Modulation of SR Ca\(^{2+}\) release by the triadin-to-calsequestrin ratio in ventricular myocytes

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In cardiac myocytes, the release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) is the crucial process for the initiation of contraction. It has been shown that the SR luminal Ca\(^{2+}\)-binding protein calsequestrin (CSQ) plays an important role in SR function by interacting with the SR Ca\(^{2+}\) release channel (36). CSQ was characterized as a highly acidic protein that binds Ca\(^{2+}\) with high capacity and undergoes major conformational changes after Ca\(^{2+}\) binding (10, 27). Polymerization occurs at high SR Ca\(^{2+}\) content and increases the Ca\(^{2+}\)-binding capacity of the protein itself (30). The importance of CSQ in regulating cardiac contractility was demonstrated by use of transgenic models. Cardiac-specific overexpression of CSQ increased SR Ca\(^{2+}\) storage capacity of myocytes but was associated with an attenuation of SR Ca\(^{2+}\) release, leading to a depressed cardiovascular function and premature death (12, 35). Moreover, these changes in SR Ca\(^{2+}\) handling were associated with downregulation of junctional SR proteins (e.g., triadin (TRN)).

It is commonly believed that CSQ acts not only as a local intra-SR Ca\(^{2+}\) buffer but as a Ca\(^{2+}\)-dependent regulator of ryanodine receptor (RYR) function (9, 19). SR Ca\(^{2+}\) release is controlled by a tetrameric protein complex that is localized at the junctional SR and consists of the RYR, TRN, junctin (JUN), and CSQ. Functional interactions among TRN, JUN, and CSQ can modulate the responsiveness of the RYR to luminal Ca\(^{2+}\) (9, 36, 37). According to this model, the binding of CSQ to TRN and JUN inhibits the RYR activity at low luminal Ca\(^{2+}\) concentrations. When luminal Ca\(^{2+}\) increases, this inhibition is gradually relieved as the Ca\(^{2+}\) binding sites on CSQ become occupied by Ca\(^{2+}\), attenuating interactions among CSQ and TRN and/or JUN. This results in an increased open probability of the RYR (3).

TRN is an integral membrane protein that is composed of a single membrane spanning domain, a short cytoplasmic NH2-terminal segment, and a long, highly positively charged COOH-terminal domain extending into the lumen of the SR (20). These molecular characteristics provide the structural basis for coordinated interactions between the proteins of the tetrameric complex during excitation-contraction (E-C) coupling. This suggests that the TRN expression level is a critical determinant of SR Ca\(^{2+}\) handling. Indeed, ablation of TRN causes the loss of Ca\(^{2+}\) release units, impaired E-C coupling, and cardiac arrhythmias (4). Moreover, the protein expression of junctional SR proteins (e.g., CSQ) was lower in TRN knockout mouse hearts, which was surprising since transgenic overexpression of TRN was associated with similar effects (15). Thus it remains to be elucidated whether the lower expression level of TRN in CSQ-overexpressing mice reflects either a protective mechanism in maintaining cardiac function or rather a negative consequence of CSQ overexpression resulting in subsequent heart failure. This led us to the question

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whether a normalization of the impaired TRN-to-CSQ protein ratio may improve the depressed SR Ca\(^{2+}\) handling in CSQ-overexpressing myocytes and restore cardiac contractility.

Therefore, the primary goal of the present study was the functional characterization of double transgenic mice with heart-directed overexpression of CSQ and TRN. The effects on SR Ca\(^{2+}\) handling and cardiac performance were tested in vivo, in whole hearts, and in isolated myocytes.

METHODS

**Experimental animals.** Generation of transgenic mouse lineages overexpressing either cardiac canine TRN1 or calsequestrin was performed by use of the cardiac-specific α-myosin heavy chain promoter as described previously (12, 21). Littermate mouse quadruplets derived from cross-breeding of both lineages, resulting in wild-type (WT), triadin-overexpressing (TGTRN), calsequestrin-overexpressing (TGCSQ), and double transgenic mice (TGC

**Histological, immunofluorescence, and electron microscopy analysis.** For histological examination of left ventricles, longitudinal cuts were made along the length of the myocardium and immediately fixed in 4% buffered formalin, dehydrated, and embedded in paraffin. Tissue sections of 5-μm thickness were deparaffinized, rehydrated, and stained by the protocol of trichrome Masson-Goldner for estimation of interstitial fibrosis. To determine whether transgenic canine TRN colocalizes with endogenous mouse JUN, we performed indirect immunofluorescence in TGTRN myocytes. A suspension of cardiac myocytes (∼100 μl) was placed into polylysine-covered chamber slides (Thermo Scientific). The cells were fixed in precolloled methanol for 5 min and then washed with PBS (±1% BSA) for 15 min. PBS was used in all following dilutions and washing steps. To block unspecific binding sites, incubation with the Fab fragment of goat anti-mouse IgG (100 μg/ml; Dianova) was performed. Myocytes were then immunolabeled with mouse monoclonal antibody 9G5 (15) raised against canine TRN (1:300) and rabbit polyclonal antibody JCN4 raised against murine JUN (1:300) for 1 h. Primary antibodies were visualized with goat anti-mouse Alexa Fluor 594 (Invitrogen; 1:300) and goat anti-rabbit Alexa Fluor 488 (1:300), respectively, using confocal microscope LSM 710 Meta (Zeiss, Germany). Finally, the red (for TRN) and green (for JUN) fluorescence components were recorded and merged for analysis. Electron microscopy was performed on small pieces of ventricular heart tissue as described previously (15).

