Disruption of a Single Copy of the SERCA2 Gene Results in Altered Ca\(^{2+}\) Homeostasis and Cardiomyocyte Function*  

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This paper is available online at http://www.jbc.org

A mouse model carrying a null mutation in one copy of the sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase isoform 2 (SERCA2) gene, in which SERCA2 protein levels are reduced by -35%, was used to investigate the effects of decreased SERCA2 level on intracellular Ca\(^{2+}\) homeostasis and contractile properties in isolated cardiomyocytes. When compared with wild-type controls, SR Ca\(^{2+}\) stores and Ca\(^{2+}\) release in myocytes of SERCA2 heterozygous mice were decreased by ~40–60% and ~30–40%, respectively, and the rate of myocyte shortening and relengthening were each decreased by ~40%. However, the rate of Ca\(^{2+}\) transient decline (α) was not altered significantly, suggesting that compensation was occurring in the removal of Ca\(^{2+}\) from the cytosol. Phospholamban, which inhibits SERCA2, was decreased by ~40% in heterozygous hearts, and basal phosphorylation of Ser-16 and Thr-17, which relieves the inhibition, was increased ~2- and 2.1-fold. These results indicate that reduced expression and increased phosphorylation of phospholamban provides compensation for decreased SERCA2 protein levels in heterozygous heart. Furthermore, both expression and current density of the sarcolemmal Na\(^+-\)Ca\(^{2+}\) exchanger were up-regulated. These results demonstrate that a decrease in SERCA2 levels can directly modify intracellular Ca\(^{2+}\) homeostasis and myocyte contractility. However, the resulting deficit is partially compensated by alterations in phospholamban/SERCA2 interactions and by up-regulation of the Na\(^+-\)Ca\(^{2+}\) exchanger.

In heart, muscle relaxation is largely dependent on the action of the sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase (SERCA) to resequester cytosolic calcium released during contraction. Increased activity of SERCA, either by transgenic overexpression of SERCA isoforms in the heart (1–3) or by ablation of its regulatory protein, phospholamban (PLB) (4), has been shown to enhance cardiac rates of contraction and relaxation (1–4). To examine the effects of decreased SERCA2 activity on cardiac function, we have recently developed a transgenic mouse model with a null allele of the SERCA2 gene (5). Although complete loss of SERCA function in homozygous animals is embryonic lethal, disruption of one copy of the SERCA2 gene results in decreased cardiac SERCA2 mRNA (~45%), protein (~35%), and SR Ca\(^{2+}\) uptake (~35%) (5). These changes are associated in vivo with impaired cardiac performance (5). Because SERCA2 activity controls both the rate of calcium removal and the amount of calcium stores available within the SR, we hypothesize that the level of SERCA2 activity is a critical determinant of cardiac contractility. Therefore, one goal of this study is to determine if reduced SERCA2 levels compromise cardiac contractility by directly altering calcium handling and contractile functions of individual myocytes during excitation-contraction coupling.

During excitation-contraction coupling, Ca\(^{2+}\) entry through the L-type Ca\(^{2+}\) channel activates Ca\(^{2+}\) release from SR Ca\(^{2+}\) stores, via the ryanodine receptor ( RyR). This rise in cytosolic Ca\(^{2+}\) initiates muscle contraction. In the relaxation phases most of the released Ca\(^{2+}\) is subsequently resequestered into the SR by SERCA, the activity of which is closely regulated by its interaction with PLB. In addition, the released Ca\(^{2+}\) is also extruded via the Na\(^+-\)Ca\(^{2+}\) exchanger (NCX) and the plasma membrane Ca\(^{2+}\)-ATPase (6). We hypothesize that chronic loss of SERCA2 in SERCA2 heterozygous hearts may be partially compensated by altered expression of other proteins involved in calcium homeostasis during muscle contraction and relaxation. To test this hypothesis, we have examined expression of several proteins known to be important in the control of calcium transients in the heart. Alternatively, decreased contractility in heterozygous hearts may be associated with changes in the expression of contractile proteins, such as switching between α- and β-myosin heavy chain (MHC) isoforms, which occurs during cardiac hypertrophy (7). Therefore, we also examined the expression of myosin heavy chain isoforms in SERCA2-deficient hearts.

We have previously demonstrated that a decreased SERCA2 level in heterozygous hearts results in impaired cardiac function in vivo (5). In this study, we show that, despite multiple changes in expression and function of other calcium-handling proteins (such as PLB, triadin, and NCX), peak calcium transients and SR calcium stores of SERCA2-deficient myocytes are significantly diminished and contractile function is impaired. These data suggest that changes in other calcium-handling...
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proteins are functionally insufficient to completely compensate for the loss of the calcium pump protein and that the level of SERCA protein is an important critical regulator of cardiac function.

