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Frequency-dependent contractile strength in mice over- and underexpressing the sarco(endo)plasmic reticulum calcium-ATPase

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Hiranandani N, Raman S, Kalyanasundaram A, Periasamy M, Janssen PM. Frequency-dependent contractile strength in mice over- and underexpressing the sarco(endo)plasmic reticulum calcium-ATPase. Am J Physiol Regul Integr Comp Physiol 293: R30–R36, 2007. First published January 25, 2007; doi:10.1152/ajpregu.00508.2006.—One of the prominent markers of end-stage heart failure at the molecular level is a decrease in function and/or expression of the sarcoplasmic reticulum ATPase protein [sarco(endo)plasmic reticulum calcium-ATPase, SERCA]. It has been often postulated that a decrease in SERCA pump activity can contribute in a major way to decreased cardiac function. To establish a functional relationship, we assessed how alterations in SERCA activity level affect basic contractile function in healthy myocardium devoid of other significant molecular changes. We investigated baseline contractile function, frequency-dependent activation, and β-adrenergic response in ultrathin trabeculae isolated from hearts of mice overexpressing SERCA (transgenic, TG), underexpressing SERCA2a (heterozygous knockout, Het), and their respective wild-type (WT) littermates. At physiological temperature and frequency, compared with their respective WT littermates, SERCA1a mice displayed increased developed force at frequencies of 4–8 Hz (~90% increase at 4 Hz) and force equal to WT mice at 10–14 Hz. Force development at 4 Hz in presence of 1 μM isoproterenol was similar in TG and WT mice. In Het mice, developed force was nearly identical at the lower end of the frequency range (4–8 Hz) but slightly depressed at higher frequency (P < 0.05 at 14 Hz). In presence of 1 μM isoproterenol, developed force at 4 Hz was equal to that in WT mice. Compared with normal levels, increased SERCA activity enhanced force development only at subphysiological frequencies. A reduction in SERCA activity only showed a depression of force at the higher frequency range. Thus generalizations regarding the correlation between SERCA activity and contractility can be highly ambiguous, because this relationship is critically dependent on other factors including stimulation frequency.

THE SARCOPLASMIC RETICULUM (SR) calcium pump [sarco(endo)plasmic reticulum calcium-ATPase, SERCA] actively resequesters calcium ions from the cytoplasm. The total activity of all SERCA pumps is a strong determinent of myocardial contractility (22), i.e., the total amount of Ca2+ pumped back into the SR each beat under steady-state conditions is both followed and preceded by an equivalent amount of calcium released via the ryanodine receptors on activation of the next (and/or previous) beat. In end-stage heart failure, the total activity of the SERCA pumps is decreased (1), via loss of number of pumps, loss of activity of the individual pumps, or a combination thereof (19, 26).

Frequency-dependent inotropic modulation largely stems from an increase in SR load established via increased total activity of SERCA. Within the physiological range, total SERCA activity generally increases with frequency, generally resulting in a higher contractility in healthy myocardium (21). This frequency-dependent activation not only can increase the force of contraction, it also speeds up the relaxation (10), so the cardiac muscle has sufficient time in diastole to maintain adequate filling before the next beat (25). The SERCA pump not only generates the intracellular calcium concentrations, activity of the pump is increased. Thus, as it pumps calcium ions back into the SR, it reduces the intracellular calcium transient and negatively feeds back on its own activity. As a result, in the time domain SERCA pump activity is cycling continuously, but with variable capacity. When interbeat duration is decreased (increased frequency), as a result of a time-averaged higher calcium concentration activity is higher per time unit, resulting in an elevation of SR load during the first few beats following a frequency increase before a new homeostasis with increased SR calcium levels sets in.