**SDS-PAGE and Western blotting.** To determine the protein expression of SR proteins, hearts were excised and immediately homogenized for 90 s at 4°C in 1 ml of medium containing 10 mM histidine and 0.25 M sucrose (pH 7.4). Protein concentrations were determined in heart homogenates (26). Thereafter, samples were solubilized in 5% SDS buffer (for composition, see Ref. 15). Fifty micrograms of homogenate protein were separated on 8% SDS-PAGE (32). To determine the protein expression of the RyR, 200 μg of homogenate protein were subjected to 5% PAGE. Separated proteins were transferred to nitrocellulose membranes and incubated with different antibodies. The primary antibodies recognizing the RyR, CSQ, TRN, JUN, L-type Ca\(^{2+}\) channel (Ca.1.2), sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase 2a (SERCA2a), or phospholamban were used as described previously (5, 7, 17). The amounts of bound primary antibodies were detected by 125I-labeled protein A (Amersham Biosciences).

**Echocardiography.** Echocardiographic measurements were performed on sedated mice (diazepam: 17.5 mg/kg body wt ip) as described previously (18). Cardiac dimensions, left ventricular wall thickness, and cardiac function were assessed by M-Mode, two-dimensional echocardiography, and Doppler measurements (18). Echocardiographic parameters were determined under basal and acute isoproterenol (ISO)-stimulated conditions (ip bolus injection: 1 mg/kg body wt).

**Left ventricular catheterization.** Left ventricular catheterization was performed in closed-chest mice (14). Briefly, the left jugular vein was cannulated with a custom-fashioned polyethylene tube connected to a microinfusion pump for drug administration. The polyethylene catheter (Micro-Med) was inserted into the aorta via the right carotid artery and advanced into the left ventricle under continuous monitoring of the pressure waveform. Analog pressure signals were obtained by using the pressure transducer TXD-310 (Micro-Med), recorded, and digitized at a sampling rate of 1,000 Hz and a sampling period of 1 min with the PowerLab system (ADInstruments), as described previously (24). Heart rate, left ventricular pressure, and the first derivatives of left intraventricular pressure (±dP/dt) were monitored continuously, recorded, and analyzed after a 10-min period of stabilization. Increasing doses of dobutamine were injected into the left jugular vein, and the effects on above parameters were determined.

**Work-performing heart preparations.** Work-performing heart preparations were utilized as described previously (15) using a perfusion system for isolated hearts (Hugo Sachs Elektronik). Mice were anesthetized intraperitoneally with urethane (2.0 g/kg body wt) and treated with 1.5 U of heparin. Hearts were removed from the opened chest and perfused retrogradely with oxygenized Krebs-Henseleit buffer (37°C) containing 118 mM NaCl, 25 mM NaHCO3, 0.5 mM Na-EDTA, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, and 11 mM glucose. During the short period of retrograde perfusion in the Langendorff modus, the pulmonary vein was cannulated. The perfusion of the heart was then changed to an anterograde mode. Heart rate and first derivatives of left intraventricular pressure (±dP/dt) were recorded at an afterload of 50 mmHg and a perfusion rate of 5 ml/min.

**Isolation of ventricular myocytes.** Ventricular myocytes were isolated enzymatically using a slightly modification of a protocol published before (15). First, the mice were pretreated with heparin (1.5 U) and asphyxiated with carbon dioxide. After bilateral thoracotomy, the heart was quickly excised and cannulated through the aorta fixed in 4% buffered formalin, dehydrated, and embedded in paraffin. Cross-breeded mice of the fifth generation or older in the F1, F2, and F3 generations were utilized as described previously (15) using a perfusion system for isolated hearts (Hugo Sachs Elektronik). Mice were anesthetized intraperitoneally with urethane (2.0 g/kg body wt) and treated with 1.5 U of heparin. Hearts were removed from the opened chest and perfused retrogradely with oxygenized Krebs-Henseleit buffer (37°C) containing 118 mM NaCl, 25 mM NaHCO3, 0.5 mM Na-EDTA, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, and 11 mM glucose. During the short period of retrograde perfusion in the Langendorff modus, the pulmonary vein was cannulated. The perfusion of the heart was then changed to an anterograde mode. Heart rate and first derivatives of left intraventricular pressure (±dP/dt) were recorded at an afterload of 50 mmHg and a perfusion rate of 5 ml/min.

**Measurement of contractile Ca\(^{2+}\) transients and myocyte contractility.** Measurements of the ventricular myocyte function were performed using a dual-emission microfluorescence system (Photon Technologies) combined with an epifluorescent microscope (Diaphot 200; Nikon), CCD camera (XC-75; Sony), and a video edge detection system (VED-105; Crescent Electronics). Myocytes were incubated with 25 μM indo-1/AM (Molecular Probes) for 15 min at RT in a chamber mounted on the microscope. This chamber allowed simultaneous perfusion with Tyrode’s solution (composition in mM: 140 NaCl, 5.8 KCl, 0.5 KH2PO4, 0.4 NaH2PO4, 0.9 MgSO4, 10 HEPES, 1 glucose, and 2 CaCl2, pH 7.45) and electrical field-stimulation. After incubation without perfusion and stimulation, the excess of indo-1/AM was washed out for 15
The cells were then superfused with Tyrode's solution and electrically paced with 0.5 Hz. Indo-1 fluorescence was recorded at RT in single myocytes under excitation at 365 nm, and the emitted fluorescence was recorded at 405 and 495 nm. The fluorescence ratio of both wavelengths was used as an index of the cytosolic Ca$^{2+}$/H$^{10}$ concentration. Because diastolic intracellular Ca$^{2+}$/H$^{10}$ concentration ([Ca$^{2+}$]i) ratios were similar among all four experimental groups, [Ca$^{2+}$]i peak amplitudes reflect only changes in cytosolic [Ca$^{2+}$]i during

