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ORIGINAL CONTRIBUTION

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Frequency-dependent changes in calcium cycling and contractile activation in SERCA2a transgenic mice

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Abstract *Objective:* This study was undertaken to investigate the mechanism of altered contractility in hearts from transgenic mice overexpressing the sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA2a). In particular, we sought to determine whether the reported increase in contractility is frequency-dependent, as might be expected if attributable to changes in SR Ca^{2+} loading. *Methods:* Intracellular [Ca^{2+}] and contractile force were measured at room temperature (22 °C) simultaneously in fura-2-loaded isometrically-contracting trabeculae dissected from the hearts of FVB/N control (n = 6) or SERCA2a transgenic (n = 6) mice. *Results:* SERCA transgenics exhibit a positive force-frequency relationship, but this was flat in age- and strain-matched controls. SERCA transgenics exhibit a sizable increase in calcium transient amplitude relative to controls, with a concomitant increase in force generation at higher frequencies of stimulation. Amplitudes of Ca^{2+} transients (transgenics: 1.56 ± 0.09 μmol/L, controls: 1.21 ± 0.14) and twitches (transgenics: 21.71 ± 0.91 mN/mm^2, controls: 13.74 ± 1.67) were significantly different at 2.0 Hz stimulation ($P < 0.05$). *Conclusion:* An increase in SERCA expression increases the ability of the sarcoplasmic reticulum to store calcium, such that more calcium is available to be released during each heartbeat at higher stimulation rates.

Key words Calcium – Ca-pump – contractile function – e-c coupling – SR

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Introduction

The sarcoplasmic reticulum (SR) plays a central role in cardiac contraction and relaxation by regulating intracellular calcium (Ca^{2+}) concentration (29). Muscle contraction is initiated by Ca^{2+} release from the SR triggered by Ca^{2+} influx through sarcolemmal Ca^{2+} channels, whereas Ca^{2+} sequestration into the lumen of the SR results in muscle relaxation (44). Calcium uptake by the SR is mediated by an ATP-dependent Ca^{2+} pump, the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) (30).

The expression of SERCA2a is altered in various pathophysiological conditions (2, 21, 24, 25, 31, 38, 40, 46). However, it is still controversial, whether decreased protein expression of SERCA2a or alterations in the phosphorylation state of phospholamban is responsible for the reduced SERCA2a function in failing human myocardium. Nevertheless, decreased Ca^{2+} uptake function of SERCA has been proposed to contribute to the abnormally flat or negative frequency dependence of contractility in end-stage heart failure (1, 8, 9, 14, 17, 18, 25, 27, 32, 34, 36, 37, 42, 43, 45, 48). The prevailing concept holds that the deficiency of calcium reuptake becomes most apparent at higher frequencies; the inability of the SR to sequester and release sufficient calcium undermines the increase in force that is normally observed at higher frequencies (i.e., the positive force-frequency relation).

An increase in SERCA expression might logically be predicted to increase the ability of the SR to store calcium, such that more calcium is available to be released during each heartbeat. Indeed, adenovirus-mediated gene transfer of SERCA into fetal/neonatal myocytes can increase Ca^{2+} transport function and enhance contractility (13, 15, 16, 22, 23). To examine the role of the SR calcium pump in vivo, transgenic mouse models have been made in which cardiac-type SERCA (SERCA2a) is overexpressed (5, 20, 28), or skeletal muscle type SERCA (SERCA1a) is co-expressed (28), in the heart. In the study of He et al., SERCA2a was overexpressed using the chicken β-actin promoter, which is not tissue-specific. To achieve cardiac-specific expression, Baker et al. and Loukianov et al. generated SERCA transgenics using the mouse α-myosin heavy chain (α-MHC) promoter. The SERCA2a transgenics exhibited high-level expression of SERCA2a mRNA resulting in an increase in the total amount of SERCA pumps specifically in the heart. Functional analysis of isolated perfused working hearts over a limited frequency range revealed that SERCA2a transgenic hearts are hyperdynamic, exhibiting increased systolic intraventricular pressure, +dP/dt and -dP/dt. Similar findings were reported in SERCA1a transgenics (28). Muscles from SERCA2a transgenic hearts exhibited a sizable increase in Ca^{2+} transient amplitude and contractility (5), but such measurements were limited to a single frequency of stimulation in 2 mM extracellular calcium. Other functional studies have been limited by reliance on surrogate parameters of force (e.g., edge position in isolated myocytes), and also suffer from having been performed over a narrow range of frequencies of stimulation and extracellular calcium concentrations (5, 15, 16, 20, 22, 28, 33). We sought to perform a more thorough investigation of frequency- and calcium-dependence to test the prediction that the changes in contractility will be most prominent at high workloads. To address this question we used trabeculae from SERCA2a transgenic mice, in which we measured intracellular [Ca^{2+}] and contractile force simultaneously over a broad range of frequencies and extracellular calcium concentrations.

