Increased SR Ca\textsuperscript{2+} cycling contributes to improved contractile performance in SERCA2a-overexpressing transgenic rats

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Abstract

Objective: Heart failure is associated with reduced function of sarcoplasmic reticulum (SR) Ca\textsuperscript{2+}-ATPase (SERCA2a) but increased function of sarcolemmal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX), leading to decreased SR Ca\textsuperscript{2+} content and loss of frequency-potentiation of contractile force. We reported that SERCA2a-overexpression in transgenic rat hearts (TG) results in improved contractility. However, it was not clear whether TG have improved contractility due to frequency-dependent improved SR Ca\textsuperscript{2+} handling.

Methods: Therefore, we characterized TG (n=35) vs. wild-type (WT) control rats (n=39) under physiological conditions (37 °C, stimulation rate <8 Hz). Twitch force, intracellular Ca\textsuperscript{2+} transients ([Ca\textsuperscript{2+}]\textsubscript{i}), and SR Ca\textsuperscript{2+} content were measured in isolated muscles. The contribution of transsarcolemmal Ca\textsuperscript{2+} influx (I\textsubscript{Ca}) through L-type Ca\textsuperscript{2+} channels (LTCC) and reverse mode NCX (I\textsubscript{Na}/Ca) to Ca\textsuperscript{2+} cycling were studied in isolated myocytes.

Results: With increasing frequency, force increased in TG muscles by 168±35% (8 Hz; P<0.05) and SR Ca\textsuperscript{2+} content increased by maximally 118±31% (4 Hz; P<0.05). In WT, there was a flat force-frequency response without changes in SR Ca\textsuperscript{2+} content. Relaxation parameters of force and [Ca\textsuperscript{2+}]\textsubscript{i} decay were accelerated at each frequency in TG vs. WT by ~10%. At prolonged rest intervals (<240 s), force and SR Ca\textsuperscript{2+} content increased significantly more in TG. Consequently, absolute SR Ca\textsuperscript{2+} content measured in myocytes was increased ~2-fold in TG. Transsarcolemmal Ca\textsuperscript{2+} fluxes estimated by I\textsubscript{Ca} (at 0 mV -10.2±1.1 vs. -16.9±1.3 pA/pF) and I\textsubscript{Na}/Ca (0.17±0.02 vs. 0.46±0.05 pA/pF) were decreased in TG vs. WT (P<0.05), whereas NCX and LTCC protein expression was only slightly reduced (P=n.s.).

Conclusion: In summary, SERCA2a-overexpression improved contractility in a frequency-dependent way due to increased SR Ca\textsuperscript{2+} loading whereas transsarcolemmal Ca\textsuperscript{2+} fluxes were decreased.

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Keywords: Calcium (cellular); Contractile function; E–C coupling; Transgenic animal models; SR (function)

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CA2a) and to a smaller extent by sarcolemmal Na+/Ca2+ exchanger (NCX). Under steady-state conditions, the same amount of Ca2+ as had been previously released from the SR is taken up by SERCA2a, while NCX extrudes that amount of Ca2+ that had previously entered the cell through LTCC [3]. Sarcolemmal Ca2+ pumps and mitochondrial Ca2+ uniporter eliminate only a small fraction of Ca2+ ions (1–2%) and may be more important in long-term Ca2+ regulation [2].

Contractile dysfunction in heart failure (HF) [4,5] was related to defective SR Ca2+ uptake in animal models [6] and humans [1]. Direct relationships between impaired force-frequency relation (FFR), intracellular Ca2+ transients ([Ca2+]i), SR Ca2+ content [9,10], and SERCA2a expression [11] were reported in human HF. SERCA2a appears to play a key role for Ca2+ homeostasis, but little information regarding subcellular consequences of SERCA2a-overexpression is available.

Previous studies with SERCA2a-overexpressing transgenic mice [12,13], adenovirus-mediated gene transfer in neonatal rat myocytes [14,15], and animal models of HF reported enhanced contractility [16–18]. However, these studies investigated contractility under baseline conditions (force, shortening, ventricular pressure at low stimulation rates) but neither isometric twitch force nor SR Ca2+ content under the most physiological experimental conditions possible (37 °C) at increasing stimulation frequencies (up to 8 Hz). No detailed quantitative electrophysiological analyses of SR Ca2+ content, L-type Ca2+ currents (Ica), and NCX function (INa/Ca) were performed, and the effects of transgenic upregulation of SERCA2a on the expression and function of other Ca2+ regulatory proteins is completely unknown. Excessive SERCA2a-overexpression may even decrease contractility due to immediate Ca2+ reuptake before troponin C binding can occur (“futile” Ca2+ cycling) [19].

