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Influence of a Constitutive Increase in Myofilament Ca^{2+} -sensitivity on Ca^{2+} -fluxes and Contraction of Mouse Heart Ventricular Myocytes

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Abstract

Chronic increases in myofilament Ca^{2+} -sensitivity in the heart are known to alter gene expression potentially modifying Ca^{2+} -homeostasis and inducing arrhythmias. We tested age-dependent effects of a chronic increase in myofilament Ca^{2+} -sensitivity on induction of altered gene expression and activity of Ca^{2+} transport systems in cardiac myocytes. Our approach was to determine the relative contributions of the major mechanisms responsible for restoring Ca^{2+} to basal levels in field stimulated ventricular myocytes. Comparisons were made from ventricular myocytes isolated from non-transgenic (NTG) controls and transgenic mice expressing the fetal, slow skeletal troponin I (TG-ssTnI) in place of cardiac TnI (cTnI). Replacement of cTnI by ssTnI induces an increase in myofilament Ca^{2+} -sensitivity. Comparisons included myocytes from relatively young (5–7 months) and older mice (11–13 months). Employing application of caffeine in normal Tyrode and in $0\text{Na}^+ 0\text{Ca}^{2+}$ solution, we were able to dissect the contribution of the sarcoplasmic reticulum Ca^{2+} pump (SR Ca^{2+} -ATPase), the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), and “slow mechanisms” representing the activity of the sarcolemmal Ca^{2+} pump and the mitochondrial Ca^{2+} uniporter. The relative contribution of the SR Ca^{2+} -ATPase to restoration of basal Ca^{2+} levels in younger TG-ssTnI myocytes was lower than in NTG ($81.12 \pm 2.8\%$ vs $92.70 \pm 1.02\%$), but the same in the older myocytes. Younger and older NTG myocytes demonstrated similar contributions from the SR Ca^{2+} -ATPase and NCX to restoration of basal Ca^{2+} . However, the slow mechanisms for Ca^{2+} removal were increased in the older NTG ($3.4 \pm 0.3\%$) vs the younger NTG myocytes ($1.4 \pm 0.1\%$). Compared to NTG, younger TG-ssTnI myocytes demonstrated a significantly bigger

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contribution of the NCX ($16 \pm 2.7\%$ in TG vs $6.9 \pm 0.9\%$ in NTG) and slow mechanisms ($3.3 \pm 0.4\%$ in TG vs $1.4 \pm 0.1\%$ in NTG). In older TG-ssTnI myocytes the contributions were not significantly different from NTG (NCX: $4.9 \pm 0.6\%$ in TG vs $5.5 \pm 0.7\%$ in NTG; slow mechanisms: $2.5 \pm 0.3\%$ in TG vs $3.4 \pm 0.3\%$ in NTG). Our data indicate that constitutive increases in myofilament Ca^{2+} -sensitivity alter the relative significance of the NCX transport system involved in Ca^{2+} -homeostasis only in a younger group of mice. This modification may be of significance in early changes in altered gene expression and electrical stability hearts with increased myofilament Ca-sensitivity.

Keywords

troponin I; arrhythmia; Ca-sensitizer; hypertrophic cardiomyopathy

INTRODUCTION

Apart from its critical role in triggering contraction of cardiac myofilaments by binding to troponin C (cTnC), Ca^{2+} regulates a variety of cellular processes involved in both long and short-term control of cardiac function. Ca^{2+} binding domains on Ca^{2+} channels and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger regulate Ca^{2+} entry into and exit from the cells. Ca^{2+} -dependent kinases also regulate Ca^{2+} transport and myofilament response to Ca^{2+} . Moreover, Ca^{2+} -calmodulin dependent kinases and phosphatases also potentially regulate transcription, translation, and metabolism [1, 2]. Homeostasis of cardiac function requires a well-integrated interplay of Ca^{2+} fluxes controlling contraction, excitation contraction and energy coupling as well as gene expression [3]. A disruption in this Ca^{2+} homeostasis is a hallmark of cardiac failure [4].