![Image](https://via.placeholder.com/150)

Table 1. Expression of Ca$^{2+}$/H$^{10}$ regulatory proteins

<table>
<thead>
<tr>
<th></th>
<th>WT (n = 6)</th>
<th>TG$^{TRN}$ (n = 6)</th>
<th>TG$^{CSQ}$ (n = 6)</th>
<th>TG$^{XT}$ (n = 6)</th>
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<tbody>
<tr>
<td>Triadin, %</td>
<td>100 ± 6</td>
<td>381 ± 41†</td>
<td>48 ± 9‡</td>
<td>285 ± 45‡</td>
</tr>
<tr>
<td>Calsequestrin, %</td>
<td>100 ± 7</td>
<td>92 ± 17</td>
<td>447 ± 107‡</td>
<td>430 ± 42‡</td>
</tr>
<tr>
<td>Junctin, %</td>
<td>100 ± 6</td>
<td>34 ± 4‡</td>
<td>60 ± 10‡</td>
<td>44 ± 11†</td>
</tr>
<tr>
<td>RyR2, %</td>
<td>100 ± 4</td>
<td>101 ± 11</td>
<td>80 ± 11</td>
<td>68 ± 14†</td>
</tr>
<tr>
<td>Ca$_{1,2}$, %</td>
<td>100 ± 4</td>
<td>101 ± 5</td>
<td>36 ± 15‡</td>
<td>66 ± 11‡‡</td>
</tr>
<tr>
<td>SERCA2a, %</td>
<td>100 ± 8</td>
<td>101 ± 7</td>
<td>101 ± 7</td>
<td>61 ± 11‡†</td>
</tr>
<tr>
<td>Phospholamban, %</td>
<td>100 ± 18</td>
<td>92 ± 11</td>
<td>160 ± 42</td>
<td>126 ± 25</td>
</tr>
<tr>
<td>Triadin-to-calsequestrin ratio</td>
<td>12.6 ± 0.6</td>
<td>68.3 ± 20.5</td>
<td>2.0 ± 0.7‡*</td>
<td>8.4 ± 1.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Levels of regulatory sarcoplasmic reticulum and sarcolemmal proteins were determined in heart homogenates of wild-type (WT), monograftic (TG$^{TRN}$ and TG$^{CSQ}$), and double transgenic (TG$^{XT}$) mice after scanning $^{125}$I-labeled immunoblots in a STORM Imager (Molecular Dynamics). For statistical analysis of the triadin-to-calsequestrin ratio, Kruskal-Wallis ANOVA test on ranks was used. All pair-wise multiple comparison procedures were done by Tukey's test. RyR2, ryanodine receptor; Ca$_{1,2}$, L-type Ca$^{2+}$/H$^{10}$ channel; SERCA2a, sarco(endo)plasmic reticulum Ca$^{2+}$/H$^{10}$-ATPase 2a. *$P < 0.05$ vs. WT; †$P < 0.05$ vs. TG$^{TRN}$; ‡$P < 0.05$ vs. TG$^{CSQ}$. 

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systole. Myocyte shortening was performed simultaneously and visualized on a monitor (Pieper), which was connected to a video integrator (Pieper) interfaced to a video camera (Pieper) attached to the microscope. The maximum contraction was normalized to resting cell length and expressed as percentage of shortening. Data were collected at 40 Hz, and acquisition and processing were supported by the Felix 1.4 software (Photon Technologies). Myocyte shortening was also examined under administration of 1 μM ISO. To determine the SR Ca\(^{2+}\) load, caffeine was applied directly to isolated myocytes. First, myocytes were stimulated at 0.5 Hz and perfused with Tyrode’s solution (composition see above). After a 30-s rest period to achieve maximal SR Ca\(^{2+}\) load, caffeine-induced Ca\(^{2+}\) release was initiated by rapid application of 10 nM caffeine using a fast solution switching system (Warner Instrument) for 60 s. The peak amplitude of caffeine-induced Ca\(^{2+}\) transients was determined.

\(^{[3]H}\)ryanodine binding. Detection of \(^{[3]H}\)ryanodine binding to RyRs was performed at 37°C for 60 min by use of 45 μg homogenate protein as described previously (15). The homogenate was incubated in a buffer containing 20 mM MOPS (pH 7.1), 1 mM CaCl\(_2\), 0.6 M NaCl, and the saturating concentration of 15 nM \(^{[3]H}\)ryanodine. Nonspecific binding was determined in the same buffer in the presence of 10 μM cold ryanodine. Samples were filtered (Millipore) and rinsed repetitively with ice-cold saline, and the remaining radioactivity on the filters was quantified in a liquid scintillation counter.

Statistics. Data are reported as means ± SE. All of our data were distributed normally, which was confirmed by Shapiro-Wilk normality test. Statistical differences between groups were calculated by ANOVA followed by Bonferroni’s t-test. \(P < 0.05\) was considered significant.