**EXPERIMENTAL PROCEDURES**

As described previously, gene targeting was used to delete part of the promoter, the transcript initiation site, and the first two coding exons of the SERCA2 gene (5). Heterozygous mice, containing a single functional allele of the SERCA2 gene, were identified by polymerase chain reaction genotyping and used in the following studies at 12–14 weeks of age.

Ribonuclease Protection Assays—

As described previously, gene targeting was used to delete part of the promoter, the transcript initiation site, and the first two coding exons of the SERCA2 gene (5). Heterozygous mice, containing a single functional allele of the SERCA2 gene, were identified by polymerase chain reaction genotyping and used in the following studies at 12–14 weeks of age.

**Ribonuclease Protection Assays—**

Ribonuclease protection assays for mouse cardiac ryanodine receptor gene (RyR2) corresponding to nucleotides 118 bp to 118 bp (150 bp) was used to synthesize 32P-labeled cRNA probes from the AUG codon. The MAXI script from Dr. Evangelia Kranias) (9), corresponding to nucleotides -1 to nucleotides 97 bp (150 bp) relative to the start codon; mouse cardiac calsequestrin cDNA (a gift from Dr. Evangelia Kranias) (9), corresponding to nucleotides +421 bp to +570 bp (150 bp) relative to the start codon; mouse triadin gene isoform 3, corresponding to nucleotides +22 bp to +170 bp (150 bp) obtained from GenBank\(^{\text{TM}}\) (accession number AA613321); mouse Na\(^+\)-Ca\(^{2+}\) exchanger isoform 1, consisting of a fragment –31 bp to +118 bp (150 bp) relative to the AUG codon (10); and mouse GAPDH gene (sequence obtained from GenBank\(^{\text{TM}}\) (accession number M33599)), corresponding to the region from –46 bp to +179 bp (225 bp) relative to the AUG codon. The MAXI script *in vitro* transcription kit (Ambion, Inc.) was used to synthesize \(^{32}\)P-labeled cRNA probes from pOl-1-linearized plasmid templates. Total RNA was isolated from hearts using the Ultraspec-II RNA isolation system (Biotex Laboratories, Houston, TX). 5 μg of total RNA (n = 6 for each genotype) was hybridized with the riboprobes described above and processed using RPA III ribonuclease protection assay kit (Ambion, Inc.), (5, 10). The protected fragments were separated by electrophoresis in a 5% denaturing polyacrylamide gel and analyzed by autoradiography.

**Quantitative Immunoblotting Analysis—**

Quantitative immunoblotting of cardiac homogenates was carried out as described previously (2, 11–13). Briefly, homogenates were prepared from hearts of wild-type or heterozygous mice (n = 6 of each genotype) in homogenate buffer (in mM: imidazole (pH 7.0) 10, sucrose 300, dithiothreitol 1, sodium mtesulfonate 1, and phenylmethylsulfonyl fluoride 0.3) at 4 °C. The homogenates were separated on 10%, 20%, and 30% SDS-PAGE gels, transferred to nitrocellulose membranes or polyvinylidene difluoride (PVDF) membranes (for RyR and α- and β-MHC). Membranes were probed with the primary antibodies: mouse monoclonal anti-PLB antibody, mouse monoclonal anti-RyR antibody, mouse monoclonal anti-triadin isoform 3 antibody, mouse monoclonal anti-Ncx antibody (all from Affinity Bioreagents Inc.), polyclonal rabbit anti-calsequestrin antibody (Swant, Switzerland), mouse monoclonal anti-serromycin antibody (Sigma), polyclonal rabbit antibody specific to SERCA2a (gift of Dr. Frank Wuytack), and mouse monoclonal antibodies specific to α-MHC and β-MHC (gift from Dr. Mark Sussman, Children’s Hospital Medical Center, Cincinnati, OH). After incubation with the primary antibodies and probing with the peroxidase-conjugated secondary antibodies, the protein bands were detected by enhanced chemiluminescence (ECL) (Kirkgaard and Perry Laboratories, Gaithersburg, MD). Signals were quantitated by densitometry using a UMAAX Astra 1200S scanner and analyzed using IMAGE software (version 6.1, National Institutes of Health).

To determine the basal phosphorylation level of PLB, polyclonal antibodies raised against a PLB peptide phosphorylated at Ser-16 (PLB-phospho-Ser16) or at Thr-17 (PLB-phospho-Thr17) (FluoScience Ltd., Reeds, UK) (15) were used. The mouse hearts were excised and immediately freeze-clamped using instruments precooled with liquid nitrogen to ensure complete and rapid freezing of the cardiac tissues. Cardiac homogenates were prepared as described (12, 13). The homogenate buffer contained (in mM): imidazole (pH 7.0) 10, sucrose 300, and EDTA 1. The buffer was supplemented with the following proteinase inhibitors: 0.3 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 2 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml aprotinin (13), and 25 mM sodium fluoride was added as phosphatase inhibitor. After separation on 15% SDS-polyacrylamide gels, the homogenate protein was transferred onto a PVDF membrane. The membranes were incubated with PLB-phospho-serine 16 (1:10,000 dilution) and PLB-phosphothreonine 17 (1:5000 dilution) antibodies. ECL and quantitation of the signals were carried out as described above. Both the high molecular weight form of PLB (PLB\(_{h}\)) and the low molecular weight form of PLB (PLB\(_{l}\)) were quantified, and the ratio of PLB\(_{h}\) to PLB\(_{l}\) was measured. To measure the relative ratio of PLB to SERCA protein, the signals of PLB\(_{h}\) and SERCA2a from the same membrane were quantitated.