As the major site of Ca^{2+} -binding in the ventricular myocyte, it is important to understand to what extent alterations in Ca^{2+} buffering by the myofilaments influence Ca^{2+} fluxes and/or gene expression. The major physiological mechanisms by which alterations in cTnC Ca^{2+} -binding appear to occur are by changes in sarcomere length and phosphorylation of troponin I (cTnI) by cAMP-dependent protein kinase (PKA). The effects of these alterations in cTnC Ca^{2+} -affinity have been reported to include alterations in the amplitude and dynamics of the Ca^{2+} -transient and contraction [5]. For example, stretch of the cardiac myocytes and the associated increase in sarcomere length, which is likely to result in an increase in cTnC Ca^{2+} -affinity [6] induces an immediate prolongation of contraction. Whether this change in Ca^{2+} -affinity affects the Ca^{2+} transient is not clear, as some reports indicate no change [7], whereas others report an increase in its amplitude or a decrease in its duration. Alteration of cTnC Ca^{2+} -affinity with EMD 57033 results in a decrease in the Ca^{2+} -transient amplitude and prolongation of its declining phase [8] with no change in the rise time. Variations in cardiac function as a consequence of ischemia, reperfusion injury and stunning also appear to involve major changes in myofilament response to Ca^{2+} [9, 10]. One might also expect that a reduction in myofilament Ca^{2+} -affinity may contribute to elevations in diastolic Ca^{2+} and to induce arrhythmias.

Whether long-term changes in myofilament sensitivity to Ca^{2+} affect gene expression and Ca^{2+} fluxes in the heart remains an important question. Mutations in sarcomeric proteins linked to familial hypertrophic cardiomyopathies (FHC) very frequently induce an increase in myofilament Ca^{2+} -sensitivity [11, 12]. This constitutive change in Ca^{2+} -sensitivity restricts the dynamic range over which sarcomeres respond to physiological regulators such as protein phosphorylation [13, 14] and sarcomere length [15]. It is apparent, for example in the case of mutations in tropomyosin [16] and troponin T (cTnT) [17], that the increase in myofilament Ca^{2+} -sensitivity may slow down relaxation and lead to diastolic abnormalities that trigger signaling of hypertrophy. Baudenbacher et al. [18] reported that increased cardiac myofilament Ca^{2+} -sensitivity induced a susceptibility to arrhythmias and a risk of developing ventricular tachycardia proportional to the extent of Ca^{2+} -sensitization induced by various myofilament modifications.

In the work presented here, we have investigated the effects of a prolonged constitutive increase in myofilament Ca^{2+} -sensitivity on cellular Ca^{2+} fluxes by employing the transgenic mouse model (TG-ssTnI) in which ssTnI completely replaces cardiac troponin I (cTnI). Unlike cTnI, ssTnI does not have sites for phosphorylation by PKA. Moreover, we have previously reported that compared to controls, TG-ssTnI cardiac myofilaments demonstrate a substantial increase in Ca^{2+} -sensitivity [19], a resistance to desensitization by acidic pH [20], and a blunted response to changes in sarcomere length [21]. Hearts of TG-ssTnI mice are also significantly resistant to ATP loss in global ischemia associated with remodeling to a relatively high glycolytic phenotype compared to controls [22]. We studied isolated cardiac myocytes from relatively young mice (5~7 months old) and relatively old mice (11~13 months old). Our results demonstrate that increased myofilament sensitivity to Ca^{2+} is likely to be an important factor, which contributes to the increased expression and activity of the NCX in younger mice and a decreased expression of SERCA2a in older mice. While a direct correlation between increased Ca-sensitivity and expression of NCX and SERCA2a is not established by our findings, our data suggest that persistent increases in myofilament Ca-sensitivity alters the time course of age dependent changes in NCX and SERCA2a protein expression.

METHODS

Transgenic animals

Mice expressing ssTnI in the heart were generated as previously described [19]. The transgene was under the control of the cardiac specific alpha-myosin heavy chain promoter and there was complete and stoichiometric replacement of cTnI by ssTnI in myofilaments from the TG-ssTnI mice. To determine time dependent alterations, we studied a group of relatively young mice (5~7 months old) and a group of older mice (11~13 months old).