RESULTS

Heart-directed overexpression of TRN and CSQ in mice. The overexpression of dog TRN and/or CSQ in myocardium was achieved by use of the α-myosin heavy chain promoter (8). Successful TRN and/or CSQ overexpression was confirmed by PCR (Fig. 1A) and quantified in transgenic mouse hearts on protein level (Fig. 1B). This analysis revealed a comparable overexpression of CSQ in TG\(^{CSQ}\) and TG\(^{CXT}\) mice (Table 1) and of TRN in TG\(^{TRN}\) and TG\(^{CXT}\) mice (Table 1). Of note, the protein level of TRN was reduced by 52% in TG\(^{CSQ}\) compared with WT mice (Table 1). After all, the TRN-to-CSQ ratio was normalized in double transgenic TG\(^{CXT}\) mice. Immunofluorescence analysis of isolated myocytes, using a mouse monoclonal antibody that recognizes exogenous canine but no endogenous mouse TRN, exhibited expression of the transgenic protein along the sarcomeric structure in TG\(^{TRN}\) myocytes (Fig. 1C). This suggests the expression of a functional relevant dog TRN in transgenic mouse hearts. Because of lack of a specific antibody directed against dog CSQ, it was not possible to determine the location of this transgenic protein. Moreover, these measurements revealed the colocalization of canine TRN and endogenous mouse JUN using confocal microscopy (Fig. 1C), suggesting normal trafficking of overexpressed TRN. Electron microscopy analysis revealed that the content of the junctional SR appears denser in TG\(^{TRN}\) (Fig. 1D, top right) compared with WT hearts (Fig. 1D, top left). This suggests that TRN overexpression holds CSQ in a condensed configuration. Transgenic overexpression of CSQ is followed by a massive swelling of SR vesicles (Fig. 1D, middle left), which are filled with a network of elongated CSQ polymers (6). Interestingly, co-overexpression results in a coexistence of small (Fig. 1D, bottom left) and wide SR vesicles (Fig. 1D, middle right). Overall, it appears that the width of junctional SR vesicles is reduced in TG\(^{CXT}\) (Fig. 1D, bottom right) compared with TG\(^{CSQ}\) hearts.

Decreased survival rate and increased fibrosis in TG\(^{CXT}\) mice. Because TG\(^{CSQ}\) mice died prematurely, we tested whether an increase in TRN expression in TG\(^{CSQ}\) mice is associated with a higher survival rate. Figure 2A shows the survival curves for all mouse lineages tested. Interestingly, we observed that TG\(^{CXT}\) mice had no benefit from the higher TRN expression compared with TG\(^{CSQ}\) mice. The double transgenic mice showed even an additional reduction of the survival rate (\(P < 0.05\)). At the age of 8 wk, >80% of TG\(^{CXT}\) mice were dead. In contrast, the same mortality rate was recorded after 24 wk of age in TG\(^{CSQ}\) mice (Fig. 2A). This was the first indication that the co-overexpression of both CSQ and TRN has detrimental effects on the viability of transgenic mice. Therefore, we studied the structural and functional mecha-
nisms of this phenomenon. First, we determined the heart weight-to-body weight ratio. Although we found an increase in this ratio in TG<sup>CxT</sup> compared with TG<sup>TRN</sup> and WT, there was no further deterioration compared with TG<sup>CSQ</sup> mice (Fig. 2B), suggesting a similar degree of myocardial hypertrophy. Histological analysis of ventricular sections using a staining protocol of trichrome Masson-Goldner detected a pronounced interstitial fibrosis in TG<sup>CxT</sup> hearts (Fig. 2C). This effect may contribute, at least in part, to the lower survival rate in double transgenic mice. In the next step, we then studied whether the decreased viability is reflected by an impaired contractile function at the whole heart or single cell level.

**Reduced fractional shortening in echocardiography after ISO administration in TG<sup>CxT</sup> mice.** The in vivo ventricular function was investigated by transthoracic echocardiography and Doppler measurements (Table 2). There were no alterations in cardiac parameters between all experimental groups under basal conditions. However, β-adrenergic stimulation by ISO revealed an increased left atrial diameter in TG<sup>CxT</sup> compared with TG<sup>CSQ</sup> mice. This dilatation might contribute to an impaired cardiac function. Alternatively, this increase reflects a high experimental variability and may have no functional consequences. The fractional shortening (FS), the mean aortic pressure gradient, and the velocity of circumferential fiber shortening were reduced in TG<sup>CxT</sup> compared with TG<sup>TRN</sup> and WT mice. FS and velocity of circumferential fiber shortening were also comparably diminished in TG<sup>CSQ</sup> mice. The acute administration of ISO led to an increase in FS in WT and TG<sup>TRN</sup> mice (by 38 and 42%, respectively) compared with basal conditions, whereas the ISO effect was lacking in TG<sup>CSQ</sup> mice compared with basal conditions. In addition, ISO stimulation was associated even with a decrease in FS in TG<sup>CxT</sup> compared with basal conditions (by 45%). This is in agreement with a 52% higher left ventricular end-systolic diameter under ISO-stimulated conditions in TG<sup>CxT</sup> mice. These results indicate an impaired β-adrenergic response of in vivo cardiac contractility in both TG<sup>CSQ</sup> and TG<sup>CxT</sup> mice.

