Ca\textsuperscript{2+} Uptake by the Sarcoplasmic Reticulum in Ventricular Myocytes of the SERCA2\textsuperscript{b/b} Mouse Is Impaired at Higher Ca\textsuperscript{2+} Loads Only

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Abstract—SERCA2a is the cardiac-specific isoform of Ca\textsuperscript{2+}-ATPase of the sarcoplasmic reticulum (SR). A reduction of SERCA2a has been implicated in the contractile dysfunction of heart failure, and partial knockout of the SERCA2 gene (Atp2a2\textsuperscript{-/-} mice) reiterated many of the features of heart failure. Yet, mice with a mutation of Atp2a2, resulting in full suppression of the SERCA2a isoform and expression of the SERCA2b isoform only (SERCA2\textsuperscript{b/b}), showed only moderate functional impairment, despite a reduction by 40% of the SERCA2 protein levels. We examined in more detail the Ca\textsuperscript{2+} handling in isolated cardiac myocytes from SERCA2\textsuperscript{b/b}. At 0.25 Hz stimulation, the amplitude of the [Ca\textsuperscript{2+}]j transients, SR Ca\textsuperscript{2+} content, diastolic [Ca\textsuperscript{2+}], and density of \( I_{\text{Ca}} \), were comparable between WT and SERCA2\textsuperscript{b/b}. However, the decline of [Ca\textsuperscript{2+}], was slower (\( t_{\text{1/2}} 154\pm7 \text{ versus } 131\pm5 \text{ ms; } P<0.05 \)). Reducing the amplitude of the [Ca\textsuperscript{2+}]j transient (eg, SR depletion), removed the differences in [Ca\textsuperscript{2+}], decline. In contrast, increasing the Ca\textsuperscript{2+} load revealed pronounced reduction of SR Ca\textsuperscript{2+} uptake at high [Ca\textsuperscript{2+}]j. There was no increase in Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange protein or function. Theoretical modeling indicated that in the SERCA2\textsuperscript{b/b} mouse, the higher Ca\textsuperscript{2+} affinity of SERCA2b partially compensates for the 40% reduction of SERCA expression. The lack of SR depletion in the SERCA2\textsuperscript{b/b} may also be related to the absence of upregulation of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange. We conclude that for SERCA isoforms with increased affinity for Ca\textsuperscript{2+}, a reduced expression level is better tolerated as Ca\textsuperscript{2+} uptake and storage are impaired only at higher Ca\textsuperscript{2+} loads. (Circ Res. 2003;92:881-887.)

Key Words: ventricular myocytes ■ transgenic mice ■ sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase ■ excitation-contraction coupling ■ Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange

The Ca\textsuperscript{2+}-transport ATPase of the sarco- and endoplasmic reticulum (SERCA) is essential for reuptake of Ca\textsuperscript{2+} into the intracellular stores. In cardiac cells, the SERCA2a splice variant maintains and regulates the Ca\textsuperscript{2+} content of the sarcoplasmic reticulum (SR). As Ca\textsuperscript{2+} release is the major source for the transient increase in [Ca\textsuperscript{2+}], during excitation-contraction coupling, the amount of Ca\textsuperscript{2+} available for release, and thus SERCA activity, are important in regulation of cardiac contractility (see review\textsuperscript{1}). SERCA activity is regulated by phospholamban (PLB), which decreases the affinity for Ca\textsuperscript{2+} and thus acts as an inhibitor (see review\textsuperscript{2}). Phosphorylation of PLB by protein kinase A (PKA) during adrenergic stimulation removes this inhibition, enhances SR Ca\textsuperscript{2+} uptake, and contributes to the positive inotropic and lusitropic effect of adrenergic stimulation (see review\textsuperscript{3}). This is accompanied by an increase in SR Ca\textsuperscript{2+} content.\textsuperscript{5} Phosphorylation of PLB, or SERCA itself, by Ca\textsuperscript{2+}/calmodulin kinase has been implicated in the enhanced Ca\textsuperscript{2+} uptake at higher stimulation frequencies.\textsuperscript{6,7}

In human heart failure, a decrease in SERCA function is likely to be important in the slowed relaxation and diastolic dysfunction,\textsuperscript{8-10} although it may not be the only mechanism.\textsuperscript{11} Reduced SERCA function can also contribute to the decreased systolic function, as it would lower SR Ca\textsuperscript{2+} content. Such a decrease in SR Ca\textsuperscript{2+} content was observed in human heart failure,\textsuperscript{12,13} and in some\textsuperscript{14,15} but not all animal models of heart failure.\textsuperscript{16} Enhanced expression of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger may help to maintain diastolic function,\textsuperscript{14,17,18} but may also enhance loss of Ca\textsuperscript{2+} from the cell as observed during overexpression in cultured rabbit cardiomyocytes.\textsuperscript{19} The importance of SERCA in heart failure is supported by experiments where overexpression of the cardiac isoform of SERCA, ie, SERCA2a, could restore systolic and diastolic function.\textsuperscript{20}

Transgenic mouse models have further explored the pivotal role of SERCA2a in excitation-contraction coupling. Overexpression of SERCA2a,\textsuperscript{21,22} or removing the inhibition of PLB on SERCA2a,\textsuperscript{23,24} enhanced cardiac function and increased SR Ca\textsuperscript{2+} content. A knockout of the Atp2a2 gene, encoding SERCA2, was lethal in utero for homozygous mice.\textsuperscript{25} Heterozygous mice, Atp2a2\textsuperscript{+/-}, had a 50% downregulation of SERCA2 protein and reduced in vivo contractility.
Isolated myocytes had smaller \([\text{Ca}^{2+}]_i\), transients and reduced SR \(\text{Ca}^{2+}\) content, but the rate of \([\text{Ca}^{2+}]_i\), decline was unaffected.\(^{26}\) It was postulated that in these mice, the observed upregulation of \(\text{Na}^+\)/\(\text{H}^+\) exchange compensated for the loss of \(\text{Ca}^{2+}\) removal by SERCA.