**[H]Ryanodine Binding Assay—**

Ryanodine radioligand binding assays were performed as described (16, 17). The binding medium contained (in mM): HEPES 20 (pH 7.1), and KCl 600, with free [Ca\(^{2+}\)] adjusted to a final concentration of 20 μM by appropriate addition of 0.5 mM EGTA using the computer algorithms of Robertson and Potter (18). Cytosolic homogenates in assay buffer (100 μg of total protein) with various concentrations (0.1–30 nM) of [H]Ryanodine (56.9 Ci/mmol, PerkinElmer Life Sciences) were incubated at 37 °C for 90 min in the presence and absence of 17 μM cold ryanodine, and reactions were terminated by filtration. Specific [H]Ryanodine binding was calculated using a radioligand binding analysis computer program by G. A. McPherson (Elsevier-BIOSOFT).

**Measurement of [Ca\(^{2+}\)]\(_{\text{i}}\) Transients and Contractile Parameters in Isolated Ventricular Myocytes—**

Adult left ventricular myocytes were isolated and resuspended in a physiological buffer at 37 °C for 15–20 min (2, 19, 20). Half of the cells from each heart were then used for mechanical studies, and the other half were used for measurements of intracellular free Ca\(^{2+}\) transient. Intracellular calcium transients were measured with fura-2, as previously described (2, 19, 20). Briefly, myocytes were loaded with 7.5 μM Fura-2 AM at 37 °C for 15 min in the dark. After loading, cells were washed and resuspended in Ca\(^{2+}\)-free Tyrode’s buffer. Cytosolic free calcium was measured by ratio imaging of 340- to 380-nm excitation fluorescence of Fura-2 AM (emission wavelength, 510 nm), using a photocam dual spectrophotometer (Photon Tech. Inc., Santa Clara, CA) coupled to an Olympus IMT-2 UV fluorescence microscope with UV-transparent optics. Cells were selected for use if they were quiescent when unstimulated, and contracted robustly upon field stimulation. Cells were perfused with Sanguinietti solution (in mM: NaCl 112, KCl 15, CaCl\(_2\) 0.5, MgSO\(_4\) 1.2, NaH\(_2\)PO\(_4\) 2, NaHCO\(_3\) 28, glucose 10), bubbled with 95% O\(_2\)/5% CO\(_2\), and field-stimulated at 0.25 Hz (Grass S9D stimulator) until twitch characteristics were repeatable, indicating stable Ca\(^{2+}\) loading of the SR. Following field stimulation protocols, cells were exposed to ionomycin, EGTA, and Mn\(^{2+}\) to determine background fluorescence and dye compartmentalization. The intracellular Ca\(^{2+}\) kinetics were analyzed using Origin 4.1 (Microcal Software).

**Assessment of SR Ca\(^{2+}\) Load in Isolated Myocytes—**

A solenoid perfusion system (W. Barry Co., Boulder, CO) was used to rapidly apply 10 mM caffeine to induce release of SR Ca\(^{2+}\) and assess the SR Ca\(^{2+}\) load (21, 22). Cells were perfused with Sanguinietti solution and field-stimulated at 0.25 Hz until twitch characteristics stabilized before each caffeine application. Caffeine was then applied for 10 s. The amplitude of the caffeine-induced Ca\(^{2+}\) transient was used as an index of SR Ca\(^{2+}\) content (23, 24).

**Measurement of Na\(^{+}\)-Ca\(^{2+}\) Exchanger Current Density—**

Na\(^{+}\)-Ca\(^{2+}\) exchanger currents were recorded using whole-cell patch-clamp techniques as described previously (25–27). Briefly, isolated cardiomyocytes were prepared, and Na\(^{+}\)-Ca\(^{2+}\) exchanger currents were recorded at room temperature. External solution contained (in mM): NaCl 135, CaCl\(_2\) 2, CsCl 10, MgCl\(_2\) 1, glucose 10, HEPES 10, pH 7.3, and pipette solution contained (in mM): CsCl 135, MgCl\(_2\) 2, NaCl 15, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA; Molecular probes) 0.05, and HEPES 10 (pH 7.2). Ryanodine (0.01 mM) and nifedipine (0.01 mM) were included in the external solution to block SR Ca\(^{2+}\) channels and Ca\(^{2+}\) influx through the L-type channels. The 