Isolation of myocytes

Adult mice were heparinized (5,000 U/kg body wt), and after 30 min anesthetized with ether. Left ventricular myocytes were isolated as previously described [23] and were studied 1–6 hrs after isolation.

Measurements of intracellular Ca^{2+} transients and cell shortening

After isolation the cells were loaded for 20 min at room temperature with 10 μM Fluo3-AM followed by a 30 min washout. Cells were excited at 480 ± 5 nm using a 75 W Xenon arc lamp via epi-illumination, and emitted fluorescence at 535 ± 20 nm was recorded using photomultipliers (PTI Corporation). Background fluorescence was measured each day as the average fluorescence from 10 cells not loaded with Fluo3-AM of the same preparation. Fluorescence values are expressed as a pseudo ratio F/F_0 , where the emitted fluorescence F is divided by the resting emitted fluorescence F_0 . Cell shortening was recorded using a video edge detection system. Length changes were expressed as percentage of the resting length.

Solutions

The modified normal Tyrode (NT) solution had the following composition (mM): 140 NaCl, 6 KCl, 1 MgCl_2 , 2 CaCl_2 , 10 glucose and 5 N-2-hydroxyethylpiperazine- N' -2-ethanesulfonic acid (HEPES). In the Na- and Ca-free solution, NaCl was replaced with LiCl, CaCl_2 was omitted and 1mM ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N' tetraacetic acid (EGTA). All solutions had pH 7.4 at room temperature.

Experimental Procedure

Cells were equilibrated for 15 min in NT and paced at 0.5 Hz by field stimulation using platinum electrodes. Once a steady-state contraction level was achieved, the control twitch contraction and Ca^{2+} transient were recorded. Electrical stimulation was then stopped before rapidly switching to NT solution containing 10 mM caffeine (CaffNT). Sustained exposure of cells to CaffNT induces release of Ca^{2+} from the sarcoplasmic reticulum (SR) and inhibition of SR Ca^{2+} re-uptake [24]. Thus, CaffNT produced a long lasting contracture. After complete relaxation of the contracture, superfusion with caffeine solution was stopped and myocytes were washed with NT for ~ 1 min before electrical stimulation was resumed. After a recovery period of 5 min, twitch contractions returned to steady-state level. Stimulation was again stopped and myocytes were bathed in Na^+ - and Ca^{2+} -free solution for 15 s before application of 10 mM caffeine in Na^+ - and Ca^{2+} -free solution (Caff00). Similarly to the previous application, caffeine in 0Na^+ and 0Ca^{2+} induces a contracture in the cell (this time stronger due to the absence of NCX in the removal process). After recovery from the contracture, myocytes were washed with NT for 1 min before stimulation was resumed.

Evaluation of contribution to Ca^{2+} fluxes during relaxation

Data from the time course of relaxation of cell length and Ca^{2+} transients in NT, CaffNT and Caff00 were used to determine the relative contribution of the major Ca^{2+} transport mechanisms involved in cardiac relaxation. During a twitch the Ca^{2+} removal systems include the SR Ca^{2+} pump (SR Ca^{2+} -ATPase), the Na/Ca exchange (NCX), and slow mechanisms (SL Ca^{2+} pump and the mitochondria uniporter). However, in the presence of CaffNT, the primary means of Ca^{2+} extrusion from the cytoplasm is the NCX. During caffeine application in $0\text{Na}0\text{Ca}$, the NCX is not able to work (and caffeine prevents SR Ca^{2+} uptake) leaving only the slow mechanisms working.