Methods

SERCA2a transgenic mice

SERCA2a transgenic mice were generated by the Transgenic Core Facility, University of Cincinnati as described before (5), and shipped to the Johns Hopkins University for physiological studies. Protein analysis by Western blotting of the mouse lines used in this study revealed that total SERCA2a protein was increased by 30 % in SERCA2a transgenics. All animal care and procedures were approved by the Johns Hopkins University Animal Care and Use Committee, and the University of Cincinnati Institutional Animal Care and Use Committee. The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Mouse muscle preparations

Mouse muscle preparations were made as described previously (11, 12). Adult mice of either sex (FVB/N strain, 12–22 weeks, 25–35 g) were anesthetized by intra-abdominal injection of sodium pentobarbital (~10–20 mg), and the hearts were rapidly excised via midsternal thoracotomy. The hearts were retrogradely perfused with modified Krebs-Henseleit (KH) buffer with high K^+ (20 mM), bubbled with 95 % O_2–5 % CO_2 gas mixture, in a dissection dish at room temperature (20–22 °C). Because good trabeculae were usually present in right ventricle near the tricuspid valve, right ventricle was first examined and muscles were dissected from there. Only about 30 % of the mouse hearts examined had muscles technically suitable for force measurements and fura-2 microinjection. When encountered, such muscles (1.03 ± 0.20 mm long, 0.26 ± 0.09 wide, and 0.13 ± 0.04 thick; mean ± SD, n = 12) were quickly dissected and mounted between a force transducer and

a micromanipulator in a perfusion bath. The muscles were superfused with Krebs-Henseleit (KH) buffer equilibrated with 95 % O_2–5 % CO_2. The KH buffer was composed of (mM): 112 NaCl, 5 KCl, 0.5 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4, 28 $NaHCO_3$, and 10 glucose, pH 7.35–7.40. The perfusion rate was ~10 ml/min and the preparations were field-stimulated at 0.5 Hz with 5 ms pulses (Grass SD9 Stimulator) unless otherwise indicated. All experiments were performed at room temperature. After stabilization, the preparations were stretched to optimal length, and the background fluorescence excited at 380 nm and 340 nm was recorded.

Measurement of intracellular $[Ca^{2+}]$ with fura-2

$[Ca^{2+}]_i$ was measured as described previously (3, 4, 10–12). Briefly, after stabilization of the preparations, fura-2 potassium salt was micro-injected iontophoretically into one cell and allowed to spread throughout the muscle via gap junctions. $[Ca^{2+}]_i$ was determined by measuring the epifluorescence of fura-2 in the cell, excited using ultraviolet light at 380 nm and 340 nm. The fluorescent light was collected at 510 nm by a photomultiplier tube (R2693, Hamamatsu, Bridgeport, NJ, USA). After equilibration of the loaded fura-2, intracellular free Ca^{2+} concentration and contractile force were measured. The outputs of the photomultiplier tube and force transducer were filtered at 100 Hz, collected by an A/D converter and stored in the computer for later analysis.