We recently reported that transgenic SERCA2a-overexpression in rat hearts (TG) leads to improved relaxation and contractility under normal and pressure overload conditions [20]. The aim of the present study was a detailed analysis of the effects of TG on E–C coupling processes under physiological conditions. Here, we demonstrate improved frequency-potentiation of force related to increased SR Ca2+ loading whereas transsarcolemmal Ca2+ cycling is reduced.

2. Methods

Ca2+ handling was investigated using TG (n = 35) and age-matched wild-type (WT) rats (n = 39) from one strain generated as reported recently [20]. SERCA2a protein level was overexpressed by 45.8 ± 6.1% in TG vs. WT. Data on heart and body weights did not differ between groups (Table 1a). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US NIH (Publication No. 85-23, revised 1996). Experiments were performed in isolated left (n = 39) and right (n = 6) ventricular trabeculae or papillary muscles obtained from 17 WT and 28 TG rat hearts.

2.1. RCC experiments

Muscles were mounted into a chamber, superfused with Krebs-Henseleit-buffer (KHB) containing (in mmol/L): NaCl 127, KCl 2.3, NaHCO3 25, KH2PO4 1.3, MgSO4 0.6, CaCl2 1.5, glucose 11, insulin 10 IU/L (37 °C), and electrically stimulated. In order to measure SR Ca2+ loading, rapid cooling contractures (RCCs) were elicited by a decrease in chamber temperature to 1 °C [10,21].

2.2. Intracellular Ca2+ transients

Ca2+ transients were assessed using aequorin which was injected into the muscles [8]. Aequorin light emission was analyzed using the amplitude (mV amplifier output) and time course of signals. Since aequorin is consumed when Ca2+ is bound we do not calibrate our signals.

2.3. Experimental protocol

FFR were tested by increasing frequency from 0.5–8 Hz. At each frequency recordings of isometric force were obtained followed by RCCs. In order to investigate the extent of which the SR gains Ca2+ during rest, rest intervals between 1–240 s were instituted from a basal frequency of 1 Hz and post-rest force or RCCs were measured. RCCs were normalized to basal values (0.5 Hz and 1 s rest, respectively) and only compared within each group. In a subset of experiments, isoproterenol (10−9 – 10−5 mol/L) was added to the solutions.

2.4. Myocyte isolation

Hearts were removed and perfused with a Tyrode’s solution containing (in mmol/L): NaCl 138, KCl 4, MgCl2 1, glucose 10, NaH2PO4 0.33, HEPES 10 (37 °C), to which collagenase (70 mg/50 mL) and protease (6 mg/50 mL) were added for myocyte isolation [22].

Table 1a

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Heart weight (g)</th>
<th>Heart weight/Body weight (mg/g)</th>
<th>Muscle cross sectional area (mm²)</th>
<th>Myocyte capacitance (pF)</th>
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<tr>
<td></td>
<td>WT</td>
<td>TG</td>
<td></td>
<td></td>
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<tr>
<td>Body weight (g)</td>
<td>383±34 (n = 11)</td>
<td>346±23 (n = 19)</td>
<td>n.s.</td>
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<td>Heart weight (g)</td>
<td>1.69±0.11 (n = 11)</td>
<td>1.44±0.07 (n = 19)</td>
<td>n.s.</td>
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<tr>
<td>Heart weight/Body weight (mg/g)</td>
<td>4.56±0.20 (n = 11)</td>
<td>4.28±0.13 (n = 19)</td>
<td>n.s.</td>
<td></td>
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<tr>
<td>Muscle cross sectional area (mm²)</td>
<td>0.026±0.003 (n = 17)</td>
<td>0.024±0.003 (n = 28)</td>
<td>n.s.</td>
<td></td>
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<tr>
<td>Myocyte capacitance (pF)</td>
<td>138±2 (n = 159)</td>
<td>175±7 (n = 68)</td>
<td>&lt;0.05</td>
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</table>
2.5. Recording techniques for patch-clamp experiments

Experiments were carried out with microelectrodes having resistances of 2–3 MΩ (37 °C). Cell capacitance was calculated by applying 5 mV steps from −80 mV in the hyperpolarizing direction and integrating the current required to charge the membrane when stepping back to −80 mV.