Calculation of the relative contribution of each system was carried out as described by Puglisi et al. [25], equating the relaxation time constant Tau (τ) of the Ca^{2+} transients to the various conditions as follows:

$$\frac{1}{\tau_{\text{twitch}}} = K_{\text{SR}} + K_{\text{NCX}} + K_{\text{slow}} \quad (1)$$

$$\frac{1}{\tau_{\text{CaffNT}}} = K_{\text{NCX}} + K_{\text{slow}} \quad (2)$$

$$\frac{1}{\tau_{\text{Caff00}}} = K_{\text{slow}} \quad (3)$$

Where K_{SR} , K_{NCX} and K_{slow} represent the rates from the SR, the NaCa Exchanger and the slow mechanisms respectively. From the value of $1/\tau_{\text{Caff00}}$, we inferred directly the rate of the slow mechanisms. Subtracting the rate of the slow mechanisms from $1/\tau_{\text{CaffNT}}$, we obtained the rate of NCX and finally subtracting the rate of the slow mechanisms and the rate of NCX from $1/\tau_{\text{twitch}}$, we obtained the rate of the SR Ca^{2+} -ATPase. From each of the rate values and the total we computed the relative participation of each system in Ca^{2+} removal during relaxation.

Effect of caffeine on cardiac myofilaments response to Ca^{2+}

Hearts were quickly excised from NTG and TG-ssTnI mice (male and female) that had been deeply anesthetized by intra-peritoneal injection of pentobarbital sodium (50 mg/kg body weight). After the hearts were placed in ice cold saline and washed free of blood, fiber bundles were dissected on a cold plate (4° C) from the left ventricular papillary muscle. These fiber bundles (approximately 150–200 μm in width and 3–4 mm in length) were extracted overnight at 4° C in a high relaxing (HR) solution 20 mM MOPS (pH 7.0), 53.5 mM KCl, 10 mM EGTA, 0.025 mM CaCl_2 , 1 mM free Mg^{2+} , 5 mM MgATP, 12 mM creatine phosphate, 10 IU/ml creatine kinase (bovine heart, Sigma), 1 mM DTT and 1% Triton X-100. A cocktail of protease inhibitors was included in all buffers (1 $\mu\text{g/ml}$ pepstatin, 5 $\mu\text{g/ml}$ leupeptin and 0.2 mM PMSF). The detergent extracted fiber bundles were mounted with fast setting glue in a previously described [26] apparatus for measurement of sarcomere length by laser diffraction and determination of isometric tension. Sarcomere length was set at 2.3 μm in HR. Maximum force was measured first in an activating solution at pCa 4.5 and containing 20 mM MOPS (pH 7.0), 33.8 mM KCl, 10 mM EGTA, 9.96 mM CaCl_2 , 5.39 mM ATP, 6.47 mM MgCl_2 , 12 mM creatine phosphate, 10 IU/ml creatine kinase and 1 mM DTT. Tension was then measured in fiber bundles incubated in HR, followed by solutions containing values between pCa 8 to pCa 4.5, calculated as previously described (26).

Quantitative RT-PCR

Gene expression analysis was conducted by real-time quantitative RT-PCR with SYBR Green detection in the LightCycler thermocycler (Roche Diagnostics). Each one-step RT-

PCR reaction employed 100 ng of total RNA, which had been extracted from the apex of the heart using TRIzol (Life Technologies) reagent. Quantification of the RT-PCR was carried out as described previously [27] by running a series of *in vitro* transcribed mRNA standards prepared for each gene alongside the unknowns. Melting curve analysis was performed on the standards prior to determine the specific temperature (the T_m of the PCR product) at which the fluorescent signal should be acquired, thereby excluding fluorescence from non-specific products and/or primer dimers, which can be detected with the SYBR Green dye. The reaction conditions for the reverse transcriptase were 55° C for 15 min, followed by 95° C for 30 sec. This was followed by a four-step PCR amplification to quantify the expression of the various genes. The steps were: 95° C for 1 sec, 55–60 ° for 1 sec (depending on primer T_m), 72° C for 10 sec with signal acquisition at 80–89° C for 2 sec (depending on T_m of amplicon) for 40 cycles. The second derivative maximum (log linear phase) for each amplification curve was determined and plotted against GAPDH expression to ensure equal loading. As a final step to ensure correct amplification, appropriate size determination was made for each amplicon by electrophoresis. Primers used were selected against regions close to the 3' end of the gene of interest as previously published (27, 30).