Ver Heyen et al.\(^{27}\) recently described a gene-targeted mouse with full substitution of the cardiac isoform SERCA2a by SERCA2b (further indicated as \(\text{SERCA2}^{b/b}\)). SERCA2b, the isoform that is found in most cell types and therefore considered to be the housekeeping form, has a nearly 2-fold higher affinity for \(\text{Ca}^{2+}\) but a lower maximal catalytic turnover rate than SERCA2a. Unexpectedly, total cardiac SERCA2 protein levels in the \(\text{SERCA2}^{b/b}\) were found to be decreased by 40% as compared with the WT, whereas phospholamban levels (PLB) were doubled. \(\text{Ca}^{2+}\)-uptake studies showed a decrease of \(V_{\text{max}}\) of 40%, but despite the increase in PLB, the affinity was significantly higher for \(\text{Ca}^{2+}\) with full substitution of the cardiac isoform SERCA2a by SERCA2b (\(K_d\) of 0.19 ± 0.01 \(\mu\)mol/L in SERCA2b versus 0.28 ± 0.02 \(\mu\)mol/L in WT). Adult \(\text{SERCA2}^{b/b}\) mice showed a mild cardiac hypertrophy and a slowed relaxation and contraction in vivo, but no signs of heart failure. Myocytes isolated from \(\text{SERCA2}^{b/b}\) mice presented a slower decline of \([\text{Ca}^{2+}]_i\), compared with WT, although the difference was small. In the present study, we further investigated \(\text{Ca}^{2+}\) handling in \(\text{SERCA2}^{b/b}\) in order to understand the mild phenotype.

### Materials and Methods

#### Cell Isolation

Wild-type mice (WT) were compared with \(\text{SERCA2}^{b/b}\) mice, expressing SERCA2b but no SERCA2a.\(^{27}\) The exclusive expression of SERCA2b, and not SERCA2a, was obtained by mutation of the \(Atp2a2\) gene. The 5’D1 donor splicing site was inactivated, and a strong polyadenylation site was added such that class 1 mRNA encoding SERCA2a, could not be produced. Single ventricular myocytes from hearts of 3- to 4-month-old mice were enzymatically isolated as described in.\(^{26}\) All animal handling was conformed with the Guide for the Care and Use of Laboratory Animals (National Institute of Health, USA), and experimental protocols were approved by the in-house ethical committee. Cells were stored at room temperature and used for experiments within 6 hours after isolation.

#### Measurements of \([\text{Ca}^{2+}]_i\) and Membrane Currents

The setup for combined fluorescence and membrane current measurements was as described before.\(^{29}\) Myocytes were studied under voltage clamp, using the ruptured whole-cell recording technique.\(^{30}\) Membrane currents were filtered at 2 kHz and sampled and digitized at 4 kHz. \([\text{Ca}^{2+}]_i\) was monitored with fluo-3, included in the pipette solution. Fluorescence values were calibrated using the approach of Cheng et al.\(^{31}\) with a \(K_a\) of 600 \(\text{nmol/L}\), and after establishing that resting \([\text{Ca}^{2+}]_i\), was similar for WT and \(\text{SERCA2}^{b/b}\).\(^{27}\) In a number of cells, \(F_{\text{max}}\) was measured at the end of the experiment by repeated depolarizing steps to +120 mV and/or gently pushing down the cell on the bottom of the chamber with the patch pipette. This resulted in a rapid increase of fluorescence and cell contracture.

#### Solutions and Experimental Protocols

To study the general properties of the \([\text{Ca}^{2+}]_i\), transient and the frequency response, the external solution was a normal Tyrode solution (in mmol/L: NaCl 137, KC1 5.4, MgCl2 0.5, CaCl2 1.8, Na-HEPES 11.8, and glucose 10; pH 7.4, temperature 37°C), and the pipette solution was \(\text{K}^+\)-based (in mmol/L: 120 K-aspartate, 20 KC1, 10 K-HEPES, 5 MgATP, 10 NaCl, and 0.05 K5-fluo-3; pH 7.2). Cells were stimulated with 25-ms depolarizing pulses from −70 to +20 mV, mimicking the short action potentials of mouse ventricular myocytes.\(^{32}\)

For all other experiments, \(\text{K}^+\) currents were blocked by replacing \(\text{K}^+\) with \(\text{Cs}^+\). The bath solution contained (in mmol/L) NaCl 130, CaCl2 10, MgCl2 0.5, CaCl2 1.8, Na-HEPES 11, and glucose 10; pH 7.4, temperature 24°C; the pipette solution contained (in mmol/L) Cs-aspartate 120, TEACl 10, Cs-HEPES 10, MgCl2 0.5, MgATP 5, and K5-fluo-3 0.05; pH 7.2.