Ni\(^{2+}\)-sensitive current was obtained by subtracting current records obtained during exposure to 5 mM Ni\(^{2+}\) from the control record, which was measured in the absence of Ni\(^{2+}\). Recordings were done using an Axopatch 200B patch clamp amplifier (Axon Instruments, Foster City, CA), interfaced to a personal computer. Data were stored in the computer for analysis using custom-written software.
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**Fig. 1.** Ca\(^{2+}\) transient and contractile parameters in isolated ventricular myocytes. A, representative Ca\(^{2+}\) transients in left ventricular myocytes isolated from wild-type and heterozygous hearts. The cells were loaded with Fura-2 AM and paced at 0.25 Hz. The peak amplitude of calcium signal was decreased, but the rate of Ca\(^{2+}\) decline was not significantly altered in heterozygous (HET) myocytes compared with wild-type (WT) controls. B, representative tracings of percentage cell shortening (upper panel), the rate of cell shortening (\(\frac{dL}{dt}\)), and the rate of cell relengthening (\(-\frac{dL}{dt}\)) (lower panel) from cardiomyocytes isolated from wild-type and heterozygous mice.

Statistical Analyses—Results are expressed as mean ± S.E. and statistically evaluated by the ANOVA test followed by the Student t test. \(p < 0.05\) was considered to be the threshold for statistical significance.

**RESULTS**

Cardiomyocytes from SERCA2 Heterozygous Hearts Have Lower Cytosolic Peak Ca\(^{2+}\) Transients—We have recently shown that disruption of one copy of the SERCA2 gene results in decreased SERCA2 mRNA (\(-45\%\)), protein (\(-35\%\)), and SR Ca\(^{2+}\) uptake (\(-35\%\)) and that these changes are associated in vivo with impaired cardiac performance (5).

To determine whether this decrease in SERCA pump level affects myocyte Ca\(^{2+}\) homeostasis on a beat-to-beat basis, cytosolic Ca\(^{2+}\) transients were measured. Left ventricular myocytes isolated from wild-type and heterozygous hearts were loaded with Fura-2 AM, and the Ca\(^{2+}\) signals during electrical pacing at 0.25 Hz were measured. The baseline cytosolic calcium level was not significantly different between wild-type and heterozygous cells. But the peak amplitudes of calcium transients were decreased by \(-30\%–40\%\) in heterozygous myocytes (Fig. 1A, Table I), indicating that the absolute amount of Ca\(^{2+}\) available for myofibrillar contractility was decreased. However, the rate of Ca\(^{2+}\) decline as fitted by exponential decay (\(\tau\)) in heterozygous myocytes was not different from that of wild-type myocytes (Fig. 1A, Table I), suggesting no significant difference in the rate of Ca\(^{2+}\) removal from the cytosol.

SR Ca\(^{2+}\) Load Is Significantly Decreased in SERCA2 Heterozygous Hearts—Because SERCA2 heterozygous myocytes showed a significant decrease in Ca\(^{2+}\) amplitude upon twitch stimulation, we next determined whether the decrease in Ca\(^{2+}\) amplitude was due to a decrease in SR Ca\(^{2+}\) stores or due to a decrease in Ca\(^{2+}\) efflux from the SR. Fura-2 AM-loaded myocytes were exposed to caffeine, and peak Ca\(^{2+}\) transients were measured. We found that amplitudes of the Ca\(^{2+}\) transients in the presence of caffeine were decreased \(-40\%–60\%\) in heterozygous myocytes relative to the transients observed in wild-type cells (Table I). Because caffeine binding opens the RyR, causing Ca\(^{2+}\) release from the SR, the amplitude of the caffeine-induced Ca\(^{2+}\) transient can be used as an index of SR Ca\(^{2+}\) content (23, 24). Our data show that reduced SERCA activity leads to significantly decreased RyR-releasable SR Ca\(^{2+}\) stores in heterozygous myocytes.

Decreased Contractility of SERCA2 Heterozygous Myocytes—To determine whether the observed changes in SR Ca\(^{2+}\) transport affect cardiac contractility, mechanical properties of isolated cardiomyocytes were examined. There was no significant difference in the resting cell length between wild-type and heterozygous myocytes. However, there was \(-30\%\) decrease in the percentage of cell shortening in heterozygous myocytes (Fig. 1B, Table I). In addition, the rate of cell shortening (\(\frac{dL}{dt}\)), as well as the rate of cell relengthening (\(-\frac{dL}{dt}\)) was significantly decreased (\(-40\%\)) in heterozygous myocytes (Fig. 1B, Table I). These data demonstrate that a reduction in SERCA2 results in decreased cytosolic Ca\(^{2+}\) transients and SR Ca\(^{2+}\) load and that these alterations can directly affect contractile function of the individual myocyte.