Western Blot Analysis

We homogenized tissue samples in ice cold buffer containing (mM) imidazole 10; sucrose 300, DTT 1, Na metabisulfite 1, EDTA 2, pH 8.2, along with a protease inhibitor cocktail (Sigma). Protein concentration was measured using the Lowry assay. Proteins (75–100 µg total) were loaded on to a 10% polyacrylamide gel and separated by electrophoresis. Proteins were transferred to nitrocellulose using a wet blot apparatus (Bio-Rad). Membranes were blocked for 1 h in 5% non-fat milk-phosphate buffered saline, 0.1% Tween (1:1000 dilution; ABR). Blots were washed for 30 min in 0.05% PBS-T and subsequently incubated in anti-mouse secondary antibody (Vector Labs). The blot was then rinsed with PBS-T and incubated in ABC mix (Vector Labs) for 1 h. Following another 30 min wash, a DAB substrate kit was used for protein detection [28]. We used the following antibodies: For NCX (R3F1 SWANT; Bellinzona (Switzerland)); for SERCA2a Thermo Scientific (Clone 2A7-A1), and the GAPDH (FL-335) antibody from Santa Cruz Biotechnology for normalization. For SERCA2a blots, we used a 12% SDS-PAGE criterion gel with 20 µg protein loaded with transfer to a 0.2µm PVDF membrane.

Statistical Analysis—Data are presented as means $\pm$ SE. The Student's t-test was used for paired observations. Statistical comparison of values between cells from TG-ssTnI and NTG was carried out using ANOVA followed by a post-hoc test. $P < 0.05$ was considered significant.

RESULTS

The experiments were designed to compare the relative contribution to relaxation of the major Ca^{2+} transport mechanisms in the cell; namely the SR Ca^{2+} -ATPase, responsible for the Ca^{2+} flux through the sarcoplasmic reticulum (SR), the NCX responsible of the main flux through the cell membrane and the slower fluxes carried out by the sarcolemma Ca^{2+} pump and the mitochondria uniporter (referred to as slow mechanisms) in TG-ssTnI and non NTG

cardiomyocytes. To determine age dependent effects on these Ca^{2+} fluxes, experiments were carried out on isolated myocytes from mice 5–7 months of age and mice from 11–13 months of age. Fluorescence and shortening traces from TG-ssTnI and NTG hearts were measured in normal Tyrode (NT), caffeine in normal Tyrode (CaffNT) and caffeine in Tyrode free of Na^+ and Ca^{2+} (Caff00). In NT, Ca^{2+} removal during relaxation of mouse heart cells occurs by the action of the three mechanisms: the SR Ca^{2+} -ATPase that resequesters Ca^{2+} back to the SR, the NCX working in the forward mode extruding Ca^{2+} of the cytoplasm, and the slow mechanisms also removing Ca^{2+} from the cytoplasm. During caffeine application in NT, SR uptake is suppressed leaving the activity of the NaCa exchange and the slow mechanisms as the means for Ca^{2+} extrusion. In Caff00 the activity of the NCX is also suppressed and the slow mechanisms are the only active Ca^{2+} removal systems.

Cardiomyocyte Studies

Figure 1 compares traces of fluorescence and shortening for myocytes from the younger (Fig. 1A–F) and older (Fig. 1G–L) groups of myocytes from TG-ssTnI and NTG mouse hearts. Although traces in Figure 1A indicate that peak amplitude of the Ca^{2+} transient was increased and decay kinetics were slower in TG-ssTnI myocytes, this difference did not turn out to be significant (Fig. 2). However, compared to NTG, the amplitude of shortening shown in Figure 1D was significantly increased in TG-ssTnI myocytes (Figs. 1D and 2). These mechanical differences reflect in part the increased sensitivity of the TG-ssTnI myofilaments to Ca^{2+} , but may also reflect altered Ca^{2+} fluxes. Figure 1B shows changes in fluorescence in a cell during caffeine application in NT and Figure 1E the corresponding cell shortening record. As expected in CaffNT without a functional SR, Ca^{2+} signal relaxation was substantially prolonged for both TG-ssTnI and NTG myocytes. In both recordings of fluorescence (Fig. 1B) and shortening (Fig. 1E) the data indicate that Ca^{2+} removal from the cytoplasm by non-SR fluxes is enhanced in TG-ssTnI compared to NTG myocytes. As summarized in Figure 2 both the decay constant τ and decay half time ($t_{1/2}$) were significantly abbreviated in TG-ssTnI versus NTG myocytes. Similar differences were recorded during Caff00 applications (Figs. 1C and 1F). These data indicate that fluxes, through the NCX and through the slow mechanisms are enhanced in the TG-ssTnI myocytes compared to the NTG from the younger group of mouse hearts.