Cells were stimulated at 0.25 Hz with a depolarizing step of 50 ms from −70 to −40 mV to inactivate \(\text{Na}^+\) current, followed by a 100-ms step from −40 to +20 mV to activate \(I_{\text{CaL}}\). \(I_{\text{CaL}}\) was measured as the inward current 15 ms after repolarization to −70 mV. Currents were normalized to cell membrane capacitance. Half-relaxation time of \([\text{Ca}^{2+}]_i\), transients (\(t_{1/2}\)) was measured from peak to half maximal relaxation.

To assess releasable \(\text{Sr}^{2+}\) content, cells were clamped at −70 mV and abruptly superfused for 8 seconds with 10 mmol/L of caffeine after a train of conditioning pulses at 0.25 Hz to induce stable loading. From the resulting inward \(I_{\text{CaL}}\), the amount of \(\text{Ca}^{2+}\) extruded was calculated and normalized to cell volume using an accessible cell volume fraction of 65%, and a correction factor for \(\text{Ca}^{2+}\) removal by other pathways.\(^{28,33}\)

High cellular \(\text{Ca}^{2+}\) loads were obtained by blocking \(\text{Ca}^{2+}\) extrusion via the Na\(^+\)/\(\text{Ca}^{2+}\) exchanger. After depleting the SR with a fast caffeine (10 mmol/L) application, cells were superfused with Na\(^+\)-free solution (in mmol/L: NMDGCl 120, TEACl 20, HEPES 11, MgCl2 0.5, CaCl2 5.4, and glucose 10; pH 7.4) and repeatedly depolarized (100-ms steps from −70 to 0 mV, at 0.25 Hz) activating \(\text{Ca}^{2+}\) entry via \(I_{\text{CaL}}\).

#### Immunoblot

Protein levels of the Na\(^+\)/\(\text{Ca}^{2+}\) exchanger were determined in total cardiac homogenates by Western blot analysis, using a polyclonal rabbit antibody (\(\alpha\)-8–10, detailed in the expanded Materials and Methods section in the online data supplement available at http://www.circresaha.org).

#### Statistics

For group comparisons, unpaired \(t\) test was used. For frequency-dependent and time-dependent changes, ANOVA for repeated measurements was used with Bonferroni post hoc testing; \(P<0.05\) was considered as significant. Results are shown as mean±SEM.

#### Results

### Slowed Relaxation, but no Decrease in SR \(\text{Ca}^{2+}\) Content

Figure 1A shows a typical example of \([\text{Ca}^{2+}]_i\), transients in a \(\text{SERCA2}^{b/b}\) versus a WT cell, using experimental conditions approaching physiological conditions. The amplitude of the \([\text{Ca}^{2+}]_i\), transient showed a negative frequency dependence for both \(\text{SERCA2}^{b/b}\) and WT and was not significantly different between the groups (Figure 1B). However, the rate of decline of the \([\text{Ca}^{2+}]_i\), transient was slower in \(\text{SERCA2}^{b/b}\) at the lower frequencies (Figure 1C), but not at the higher frequencies where the amplitude of the \([\text{Ca}^{2+}]_i\), transient was smaller.

At lower temperature and with \(\text{K}^+\)-free solutions (see Materials and Methods), the difference in rate of \([\text{Ca}^{2+}]_i\), decline between \(\text{SERCA2}^{b/b}\) and WT remained (Figure 2A). A slower rate of \(\text{Ca}^{2+}\) uptake into the SR could lead to a decreased SR \(\text{Ca}^{2+}\) content. However, as shown in Figure 2B, there was no difference of SR \(\text{Ca}^{2+}\) content. Differences in \(\text{Ca}^{2+}\) influx via \(I_{\text{CaL}}\) could affect SR \(\text{Ca}^{2+}\) loading, but we could not detect a significant difference in peak current densities at +20 mV (4.17±0.73 and 3.73±0.3 pA/pF for
respectively SERCA2\textsuperscript{bh} and WT). Consistent with this absence of differences in $I_{\text{CaL}}$ and SR content, the amplitude of the $[\text{Ca}^{2+}]_{i}$ transient was not significantly different (Figure 2C).

### Function and Expression of the Na\textsuperscript{+}/Ca\textsuperscript{2+} Exchanger

In the $Atp2a2^{+/+}$ mouse studied by Periasamy and coworkers, \textsuperscript{25} Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange was upregulated. We evaluated Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange function in the SERCA2\textsuperscript{bh} mice from the rate of Ca\textsuperscript{2+} removal from the cytosol in the presence of 10 mmol/L caffeine, which prevents sequestration of Ca\textsuperscript{2+} in the SR. Figure 3A shows a typical example of a $[\text{Ca}^{2+}]_{i}$ transient in this condition. The decline of the $[\text{Ca}^{2+}]_{i}$ transient was fitted with a single exponential. The values for $\tau$ are shown in Figure 3B and do not differ between the groups.