2.6. SR Ca²⁺ content

Absolute SR Ca²⁺ content was determined by measuring the integral of caffeine-induced inward $I_{Na/Ca}$. Myocytes were held at −80 mV with an electrode containing (mmol/L): NaCl 5, CsCl 110, tetraethylammonium chloride (TEA) 20, MgCl₂ 1, MgATP 2, HEPES 10, EGTA 0.05. The cell was superfused in a microstream containing (mmol/L): NaCl 135, MgCl₂ 1, CaCl₂ 2, CsCl 10, glucose 10, HEPES 10. After a sequence of conditioning pulses (eight 200 ms pulses to 0 mV), followed by a 60 s rest to load the SR maximally with Ca²⁺, the cell was abruptly superfused for 6 s with caffeine (10 mmol/L) to release SR Ca. The resulting inward $I_{Na/Ca}$ was integrated (nA * ms = pC) and normalized to cell volume (pC/pL) and capacitance (pC/pF).

2.7. $I_{Ca}$

Myocytes were superfused with solution containing (mmol/L): 135 NaCl, 10 CsCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and voltage-clamped [23] with an electrode filled with (mmol/L) 120 CsCl, 20 TEA, NaCl 1, MgCl₂ 1, EGTA 10, MgATP 2, HEPES 10. Myocytes were held at −80 mV and a prepulse to −45 mV inactivated Na⁺ channels. $I_{Ca}$ was then activated by stepwise depolarizing from −30 to +80 mV (200 ms). Steady-state inactivation curves were determined by depolarizing the membrane (−60 to +60 mV) with conditioning pulses of 2 s, followed by a pulse to +10 mV. The current amplitudes during the second pulse were

![Fig. 1](https://via.placeholder.com/150)
normalized to the maximal current amplitude, plotted as a function of the preceding membrane potential (Boltzmann function). Time constants of channel inactivation \((\tau_1, \tau_2)\) were determined by biexponential function fitting the current evoked during a pulse to 0 mV.

2.8. \(I_{Na/Ca}\)

For recordings of NCX activity, reverse-exchange current was measured. Myocytes were held at \(-40\) mV. The pipette contained (mmol/L): CsCl 130, TEA 20, NaCl 20, MgCl\(_2\) 1, EGTA 14, MgATP 2, HEPES 10, CaCl\(_2\) 0.1. To activate outward currents, cells were superfused in a microstream containing (mmol/L): 145 NaCl, CsCl 10, 1 MgCl\(_2\), 2 CaCl\(_2\), 10 glucose, 10 HEPES. Outward exchange current was activated when the cell was immersed for 5 s in an adjacent microstream of solution containing Li\(^+\) instead of Na\(^+\).

2.9. Western blots

For measuring protein expression of NCX and LTCC we performed standard Western blot techniques [11,19]. Briefly, equal amounts of protein were subjected to SDS-PAGE and blotted to nitrocellulose. The blots were blocked in 5% nonfat milk dissolved in TBS (20 mmol/L Tris–Cl, pH 7.5, 50 mmol/L NaCl), then probed with NCX antibodies (ABR), antibodies against the \(\alpha_{1C}\) subunit of LTCC (Alamone), or GAPDH (Bio Trend) in TBS containing 0.5% nonfat milk and 0.1% Tween 20. The membranes were incubated for 1 h with horse radish peroxidase-labeled antibody (Amersham). Immunoreactive bands were visualized and exposed to x-ray film.

<table>
<thead>
<tr>
<th>Table 1b Force-frequency data</th>
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<tr>
<td>Frequency (0.5 Hz)</td>
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<tr>
<td>Force (mN/mm(^2))</td>
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<tr>
<td>Twitch TP (ms)</td>
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<tr>
<td>Twitch TT (ms)</td>
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<td>(\Delta) aequorin light signal (%)</td>
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Wild-type (WT), SERCA2a-overexpressing rats (TG). Time to peak force (TP), total time (TT). \(* P<0.05\) 8 Hz vs. 0.5 Hz. \({}^\# P<0.05\) WT vs. TG.

Fig. 2. Influence of frequency on kinetics of twitches and \(Ca^{2+}\) transients. A. Normalized twitches and \(Ca^{2+}\) transients in muscles from a WT and TG heart (1 Hz). B. Average values for 50% and 90% relaxation (RT\(_{50\%}\), RT\(_{90\%}\)) of force (WT \(n=15\), TG \(n=19\)), and \(Ca^{2+}\) transients (inset: Aequorin RL\(_{90\%}\); WT \(n=5\), TG \(n=8\)) at increasing frequencies. \(* P<0.05\) vs. 0.5 Hz, \({}^\# P<0.05\) WT vs. TG.
2.10. Statistics

Data are expressed as mean±S.E.M. Statistical analysis was performed with Student’s unpaired t-test and one or two-way ANOVA followed by Student–Newman–Keuls test where appropriate. Statistical significance was taken as $P<0.05$.