Data from a series of experiments with the relatively young myocytes are summarized in Figure 2 (Ca^{2+} transients) and Figure 3 (shortening). In NT, the rate of removal in either Ca^{2+} transients or shortening traces did not differ significantly. Ca^{2+} transient amplitude did not show any statistically difference between TG-ssTnI and NTG cells, but shortening was significantly bigger in the TG-ssTnI myocytes..

In CaffNT and Caff00 there were no significant differences between the Ca^{2+} transient amplitude or in percent shortening. But the rate constants for both caffeine applications were significantly faster in TG-ssTnI. These data point out that during relaxation, the contribution of NCX and the slow mechanisms are relatively greater in TG-ssTnI versus NTG in the younger group (Tables I and II).

Figure 1 (G–L) shows data from the older group of myocytes using the same protocol applied to the younger group. In the older mice, normal twitch during NT did not show any

statistically significant difference in Ca^{2+} transients amplitude but significantly bigger shortening amplitude in TG-ssTnI vs NTG (Fig 3G) similar to the younger group. During CaffNT and Caff00 applications Ca^{2+} transients amplitude were the same for both groups (Figs. 2G, H, I). However, the myocytes relaxed more slowly in TG-ssTnI myocytes during applications of both CaffNT and Caff00 (Fig. 2K & L). This result is opposite to that obtained in the younger group in which Ca^{2+} transients relaxed more *quickly* in TG-ssTnI during CaffNT or Caff00. The older group of TG-ssTnI myocytes also demonstrated a significant increase in amplitude of cell shortening in CaffNT but not in Caff00 in which both TG-ssTnI and NTG myocytes shortened to the same extent. Data in Figure 4 depict the relative contribution of Ca^{2+} transporters to relaxation in the younger and older group of TG-ssTnI and NTG myocytes. No differences in percent contribution of transporters were evident in the older group of both TG-ssTnI and NTG myocytes. Nevertheless there were significant differences in percent contribution of Ca^{2+} transport by the SR Ca^{2+} -ATPase, NCX and the slow mechanisms in the younger group. In this case, there is a reduction in the contribution by the SR Ca^{2+} -ATPase, which is offset by increased contribution of NCX and slow mechanisms.

Comparison of effects of caffeine on myofilaments response to Ca^{2+}

Although we have previously demonstrated that caffeine does not affect Ca^{2+} binding to cardiac TnC [29], it was important to compare the effects of caffeine on the Ca^{2+} sensitivity of myofilaments from TG-ssTnI and NTG hearts. Figure 5 shows data from experiments in which we determined the relation between pCa ($-\log$ of molar Ca concentration) and tension in detergent extracted fiber bundles. As shown the increase in Ca^{2+} sensitivity was similar in both TG-ssTnI and NTG myofilaments. Caffeine induced a 0.21 unit shift in the pCa value at 50% activation (pCa50) in the NTG myofilaments and a 0.28 pCa unit shift in the TG-ssTnI myofilaments.