To understand, and quantify, the frequency-dependent SERCA pump function, we set out to investigate frequency-dependent activation in isolated cardiac tissue with normal, increased, and reduced SERCA activity levels. Our working hypothesis was that increased SERCA levels promote increased force, and because the maximum force-generating capacity is limited by the myofilaments, the increases in SERCA level at low frequency may increase calcium handling and contractile force, resulting in a reduced capacity to increase further, or possibly may result in such a high baseline force that no increases are observed at higher frequencies. To test whether SERCA function affects basal contractility to such an extent that the cardiac reserve is significantly compromised, we investigated isometric developed force generation in ultrathin trabeculae isolated from hearts of SERCA1a-overexpressing mice (higher baseline SERCA function) (2, 20) and SERCA2a heterozygous knockout (Het) mice (reduced SERCA function) (23), as well as from respective wild-type (WT) animals. Our results indicate that enhanced baseline SR function in healthy myocardium devoid of other significant molecular changes. We investigated baseline contractile function, frequency-dependent activation, and β-adrenergic response in ultrathin trabeculae isolated from hearts of mice overexpressing SERCA (transgenic, TG), underexpressing SERCA2a (heterozygous knockout, Het), and their respective wild-type (WT) littermates. At physiological temperature and frequency, compared with their respective WT littermates, SERCA1a mice displayed increased developed force at frequencies of 4–8 Hz (~90% increase at 4 Hz) and force equal to WT mice at 10–14 Hz. Force development at 4 Hz in presence of 1 μM isoproterenol was similar in TG and WT mice. In Het mice, developed force was nearly identical at the lower end of the frequency range (4–8 Hz) but slightly depressed at higher frequency (P < 0.05 at 14 Hz). In presence of 1 μM isoproterenol, developed force at 4 Hz was equal to that in WT mice. Compared with normal levels, increased SERCA activity enhanced force development only at subphysiological frequencies. A reduction in SERCA activity only showed a depression of force at the higher frequency range. Thus generalizations regarding the correlation between SERCA activity and contractility can be highly ambiguous, because this relationship is critically dependent on other factors including stimulation frequency.

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function leads to enhanced contractile strength. However, the higher baseline strength prohibits further increases in frequency-dependent inotropy observed normally in WT animals.

METHODS

Transgenic mouse models. All mouse models have been published previously. SERCA-overexpressing (transgenic, TG) mice are mice expressing the (skeletal) SERCA1a isoform and have a 2.5-fold increase in the total amount of SERCA and a ~2-fold increase in SR Ca\(^{2+}\) uptake function (15, 20). We chose the SERCA1a-overexpressing mice for this proof-of-principle study; SERCA1a possesses faster Ca\(^{2+}\) transport kinetics, and therefore possible effects of enhanced SERCA activity (2) would be possibly most pronounced. SERCA2a heterozygous knockout (Het) mice have one allele mutated, and as a result have an ~40% reduction in expression of SERCA2a (23), resulting in a decreased SR calcium reuptake (8, 14, 23). Protein expression levels of SERCA1a and SERCA2a were regularly checked to ensure altered protein expression via standard Western blotting techniques. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Muscle preparation and experimental setup. Mice weighing 20–25 g were anesthetized with the inhalation anesthetic halothane. After 95% O\(_2\)-5% CO\(_2\). Additionally, 20 mM 2,3-butanedione monoxime (BDM) was added to the dissection buffer to prevent cutting injury. The effects of BDM after brief exposure have been found to be reversible (12, 21). Hearts were cannulated via the ascending aorta and retrograde perfused with the same buffer for several minutes. The dimensions of the muscles were measured with a calibration reticule in the ocular of the dissection microscope (×40, resolution ~10 μm). Cross-sectional area was calculated, assuming an ellipsoid cross-sectional shape. Suitable trabeculae (thickness not exceeding 200 μm) were dissected and contractile parameters assessed in identical setups. However, only one muscle per heart was randomly selected to be included in the analysis of contractile properties. All experimental protocols conformed to institutional guidelines regarding the use and care of animals.