**Decreased basal and dobutamine-stimulated contractility in catheterized TG<sup>CxT</sup> mice.** To characterize in depth the deterioration of the heart function in TG<sup>CxT</sup> mice, we also studied cardiac contractility by left ventricular catheterization. The rate of ventricular contraction (+dP/dt) was impaired in all transgenic groups compared with WT mice (Fig. 3A). TG<sup>TRN</sup> showed a slight reduction of +dP/dt under basal conditions compared with WT mice. TG<sup>CSQ</sup> mice demonstrated a further impairment of basal contractility compared with TG<sup>TRN</sup>. In TG<sup>CxT</sup> mice, +dP/dt was reduced compared with all other groups tested (by 65% vs. WT, by 49% vs. TG<sup>TRN</sup>, and by 20% vs. TG<sup>CSQ</sup>). The rates of ventricular relaxation (−dP/dt) were depressed similarly under basal conditions in all transgenic groups (Fig. 3B). However, there was no difference of this parameter between TG<sup>CSQ</sup> and TG<sup>CxT</sup> mice. Cardiac performance was also examined under β-adrenergic stimulation by dobutamine administration. In the present study, we missed an increase of contractility in TG<sup>CxT</sup> mice compared with WT and TG<sup>TRN</sup> mice for almost all dobutamine concentrations (Fig. 3C). The rate of pressure development was decreased by 66% under the maximum concentration of dobutamine in TG<sup>CxT</sup> compared with TG<sup>CSQ</sup> mice. The rate of pressure decline and the heart rate (data not shown) exhibited a comparable behavior for all groups tested. Thus these data confirmed the reduced contractile responsiveness to β-adrenergic stimulation in TG<sup>CxT</sup> mice observed by echocardiography.

**Impaired contractile parameters in work-performing heart preparations of TG<sup>CxT</sup> mice.** To test whether the reduction of cardiac performance in TG<sup>CxT</sup> mice under basal and ISO-stimulated conditions is also observed ex vivo, we used isolated work-performing heart preparations eliminating a potential neuronal and/or humoral contribution to the contractile effects. Under basal loading conditions (i.e., 50 mmHg after

Table 2. **Echocardiographic characterization**

<table>
<thead>
<tr>
<th></th>
<th>WT (n = 5)</th>
<th>TG&lt;sup&gt;TRN&lt;/sup&gt; (n = 5)</th>
<th>TG&lt;sup&gt;CSQ&lt;/sup&gt; (n = 5)</th>
<th>TG&lt;sup&gt;CxT&lt;/sup&gt; (n = 5)</th>
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</thead>
<tbody>
<tr>
<td><strong>Basal</strong></td>
<td></td>
<td></td>
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<tr>
<td>Heart rate, beats/min</td>
<td>460 ± 41</td>
<td>454 ± 15</td>
<td>449 ± 31</td>
<td>456 ± 42</td>
</tr>
<tr>
<td>LA, mm</td>
<td>1.9 ± 0.2</td>
<td>1.8 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>3.4 ± 0.2</td>
<td>3.2 ± 0.1</td>
<td>3.7 ± 0.1*</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>FS, %</td>
<td>46 ± 2</td>
<td>50 ± 3</td>
<td>43 ± 2</td>
<td>40 ± 7</td>
</tr>
<tr>
<td>AoPG&lt;sub&gt;mean&lt;/sub&gt;, mmHg</td>
<td>2.4 ± 0.4</td>
<td>2.3 ± 0.1</td>
<td>2.1 ± 0.1*</td>
<td>2.5 ± 0.6</td>
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<tr>
<td>MVV&lt;sub&gt;mean&lt;/sub&gt;, cm/s</td>
<td>80 ± 5</td>
<td>78 ± 5</td>
<td>77 ± 4</td>
<td>68 ± 5</td>
</tr>
<tr>
<td>MVP&lt;sub&gt;mean&lt;/sub&gt;, mmHg</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>0.7 ± 0.1</td>
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<tr>
<td>Vcf, mmHg</td>
<td>7.5 ± 0.7</td>
<td>7.6 ± 0.3</td>
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<td>7.6 ± 1.2</td>
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<tr>
<td><strong>ISO</strong></td>
<td></td>
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<tr>
<td>Heart rate, beats/min</td>
<td>579 ± 29§</td>
<td>572 ± 10§</td>
<td>578 ± 22§</td>
<td>604 ± 25§</td>
</tr>
<tr>
<td>LA, mm</td>
<td>1.9 ± 0.2</td>
<td>1.8 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>2.3 ± 0.1*†</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>3.1 ± 0.3</td>
<td>2.7 ± 0.1§</td>
<td>4.2 ± 0.4*</td>
<td>4.8 ± 0.4*†</td>
</tr>
<tr>
<td>FS, %</td>
<td>12 ± 0.2§</td>
<td>0.8 ± 0.1§</td>
<td>2.9 ± 0.6**</td>
<td>3.8 ± 0.6*†</td>
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<tr>
<td>AoPG&lt;sub&gt;mean&lt;/sub&gt;, mmHg</td>
<td>63 ± 4§</td>
<td>71 ± 4§</td>
<td>33 ± 9**</td>
<td>22 ± 7††</td>
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<tr>
<td>MVV&lt;sub&gt;mean&lt;/sub&gt;, cm/s</td>
<td>5.2 ± 0.78</td>
<td>4.6 ± 0.48</td>
<td>3.3 ± 0.7</td>
<td>2.2 ± 0.4**†</td>
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<tr>
<td>MVP&lt;sub&gt;mean&lt;/sub&gt;, mmHg</td>
<td>29 ± 5</td>
<td>79 ± 5</td>
<td>73 ± 4†</td>
<td>71 ± 3†</td>
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<tr>
<td>Vcf, mmHg</td>
<td>14.0 ± 1.2§</td>
<td>14.5 ± 1.4§</td>
<td>5.7 ± 1.3**</td>
<td>4.5 ± 1.2†</td>
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Values are means ± SE. Parameters were determined at basal and isoproterenol (ISO)-stimulated conditions in 5-wk-old mice. LA, left atrium; LVESD, LV end-diastolic diameter; MVP<sub>mean</sub>, mean pressure gradient of mitral valve; Vcf, velocity of circumferential fiber shortening. *P < 0.05 vs. TG<sup>TRN</sup>; †P < 0.05 vs. WT; ‡P < 0.05 vs. TG<sup>CSQ</sup>; §P < 0.05 vs. basal.