Western blot analysis showed that the protein levels of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger were also not different between the groups (Figure 4A). We also measured peak current densities of $I_{\text{NCX}}$, as the inward current on repolarization to $-70$ mV, normalized to cell capacitance (Figure 4B) and to $[\text{Ca}^{2+}]_{i}$ at the time of peak current (Figure 4C), and found no significant differences. These data indicate that in contrast to the findings in the $Atp2a2^{+/+}$ mice, the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is not upregulated in the SERCA2\textsuperscript{bh}.
Ca\(^{2+}\) Removal in Cells Heavily Loaded With Ca\(^{2+}\)

Although there are significant differences in the rate of Ca\(^{2+}\) removal between SERCA2\(^{b/b}\) and WT, at least at the low frequencies of stimulation, these differences are not very pronounced despite the reduction of SERCA protein by 40%.\(^{27}\) We hypothesized that this relatively mild effect was related to the documented higher Ca\(^{2+}\) affinity of SERCA2b compared with SERCA2a.\(^{27}\) Indeed, a leftward shift in the Ca\(^{2+}\)-activation curve of SERCA2b compared with SERCA2a would compensate for decreased levels of SERCA2 at lower [Ca\(^{2+}\)]. However, one can expect a more severe impairment of Ca\(^{2+}\) removal at higher Ca\(^{2+}\) loads (see Ca\(^{2+}\) uptake curve of Figure 3C in Ver Heyen et al\(^{27}\)). We therefore examined the decline of [Ca\(^{2+}\)], transients during Ca\(^{2+}\) loading in Na\(^{-}\)-free solutions with high [Ca\(^{2+}\)], (5.4 mmol/L). A typical example is shown in Figure 5A with superimposed [Ca\(^{2+}\)], transients for a WT and a SERCA2\(^{b/b}\) cell. The amplitude of the [Ca\(^{2+}\)], transients increased with successive pulses (Figure 5B), due to cumulative influx via \(I_{\text{Ca}}\), which was comparable for both cell types (Figure 5C). [Ca\(^{2+}\)], transients at high Ca\(^{2+}\) loads had a very slow initial decline of [Ca\(^{2+}\)], followed by a more rapid phase. We ruled out that dye saturation distorted the time course of the [Ca\(^{2+}\)], transient as \(F_{\text{max}}\), measured immediately after the Na\(^{-}\)-free loading protocol was clearly higher than the fluorescence values obtained during the experiment. As a first approach to compare the differences in Ca\(^{2+}\) removal, we measured the half-time of [Ca\(^{2+}\)], decline from the onset of repolarization for successive pulses. The half-time for the SERCA2\(^{b/b}\) increased well above the half-time of the controls, and the curves diverged as the amplitude of [Ca\(^{2+}\)], increased (Figure 6A, compare to Figure 5C for [Ca\(^{2+}\)]). A plot of the half-time values versus the peak [Ca\(^{2+}\)], for all individual traces confirms that the half-time of SERCA2\(^{b/b}\) is longer in particular for the larger values of [Ca\(^{2+}\)], (Figure 6B). We next measured the actual SR Ca\(^{2+}\) flux during a single large [Ca\(^{2+}\)], transient (see online data supplement). Figure 6C shows the time course for total [Ca\(^{2+}\)] after 12 seconds in Na\(^{-}\)-free solution. The derivative of \(\text{[Ca}^{2+}]_{\text{tot}}\), after subtraction of the sarcolemmal flux, is a direct indicator of the SR Ca\(^{2+}\) flux (Figure 6D). Shortly after repolarization, when [Ca\(^{2+}\)], is still high, the rate of Ca\(^{2+}\) uptake is clearly lower in the SERCA2\(^{b/b}\) (a, corresponding to a free [Ca\(^{2+}\)], of 450 nmol/L), but as [Ca\(^{2+}\)], declines, the rate becomes similar (b, corresponding to a free [Ca\(^{2+}\)], of \(\approx\)200 nmol/L, consistent with the Ca\(^{2+}\)-uptake curve in Ver Heyen et al\(^{27}\)). The somewhat lower SR Ca\(^{2+}\) release in SERCA2\(^{b/b}\) in Figure 6D suggests that at this time SR Ca\(^{2+}\) loading is also compromised. These data are consistent with the hypothesis that Ca\(^{2+}\) reuptake in SERCA2\(^{b/b}\) is more impaired at higher Ca\(^{2+}\) loads.

Model for Ca\(^{2+}\) Removal by SERCA2b Versus SERCA2a

We further explored the effects of substituting the WT SERCA2a by a high-affinity SERCA2b isoform in a theoretical model (detailed in the online data supplement available at http://www.circresaha.org). The model consists of Ca\(^{2+}\) influx into the cytosol via \(I_{\text{Ca}}\), for a fixed duration and an amplitude that decreases linearly with time. Ca\(^{2+}\) release is of fixed duration, and the rate is a linear function of the SR Ca\(^{2+}\) content. Ca\(^{2+}\) uptake by SERCA and Ca\(^{2+}\) extrusion by the Na\(^{-}\)-Ca\(^{2+}\) exchanger and PMCA pumps depends on the respective \(K_{\text{m}}\) and \(V_{\text{max}}\) values. The Hill coefficient is 2 for

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Figure 5. Induction of high Ca\(^{2+}\) loads into the cell. A, Typical examples of \(I_{\text{Ca}}\) and [Ca\(^{2+}\)], transients recorded during the first 5 pulses in a WT and SERCA2\(^{b/b}\) cell (top inset, pulse protocol). Arrows on \(I_{\text{Ca}}\) indicate the increasing rate of inactivation of \(I_{\text{Ca}}\), B, Average amplitude of [Ca\(^{2+}\)], during successive pulses in WT (n=8) and SERCA2\(^{b/b}\) (n=9), D, Ca\(^{2+}\) entry via \(I_{\text{Ca}}\), calculated as the cumulative integral of successive pulses normalized to accessible cell volume.