Expression Levels of Myosin Heavy Chain Isoforms Are Unchanged in SERCA2 Heterozygous Hearts—Alterations in the expression levels of myosin heavy chain isoforms \(\alpha\) and \(\beta\)-MHC can be an important determinant of the dynamic function of the myocardium and are correlated with the maximum velocity of muscle shortening (28, 29). Therefore, it can be argued that the impaired myocyte contraction and relaxation in heterozygous hearts is also due to a switch in myosin heavy chain isoform expression. In addition, induction of \(\beta\)-MHC has been reported to be a marker of cardiac hypertrophy (7), a condition that is also associated with decreased levels of SERCA pump expression (30). In this study, we examined the expression levels of \(\alpha\)-MHC and \(\beta\)-MHC in SERCA2 heterozygous hearts. Our results showed that the expression levels of both isoforms were unchanged (\(\alpha\)-MHC, 107 ± 2\%; \(\beta\)-MHC, 107 ± 1\%) in heterozygous hearts compared with wild-type controls, 100\% (\(n = 4\) of each genotype) (Fig. 2). These results indicate that the impaired contractile function in heterozygous myocyte is not due to a change in myosin heavy chain isoforms. Gravimetric analysis showed similar heart:body-weight ratios between wild-type and heterozygous hearts (4.10 ± 0.05 mg/g, heterozygous; 4.12 ± 0.54 mg/g, wild-type; \(n = 6\) of each genotype). Therefore, there is no apparent indication of cardiac hypertrophy in SERCA2 heterozygous mice.

The Amount of PLB Protein Is Decreased but Its Phosphorylation Status Is Increased in SERCA2 Heterozygous Mice—Despite the decreased level of SERCA2 expression, the apparent rate of Ca\(^{2+}\) decline (\(\tau\)) during Ca\(^{2+}\) transients was unaltered (Fig. 1A). This result suggests that the decrease in SERCA2 pump activity might be compensated by SERCA2/PLB interaction in myocytes of heterozygous mice. Recent studies have shown that both the PLB:SERCA2 protein ratio and the PLB phosphorylation status regulate SERCA pump function (4, 19, 31, 32). To determine whether PLB regulation of SERCA activity is altered in SERCA2 heterozygous hearts, we examined the amount of PLB protein, as well as its phosphorylation status, using quantitative immunoblot analysis. To maintain the relative ratios of both high molecular weight and low molecular weight PLB forms, some samples were analyzed with-
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Table I

| Parameter                      | Wild-type | Heterozygous |
|--------------------------------|-----------|--------------|
| Calcium transient              |           |              |
| Calcium amplitude (340 nm/380 nm, units) | 1.21 ± 0.10 | 0.86 ± 0.08* |
| τ of Ca$^{2+}$ decay (s)       | 0.36 ± 0.05 | 0.37 ± 0.02  |
| SR Ca$^{2+}$ load              |           |              |
| CAC* (340 nm/380 nm, units)    | 2.85 ± 0.38 | 0.95 ± 0.10* |
| Contractile parameters        |           |              |
| Cell length (μm)               | 138.60 ± 8.30 | 131.60 ± 4.87 |
| PC (%)                        | 11.80 ± 1.67  | 8.50 ± 0.78* |
| +dL/dt (μm/s)                 | 194.10 ± 15.35 | 109.40 ± 11.60* |
| −dL/dt (μm/s)                 | 171.10 ± 7.92  | 82.70 ± 9.76* |

*CAC, calcium amplitude in the presence of 10 mM caffeine. PC, percentage of cell shortening.

Fig. 2. Quantitation of α- and β-myosin heavy chain proteins in wild-type and heterozygous hearts. Four individual hearts were used for each group. 3 μg of homogenate protein was subjected to SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and probed with specific antibodies. The signals for α- and β-myosin heavy chain in hearts from wild-type (W) and heterozygous (H) mice were quantitated relative to that of α-sarcinonic actin.

out boiling (which reduces PLB to monomer) prior to gel electrophoresis. As shown in Fig. 3, A and B, PLB protein (both high molecular weight PLB (PLB$^H$) and low molecular weight PLB (PLB$^L$)) levels were significantly decreased in SERCA2 heterozygotes (PLB$^H$, 60 ± 7% ; PLB$^L$, 45 ± 6%; wild-type, 100%; n = 6 of each genotype). Moreover, the relative ratio of PLB$^H$ to PLB$^L$ was increased −1.4-fold (0.99 in wild-type; 1.40 in heterozygous; Fig. 3A), suggesting a shift toward the oligomeric form of PLB in heterozygous hearts.

We recently showed that the PLB mRNA level was similar between wild-type and heterozygous hearts (5). However, our protein analyses show that the PLB$^H$ protein is decreased and its level is comparable to the decreased SERCA2 protein level (−35%) (5). To confirm these findings, we further quantitated PLB$^H$ and SERCA2 protein levels and determined the PLB$^H$: SERCA2 protein ratio. In comparison to wild-type hearts (relative ratio of PLB$^H$ to SERCA2 was set as 1.0), the PLB$^H$ to SERCA2 ratio was 1.1 ± 0.1 in heterozygous hearts (n = 6 of each genotype, p > 0.05), indicating no significant change in heterozygous hearts. Thus, our data suggest that the SERCA2 protein level may affect the steady-state level of PLB protein to maintain an optimal PLB:SERCA2 ratio in SERCA2 heterozygous mice.