With the dissection microscope, muscles were mounted between a platinum-iridium basket-shaped extension of a force transducer (tissue block end) and a hook (valve end) connected to a micromanipulator. This method has been shown (3, 10, 11, 18, 34) to minimize end-damage compliance of the muscle and to prevent excessive loss of force throughout the experimental protocols. Muscles were superfused with the same buffer at 37°C as above (with the exception that BDM was omitted) and stimulated at 4 Hz. Extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) was raised to 1.5 mM, and muscles were allowed to stabilize for at least 30 min before the experimental protocol was initiated. Muscles were stretched to a length at which a small increase in length resulted in about equal increases in resting tension and active developed force. This is a length (optimal length) that is slightly below the length at which active force development is maximal, and it was selected to be comparable to the maximally attained length in vivo at end of diastole (~2.2-μm sarcomere length) (28).

Determination of contractile properties. To assess the effect of muscle length on developed force and relaxation time, the following protocol was performed. First, slack length (length of muscle without any preload) and optimal length were determined. The difference was divided into three equal steps, and the muscle was stretched sequentially, with each step increasing the length of the muscle until the baseline length was achieved. Parameters were recorded when the muscle had stabilized at each length. Thereafter, we assessed the effects of increasing stimulation frequencies between 2 and 14 Hz. At each frequency, forces were allowed to reach steady state before data were recorded. The effects of b-adrenergic stimulation were assessed in a subset of muscles by a concentration-response curve with isoproterenol (10\(^{-7}\)–10\(^{-6}\) M) at a baseline stimulation frequency of 4 Hz. In a subset of experiments at the in vivo resting frequency of 8–10 Hz (4, 16, 36), the effect of maximal isoproterenol stimulation was also tested. To assess the size of SR calcium load, rapid cooling contracture (RCC) amplitude was measured. After the stabilization of muscle, stimulation was stopped and muscles were rapidly cooled to 0°C in <1 s. After a steady force was reached, the temperature was switched back to 37.5°C. This protocol was repeated at different frequencies. After stabilization at baseline of 4 Hz, in a set of muscles we measured the postrest potentiation (PRP), because it is an important determinant of force-frequency response. In this protocol the stimulation interval was sequentially set to 2, 4, 6, 10, 15, 50, 60, and 120 s from the basic frequency of 4 Hz.

Data analysis and statistics. In all the experiments performed, the parameters of developed force (F\(_{\text{dev}}\)) and diastolic force were determined and normalized to the cross-sectional area of the muscle. Additionally, as a model-independent parameter of force decay kinetics, time from peak force to 50% relaxation (RT\(_{50%}\)) was determined. Parameters were calculated offline and also online to facilitate immediate judgment of preparation quality. Preparations that displayed excessive rundown of F\(_{\text{dev}}\) (>10%/h) were excluded. In muscles that underwent the length, force-frequency, isoproterenol, and RCC or PRP protocols, total protocol time was nearly 4 h.

One-way repeated ANOVA was used to determine significant differences between the groups, with Newman-Keuls post hoc t-test when appropriate. A two-sided P value of <0.05 was considered significant. All values are expressed as means ± SE.

RESULTS

First we verified the protein phenotype of our mice. In Fig. 1, we show that in the SERCA1a mouse this protein was indeed robustly expressed, whereas in the SERCA2aHet mice the SERCA2a protein level was reduced compared with the respective WT mouse. This is in close agreement to the initial studies on these mice that showed protein expression patterns nearly identical to those we observed in these generations (20, 23). It is already known that phospholamban (PLB) levels are unchanged in SERCA1a mice (20). Likewise, it has been shown that in the SERCA2aHet mouse PLB is decreased, whereas PLB-P at Ser16 is increased at 16 Hz (7). We very recently (7) verified several of these key protein expression patterns and again specifically verified PLB phosphorylation in the mice used for this study, and our results were consistent with the previously published protein levels. The respective phosphorylation status of Ser16 and Thr17 is depicted in Fig. 1B, and it indicated that Ser16 was increased in SERCA2a Het and unchanged in SERCA1a mice, while no significant differences were found in levels of Thr17 phosphorylation.