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Figure 6. Ca\(^{2+}\) removal at high Ca\(^{2+}\) loads. A, Pooled data of half-time of [Ca\(^{2+}\)], transients during successive depolarizations in Na\(^{-}\)-free solutions in WT (n=8, ■) and SERCA2\(^{b/b}\) (n=9, ◇). *P<0.05 for SERCA2\(^{b/b}\) vs WT. B, Half-time values vs peak [Ca\(^{2+}\)], for all individual traces (●, WT; ○, SERCA2\(^{b/b}\)). C, Time course of total [Ca\(^{2+}\)] for the pulse at 12 seconds in Na\(^{-}\)-free solution (black trace is average of 8 WT, and red trace of 9 SERCA2\(^{b/b}\) cells). C, Net SR Ca\(^{2+}\) flux (color code as in B). See text for a and b.
SERCA and 1 for Na\(^+-\)Ca\(^{2+}\) exchanger and PMCA. [Na\(^+\)] is assumed to be constant.

Figure 7A shows superimposed simulated [Ca\(^{2+}\)] transients during steady-state stimulation at 1 Hz for various properties of SERCA. Without further compensatory mechanisms, a slight increase of the affinity of the SERCA pump for Ca\(^{2+}\) (decrease of K\(_s\) from 0.28 to 0.25 \(\mu\)mol/L, dotted line) results in a dramatic increase of the Ca\(^{2+}\) signal. The peak amplitude can be restored to that of the WT signal by decreasing the V\(_{\text{max}}\) of the Ca\(^{2+}\) pump from 1000 to 640 (\(\mu\)mol/L \(\cdot\) s\(^{-1}\)) (dashed line). This simultaneously leads to a small but significant slowing of the decline of [Ca\(^{2+}\)]. The steady-state SR Ca\(^{2+}\) content was practically unchanged (−0.7%). A similar effect on the amplitude could be obtained by reducing the Ca\(^{2+}\) influx from 9 to 5.7 \(\mu\)mol/L, but this accelerated the decline of [Ca\(^{2+}\)]. (data not shown). Thus, the first approach fits better with the experimentally observed Ca\(^{2+}\) transients in SERCA\(^{2b}\) mice and is also in agreement with the observed decrease of the number of pump sites.

Figure 7B simulates the experiments of Figure 5 by reducing the capacity of the Na\(^--\)Ca\(^{2+}\) exchanger by 90% and setting the initial values for SR content low. It can be seen that at the low Ca\(^{2+}\) load, the decline of [Ca\(^{2+}\)], for SERCA\(^{2b}\) is not markedly different, but that Ca\(^{2+}\) removal is most impaired at the higher [Ca\(^{2+}\)] transient amplitudes. For the high values of SR content, the actual onset of decline of [Ca\(^{2+}\)], in the SERCA\(^{2b}\) is also delayed, as in Figure 5.

We also examined the effects of a SERCA2-isomorph switch in the more elaborated guinea-pig myocyte model, developed by Rudy et al.\(^{35,36}\) Similar results were obtained (data not shown). Reduction of the K\(_s\) increased the amplitude of the [Ca\(^{2+}\)] transient, and simultaneous reduction of the V\(_{\text{max}}\) by 40% brought the amplitude back to control levels, although with slowed relaxation, as experimentally observed. In neither of the models, Na\(^--\)Ca\(^{2+}\) exchange function had to be altered to reproduce the experimentally observed results.

**Discussion**

We have investigated Ca\(^{2+}\) handling in SERCA\(^{2b}\) mice, which express the SERCA2b isoform in the heart instead of the SERCA2a isoform. These mice have a reduction of total cardiac SERCA protein of 40%.\(^{27}\) In SR vesicles, maximal Ca\(^{2+}\)-uptake rate is reduced, but Ca\(^{2+}\) affinity is increased.\(^{27}\) In single ventricular myocytes of SERCA\(^{2b}\) mice, [Ca\(^{2+}\)] decline is slower, but SR Ca\(^{2+}\) content and amplitude of [Ca\(^{2+}\)], transients are not significantly different. There is no increase in Na\(^--\)Ca\(^{2+}\) exchange protein or function. However, at high Ca\(^{2+}\) load, Ca\(^{2+}\) removal by the SR in myocytes of SERCA\(^{2b}\) is severely compromised. Simulations of [Ca\(^{2+}\)] transients can reproduce these observations by incorporating into the model (1) the specific properties of SERCA2b, (2) the downregulation of the total number of pumps, and (3) the absence of upregulation of Na\(^--\)Ca\(^{2+}\) exchange.

**Phenotype of the SERCA\(^{2b}\) Heart: Why Cellular Function Is Not More Impaired**

In the present concept of cellular mechanisms of heart failure, a decrease in SERCA protein is a pivotal event, responsible for slowing of relaxation, decreased SR content, and smaller [Ca\(^{2+}\)], transients. Although the total amount of SERCA2 protein in the SERCA\(^{2b}\) is decreased by 40%, the slowing of decline of [Ca\(^{2+}\)], in baseline conditions is not very pronounced. In the SERCA\(^{2b}\), we propose that the nearly 2-fold increase in affinity of the SERCA2b isoform, which makes up all of the SERCA2 protein in the mouse ventricle, compensates for the reduction of total pump capacity at lower Ca\(^{2+}\) loads. Consistent with this postulate, Ca\(^{2+}\) removal is not impaired for small [Ca\(^{2+}\)], transients, as seen at the higher stimulation frequency (Figure 1), or after depletion of the SR following a caffeine application (Figure 6A, onset of stimulation in Na\(^--\)-free). Conversely, Ca\(^{2+}\) removal at the higher Ca\(^{2+}\) loads is more significantly reduced (Figures 6B through 6D). This effect of the increased affinity could be reproduced during model simulations, supporting the hypothesis.