Interactions between PLB and SERCA2 are also regulated by the phosphorylation status of PLB. Phosphorylation of PLB occurs at two sites: serine 16 (by cAMP-dependent protein kinase) and threonine 17 (by Ca$^{2+}$/calmodulin-dependent protein kinase, CaM kinase II) (33, 34). To determine whether the phosphorylation status of PLB is altered subsequently to decreased SERCA pump level, we analyzed PLB phosphorylation in vivo under basal conditions using phospho-specific PLB antibodies and Western blot analysis. There is increased basal PLB$^H$ phosphorylation at Ser-16 (−200%) and Thr-17 (210%) residues in SERCA2 heterozygous hearts compared with wild-type hearts (set to 100%, for wild-type, n = 6 of each genotype, Fig. 3A). These data were further confirmed by analyzing boiled samples, which reduces PLB to the monomeric form, PLB$^L$ (Fig. 3B). When normalized to the decreased PLB$^H$ protein level, phosphorylation at PLB$^H$ Ser-16 and Thr-17 sites was increased −3.3- and −3.5-fold in heterozygous hearts, respectively. In addition, the ratio of both Ser-16 and Thr-17 phosphorylation in the pentameric form PLB$^H$ to the monomeric form PLB$^L$ was increased −1.4- and −1.3-fold, respectively, in heterozygous mice (Fig. 3A). These results suggest that decreased SERCA2 pump activity results in multiple compensatory changes in PLB, including decreased PLB protein level, increased expression of the oligomer form of the protein, and increased basal PLB phosphorylation.

The Na$^+$-Ca$^{2+}$ Exchanger Protein Expression and Function Are Up-regulated in SERCA2 Heterozygous Hearts—SR Ca$^{2+}$ uptake is thought to be the dominant mechanism responsible for the rapid decrease in cytosolic free Ca$^{2+}$ (35, 36). However, Ca$^{2+}$ can also be extruded from the cell via the sarcolemmal Na$^+$-Ca$^{2+}$ exchanger (NCX) (6, 35). Therefore, to determine whether a decreased amount of SERCA2 resulted in compensatory alterations in NCX function, we measured NCX expression levels and determined currents by patch-clamp analysis.

RNase protection analysis showed that the level of NCX mRNA in heterozygous hearts (0.50 ± 0.02%) was not significantly different from that of wild-type hearts (0.49 ± 0.02%, n = 6, p > 0.05; Fig. 4A). However, quantitative immunoblot analyses showed that NCX protein expression was increased by −38% in heterozygous mice (138 ± 9% versus 100% in wild-type mice; Fig. 4B), suggesting post-transcriptional regulation of NCX protein.

Patch-clamp analysis was used to analyze NCX current in heterozygous myocytes. Ni$^{2+}$-sensitive current (which represents the NCX current) was determined by subtracting control current records from those obtained during exposure to 5 mM Ni$^{2+}$. As shown in Fig. 5 (A and B), the sustained outward current was reduced in wild-type and heterozygous myocytes that were exposed to Ni$^{2+}$, suggesting a decreased NCX current. Furthermore, the Ni$^{2+}$-sensitive current was significantly reduced in wild-type and heterozygous myocytes exposed to Ni$^{2+}$, indicating a decrease in NCX function. This decrease in NCX function may contribute to the maintenance of Ca$^{2+}$ homeostasis in SERCA2 heterozygous hearts.
current was increased by \( \sim 40\% \) (0.65 \( \pm \) 0.07 mV in wild-type versus 0.91 \( \pm \) 0.11 mV in heterozygous, \( p < 0.05 \)), and the inward tail current was increased by \( \sim 43\% \) (0.60 \( \pm \) 0.07 mV versus 0.86 \( \pm \) 0.10 mV, \( p < 0.05 \)). These data confirm that increased NCX protein levels result in increased functional NCX activity, both in forward and reverse current direction.

**SERCA2 Gene Ablation Differentially Alters the Expression**
Fig. 5. Na⁺-Ca²⁺ exchanger currents in wild-type and heterozygous mice. A, representative tracings of Na⁺-Ca²⁺ exchange currents from myocytes during 1.5-s depolarizing pulses from a holding potential of −60 mV to +80 mV. The Na⁺-sensitive currents (c) were obtained by subtracting current records obtained during exposure to 5 mM Ni²⁺ (b) from the control record (a). B, Na⁺-Ca²⁺ exchanger current density (normalized to the cell capacitance) shows a significant increase in the sustained outward current and peak tail current density of the Ni²⁺-sensitive current from six heterozygous (HET) mice as compared with five wild-type (WT) mice. One to three cells were selected from each heart, “n” in parentheses represents the numbers of cells in wild-type and heterozygous hearts. Data represent mean ± S.E. *, p < 0.05.