Intracellular $[Ca^{2+}]$ was given by the following equation (after subtraction of the autofluorescence of the muscle):

$$[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R) \quad (1)$$

where R is the observed ratio of fluorescence (340 nm/380 nm), K_d is the apparent dissociation constant, R_{max} is the ratio of 340 nm/380 nm at saturating $[Ca^{2+}]$, and R_{min} is the ratio of 340 nm/380 nm at zero $[Ca^{2+}]$. The values used for K_d, R_{max}, and R_{min} were 3.5 μM, 7.20, and 0.47, respectively, determined by Gao et al. in their *in vivo* calibrations with mouse ventricular muscles (11).

Statistics

All the data were analyzed by 2 way ANOVA followed by Fisher's PLSD post-hoc test using StatView software (Ver. 4.54, Abacus Concepts, Inc., Berkeley, CA, USA). A value of $P < 0.05$ was considered to indicate significant differences. Unless otherwise indicated, pooled data are expressed as mean ± SE.

Results

Force development at varied $[Ca^{2+}]_o$

Remarkably, there was little difference between controls and transgenics when the muscles were stimulated at a low rate. Figure 1 shows pooled data for $[Ca^{2+}]_i$ (left) and force (right) from 6 muscles each in the control and SERCA2a transgenic groups, at 0.5 Hz stimulation. Ca^{2+} transients and twitch force increased almost monotonically as $[Ca^{2+}]_o$ increased up to 2.0 mM; the diastolic levels of $[Ca^{2+}]_i$ and force were unaf-

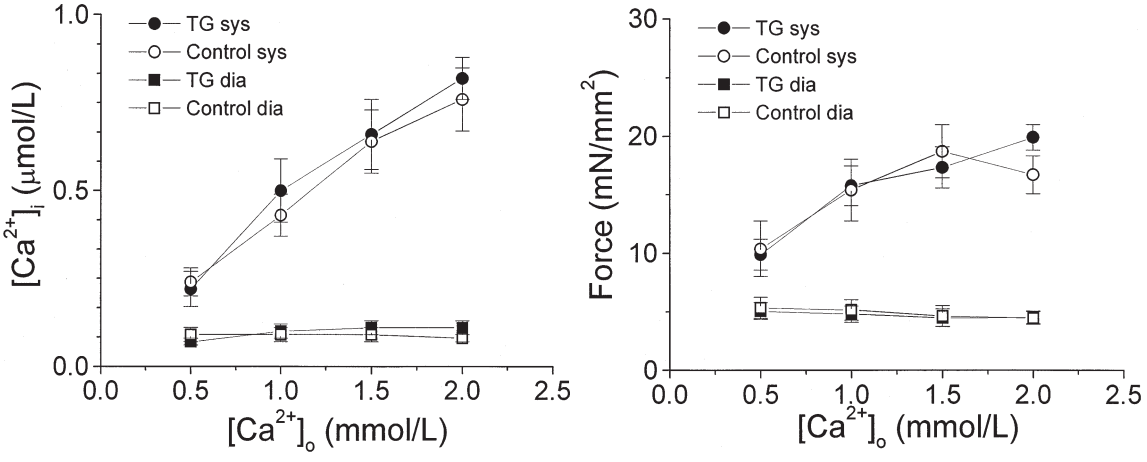


Fig. 1 Effects of $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ and twitch force of cardiac muscle from control and SERCA2a transgenic mice. Pooled data for systolic (sys) and diastolic (dia) $[Ca^{2+}]_i$ (left) and force (right) from 6 muscles in each group are plotted at varied $[Ca^{2+}]_o$. Systolic $[Ca^{2+}]_i$ and force increased similarly in both groups as $[Ca^{2+}]_o$ increased. All muscles were stimulated at 0.5 Hz. *TG* SERCA2a transgenics; *Control* controls.

fected. None of the differences were consistent or significant, nor were there differences in twitch decay kinetics between the two groups (not shown). Thus, SERCA overexpression has no clear functional effect at low stimulation rates.

Effects of stimulation frequency on $[Ca^{2+}]_i$ and contraction

We next investigated the effects of increasing the rate of stimulation. Figure 2 shows representative records of Ca^{2+} transients (top) and corresponding twitch force (bottom) measured at 2.0 mM $[Ca^{2+}]_o$ and 2.0 Hz stimulation in muscles from control (left) and SERCA2a transgenic hearts (right). The transgenics exhibited a sizable increase in Ca^{2+} transient amplitude relative to the normals, with a concomitant increase in force generation.