Next we examined the contractile response of isolated cardiac trabeculae under isometric conditions at 37.5°C and preload resembling end-diastolic values. We observed that compared with the respective WT muscles, at a stimulation frequency of 4 Hz, SERCA1a-expressing trabeculae displayed a significantly higher level of active developed force (36.4 ± 5.1 vs. 19.2 ± 3.5 mN/mm²; P < 0.05). This was accompanied by a faster relaxation: RT\(_{50%}\) was significantly faster in
SERCA1a-expressing trabeculae (21.8 ± 1.3 vs. 26.8 ± 2.2 ms; *P < 0.05). Similarly, time to peak tension was faster in SERCA1a muscles (47.2 ± 2.1 vs. 54.5 ± 1.8 ms; *P < 0.05). Another often-used index of contractility and relaxation, the first derivative of force (dF/dt), mirrored these findings; the maximum and minimum dF/dt values were indicative of increased contractility (1,500 ± 199 vs. 1,092 ± 333 and 1,210 ± 165 vs. 977 ± 299 mN·mm⁻²·s⁻¹, respectively).

Next we investigated the contractile response to increased stimulation frequencies. Starting from the 4-Hz baseline, the frequency was increased in steps of 2 Hz up to 14 Hz. At each frequency, the contractile parameters were allowed to stabilize before they were recorded and the next frequency was applied. In Fig. 2, active Fdev for frequencies ranging from 4 to 14 Hz is depicted: raw force tracings from individual experiments at 4, 8, and 12 Hz (Fig. 2A) as well as average values at 4–14 Hz (Fig. 2B) are shown. At the low end of the range, 4 and 6 Hz, the SERCA1a muscles are significantly stronger (SERCA 1a 36.4 ± 5.1 and 35.7 ± 5.3 mN/mm² vs. WT 19.2 ± 3.5 and 21.7 ± 4.2 mN/mm² at 4 and 6 Hz, respectively) At 8 Hz the difference still persists but is no longer significant, and from 10 to 14 Hz there is no significant difference compared with the WT muscles. The response to isoproterenol in SERCA1a-expressing muscles is depicted in Fig. 3. With increasing isoproterenol concentrations, force development increases. At concentrations of isoproterenol 3 × 10⁻⁸ M and above, the difference between the two groups is no longer significant. Thus the absolute response to isoproterenol is greater in WT muscles, because of the lower baseline contractility. However, the levels of Fdev under maximal isoproterenol stimulation are similar between the two groups.

SERCA1a expression not only elevated basal contractility, it also accelerated the contractions. At 4 Hz, RT₅₀% was significantly shorter in SERCA1a muscles versus WT muscles (Fig. 4). At the highest stimulation frequency used, 14 Hz, or in the presence of 1 μM isoproterenol, the difference between the two groups was no longer present.

Using an identical protocol, we proceeded to investigate the same contractile parameters in SERCA2a mice (SERCA2a Het) in which one of the two alleles is mutated. The expression...
level of SERCA2a in these muscles is reduced by ~40%. The background strain for these mice is C57BL/6. This is a different strain than the SERCA1a mice (FVBN), and hence we used different WT controls. We recently reported (32) that within the physiological frequency range three different strains possessed very similar contractile properties. This was also the case in the present study. However, similarly to our previous study, at lower frequency ranges strain differences could be observed. We indeed observed a significantly higher basal contractility in the WT mice of the SERCA2a Het background strain versus the SERCA1a background strain.

In SERCA2a Het muscles, basal contractility at 4 Hz appeared reduced, but this was not quite significant (P = 0.11). As frequency was increased, both SERCA2a Het and WT mice behaved similarly up to 12 Hz. At 14 Hz, which is the upper limit of the in vivo murine heart rate (4), the difference was significant (P < 0.05); SERCA2a Het mice had a lower F\textsubscript{dev} compared with WT littermates (16.2 ± 3.5 vs. 24.4 ± 2.2 mN/mm\textsuperscript{2}; Fig. 5). The isoproterenol response at a baseline frequency of 4 Hz was not different between the groups (Fig. 6). Relaxation kinetics were slightly slower in SERCA2a Het muscles, but this was not significant. Similar results regarding RT\textsubscript{50%} were observed for 14 Hz and 1 μM isoproterenol (Fig. 7).