A comparison with the heterozygous SERCA KO mouse, Atp2a2\(^{+/−}\), yields some interesting perspectives. In this latter model, the total SERCA protein is reduced to similar levels as in our SERCA\(^{2b}\), but cardiac myocytes from Atp2a2\(^{+/−}\) did not show slowing of the decline of [Ca\(^{2+}\)]. This could be explained by a compensatory upregulation of Na\(^--\)Ca\(^{2+}\) exchange.\(^{26}\) In this same Atp2a2\(^{+/−}\) model, SR Ca\(^{2+}\) content was reduced, and the amplitude of [Ca\(^{2+}\)], transients was smaller. It is tempting to speculate that this is due to the concomitant upregulation of Na\(^--\)Ca\(^{2+}\) exchange, which will favor Ca\(^{2+}\) extrusion. Such upregulation is absent in the SERCA\(^{2b}\), and we do not see a decrease in SR content, suggesting that the lack of upregulation of Na\(^--\)Ca\(^{2+}\) exchange may protect the cells from Ca\(^{2+}\) loss. It is also
important to note that in the transgenic mouse with overexpression of Na⁺-Ca²⁺ exchanger, there was no reduction of SR Ca²⁺ content. This suggests that in the Atp2a2⁻/⁻, it is the combination with the reduced SERCA, and not Na⁺-Ca²⁺ exchange alone, which leads to increased loss of Ca²⁺. In contrast, in cultured rabbit ventricular myocytes, overexpression of Na⁺-Ca²⁺ exchange could by itself reduce SR Ca²⁺ content and amplitude of [Ca²⁺], transients. Differences in [Na⁺], between mouse and rabbit, and/or alterations in SERCA with culture, could explain these differences.

The contractile function in vivo of the SERCA2b⁻/⁻ mice is more impaired than might be expected from the properties of the isolated myocytes reported here. Several factors could explain this apparent discrepancy. The presence of adrenergic stimulation in vivo will increase the Ca²⁺ load and amplitude of the [Ca²⁺] transients, with consequently more impaired relaxation and potentially reduced SR Ca²⁺ content (Figure 6D). In addition, the presence of hypertrophy will further impair ventricular relaxation and filling. This hypertrophy is currently unexplained, but may be a consequence of the slower Ca²⁺ removal.

**Dissociation Between Downregulation of SERCA and Upregulation of Na⁺-Ca²⁺ Exchange**

It has been proposed that in heart failure there is a reexpression of the fetal gene pattern, which includes a lower level of SERCA and upregulation of Na⁺-Ca²⁺ exchange. A review of available literature data however indicates that upregulation of Na⁺-Ca²⁺ exchange is not a consistent finding, although the data on SERCA downregulation are more consistent. The present observations again show that low expression levels of SERCA are not necessarily associated with increased expression of Na⁺-Ca²⁺ exchange, even in the presence of hypertrophy. This indicates that regulation of expression of these Ca²⁺-handling proteins can be controlled by independent signaling pathways, at least in hypertrophy, and is consistent with the recently demonstrated complexity of the signaling in hypertrophy. This does not exclude that certain stimuli may affect the Na⁺-Ca²⁺ exchanger and SERCA in a concerted way, as was demonstrated for the thyroid hormone.

We can as yet not explain why the total SERCA protein is decreased in the SERCA2b⁻/⁻ mouse. This may, at least partially, result from a lower stability of the SERCA2b mRNA versus the SERCA2a mRNA. A provocative hypothesis is that this downregulation could actually be a protective mechanism. As the modeling results show, an increase in SERCA Ca²⁺ affinity without concomitant downregulation of protein expression would lead to an increase in the [Ca²⁺] transients and the SR Ca²⁺ content.

Our findings underline the importance of the SERCA Ca²⁺ affinity in regulating the SR Ca²⁺ uptake. With a higher affinity, a lower number of pumps can maintain Ca²⁺ homeostasis, at least at low to moderate Ca²⁺ loads. This predicts that for eg. SERCA1, modest expression levels may be sufficient. The feasibility of introducing SERCA1, as well as the importance of controlling expression levels were recently reported.

**Conclusions**

Our findings in the SERCA2b mouse illustrate that a reduced expression level of SERCA isoforms with increased affinity for Ca²⁺ is better tolerated than for SERCA2a as Ca²⁺ uptake and storage are impaired only at higher Ca²⁺ loads. The lack of upregulation of Na⁺-Ca²⁺ exchange may help to minimize Ca²⁺ loss, and indicates that expression levels of SERCA and of Na⁺-Ca²⁺ exchange are independently regulated. Expression of higher-affinity SERCA isoforms may have strategic advantages as efficient Ca²⁺ removal can be obtained at lower expression levels.