DISCUSSION

SERCA Pump Level Is a Critical Determinant of Both Relaxation and Contraction of Cardiomyocytes—A major goal of this study was to investigate whether a decrease in SERCA pump level is sufficient to alter intracellular Ca²⁺ homeostasis (SR Ca²⁺ stores, Ca²⁺ release, and Ca²⁺ removal) and cardiomyocyte contractility. Our data demonstrate that both the peak amplitudes of the cytosolic Ca²⁺ transients and SR Ca²⁺ stores (as measured by caffeine-induced Ca²⁺ release) were decreased by ∼30–40%, and ∼40–60%, respectively, in myocytes of SERCA2 heterozygous mice. Functional studies at the myocyte level (current study), as well as in intact heart in vivo (5) revealed that not only the rate of relaxation is decreased but also the rate of contraction. Importantly, these changes occur in the absence of a switch from α-MHC to β-MHC (Fig. 2).

These data, taken together, suggest that a decreased SERCA pump leads to decreased SR Ca²⁺ uptake function, thereby resulting in decreased SR Ca²⁺ store and Ca²⁺ release. This decreased SR Ca²⁺ release, hence, limits the Ca²⁺ availability for contractile protein activation. We have recently shown that, in SERCA-overexpressing mice, an increase in SERCA pump leads to enhanced rates of contraction and relaxation (1–3). These results demonstrate that the SERCA pump is a critical regulator of both contraction and relaxation of cardiomyocytes.

SERCA/PLB Interaction Is Modified to Compensate for Decreases in SERCA Pump Level and Activity—In this study, we found that the rate of Ca²⁺ decline (τ) in heterozygous myocytes was not different from that of wild-type myocytes (Fig. 1A, Table I), suggesting that the Ca²⁺ removal function is compensated in SERCA2 heterozygous hearts. A novel finding of this study is that a decrease in SERCA activity is partially compensated by adjusting PLB expression level. Using Western blot analysis, we showed that PLBα protein levels were decreased by ∼40% in SERCA2 heterozygous hearts, which is comparable to that of the decrease in SERCA pump levels (−35%) (5). Therefore, the PLB:SERCA2 ratio, which has been shown to be one of the main determinants of the affinity of SR Ca²⁺ release and Ca²⁺ storage proteins—To determine whether SERCA2 gene ablation affects the expression of SR Ca²⁺ release and storage proteins, mRNA levels of RyR, triadin, and calsequestrin were analyzed by RNase protection. As shown in Fig. 6A, the mRNA levels of RyR, triadin, and calsequestrin were not significantly different between wild-type and heterozygous mice. Quantitation of the levels of RyR and calsequestrin protein showed no significant change in SERCA2 heterozygous mice compared with wild-type mice (Fig. 6B). We also performed radioligand receptor binding studies to measure functional ryanodine receptor levels. Scatchard plot analysis showed the $B_{\text{max}}$ of the RyR binding was decreased by −6% (105.11 ± 1.89 pmol/mg in wild-type versus 111.51 ± 2.06 pmol/mg in heterozygous, n = 6, p = 0.022), whereas the dissociation constant of RyR binding ($K_{d}$) remained unchanged (2.24 ± 0.18 nM, wild-type versus 2.28 ± 0.04 nM, heterozygous; p > 0.05) (Fig. 7).

Quantitative immunoblot analysis showed that the amount of triadin isoform 3 (37, 38) was increased by −40% (140 ± 3%, heterozygous; with wild-type set to 100%) (Fig. 6B). Although ryanodine receptor and calsequestrin levels were not significantly altered, up-regulation of triadin levels suggest a potential role for this protein in SR Ca²⁺ release.
SERCA pumps for Ca\textsuperscript{2+} (4, 31), was not significantly altered in heterozygous mice compared with wild-type mice. Our results from Ca\textsuperscript{2+} uptake measurements provided additional supporting evidence that the apparent pump affinity for Ca\textsuperscript{2+} (K\textsubscript{m}) is unchanged in heterozygous hearts (5). This result may suggest that changes in the SERCA pump level can affect PLB protein level. Although our earlier studies showed that PLB mRNA levels were unchanged in heterozygous hearts (5); here we demonstrate a clear reduction in PLB protein level. Thus, our data indicate that PLB protein level can be regulated post-transcriptionally, possibly by alterations in the rate of protein translation.