3. Results

3.1. Frequency-dependent effects on force and SR Ca$^{2+}$ content

Fig. 1A shows representative isometric twitches and RCCs in muscles from WT and TG. When the muscles were rapidly cooled from 37 to 1 °C, Ca$^{2+}$ was released from the SR and RCCs developed rapidly [2,24]. Twitch force marginally increased upon raising frequency in WT without changes in RCCs. In contrast, force and RCCs greatly increased in TG showing a frequency-dependent increase in SR Ca$^{2+}$ load in TG but not WT.

Average data for twitch force and RCCs in WT (Fig. 1B) did not change significantly when stimulation frequency was increased. In contrast, in TG force frequency-dependently increased by $168 \pm 35\%$ at 8 Hz (vs. 0.5 Hz; $P<0.05$). This positive FFR was accompanied by an increase in RCC amplitude by $88 \pm 31\%$ ($P<0.05$). Interestingly, absolute force values for twitches at 0.5 Hz did not differ significantly between WT and TG. However, increasing frequency to 8 Hz leads to higher force in TG compared to WT (Table 1b). It should be mentioned that WT rat myocardium usually shows a flat or only slightly positive FFR, and even negative FFR were reported [2,21,24].

3.2. Increasing stimulation rate accelerates twitch contractions and Ca$^{2+}$ transients

The time course of twitches and Ca$^{2+}$ transients critically depends on SR Ca$^{2+}$ uptake and is physiologically accelerated at high stimulation rates [2,25]. From typical twitch contractions and Ca$^{2+}$ transients at 1 Hz (Fig. 2A) it can be seen that time to peak amplitudes is decreased, and relaxation and Ca$^{2+}$ transient decline are shortened in TG. Fig. 2B shows average results for 50% and 90% relaxation of twitch force (RT$_{50\%}$, RT$_{90\%}$). At each frequency relaxation was faster by $\sim 10\%$ in TG compared to WT. RT$_{50\%}$ decreased in WT from $35.1 \pm 0.9$ ms at 0.5 Hz to $26.4 \pm 1.6$ ms at 8 Hz ($P<0.05$), and in TG from $32.2 \pm 0.6$ to $25.1 \pm 1.5$ ms ($P<0.05$), and similarly in RT$_{90\%}$. In addition, values for 90% decay of Ca$^{2+}$ transients are presented for WT and TG showing muscles with significant faster Ca$^{2+}$ transient decay in TG at 0.5 Hz ($44.0 \pm 1.3$ vs. $35.0 \pm 1.1$ ms, $P<0.05$) and 8 Hz ($42.4 \pm 1.1$ vs. $30.8 \pm 0.6$ ms, $P<0.05$). Similarly, the time to peak tension and total twitch time decreased with increasing stimulation rates (Table 1b). Again, at all frequencies parameters were smaller in TG vs. WT.

3.3. $\beta$-adrenergic stimulation increases force and accelerates Ca$^{2+}$ transients

Fig. 3 summarizes the effects of $\beta$-adrenergic stimulation. TG and WT showed a concentration-dependent increase in force with isoproterenol ($10^{-5}$ mol/L vs. control) with a maximum of 199$\%$ for WT and 279$\%$ for TG ($P<0.05$). This inotropic response is associated with typical acceleration of relaxation parameters of force as well as Ca$^{2+}$ transient decay. RT$_{90\%}$ in WT decreased from

![Twitch Amplitude](image)

![Twitch RT](image)

Fig. 3. Influence of isoproterenol on amplitude and kinetics of twitches and Ca$^{2+}$ transients. Average values for twitch amplitude, RT$_{90\%}$ of force (WT $n=15$, TG $n=22$), and Ca$^{2+}$ transients (inset: Aequorin RL$_{90\%}$; WT $n=6$, TG $n=6$) when increasing isoproterenol concentration. *$P<0.05$ vs. 0.5 Hz. **$P<0.05$ WT vs. TG.
70.3 ± 2.9 ms at control to 50.9 ± 2.5 ms with 10^{-5} \text{ mol/L} isoproterenol (P < 0.05), and in TG from 65.9 ± 2.6 to 43.4 ± 1.9 ms (P < 0.05) with similar changes in RL_{90\%}. At each concentration of isoproterenol time parameters were shorter in TG vs. WT rats (by ~10%). These differences were even more pronounced at higher isoproterenol concentrations (~15%).