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load), WT and TG\textsuperscript{TRN} mice exhibited similar spontaneous heart rates, whereas TG\textsuperscript{CSQ} and TG\textsuperscript{CXT} mice were characterized by reduced heart rates (Fig. 4\textsuperscript{A}). This reduction is in contrast to echocardiographic and invasive hemodynamic measurements, where no alterations in heart rate were observed among all groups, suggesting the activation of the sympathetic nervous system in the intact organism. The rate of contraction (\(\frac{dP}{dt}\)) was decreased by 57\% in isolated TGC\textsuperscript{xT} compared with WT hearts (Fig. 4\textsuperscript{B}). Moreover, the rate of relaxation (\(-\frac{dP}{dt}\)) was reduced by 55\% in TGC\textsuperscript{xT} and by 34\% in TG\textsuperscript{TRN} compared with WT hearts (Fig. 4\textsuperscript{C}), while we found an unchanged contractile performance in TG\textsuperscript{CSQ} hearts despite a similar decrease of spontaneous heart rates in TG\textsuperscript{CSQ} and TGC\textsuperscript{xT}.

Reduced shortening but unchanged [Ca\textsubscript{i}], peak amplitude in TG\textsuperscript{CXT} myocytes. To test whether the impaired cardiac function in TG\textsuperscript{CXT} mice is accompanied by parallel changes at the cellular level, we measured simultaneously relative shortening and [Ca\textsubscript{i}] in isolated myocytes of all mouse groups. For this purpose, myocytes were loaded with indo-1 and stimulated at 0.5 Hz. Edge detection recordings (Fig. 5\textsuperscript{A}) exhibited a comparable impairment of relative cell shortening in both TG\textsuperscript{CSQ} and TG\textsuperscript{CXT} (by 88 and 86\%, respectively) compared with WT myocytes. In contrast, the time of 90\% relaxation was not different between all experimental groups (Fig. 5\textsuperscript{C}). Although the application of ISO was associated with an increase in cell shortening and a reduction in time of relaxation (TOR\textsubscript{90\%}) compared with basal values (Fig. 5, B and C), we found no differences in these parameters between all experimental groups. There was only a tendency for a prolonged TOR\textsubscript{90\%} both in TG\textsuperscript{CSQ} and TG\textsuperscript{CXT}. Simultaneous measurements of Ca\textsuperscript{2+} transients (Fig. 5D) revealed a concomitant decrease of the twitch [Ca\textsubscript{i}], peak amplitude in TG\textsuperscript{CSQ} myocytes (by 25\% vs. WT). However, this decrease was normalized in TG\textsuperscript{CXT} myocytes (Fig. 5E), suggesting an uncoupling of the Ca\textsuperscript{2+}-induced contraction process in double transgenic hearts. Analysis of the [Ca\textsubscript{i}], 50\% decay exhibited a similar prolongation in TG\textsuperscript{CSQ} and TG\textsuperscript{CXT} (by 131 and 139\%, respectively) compared with WT myocytes (Fig. 5F).

**Impaired SR Ca\textsuperscript{2+} handling in TG\textsuperscript{CXT} hearts.** To investigate whether an altered SR Ca\textsuperscript{2+} load contributes to the normalized twitch [Ca\textsubscript{i}], peak amplitude in TG\textsuperscript{CXT} myocytes, we measured caffeine-induced Ca\textsuperscript{2+} transients (Fig. 6A). Here, we found comparable caffeine-induced [Ca\textsubscript{i}], peak amplitudes in TG\textsuperscript{TRN} and WT myocytes. TG\textsuperscript{CSQ} myocytes exhibited an increase in the caffeine-induced [Ca\textsubscript{i}], peak amplitude (by 618\% vs. WT), suggesting a higher SR Ca\textsuperscript{2+} content in this group. This is in line with previous studies (12) using isolated murine myocytes with CSQ overexpression. Increased caffeine-triggered Ca\textsuperscript{2+}...
transients were also detected in TGCxT myocytes (by 727% vs. WT). However, there was no difference between myocytes from TGCSQ and TGCxT mice. These data led to the question whether an altered expression of SR proteins causes the different intracellular Ca\(^{2+}\) handling observed in TGCxT myocytes (Fig. 6A and Table 1). The protein level of JUN was reduced in all transgenic groups (by 66% in TGTRN, by 41% in TGCSQ and by 56% in TGCxT) compared with WT hearts. Furthermore, the protein level of the RyR was decreased only in TGCxT (by 32% compared with WT hearts). This reduction was confirmed by \(^{3}\text{H}\)ryanodine binding under saturated conditions. \(^{3}\text{H}\)ryanodine binding was not altered in TGTRN (B\(_{\text{max}}\) = 184 ± 23 fmol/mg) but reduced in TGCSQ (148 ± 15 fmol/mg) and TGCxT (141 ± 22 fmol/mg) compared with WT hearts (232 ± 28 fmol/mg, P < 0.05). The protein expression of the sarcoplasmic Ca\(_{\text{1.2}}\) was decreased by 64% in TGCSQ and by 34% in TGCxT compared with WT (Table 1) but was higher in double transgenic compared with TGCSQ hearts. In addition, protein expression of SERCA2a was downregulated by 32% in