In addition to a reduction in total PLB protein level, we found that the ratio of oligomeric PLB (PLB\textsubscript{12}) to monomeric PLB (PLB\textsubscript{1}) increased ~1.4-fold in heterozygous mice. This shift in expression to predominantly more PLB\textsubscript{12} in heterozygous hearts may be another important compensatory alteration, because PLB\textsubscript{12} has been shown to be a more effective inhibitor of the SERCA pump than PLB\textsubscript{1} (39–41). Additionally, equilibrium between these two states may be regulated by PLB phosphorylation status (39) and SERCA pump level (42). A recent study using fluorescence energy transfer to study reconstitution of PLB with purified Ca\textsuperscript{2+}-ATPase provided in vitro evidence that an increased SERCA:PLB protein ratio favors the shift from PLB oligomer to PLB monomer, thereby altering the interaction of PLB to SERCA (42). Our data demonstrate for the first time that decreased SERCA pump level can influence the oligomer:monomer ratio, suggesting that an optimal PLB\textsubscript{12}:SERCA interaction is important for the regulation of SERCA pump activity in vivo.

Another important finding of this study is that the basal phosphorylation status of PLB (in vivo) in SERCA heterozygous hearts is significantly enhanced at both Ser-16 and Thr-17 despite decreased PLB protein level. It has been well established that phosphorylation of PLB relieves the inhibition and accelerates SERCA pump activity (4, 31, 32). Phosphorylation of PLB occurs at both Ser-16 and Thr-17 sites by the action of protein kinase A and CaM kinase II, respectively, in response to \(\beta\)-adrenergic stimulation (4, 43). Therefore, one plausible mechanism for the increased PLB phosphorylation is an increase in basal sympathetic drive in heterozygous mice leading to increased activation of protein kinase A as well as CaM kinase II. Alternatively, SR Ca\textsuperscript{2+} load might itself directly regulate PLB phosphorylation, as suggested by the recent study by Bhogal and Colyer (44). They have shown that depletion of Ca\textsuperscript{2+} from cardiac SR stimulates an SR intrinsic protein kinase to phosphorylate PLB at Ser-16, promoting the refilling of SR Ca\textsuperscript{2+} stores through increased SERCA pump activity.

Taken together, our data suggest that a decrease in PLB protein level, a shift from PLB monomer to oligomer, and an increase in PLB phosphorylation status work concomitantly to enhance SERCA pump activity. As a result, SERCA pump activity is stimulated in heterozygous hearts to partially compensate for the decrease in SERCA pump level and Ca\textsuperscript{2+} uptake function.

The Up-regulation of Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger May Play a Compensatory Role in SERCA2 Heterozygous Hearts—It is known that the sarcolemmal Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger is an important Ca\textsuperscript{2+} extrusion mechanism that contributes to cardiac relaxation (35). Therefore, reduced levels of SERCA2 may increase the dependence of SERCA2 heterozygous myocytes on NCX activity to maintain a normal rate of Ca\textsuperscript{2+} removal. Consistent with this hypothesis, we found increases in the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger protein level, as well as increased exchanger current density in heterozygous hearts, suggesting that it plays a compensatory function in Ca\textsuperscript{2+} handling. Decreased SERCA2 levels have been associated with increased expression of NCX in a number of different studies. For example, during heart development as well as in heart failure, Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger expression levels are up-regulated, whereas SERCA pump levels are down-regulated (45–47). Similar results were observed in a recent study using hypothyroid mice (10). These data, taken together, suggest that the increased Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger expression is a compensatory response to the reduction in SERCA pump level.

The Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger has been shown to transport Ca\textsuperscript{2+} in either direction and, hence, promote Ca\textsuperscript{2+} entry as well as Ca\textsuperscript{2+} extrusion (6). If Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger activity was increased only in the forward mode, which promotes Ca\textsuperscript{2+} extrusion, it might lead to a further decrease in SR Ca\textsuperscript{2+} load. Therefore, we hypothesized that the reverse mode of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger is also up-regulated to maintain the balance between Ca\textsuperscript{2+} extrusion and Ca\textsuperscript{2+} entry. In this study, we found that both forward and reverse mode Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger currents are increased in SERCA2 heterozygous hearts. There is evidence showing that the enhanced Ca\textsuperscript{2+} entry via the reverse mode of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger can trigger SR Ca\textsuperscript{2+} release (48) and may provide inotropic support for the myocytes (49, 50). Our data suggest that an increase in Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger expression/activity in forward mode and reverse mode may stimulate Ca\textsuperscript{2+} extrusion as well as Ca\textsuperscript{2+} entry to maintain intracellular Ca\textsuperscript{2+} homeostasis.

In conclusion, our studies show that a decrease in SERCA pump level can result in alterations in Ca\textsuperscript{2+} homeostasis and a decrease in myocyte contractility. Although several compensatory mechanisms have been suggested to maintain Ca\textsuperscript{2+} homeostasis, our data are consistent with the hypothesis that the decrease in SERCA pump activity is a critical determinant of cardiac contractility.

Acknowledgment—We thank Dr. Evangelia G. Kranias for her suggestions.

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*J. Biol. Chem.* 2000, 275:38073-38080.
doi: 10.1074/jbc.M004804200 originally published online September 1, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M004804200

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