RT-PCR Analysis and Western Blot Analysis

Figure 6 summarizes experiments in which the expression levels of genes important in cellular Ca^{2+} fluxes (NCX, SERCA2a, L-type Ca^{2+} channel), and sarcomeric proteins (skeletal alpha-actin, alpha and beta myosin heavy chain) were measured. As illustrated in Figure 6, no significant difference in the NCX, SERCA2a, or L-type Ca^{2+} channel mRNA expression existed between the TG-ssTnI and NTG hearts. The lack of difference in SERCA2a expression fits with our earlier data [30], demonstrating no difference in SR Ca^{2+} uptake rate into cardiac SR vesicles in homogenates from TG-ssTnI hearts and NTG controls. The myosin heavy isoform showed characteristic shifts with age, a decrease in alpha-myosin heavy chain expression associated with a reciprocal increase in beta myosin heavy chain. However, the skeletal alpha-actin isoform demonstrated both an age and ssTnI transgene related decline in expression. This isoform was expressed at significantly lower levels in both young and older TG-ssTnI hearts compared to NTG hearts. Data in Figure 7 compare the levels of protein expression of NCX in younger and older hearts from TG-ssTnI and NTG hearts. The young NTG hearts showed significantly lower NCX protein expression than the young TG-ssTnI hearts. Interestingly, the older NTG hearts showed higher NCX expression levels (but not statistically significant) than the older hearts from TG-ssTnI hearts. These results fit with the changes in activity seen in these hearts. In the case of

younger hearts there was no difference in SERCA2a expression between NTG controls and TG-ssTnI hearts. However, in the older group, expression of SERCA2a was relatively low in TG-ssTnI hearts compared to controls. Despite this difference, we did not see a difference in the per cent contributions of the SR- Ca^{2+} to relaxation in young versus older TG myocytes.

DISCUSSION

Our data provide the first evidence of an age dependent modification in the percent contribution of the SR Ca^{2+} -ATPase, NCX, and slow mechanisms to restoration of basal Ca^{2+} levels after stimulation of cardiac myocytes with constitutive myofilament sensitization to Ca^{2+} . Although we found no differences in expression in the older group, the younger group demonstrated a significant increase in NCX expression in TG-ssTnI versus controls. Moreover, there was a decrease in expression of SERCA2a between younger and older TG-ssTnI hearts..

We have no detailed signaling mechanism for this difference, but it is apparent that with time the controls catch up with the ssTnI in terms of NCX expression. Moreover, the data indicate that chronic administration of agents acting as Ca-sensitizers (8) may induce altered gene expression.. The data are also highly relevant to FHC. As emphasized in a review by Tardiff (17) there is a need to determine the most proximal disorders, and to design appropriate therapies in the early stages of FHC. In our case, the results indicate early changes in NCX that do not occur in the older myocytes. To our knowledge this possibility has not been well documented or incorporated into ideas regarding the relation between genotype and phenotype in FHC.

Our approach toward dissecting the contribution of each Ca^{2+} transport system by means of caffeine application has been successfully used to determine the relative participation of the SR Ca^{2+} -ATPase and NCX in relaxation of rabbit and rat cardiac cells [31], with development of the rat heart [32], and at different temperatures [25]. These studies concluded that rabbit hearts depend more on NCX for extrusion of Ca^{2+} than rat hearts [31], and relaxation dependence on the exchanger diminishes with age in the rat [32]. On the other hand the relative contribution of the SR Ca^{2+} -ATPase and NCX remain about the same as temperature increases from 25 to 35° C. This technique has also been applied in experiments comparing wild type (WT) and mice in which phospholamban was ablated (PLBKO) [33]. In WT myocytes 90% of Ca^{2+} removal during relaxation was due to the SR Ca^{2+} -ATPase and 8% to the NCX [33]. Results for the NTG myocytes presented here are consistent with these data. In PLBKO mice the SR Ca^{2+} -ATPase values were 96% and NCX values 3.4%. However both PLBKO and WT hearts expressed the same levels of NCX protein and mRNA [34]. In contrast, the mechanism for the difference in NCX activity reported here for the TG-ssTnI and NTG myocytes appear to be due to differences in the amounts of protein expressed in the young and old mice. As reported here, the relative abundance of exchanger protein correlates with the differences we measured in NCX activity.