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Ca\textsuperscript{2+} Uptake by the Sarcoplasmic Reticulum in Ventricular Myocytes of the \textit{SERCA2\textsuperscript{b/b}} Mouse Is Impaired at Higher Ca\textsuperscript{2+} Loads Only

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METHODS

Immunoblotting
Protein levels were determined in tissue homogenates by Western-blot analysis, essentially as described previously\(^1\). Tissue isolated from adult mice were homogenized in 10 mmol/L imidazole (pH 7), 300 mmol/L sucrose, 1 mmol/L dithiotreitol, 1 mmol/L sodiummetabisulfite, 0.3 mmol/L phenylmethylsulfonyl fluoride, 5 \(\mu\)g/mL leupeptin, 5 \(\mu\)g/mL aprotinin, 5 \(\mu\)g/mL pepstatin A. Equal amounts of homogenates were separated on 3-12% Tris-glycine gradient gel, and blotted onto PVDF membranes (Immobilon-P, Millipore). Membranes were probed with a polyclonal rabbit antibody (\(\pi\)8-10) against the Na/Ca exchanger. Detection was performed using a secondary anti-rabbit IgG antibody coupled to alkaline phosphatase, and using Vistra ECF substrate (Amersham Pharmacia Biotech). The fluorescent signals were quantified on the Storm840 FluorImager with ImageQuant Software (Molecular Dynamics). The linearity of the signal was tested by loading increasing concentrations of protein.

Calculation of total [Ca\(^{2+}\)] and SR Ca\(^{2+}\) flux
We used the approach as described in\(^5\). During a caffeine application, the Na/Ca exchange current was used to calculate total [Ca\(^{2+}\)]. This curve of total [Ca\(^{2+}\)] can then be plotted as a function of the free [Ca\(^{2+}\)], to obtain the buffering capacity of the cell. Data of 6 WT cells and 6 SERCA2\(^{b/b}\) cells were pooled and the plot of the mean data was fitted with the function

\[
[Ca]_{tot} = a + (B_{max} [Ca^{2+}]_i / (K_d + [Ca^{2+}]_i))
\]

The values for \(B_{max}\) and \(K_d\) were comparable for WT and SERCA2\(^{b/b}\); \(B_{max}\) was 195 \(\mu\)mol/L for WT and 198 \(\mu\)mol/L for SERCA2\(^{b/b}\) and \(K_d\) was 0.59 \(\mu\)mol/L for WT and 0.58 \(\mu\)mol/L for SERCA2\(^{b/b}\). Using these data we calculated the [Ca\(^{2+}\)]\(_{tot}\) for the [Ca\(^{2+}\)]\(_i\) transients at 12 s in Na\(^+\)-free solutions (Fig. 6C). The derivative of this [Ca\(^{2+}\)]\(_{tot}\) curve then reflects the sum of the SR Ca\(^{2+}\) flux and the sarcolemmal flux. Since we are in Na\(^+\)-free solutions, the only relevant sarcolemmal component on this time scale is \(I_{Ca\_L}\) (see \(^3\)). After conversion of \(I_{Ca\_L}\) to Ca\(^{2+}\) flux in \(\mu\)mol/L accessible cell volume/s, this component can be subtracted to yield the net SR flux (Fig. 6D). To reduce the noise on the derivative, the [Ca\(^{2+}\)]\(_{tot}\) record was low-pass filtered with a cut-off frequency of 50 Hz for the rising phase, and 20 Hz for the declining phase. The filtered signals are superimposed on the original records in Fig. 6C.
Modeling of Ca$^{2+}$ handling

In order to find out whether the observed similarities and differences in the Ca$^{2+}$ transients between wild-type and SERCA2$^{b/b}$ mice can be explained solely by the measured differences in the expression level and in the $K_m$ for Ca$^{2+}$ of the respective Ca$^{2+}$ pumps, computer simulations of Ca$^{2+}$ transients were carried out. Computer simulations were programmed in Delphi$^\text{TM}$. The differential equations were solved using the Runge-Kutta 4th-order algorithm, with a fixed step size of 0.1 ms. The essential features of the model and the parameter values are given below.

**Ca$^{2+}$ fluxes**

To simulate the effect of the $V_{\text{max}}$ and $K_m$ values of the SERCA pump on the [Ca$^{2+}$]$_i$ transients, the computer model was kept as simple as possible while preserving the most essential features of the different Ca$^{2+}$ fluxes involved. The Ca$^{2+}$ flux components of the model are influx, efflux, uptake into the SR by the SERCA Ca$^{2+}$ pump, and Ca$^{2+}$ release. The Ca$^{2+}$ buffering components are low-affinity Ca$^{2+}$ binding proteins in the lumen of the SR, and both low- and high-affinity buffers in the cytosol.

**Ca$^{2+}$ influx.** The time course of Ca$^{2+}$ influx is approximated by a rectangular triangle. Ca$^{2+}$ influx is maximal at the time of stimulation and then decreases linearly with time till zero at time StopInflux, typically 100 ms. The total amplitude of the influx (the surface of the triangle) and the time point StopInflux can be varied independently. In steady-state conditions total influx is set at approximately 10% of the amount of Ca$^{2+}$ released from the SR$^2$.$^3$.

