA2 levels would depress cardiac relaxation and contractility even more. Additional studies are in progress to determine whether this effect could be compensated for by an altered phosphorylation status of PLB.

TABLE 2. In Vivo Hemodynamic Measurements

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<th>Baseline</th>
<th>Maximum Dobutamine</th>
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<tr>
<td>HR, bpm</td>
<td>530±14</td>
<td>520±10</td>
</tr>
<tr>
<td>LVPs, mm Hg</td>
<td>92±4.8</td>
<td>87±4.3</td>
</tr>
<tr>
<td>EDP, mm Hg</td>
<td>48±1.5</td>
<td>59±2.8</td>
</tr>
<tr>
<td>+dP/dt, mm Hg/s</td>
<td>8100±101</td>
<td>7100±190*</td>
</tr>
<tr>
<td>−dP/dt, mm Hg/s</td>
<td>4600±197</td>
<td>3600±78*</td>
</tr>
<tr>
<td>TtP, ms</td>
<td>26±1.3</td>
<td>36±3.6*</td>
</tr>
<tr>
<td>RT50, ms</td>
<td>19±1.2</td>
<td>27±0.9*</td>
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In vivo hemodynamic measurement at baseline and after stimulation with dobutamine. HR indicates heart rate; LVPs, left ventricular systolic pressure; EDP, end-diastolic pressure; ±dP/dt, the maximal rates of left ventricular pressure development and decline; TtP, time to peak pressure; and RT50, half-relaxation time. Values are mean±SEM of n mice (WT n=5, −/− n=6).

*P<0.05 vs WT.
The \(V_{max}\) of the \(\text{Ca}^{2+}\) uptake in cardiac homogenates of SERCA2a\(^{-/-}\) mice was reduced to roughly the same extent as the SERCA2 protein levels, confirming a direct relationship between SERCA2 protein levels and the maximal rate of \(\text{Ca}^{2+}\) sequestration. This suggests that there is no intrinsic in vivo difference in the maximal enzymatic turnover rate between both isoforms, in contrast to the previous in vitro experiments in the COS cell system.\(^4\) In line with the reported in vitro differences in \(\text{Ca}^{2+}\) dependence between the isoforms, we also observed a higher apparent \(\text{Ca}^{2+}\) affinity for SERCA2b in cardiac homogenates of \(-/-\) mice.

**Replacement of SERCA2a With SERCA2b Affects Embryonic and Postnatal Development**

Substituting SERCA2b for SERCA2a leads to a marked increase in embryonic and early neonatal mortality resulting from cardiac malformations in many but not all littermates. Our whole-mount immunostaining on E9.5 embryos confirmed the early expression of SERCA2a in the embryonic heart tube of WT and its replacement with SERCA2b in SERCA2a\(^{-/-}\) embryos (data not shown). Because similar increases in mortality and malformations are not seen in the SERCA2a\(^{-/-}\) embryos which, as already pointed out, express roughly equally reduced cardiac levels of SERCA2, but of the SERCA2a isoform instead of SERCA2b, we must again conclude that the phenotype depends on the isoform of the pump and not on its expression levels.\(^12\),\(^19\)

**Adult Phenotype Shows Concentric Cardiac Hypertrophy and Impaired Contractile Function**

SERCA2a\(^{-/-}\) mice that survived the critical postnatal period developed to adulthood and apparently managed to compensate for the loss of the muscle-specific SERCA2a isoform (adult phenotype). Nevertheless, these \(-/-\) mice showed a remarkably reduced spontaneous nocturnal activity. They developed a mild concentric cardiac hypertrophy. The reduced levels of total SERCA2 protein and the corresponding lowered \(\text{Ca}^{2+}\)-transport activity in cardiac homogenates of \(-/-\) mice could compromise cardiac function. Consistent with this expectation, the absolute values of both \(+dP/dt\) and \(-dP/dt\) were significantly lower in \(-/-\) mice, under basal conditions and after \(\beta\)-adrenergic stimulation. Fractional shortening during ventricular ejection was, however, preserved.

In the isolated, mechanically unloaded cells, the decrease in SR \(\text{Ca}^{2+}\) uptake was evident from significantly reduced rates of \(\text{Ca}^{2+}\) removal and relaxation. These findings suggest that the in vivo contractile impairment observed in the SERCA2a\(^{-/-}\) mice could be a result of impaired \(\text{Ca}^{2+}\) handling and structural ventricular changes. Hypertrophy could adapt a response to the impaired contractility or altered \(\text{Ca}^{2+}\) homeostasis.

**Reducing SERCA Pump Expression to Nearly Half Its Original Level in Mouse Heart Does Not Necessarily Lead to Heart Failure**

Studies on human failing/diseased hearts have revealed reductions in the levels of SERCA2a mRNA, SERCA2a protein, and the activity.\(^8\),\(^23\) Similar observations have been made in animal models of heart failure and hypertrophy.\(^24\) On the basis of these and other studies, it has been suggested that SR dysfunction resulting from decreased levels of the SR \(\text{Ca}^{2+}\) pump significantly contributes to the pathogenesis of heart disease. In view of these observations it is remarkable that cardiac performance in an earlier reported mouse model\(^12\),\(^19\) and in the model presented here (each of which showed a considerable but roughly similar reduction in SERCA2 expression [35% to 50%]), was relatively mildly affected. Compensatory changes may help to maintain \(\text{Ca}^{2+}\) transport and contractile function.\(^19\)

Taken together, our data indicate that a decrease in SERCA2 protein expression and activity levels is as such not a sufficient perturbation to cause heart failure in mice. However, mouse myocardium is considerably different from human myocardium regarding excitation-contraction coupling and contractile protein isoforms.\(^25\) Among others, mouse myocardium appears to be much more tolerant to \(\text{Ca}^{2+}\) overload than other species.
Conclusions

Our data indicate that SERCA2b can be efficiently targeted to its normal location in cardiac SR. In the SR, SERCA2b can replace the function of SERCA2a remarkably well, but it shows a slightly higher Ca$^{2+}$ affinity despite the 2-fold posttranscriptional upregulation of PLB, which would tend to decrease the affinity toward that of SERCA2a. The SERCA2a isoform is of prime importance for normal cardiac development. About 20% of the animals expressing SERCA2b instead of SERCA2a die in utero. Of the remainder, another 20% show severe cardiac malformations and die during the first days after birth. In all surviving adults, the hearts show mild concentric hypertrophy with impaired in vivo relaxation and contraction kinetics, indicating that also in the adults SERCA2b can only partially substitute for SERCA2a.

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