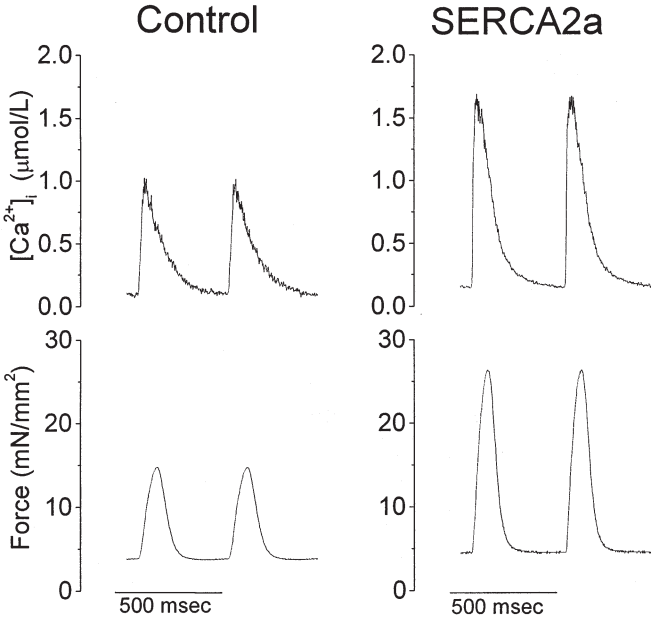


Fig. 2 Representative records of Ca^{2+} transients and corresponding twitch force in muscles from control and SERCA2a transgenic mice. $[Ca^{2+}]_o$ was 2.0 mM and muscles were stimulated at 2.0 Hz. The transgenics (right) exhibited a sizable increase in Ca^{2+} transient amplitude relative to the normals (left), with a concomitant increase in force generation.

The differences in calcium and force were graded with increasing stimulation frequency. Figure 3 and Table 1 show pooled data for Ca^{2+} transients and twitch force in control and SERCA2a transgenics (n = 6 muscles each) as a function of stimulation frequency. These experiments were performed at 2.0 mM $[Ca^{2+}]_o$. The amplitude of Ca^{2+} transients increased significantly as stimulation rate increased, with a slight increase in the diastolic values. SERCA2a transgenics exhibited a positive force-frequency relationship, whereas this relationship was flat in controls. Differences in both amplitude of Ca^{2+} transients and twitches were significant at 2.0 Hz stimulation ($P < 0.05$).

Effects of stimulation frequencies on dynamics of twitches and Ca^{2+} transients

The time-to-peak force decreased ($P < 0.01$) and the time-to-peak Ca^{2+} ($P < 0.05$) also decreased slightly but significantly as stimulation rate increased. Although there were no resolvable differences in the time-to-peak Ca^{2+} between controls and

Fig. 3 Effects of stimulation frequency on $[Ca^{2+}]_i$ and twitch force of cardiac muscle from control and SERCA2a transgenic mice. Pooled data for systolic (sys) and diastolic (dia) $[Ca^{2+}]_i$ (left) and force (right) from 6 muscles in each group are plotted at varied stimulation frequencies at $[Ca^{2+}]_o$ of 2.0 mM. Systolic $[Ca^{2+}]_i$ increased similarly in both groups as stimulation frequency increased. Note that SERCA2a transgenics exhibited a positive force-frequency relationship, whereas this relationship was flat in controls. Differences in both amplitude of Ca^{2+} transients and twitches were significant at 2.0 Hz stimulation (*$P < 0.05$, **$P < 0.01$ vs. controls). *TG* SERCA2a transgenics; *Control* controls.