### 3.4. Increasing rest intervals raises SR Ca^{2+} content

Since RCCs are a semiquantitative measure of SR Ca^{2+} content, quantitative SR Ca^{2+} load was assessed using caffeine-induced inward $I_{\text{Na}/\text{Ca}}$ [2]. Fig. 4A shows representative traces for $I_{\text{Na}/\text{Ca}}$ with increased current in TG vs. WT. On average, $I_{\text{Na}/\text{Ca}}$ was 4.98 ± 0.43 pA/pL in WT and increased in TG to 8.80 ± 0.20 pA/pL (P < 0.05). The integrated charge transferred was also significantly higher in TG (8.13 ± 1.38 pC/pL or 1.28 ± 0.22 pC/pF) compared to WT (3.99 ± 0.28 pC/pL or 0.73 ± 0.05 pC/pF; P < 0.05). When normalizing to cell volume, absolute SR Ca^{2+} load was 84.3 ± 14.3 in TG vs. 41.4 ± 2.9 µmol/L cytosol in WT (P < 0.05). These results suggest that there is not only a frequency-dependent increase in SR Ca^{2+} load (as measured by RCCs), but also the absolute [Ca^{2+}] in the SR is higher in TG.

We also analyzed the potential of the SR to accumulate Ca^{2+} during rest periods (Fig. 4B). Rats typically show

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**Fig. 4.** Effects on SR Ca^{2+} content. A. Quantitative assessment of SR Ca^{2+} load measured as caffeine-induced inward $I_{\text{Na}/\text{Ca}}$ in myocytes from WT (n=10) and TG (n=6) hearts. The inset shows the average SR Ca^{2+} load. *P < 0.05 WT vs. TG. B. Average values for rest-dependent changes in twitch and RCC amplitudes in WT (n=10) and TG (n=10) muscles (% basal value 1 s rest). *P < 0.05 vs. 1 s. # P < 0.05 WT vs. TG.
potentiation of force after increasing rest intervals [2,21]. In WT, post-rest force increased by 126±44% (P<0.05) after 240 s, but RCCs did not change. In contrast, TG showed much larger post-rest twitches at any rest interval (after 240 s by 198±52%; P<0.05) associated with clear increases in RCCs (by 153±55%; P<0.05). This suggests large rest-dependent SR Ca2+ accumulation in TG.

3.5. Decreased $I_{Ca}$ and NCX function

Representative current–voltage relations show reduced current density at most voltages applied in TG vs. WT (Fig. 5A). Average data (Fig. 5B) depicts decreased peak $I_{Ca}$ density at 0 mV of $-10.2±1.1$ for TG vs. $-16.9±1.3$ pA/pF for WT myocytes (P<0.05). However, Western blot analysis revealed no change in LTCC protein expression in TG (n=10) vs. WT (n=9) hearts ($-3.8±6.7\%$). Inactivation parameters did not differ between WT and TG (Table 2). However, there is a clear trend of faster $I_{Ca}$ decline in TG which might be attributable to feedback through the increased SR Ca2+ content in TG.

![Fig. 5. Effects on L-type Ca2+ currents ($I_{Ca}$). A. Representative $I_{Ca}$ in WT and TG myocytes when depolarizing stepwise from $-30$ to $+80$ mV. B. IV relation with average values for WT (n=24) and TG (n=6) myocytes. *P<0.05 WT vs. TG.](image-url)

<table>
<thead>
<tr>
<th>$I_{Ca}$ parameters</th>
<th>WT</th>
<th>TG</th>
<th>$P$-value</th>
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<tbody>
<tr>
<td>$\tau_1$ (ms)</td>
<td>45.1±3.1 (n=24)</td>
<td>40.7±1.1 (n=6)</td>
<td>n.s</td>
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<tr>
<td>$\tau_2$ (ms)</td>
<td>8.7±0.7 (n=24)</td>
<td>6.8±0.5 (n=6)</td>
<td>n.s</td>
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<tr>
<td>$V_{0.5}$ (mV)</td>
<td>-34.9±0.7 (n=16)</td>
<td>-34.5±5.7 (n=5)</td>
<td>n.s</td>
</tr>
<tr>
<td>$k$</td>
<td>5.1±0.1 (n=16)</td>
<td>4.9±0.2 (n=5)</td>
<td>n.s</td>
</tr>
</tbody>
</table>