Calcium handling was tested with SR constant estimations via RCC and a PRP protocol. There was no change in RCC amplitude between SERCA2a Het and WT mice at the baseline frequency of 4 Hz or at the higher frequency (12 Hz) (Fig. 8A). Figure 8B shows the PRP behavior in SERCA2a Het and WT mice. During the longest rest durations (60–120 s) the amount of PRP is significantly higher in SERCA2a Het compared with WT, but for the short durations PRP behavior is similarly positive for both groups.

**DISCUSSION**

The present study clearly demonstrates that any correlation between SERCA activity and contractility is critically depen-
dent on stimulation frequency. Previous studies (22) showed that SERCA is one of the decisive determinants of cardiac contractility (22). A decrease in SERCA pump expression and activity was observed under a variety of pathological conditions. It has been postulated that decreased SERCA activity reduces the SR Ca\(^{2+}\) content and contractility (14, 23), while increased SERCA expression improves SR Ca\(^{2+}\) content and hence myocardial function and contractility (2, 6). At the lower frequency range (4 – 6 Hz) trabeculae expressing SERCA1a indeed revealed significantly higher active F\(_{dev}\) and relaxation rate compared with WT, but at the higher frequency range (10 – 12 Hz) there was no persistent difference between WT and SERCA1a trabeculae. These data indicate that in normal WT mice the enhanced stimulation of SERCA appears sufficient to elicit a maximal contractile response at high frequency.

At a baseline frequency of 4 Hz, SERCA2a Het mice showed slightly (nonsignificant) reduced basal contractility and relaxation kinetics and a similar isoproterenol response, but this difference became significant when frequency was in the range of 12–14 Hz, compared with WT. Clearly, interpretation of whether, and to what extent, SERCA expression changes contractility is critically dependent on frequency or range of frequencies.

At subphysiological frequencies, SERCA1a-overexpressing trabeculae possessed a higher basal contractility and a faster relaxation rate compared with WT. These findings are consistent with previous studies (17, 20, 37). Interestingly, at higher (physiological) frequencies response of SERCA1a-overexpressing trabeculae were no longer significantly different from the WT trabeculae. This likely indicates that at lower frequencies, because of higher SERCA activity in the SERCA1a trabeculae, there is increased reuptake of Ca\(^{2+}\) into the SR, thereby decreasing the time to peak and relaxation time. At higher frequencies there is little or no room for any increase compared with WT, because at these higher frequencies WT mouse SERCA activity is enhanced per se. Previous studies showed that greater SERCA expression can limit the calcium transient by competing with troponin C for calcium binding and by curtailing its peak (20, 35). We know that the SERCA pump can increase the SR Ca\(^{2+}\) load, but only up to a certain limit. Once that SR Ca\(^{2+}\) storage limit is reached, SERCA pump activity cannot increase the SR Ca\(^{2+}\) load further (5, 30, 31). This possibly explains the finding that an increase in contractility in SERCA1a-overexpressing trabeculae is observed mainly at subphysiological frequencies where the maximal SR load has not yet been reached.

At a 4-Hz stimulation rate, relaxation was significantly faster (shorter) in SERCA1a versus WT muscles, but at the high frequency range this difference was no longer present. As we learned from previous studies (9), there are two main factors responsible for cardiac relaxation: decrease of intracellular [Ca\(^{2+}\)] and myofilament properties. These findings support the idea that at subphysiological frequencies relaxation rate may mainly depend on Ca\(^{2+}\) reuptake by SERCA, while at

![Fig. 6. Comparison of isoproterenol response between SERCA2a Het and WT mice. At baseline frequency of 4 Hz there is no difference in isoproterenol response between SERCA2a Het and WT mice. Temperature was 37°C throughout the experiment.](http://ajpregu.physiology.org/)

![Fig. 7. Changes in RT50% at different frequencies (A) and in the presence of isoproterenol (B) in SERCA2a Het and WT mice. Relaxation kinetics are slightly slower in SERCA2a Het mice, but this was not significant. Temperature was 37°C throughout the experiment. **Difference at \(P < 0.05\) between conditions in the same group.](http://ajpregu.physiology.org/)
physiological frequencies intrinsic myofilament properties are the major determinant of relaxation rate. Reuptake of Ca\(^{2+}\) into the SR may thus possibly act as a rate-limiting step in relaxation at low stimulation frequencies; however, at physiologically frequencies myofilament properties likely govern the relaxation rate, in accordance with our previous work (10).