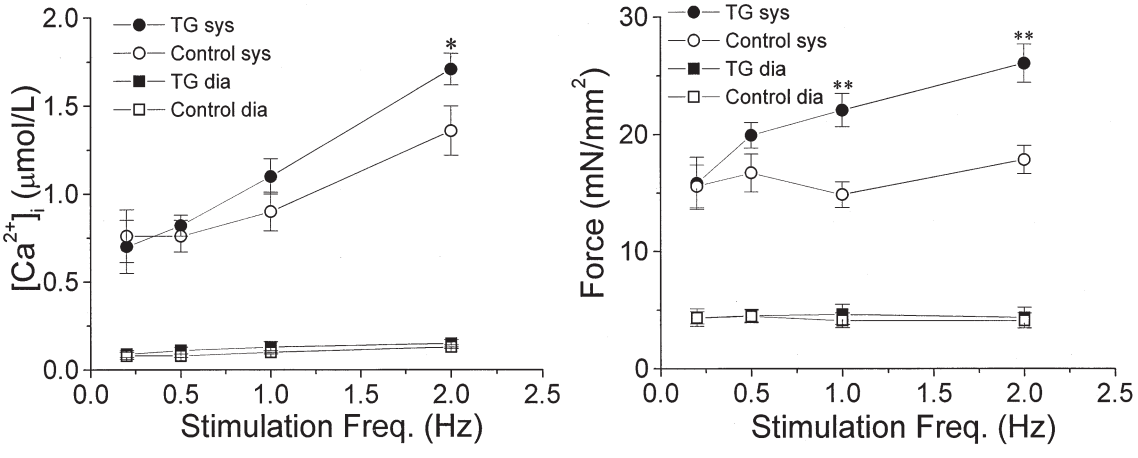


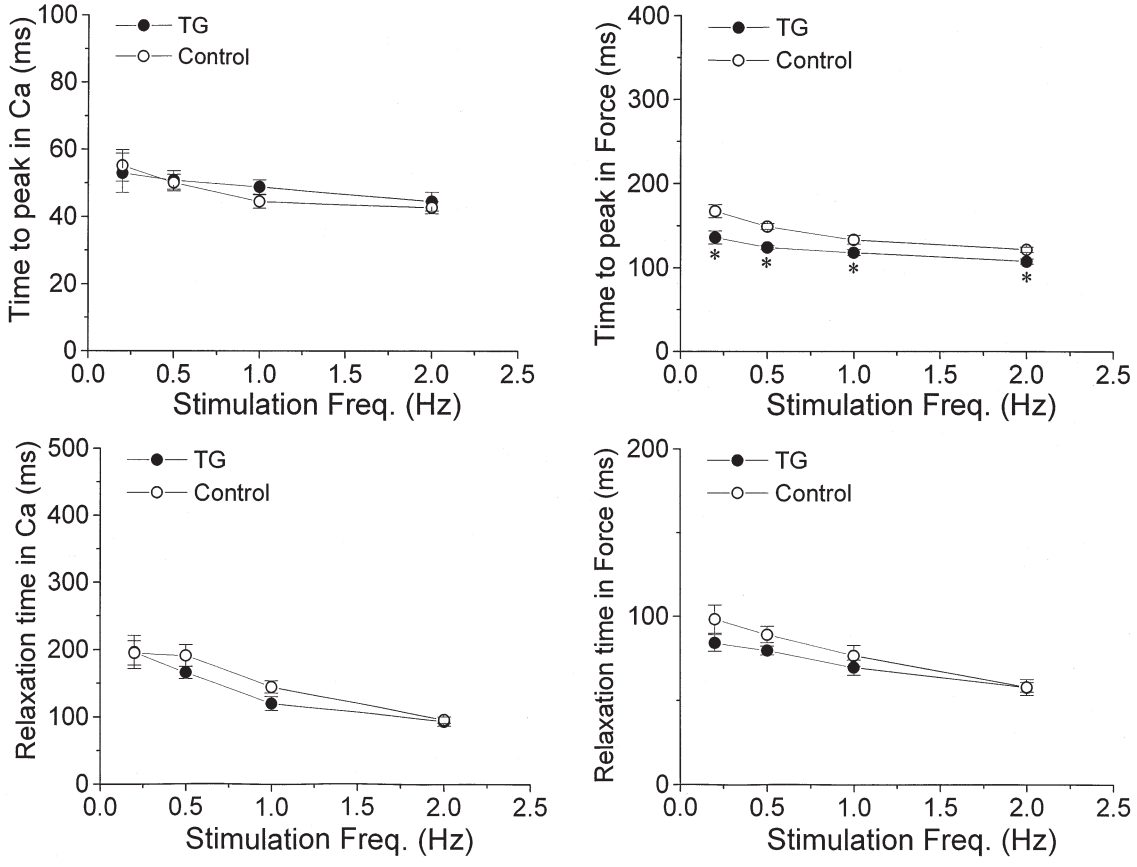
Table 1 Effects of stimulation frequency on $[Ca^{2+}]_i$ and twitch force of cardiac muscles from SERCA2a transgenic and control