Wild-type (WT), SERCA2a-overexpressing rats (TG). Time constants ($\tau_1$, $\tau_2$) of $I_{Ca}$ inactivation. Steady-state activation with $V_{0.5}$ as membrane potential for half-maximal activation, $k$ as slope-factor.
Representative traces for \( I_{Na/Ca} \) density measured by \( I_{Na/Ca} \) reverse mode were clearly reduced in TG compared to WT (Fig. 6A). Average data (Fig. 6B) show significantly lower \( I_{Na/Ca} \) outward current density in TG (0.17 \pm 0.02 pA/pF) vs. WT (0.46 \pm 0.05 pA/pF). This underlines that NCX function is reduced in TG vs. WT. Interestingly, we found only a slight reduction in NCX protein expression in TG vs. WT (\( n = 10 \)) vs. WT (\( n = 9 \)) hearts of \(-12.0 \pm 7.4\%\).

### 4. Discussion

This is the first detailed report on E–C coupling processes after transgenic SERCA2a-overexpression in rat myocardium under physiological conditions. Our main findings are that 1) a \(~45\%\) increase in SERCA2a protein levels is associated with improved contraction and relaxation kinetics and pronounced frequency or rest potentiation of force; 2) the functional effects were related to enhanced SR \( Ca^{2+} \) content and increased \([Ca^{2+}]_i\); and 3) \( I_{Ca} \) and NCX activity are reduced indicating a shift from transsarcolemmal to intracellular \( Ca^{2+} \) cycling.

#### 4.1. FFR and relaxation

Depending on experimental conditions or strain, rat FFR can be negative, positive, or flat due to differences in SR \( Ca^{2+} \) handling [26,27]. In the present study, force and SR \( Ca^{2+} \) content in trabeculae of WT rats did not increase with increasing frequencies. This indicates that despite frequency-induced increases in \( I_{Ca} \) (due to increased frequency of action potentials) [2], the SR of WT rats is unable to respond to this extra amount of \( Ca^{2+} \) with increased \( Ca^{2+} \) release. This phenomenon was previously explained with the inability of WT rat heart to overcome the negative effects of refractoriness of SR \( Ca^{2+} \) release at high stimulation rates [24]. In contrast, in TG myocardium refractoriness can be easily overcome due to increased \( Ca^{2+} \) load resulting in a clear positive FFR. Most likely the increase in SR \( Ca^{2+} \) load is attributable to a frequency-dependent activation of SERCA2a (as previously shown by paired RCCs [10]), e.g. by CaMKII in combination with a decrease of \( Ca^{2+} \) extrusion via NCX (with less activity in TG) as a consequence of increased \([Na^+]_i\) [28]. Another aspect is that phospholamban (PLB) expression is not altered in TG vs. WT [20]. Therefore, SERCA2a/PLB ratio is greatly increased with less PLB inhibiting SERCA2a leading to increased SR \( Ca^{2+} \) uptake in TG. Consequently, SERCA2a-overexpression leads to a greatly enhanced SR \( Ca^{2+} \) uptake and subsequent positive FFR.

Similarly, Hajjar et al. [29] reported for isolated rat myocytes that SERCA2a-overexpression can restore impaired FFR initially induced by PLB overexpression. The present data also agree with our observations in failing human myocardium that the FFR may be improved by low concentrations of forskolin [30] or isoproterenol [31]. However, stimulation of the cAMP–PKA axis not only stimulates SERCA2a activity by PLB phosphorylation, but has multiple further effects such as phosphorylation of LTCC, ryanodine receptors (RyR), or troponin C. Therefore, in our previous studies stimulation of the cAMP–PKA axis was far from normalizing contractility, and higher concentrations of forskolin or isoproterenol even had detrimental effects related to \( Ca^{2+} \) overload. In contrast, specific overexpression of SERCA2a protein selectively improves SR \( Ca^{2+} \) uptake [29].

It is important to note that the beneficial effect of SERCA2a-overexpression on contractility is most prominent at higher stimulation rates. This was previously only incompletely appreciated in muscles from transgenic mice at 22 \(^\circ\)C which were stimulated up to 2 Hz [32]. Since our
experiments in TG were performed at physiological temperature and frequencies (37 °C, up to 8 Hz), we believe that our results of a frequency-dependent increase in twitch force (and SR Ca²⁺ content) are of major importance for the understanding of the physiological consequences of SERCA2a-overexpression.