**DISCUSSION**

Structural and functional defects of proteins forming the junctional SR Ca\(^{2+}\) release complex represent an important feature of cardiac hypertrophy, heart failure, or ventricular arrhythmias (11, 23, 33, 34). The exact mechanisms, underlying the mode of interaction between CSQ and associated proteins within this complex, have not been understood in total.

Studies with heart-specific overexpression of CSQ reported the presence of hypertrophy, impaired cellular Ca\(^{2+}\) handling, depressed contractility, and downregulation of TRN, JUN, and RyR protein expression (12). Interestingly, ablation of CSQ in mice was associated with normal basal cardiac function but led
to a propensity for stress-induced ventricular arrhythmias and downregulation of TRN and JUN as well (19). This suggests that an altered stoichiometry between CSQ and its interacting partner proteins may result in an impaired SR Ca²⁺ release process. Thus we tested whether an additional overexpression of TRN can restore depressed cardiac function in TGCSQ mice. On the basis of individual TRN-to-CSQ ratios and functional data, a model is provided from which it can be deduced, at least in part, changes in Ca²⁺ handling and contractility loss (Fig. 7).

The importance of an intermolecular regulation inside the SR Ca²⁺ release complex was underlined by several studies (4, 12, 15, 16, 19) showing that overexpression or deletion of junctional SR proteins was associated with a reduction in their partner proteins. However, it was not clear from these studies whether this altered expression has protective or detrimental effects on the SR Ca²⁺ release process. In the present study, an increase in the TRN-to-CSQ ratio had no functional effects on [Ca], peak amplitude or cellular contractility in TGTRN mice. Normally, TRN is required to physically link the RyR with vesicles and a higher SR Ca²⁺ TRN-to-CSQ ratio. Shown is the cellular Ca²⁺ proteins of interest. SL, sarcolemma; CS, cell shortening. Processes. Number of proteins does not reflect the exact stoichiometry between partner proteins. However, it was not clear from these studies whether this altered expression has protective or detrimental effects on the SR Ca²⁺ release process. In the present study, an increase in the TRN-to-CSQ ratio had no functional effects on [Ca], peak amplitude or cellular contractility in TGTRN mice. Normally, TRN is required to physically link the RyR with CSQ (9). Thus the excess of TRN in the junctional SR membrane seems to hold CSQ in a condensed configuration appearing as a dense structure in electron microscopy analysis of TGTRN hearts (6). Moreover, the reduced expression of JUN, which can also function as a CSQ-anchoring protein at the junctional SR, may compensate for the TRN overexpression. This helps to maintain a normal SR Ca²⁺ content and Ca²⁺ release.

In TGCSQ hearts, the decrease of the TRN-to-CSQ ratio was paralleled by a lower [Ca], peak amplitude and depressed cell shortening. Under physiological conditions in WT hearts, a rise in the SR luminal Ca²⁺ leads to weakened interactions between CSQ and TRN, relaxes inhibitory effects of CSQ, and increases RyR open probability (10, 37). The binding of CSQ to TRN inhibits the activity of the RyR (36). Thus the lower TRN expression in TGCSQ hearts may also result in a higher RyR activity. This may cause a constant diastolic SR Ca²⁺ leak and at least an impaired cellular Ca²⁺ handling. However, diastolic [Ca], was not changed in TGCSQ myocytes (data not shown). This may be also due to the increased JUN expression compared with TGTRN hearts because JUN appears to act as an inhibitor of the RyR activity at high SR-luminal Ca²⁺ (1). Therefore, we rather suggest that the downregulation of TRN resulted in an uncoupling of CSQ and the RyR, i.e., despite an increase of total SR Ca²⁺ load there is less luminal Ca²⁺ available in the proximity of single Ca²⁺ release complexes (Fig. 7). It needs to be elucidated which process(es) is(are) responsible for the initial reduction of TRN expression in TGCSQ hearts. On the other hand, the prolonged decay of [Ca], may be caused by an increased expression of phospholamban.