Stimulation Freq. (Hz)	Peak $[Ca^{2+}]_i$ (μmol/L)		Diastolic $[Ca^{2+}]_i$ (μmol/L)		Peak Force (mN/mm^2)		Diastolic Force (mN/mm^2)	
	TG	Control	TG	Control	TG	Control	TG	Control
0.2	0.70 ± 0.15	0.76 ± 0.15	0.09 ± 0.02	0.08 ± 0.02	15.82 ± 2.24	15.56 ± 1.83	4.36 ± 0.75	4.33 ± 0.52
0.5	0.82 ± 0.08	0.76 ± 0.13	0.11 ± 0.02	0.09 ± 0.02	19.92 ± 1.52	16.71 ± 1.06	4.53 ± 0.80	4.46 ± 0.81
1.0	1.10 ± 0.10	0.90 ± 0.11	0.13 ± 0.03	0.10 ± 0.01	22.07 ± 1.42**	14.85 ± 1.10	4.65 ± 0.86	4.11 ± 0.63
2.0	1.71 ± 0.09*	1.34 ± 0.13	0.15 ± 0.02	0.13 ± 0.01	26.05 ± 1.62**	17.83 ± 1.21	4.35 ± 0.88	4.09 ± 0.64

*: P < 0.05, **: P < 0.01 vs. Control, *TG* SERCA2a transgenics (n = 6), *Control* controls (n = 6)

SERCA2a transgenics, the time-to-peak force was always shorter in SERCA2a transgenics (Fig. 4, top). Excitation-contraction coupling during twitch contractions is a very dynamic process. The relationship between time-to-peak $[Ca^{2+}]_i$ and time-to-peak force is affected by multiple factors (3). The difference of these relationships between transgenics and non-transgenics may reflect delicate differences in the experimental conditions or there may be some intrinsic difference in the Ca^{2+}-force kinetics between these two groups. When comparing relaxation by the time from peak to 50 %

Fig. 4 Effects of stimulation frequencies on dynamics of twitches and Ca^{2+} transients. Pooled data from 6 muscles of normal and SERCA2a transgenics are plotted at varied stimulation frequencies at $[Ca^{2+}]_o$ of 2.0 mM. The change in time-to-peak force (top right) in each group and its difference between controls and transgenics were significant (P < 0.01 and *P < 0.05, respectively), although there were no differences in the time-to-peak Ca^{2+} (top left) between controls and SERCA2a transgenics. There was a significant acceleration of relaxation of Ca^{2+} transients (bottom left) and twitches (bottom right) with increasing rate in each group (P < 0.001 and P < 0.01, respectively), but there were no significant differences between these two groups. *TG* SERCA2a transgenics; *Control* controls.



relaxation (relaxation time), there was a significant acceleration of relaxation of Ca^{2+} transients as stimulation rate increased ($P < 0.001$), with relaxation of Ca^{2+} transients being consistently faster than that of force (Fig. 4, bottom), but there were no significant differences between the two groups. Thus, the major effect of SERCA overexpression is on SR calcium loading, rather than on the kinetics of calcium removal.