Twitch parameters at all frequencies were accelerated in TG rats. This observation can be explained by increased amounts of SERCA2a proteins in TG and CaMKII-dependent activation of SR Ca²⁺ uptake [33]. This effect was also observed in studies using PLB knockout mice [34] or SERCA2a transfected rabbit myocytes [35]. Intrinsic myofilament properties might also change relaxation kinetics [36].

β-adrenergic stimulation increases force and shortens activation and relaxation parameters of force and Ca²⁺ transients [37]. Similarly, in the present study force increased while relaxation parameters decreased. In addition, relaxation at each isoproterenol concentration was accelerated in TG vs. WT and increased concentration-dependently (from ~10% to ~15%). Interestingly, RT₉₀₋₉₀ at the highest isoproterenol concentration was faster as compared to the relaxation at the highest stimulation frequency. Similar results were recently shown in SERCA2a transfected rabbit myocytes [38].

4.2. SR Ca²⁺ content

During rest Ca²⁺ is removed from the cytosol into the SR by SERCA2a and across the sarcolemma mainly by NCX [2]. There is a finite rate of SR Ca²⁺ leak which can either be taken up by the SR (resulting in constant SR Ca²⁺ load) or it can be partly removed from the cytosol by NCX (decreasing SR Ca²⁺ content). In the absence of SR Ca²⁺ depletion rest potentiation of twitches appears to be normal in rat myocardium. This has been interpreted as a recovery of E–C coupling mechanism from a refractory state with increased fractional SR Ca²⁺ release [39,40].

The current results indicate that WT rat myocardium shows some increase in post-rest force associated with a marginal increased SR Ca²⁺ content. It appears that this rest potentiation of force is due to a very slow phase of recovery of SR Ca²⁺ release processes from a previous activation. This may be related to a slow phase of RyR recovery of normal Ca²⁺ sensitivity from an adapted or inactivated state and has been observed at the level of Ca²⁺ spark availability in rat and rabbit myocytes [2]. In contrast, TG is characterized by pronounced increases in post-rest force associated with marked increases in SR Ca²⁺ content and release. The reason for increased SR Ca²⁺ content during rest remains controversial. One reason could be that the SR content increases Ca²⁺ by means of decreased Ca²⁺ efflux via NCX [2]. While a slightly higher intracellular [Na⁺] in the cells from TG rats (even by 1 mM) could also explain this in principle, there is no evidence indicating that this is the case. Nevertheless, increased SERCA2a protein expression results in more effective SR Ca²⁺ reloading during rest. Similarly we could recently show that positive inotropic compounds which stimulate SERCA2a can improve the impaired post-rest behavior in failing human myocardium [31].

To supplement the semiquantitative RCC results with more quantitative analysis we quantified SR Ca²⁺ load by patch-clamp experiments. We confirmed that SR Ca²⁺ load in TG vs. WT is ~2-fold increased. This is in agreement with previous findings in SERCA2a transgenic mice [41]. However, the difference in SR Ca²⁺ load reported by these authors between TG vs. WT (~25%) was much smaller to our study. The most important differences between the two studies may be the different levels of SERCA2a-overexpression (20% vs. 46% in our study), the experimental conditions, as well as species. Yao et al. [41] used SERCA2a transgenic mice and measured at low temperature (25 °C) and low [Ca²⁺]o (1 mM). In the present experiments we used myocytes from SERCA2a transgenic rats at 37 °C and [Ca²⁺]o of 2 mM.

As a limitation of the study, it should be pointed out that the total amount of Ca²⁺ extruded by the NCX even might underestimate SR Ca²⁺ content since Ca²⁺ removal by sarcolemmal Ca²⁺ pumps and mitochondrial Ca²⁺ uptake were not taken into account [2]. Unfortunately, we do not have any information about these pathways in TG. Of note, decreased NCX activity measured in TG (although integral Iₛₐ/Cₐ was measured) might contribute to a possible underestimated SR Ca²⁺ content. Thus, NCX current measurements are qualitative in nature and may not always exactly reflect quantitative changes. Finally, a contribution of Ca²⁺ activated Cl currents and non-selective cation currents cannot be ruled out. However, when applying nickel to block NCX the current was completely abolished. Moreover, since no differences in these currents are expected to occur in TG vs. WT we do not believe that this has an impact on our main conclusions.