At a baseline frequency of 4 Hz, SERCA2a Het mice show a slightly lower \(F_{\text{dev}}\) compared with WT mice, and this difference becomes significant at higher frequencies. In line with results from previous studies (32, 33), our results demonstrate that WT trabeculae show a positive force-frequency response between 4 and 8 Hz, while in SERCA2a Het mice the contractile response was impaired compared with WT at high frequency. At baseline, \(F_{\text{dev}}\) was less; decreased SERCA expression at the low end of the frequency range causes reduced SR Ca\(^{2+}\) uptake. At higher frequencies there is less time to transport calcium; therefore the decreased capacity of SR Ca\(^{2+}\) reuptake and SR Ca\(^{2+}\) loading becomes more prominent. Previous studies have shown that this alteration in force-frequency response can be due to a decrease in calcium transient with increased frequency (24, 25). In our study, the isoproterenol response was similar in WT and SERCA2a Het mice, indicating no significant change in \(\beta\)-adrenergic response due to decreased SERCA activity. This is consistent with previous studies showing that in SERCA2a Het mice there is no altered activation of the sympathetic system as levels of norepinephrine in plasma and cardiac were not changed (14).

Although in all mammals generally a positive force-frequency relationship (FFR) is observed under physiological conditions, it is well known that this response is most prominent in larger mammals. Although in rats and mice this FFR is positive as well under physiological conditions, it is often extremely small (4). FFR plays a prominent role in large mammals; the difference between resting heart rate and maximal heart rate is 300–400% in humans, whereas it is only 30–50% at most in mice and rats (16). Moreover, in isolated myocardium, it is only positive in mice in ultrathin trabeculae, where core hypoxia is virtually absent. Previous studies have shown that in isolated rat trabeculae a hypoxic core unavoidably develops at a muscle thickness of 250 \(\mu\)m at room temperature (29) and already at a muscle thickness exceeding 150 \(\mu\)m at body temperature at 8 Hz (27). Thus for mice, which even have faster heart rates, core hypoxia may potentially occur at even thinner muscles. Although in this study we used ultrathin muscles, with diameters around 150 \(\mu\)m, we cannot altogether exclude some minimal levels of core hypoxia, and this may be a limitation of our studies in the high frequency ranges. The levels of \(F_{\text{dev}}\), however, indicate that this possible core hypoxia was rather modest in these studies; even at the most demanding inotropic conditions (maximum isoproterenol response), force development was in the range of 40–50 mN/mm\(^2\), which is significantly larger (up to more than a full order of magnitude) than regularly reported values for murine papillary muscles. The decrease in contractile force at the higher frequencies in all groups may be partially due to insufficient energetic supply. However, this is not the only reason, because at these very high frequencies the extremely rapid calcium removal from the cytosol may decrease the time that calcium levels are above troponin C threshold, limiting contractile activation. This latter process may thus partially, and likely mainly, be responsible for the reduced contractile forces at 10–12 Hz.

In conclusion, the relation between SERCA activity and cardiac contractility is critically dependent on stimulation frequency. Although at subphysiological frequencies SERCA1a-overexpressing trabeculae exhibited higher basal contractility, relaxation rate, and isoproterenol response, there is a limit after which there will be no further increase in calcium transient, resulting in a contractile response similar to WT mice. Therefore at higher frequencies there is no room for further increases in contractility due to reduction in the contractile reserve because basal contractility was already increased. Small increases in SERCA expression may thus improve cardiac function, but too much SERCA activity may lead to loss of contractile reserve and could cause negative effects on cardiac contractility.

GRANTS

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