Discussion

SERCA2a transgenics and controls showed almost identical intracellular Ca^{2+} handling and force generation at 0.5 to 2.0 mM $[Ca^{2+}]_o$ when stimulated at a frequency of 0.5 Hz. Under these conditions, modest overexpression of SERCA has no obvious net effects on Ca^{2+} cycling. However, SERCA2a transgenics exhibit a sizable increase in calcium transient amplitude relative to controls, with a concomitant increase in force generation, at higher frequencies of stimulation. As heart rate increases, the Ca^{2+} load of the cell increases. This added calcium is either sequestered by the SR or extruded during diastole. The increase in SERCA expression appears to tip the balance towards sequestration, such that more Ca^{2+} is available for subsequent intracellular release as activator Ca^{2+}. It is reported that the contraction-relaxation cycle is more sensitive to thapsigargin or cyclopiazonic acid at higher frequencies of stimulation and less sensitive at low stimulation frequencies (6, 51). It may be also the case that the alterations in SERCA function will have small effects when the stimulation frequency is slow, as if SERCA had a frequency-dependent activation effect. SERCA transgenics exhibited a clearly positive force-frequency relationship, while age- and strain-matched controls had a relative flat response. Mouse has been described by Gao et al. to have a property of frequency-dependent myofilament calcium sensitization (11), and this appears to be exaggerated in SERCA2a transgenic mice.

Although Loukianov et al. and He et al. have reported that Ca^{2+} handling is augmented in cells from hearts overexpressing SERCA1a (28) or SERCA2a (20), neither of these reports measured $[Ca^{2+}]_i$ or contractile force directly. Instead, these investigators measured uncalibrated fluorescence changes and cell shortening. Such technical differences may explain the differences in decay kinetics between previous studies and ours. He et al. (20) compared indo-1 fluorescence transients and cell shortening in isolated myocytes from controls and SERCA2a transgenics, and found that the decay of Ca^{2+} transients and relengthening were significantly faster in the transgenics. We find that the decay rates of Ca^{2+} transients and force were somewhat faster in SERCA2a transgenics, especially around 0.5 Hz, but the differences did not reach statistical significance. This may be explained by the difference between Ca^{2+} release and sequestration processes. The amount of Ca^{2+} sequestered into and released from SR is larger in transgenics due to the increased amount of SERCA protein. However, the amount of Ca^{2+} sequestered per SERCA pump would be same as that of controls. Therefore, the decay rates settle down to similar values. While we cannot rule out genuine differences between the two SERCA strains, the difference may simply reflect the fact that He et al. measured unloaded cell shortening whereas this study measured genuine contractile force. Force per cross sectional area is exquisitely sensitive to the number of contractile units, whereas isotonic shortening is relatively insensitive to this parameter. Naturally, as Neumann et al. (39) pointed out, *in vitro* findings obtained with methods like ours must be carefully extrapolated to cardiac function *in situ,* because only a few conditions are artificially held constant in *in vitro* experiments. However, contraction and relaxation of in situ SERCA2a transgenic heart have not been studied. So, further investigations are necessary to confirm *in situ* mechanical characteristics of transgenic hearts.

SERCA2a is reported to be regulated by multiple pathways such as phosphorylation state of phospholamban through PKA or CaM kinase (decreasing K_m) (7, 23, 26, 35, 41, 47), and direct phosphorylation by CaM kinase (increasing V_{max}) (19, 49, 50). Although it is still a matter of debate that protein expression level of SERCA2a is decreased or phosphorylation state of phospholamban is altered, SERCA2a Ca^{2+} transport capacity is reduced in end-stage human heart failure. Therefore, increasing the number of SERCA2a may be advantageous in augmentation of Ca^{2+} uptake capacity of SR.

In conclusion, we have shown that an increase in SERCA expression increases the ability of the SR to store calcium, such that more calcium is available for release during each cycle with concomitant increases in force generation. This effect is prominent only at higher frequencies of stimulation. One of the most striking and consistent changes in the contractile phenotype of failing heart muscle is the loss of the normal potentiation of twitch force at higher stimulation frequencies. Our observations lend support to the emerging consensus that enhancement of SR calcium pumping capacity represents a logical strategy to reverse the functional abnormalities of failing myocardium.

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