4.3. IᵥCa and NCX

It was previously reported that IᵥCa was unchanged in TG vs. WT mice, whereas Iₛₐ/Cₐ was decreased by ~10% [41]. We suggest in the present report that Iₛₐ/Cₐ is reduced even ~2.5-fold and IᵥCa by ~40% in TG vs. WT rats. As discussed in the previous section, these discrepancies may be due to differences in animal models and experimental conditions. However, these data also demonstrate that even in rat myocardium where Ca²⁺ cycling already in WT predominantly depends on SR Ca²⁺ transport [2], SERCA2a-overexpression further enhances intracellular vs. transsarcolemmal Ca²⁺ cycling.

When measuring Ca²⁺ influx and efflux and comparing these data we found very similar values. Not surprisingly to us, when calculating absolute values we found that IᵥCa (Ca²⁺ influx) was 17.4 μmol/L for WT and 7.3 μmol/L for TG, i.e. the amount of Ca²⁺ entering the cell in TG via LTCC was
only 42% compared to WT. Given the balance of fluxes, Ca^{2+} efflux via NCX should meet Ca^{2+} entry via LTCC. Indeed, when NCX outward current density (I_{Na}/Ca) was measured, there was a reduction of the transport capacity to 37% in TG vs. WT. Although not measured during regular E–C coupling, the latter result suggests the NCX activity seems to be reduced to a very similar amount as compared to the reduced I_{Ca}. In consequence, in the light of unchanged protein expression for NCX and LTCC, functional data shows reduced transsarcolemmal Ca^{2+} cycling vs. SR function. Nevertheless, further investigations in the presence of a functional SR are needed to fully understand the consequences of SERCA2a-overexpression.

Interestingly, there are striking similarities between our study (decreased I_{Ca} and increased SR Ca^{2+} content) and a study by Henderson et al. [42] who showed a 50% reduction in I_{Ca} in NCX-knockout mice. It may be that the same general phenomenology is occurring in our TG rats. Specifically, increased SR Ca^{2+} loads will lead to reduced Ca^{2+} inactivation of LTCC (i.e. faster Ca^{2+} efflux from the SR might promote rapid Ca^{2+} inactivation). However, neither in our study nor in their study differences in inactivation kinetics were found (although we saw an almost significant trend with faster kinetics in TG vs. WT). Most likely, Ca^{2+} induced inactivation from increased SR Ca^{2+} release in TG does play a role. However, Henderson et al. speculated that the cells rather limit Ca^{2+} influx than upregulate Ca^{2+} efflux. Increased cytosolic/SR Ca^{2+} cycling might activate Ca^{2+} dependent transcription pathways leading to synthesis of non-functional channel protein as a means of protection from Ca^{2+} overload. However since this is completely speculative the unknown mechanisms of I_{Ca} and I_{Na}/Ca reduction are a limitation of the present study.

4.4. Clinical relevance for HF

SERCA2a-overexpression might be useful in human HF [43] especially in patients with a negative FFR or diastolic dysfunction due to cytosolic Ca^{2+} overload. Because disturbed Ca^{2+} handling seems to play a role in the pathophysiology of HF, the relevance of SERCA2a-overexpression to rescue disturbed Ca^{2+} cycling and myocardial function is of particular importance. As shown by del Monte et al. [44] gene transfer of SERCA2a can improve contractility and can restore the impaired FFR in myocytes from failing human hearts. Nevertheless, further experimental work will be necessary to investigate the effects of SERCA2a-overexpression in transgenic animal models and SERCA2a gene transfer in human cardiac tissue on Ca^{2+} handling and myocardial performance.

However, an increased amount of SR Ca^{2+} may also be detrimental due to an increased potential for Ca^{2+} leak through RyR, potentially resulting in delayed afterdepolarizations and arrhythmias. This is even more important in HF where RyR may be hyperphosphorylated [33,45]. Indeed, we found increased mortality of SERCA2a TG rats after myocardial infarction, as reported recently by our group [46]. Therefore, SERCA2a-overexpression may improve Ca^{2+} handling but also induce arrhythmias under certain conditions. Future therapeutic approaches in HF may therefore be targeted to both improved SR Ca^{2+} uptake and stabilization of RyR.

In summary, whether and to what extent overexpressing SERCA2a might be beneficial in the treatment of HF has to be further investigated, since recent reports by Teucher et al. [19] and Chen et al. [46] in contrast to earlier work [12–18] showed negative effects on E–C coupling and increased arrhythmias under certain conditions.

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References