Regarding to our mechanistic model, an additional expression of TRN in TGCSQ hearts may cause a normalized ratio of both proteins, although the exact stoichiometry is not known, and therefore a better coupling between CSQ and the RyR (Fig. 7). As a consequence more SR-luminal Ca²⁺, which is buffered by TRN-bound CSQ in the vicinity of RyR release sites, is now available for the release from the SR resulting in the normalization of the [Ca], peak amplitude in TGCSQ myocytes, i.e., the reduced expression of TRN in TGCSQ has rather detrimental effects on the SR Ca²⁺ release process. More evidence for this suggestion is coming from electron microscopy studies by us and others (6) showing condensation/clustering of CSQ in the junctional SR membrane that faces T tubules in TGCSQ compared with TGCSQ hearts. This model can also explain the coexistence of small and wide SR vesicles, which may contain coupled and uncoupled CSQ, respectively, on the background of an unchanged total SR Ca²⁺ content in TGCSQ hearts. Although the expression of the RyR is reduced, we found a normalization of the [Ca], peak amplitude. This suggests that either the activity of each single RyR is increased or more (now coupled) RyRs are recruited in each cluster because a normal contractile twitch requires only a small fraction of cellular RyR (3). This remains to be elucidated in future studies on Ca²⁺ spark recordings or single channel recordings of the RyR. Alternatively, the [Ca], peak amplitude is normalized without any expression changes of the RyR at the single myocyte level, because the reduction of the channel protein was measured in crude TGCSQ heart homogenates containing both nonfibrotic and fibrotic cells. It is also conceivable that the increase in Ca₅,₁,₂ protein expression compared with TGCSQ may lead to a potentially higher sarcolemmal Ca²⁺ influx and to an improved Ca²⁺-induced SR Ca²⁺ release resulting in the normalization of the [Ca], peak amplitude in TGCSQ myocytes. In contrast to the normalized [Ca], peak amplitude, the Ca²⁺ transient kinetics are still prolonged in TGCSQ, corresponding to the reduced expression of SERCA2a. In line with our study, a lower SERCA2a expression in heterozygous SERCA2a-knockout mice resulted in depressed SR Ca²⁺ uptake and cardiovascular performance.
The fact that the lower SERCA2a expression was not associated with a reduction of the SR Ca\(^{2+}\) load in TG\(^{C^T}\) suggests that the decreased RyR2 expression is responsible for preservation of high Ca\(^{2+}\) levels within the SR. Alternatively, an altered phospholamban phosphorylation or the CSQ overexpression per se compensates for the depressed Ca\(^{2+}\) pump function.

Interestingly, in contrast to the improved cellular Ca\(^{2+}\) transients, TG\(^{C^T}\) mice were characterized by further deterioration in survival and myocardial contractility at basal conditions and upon ISO administration compared with TG\(^{CSQ}\) mice. At baseline, the depressed contractile function was accompanied by a plethora of structural (e.g., extensive fibrosis) and biochemical alterations (e.g., downregulation of SERCA2a). A similar discrepancy between cellular Ca\(^{2+}\) regulation and contractile phenotype was observed in several studies. Myocytes from aortic-banded rats displayed increased systolic [Ca], but a depressed myocyte shortening, indicating an impaired responsiveness to cytosolic Ca\(^{2+}\) (13). Skinned fibers from these rats exhibited normal Ca\(^{2+}\) sensitivity of the myofilaments, although the maximum Ca\(^{2+}\)-activated force was depressed. In addition, an increase in systolic [Ca], and a concomitant depression of the contractile function were measured in papillary muscles of rats suffering on heart failure due to infarction (25).

In this model, authors assume that hyperphosphorylation of myofilament proteins could contribute to a lack of contractile increase despite an elevation of systolic [Ca]. This was attributed to an increase in PK\(\alpha\) activity (2), which was also observed in transgenic mice with heart-directed overexpression of the \(\alpha_1\) subunit of the L-type Ca\(^{2+}\) channel (28, 29). The higher expression of Ca,1.2 in TG\(^{C^T}\) compared with TG\(^{CSQ}\) might also contribute to such an activation of Ca\(^{2+}\)-dependent signaling cascades. Moreover, these authors reported the development of cardiac hypertrophy in combination with a striking loss of the ISO effects on myocardial contraction, as observed in the present study.

The blunted contractile response to ISO represents a common feature of all heart failure models. In the present study, the application of ISO failed to increase several contractile parameters in echocardiographic and hemodynamic measurements in TG\(^{C^T}\) mice. Cardiac catheterization exhibited even a reduced contractility after maximum doses of ISO in TG\(^{C^T}\) compared with TG\(^{CSQ}\). We suggest that the massive increase in cardiac fibrosis may contribute to the loss of ISO effects on cardiac performance (22) because isolated myocytes exhibited comparable ISO effects on mechanical shortening. However, it is conceivable that an upregulation of BARK1 (14), diminished phospholamban phosphorylation (14), and/or SERCA2a downregulation, as found in the present study, may be involved in the loss of inotropic response to ISO administration in TG\(^{C^T}\) hearts. Moreover, we suggest that sympathetic stimulation at basal conditions both in TG\(^{CSQ}\) and TG\(^{C^T}\) mice is so high that the intracellular Ca\(^{2+}\) signaling cannot proper respond to \(\beta\)-agonists. This is supported by the observation that heart rates are reduced in isolated working heart preparations but unchanged in echocardiographic in vivo experiments.

In summary, co-overexpression of CSQ and TRN led to a normalization of the [Ca\(^{2+}\)] peak amplitude compared with TG\(^{CSQ}\) myocytes. This effect could be due to either a better coupling of the proteins of the SR Ca\(^{2+}\) release complex or an increased Ca\(^{2+}\) influx through the L-type Ca\(^{2+}\) channel. However, the improved cellular Ca\(^{2+}\) signaling in TG\(^{C^T}\) myocytes was not associated with a restoration of the impaired contractility in TG\(^{CSQ}\) mice. Thus the simple normalization of the impaired TRN-to-CSQ protein ratio in TG\(^{C^T}\) myocytes is apparently not sufficient for restoring the depressed excitation-contraction coupling in TG\(^{CSQ}\) mice.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