**Ca$^{2+}$ efflux.** Because Ca$^{2+}$ efflux via NCX does not occur during the initial phase of the action potential, Ca$^{2+}$ efflux was set at zero from the onset of the stimulus till the time point StartEfflux, typically 50 ms. After StartEfflux, Ca$^{2+}$ efflux via NCX depends on [Ca$^{2+}$]$_i$, according to the simple relation $\text{ncx}V_{\text{max}}/(1 + \text{ncx}K_m/[\text{Ca}^{2+}]_i)$ if [Ca$^{2+}$]$_i$ > 60 nmol/L. Otherwise, Ca$^{2+}$ efflux occurs only via the PMCA Ca$^{2+}$ extrusion pump according to $\text{pmca}V_{\text{max}}/(1 + \text{pmca}K_m/[\text{Ca}^{2+}]_i)$. The membrane potential was not taken into account.

**SR Ca$^{2+}$ uptake.** The SERCA Ca$^{2+}$ pump is continuously active. The rate of pumping is given by $\text{V} = \text{serca}V_{\text{max}}/(1 + (\text{serca}K_m/[\text{Ca}^{2+}]_i)^2)$. The Ca$^{2+}$ pump is inhibited by lumenal Ca$^{2+}$ with half-maximal inhibition at 200 µmol/L free Ca$^{2+}$.

**SR Ca$^{2+}$ efflux.** Ca$^{2+}$ release from the SR is a linear function of the free Ca$^{2+}$ concentration in the lumen. The stimulated efflux starts at the beginning of the stimulation. Both the rate constant and the duration of the efflux can be varied. After this period, a much smaller rate constant determines the leak rate.
**Ca\textsuperscript{2+} buffering.** Ca\textsuperscript{2+} binding in the lumen of the SR occurs at one class of low-affinity sites (capacity SRB\textsubscript{max}, dissociation constant SRK\textsubscript{d}). Ca\textsuperscript{2+} binding was considered instantaneous. Therefore, \([Ca\textsuperscript{2+}]_{SR}\) was calculated from the total Ca\textsuperscript{2+} concentration and from the binding parameters. Ca\textsuperscript{2+} binding in the cytosol occurs at two classes of binding sites, one of high affinity (Cyt HK\textsubscript{d}, typically 0.42 µmol/L) and one of low affinity (Cyt LK\textsubscript{d}, typically 79 µmol/L).\textsuperscript{4} The rate of Ca\textsuperscript{2+} binding to (Hk\textsubscript{on} and Lk\textsubscript{on}) and release from these sites was included in the equation for calculating \([Ca\textsuperscript{2+}]_{i}\). To improve the resemblance to measurements with Ca\textsuperscript{2+} indicators, the \([Ca\textsuperscript{2+}]_{i}\) values plotted in the figures were calculated from the simulated concentrations of bound and free high-affinity sites at any instant, assuming equilibrium binding.

**Parameters**

The following parameter values were used for the steady-state simulation of Fig. 7A. If other values are used, they are indicated in the respective Figure legends. In order to get an idea of the robustness of the outcome of the simulations, simulations were carried out with various combinations of different parameter values. Results qualitatively very similar to those shown in the figures were obtained with various combinations of Ca\textsuperscript{2+} flux and/or binding characteristics. However, the eventual numbers used, and shown below, correspond to a large extent to published values.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Ca\textsuperscript{2+} influx/beat</td>
<td>9 µmol/L</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+} efflux NCX V\textsubscript{max}</td>
<td>55 (µmol/L).s\textsuperscript{-1} if ([Ca\textsuperscript{2+}]_{i} &gt; 60) nmol/L, otherwise 0.</td>
</tr>
<tr>
<td>NCX K\textsubscript{m}</td>
<td>0.316 µmol/L</td>
</tr>
<tr>
<td>PMCA V\textsubscript{max}</td>
<td>1.4 (µmol/L).s\textsuperscript{-1}</td>
</tr>
<tr>
<td>PMCA K\textsubscript{m}</td>
<td>0.158 µmol/L</td>
</tr>
<tr>
<td>SR Ca\textsuperscript{2+} pump V\textsubscript{max}</td>
<td>1000 (WT) or 640 (SERCA2\textsuperscript{b/b}) (µmol/L).s\textsuperscript{-1}</td>
</tr>
<tr>
<td>K\textsubscript{m}</td>
<td>0.28 (WT) or 0.19 (SERCA2\textsuperscript{b/b}) µmol/L</td>
</tr>
<tr>
<td>K\textsubscript{i} (lumen)</td>
<td>200 µmol/L</td>
</tr>
<tr>
<td>SR release k\textsubscript{r}</td>
<td>700 s\textsuperscript{-1}</td>
</tr>
<tr>
<td>SR leak k\textsubscript{l}</td>
<td>0.1 s\textsuperscript{-1}</td>
</tr>
<tr>
<td>SR volume</td>
<td>3% of cell volume</td>
</tr>
<tr>
<td>Buffers SR B\textsubscript{max}</td>
<td>500 µmol /L cell water</td>
</tr>
<tr>
<td>SR K\textsubscript{d}</td>
<td>120 µmol/L</td>
</tr>
<tr>
<td>Cyt HK\textsubscript{d}</td>
<td>0.42 µmol/L</td>
</tr>
</tbody>
</table>
Cyt HB$_{\text{max}}$ 215 µmol/L
Hk$_{\text{on}}$ 8 x 10$^7$ (mol/L)$^{-1}$.s$^{-1}$
Cyt LK$_{\text{d}}$ 79 µmol/L
Cyt LB$_{\text{max}}$ 400 µmol/L
LK$_{\text{on}}$ 2 x 10$^7$ (mol/L)$^{-1}$.s$^{-1}$

REFERENCES