Our results support the idea that enhanced myofilament sensitivity to Ca^{2+} may provoke increased expression of the NCX in the younger group of mice. An increase in the expression levels of the exchanger has been reported to occur in hearts at end-stage failure [35] and in animal models of heart failure [36, 37]. These models display abnormalities associated with decreased activity of the SR Ca^{2+} -ATPase together with increased myofilaments sensitivity to Ca^{2+} . Interestingly, the increase NCX activity may be a transient compensation as measurements in hearts of patients apparently closer to death than those studied by Hasenfuss et al. [35] did not display increased NCX activity [38]. The increase in myofilament Ca^{2+} sensitivity in human heart failure has been attributed to down regulation of signaling and signal transduction through beta-adrenergic receptors, and a relative dephosphorylation at PKA phosphorylation sites of key myofilament proteins compared to non-failing controls [39]. Myofilaments from hearts of dogs subjected to pacing induced heart failure also demonstrate increased response to Ca^{2+} by a mechanism possibly also involving dephosphorylation of myosin light chain 2 [40]. In the case of the ssTnI-TG mice, the region of TnI responding to PKA is not present and thus the myofilaments are not only intrinsically more sensitive to Ca^{2+} than the controls, but also the myofilaments do not desensitize in response to adrenergic stimulation as occurs in the controls. This restriction of a dynamic range of Ca-sensitivity and thus Ca^{2+} buffering is also likely to be an important aspect of the altered gene expression at least in the younger ssTnI-TG mice in the case of NCX, and in the older group of mice in the case of SERCA2a expression.

Our results are also relevant to mechanisms by which mutations in sarcomeric proteins give rise to hypertrophic cardiomyopathies [13, 17, 41–43]. These mutations are linked to a phenotype exhibiting asymmetric hypertrophy, myocyte disarray and fibrosis. Heart failure is common as are arrhythmias and sudden death [44, 45]. In most cases, the sarcomeric mutations are associated with an increase in myofilament Ca^{2+} sensitivity [46, 47] similar to that demonstrated by the myofilaments from the TG-ssTnI mouse hearts. As with the hearts expressing ssTnI, hearts expressing mutant forms of thin filament proteins such as cTnI [48, 49], cTnT [50, 51] and Tm [52] also demonstrate diastolic abnormalities. One hypothesis is that the increase in Ca^{2+} -sensitivity results in a dominant negative effect on relaxation, causing diastolic abnormalities and triggering of the hypertrophic signaling [53]. Another is that altered disposition of Ca^{2+} in the myocytes signals the hypertrophic process [54].

Although hearts of TG-ssTnI mice do not show hypertrophy or myofilament disarray, changes in SR Ca^{2+} load and up-regulation of NCX in the younger group of TG-ssTnI mice provide clues as to the processes connecting sarcomeric mutations to sudden death. There is ample evidence that an enhanced activity of the NCX in non-ischemic heart failure is the basis for an arrhythmogenic transient inward current. The reduction in SR Ca^{2+} load associated with up-regulation of NCX is also likely to be responsible for a reduction in Ca^{2+} delivery to the myofilaments and depression of contractility in heart failure. In the case of TG-ssTnI hearts, it is apparent that the functional effects of this decrease in Ca^{2+} released to the myofilaments are offset by an enhanced myofilament response to Ca^{2+} . We propose that the enhanced myofilament sensitivity to Ca^{2+} , as occurs in FHC sarcomeric mutations, leads to increased expression of NCX, as a compensatory mechanism, and thus is an important potential factor in triggered arrhythmias. There have been reports of upregulation of NCX in FHC associated with an E334K mutation in myosin binding protein C [55] and a R92W in

cTnT [56]. In the case of the MyBP-C mutation the change was not linked to increased myofilament Ca^{2+} -sensitivity, and in the TnT mutation there was no correlation with altered activity of the NCX.

Findings reported here are of significance with regard to a recent novel hypothesis linking increased myofilament Ca^{2+} sensitivity to triggered arrhythmias. Huke et al. [57] reported that myofilaments of an FHC model with relatively high Ca-sensitivity induce a localized, focal increase in ATP demand resulting in a loss of energy supply and a pro-arrhythmic reduction in gap junction coupling. These effects occurred in a cTnT-I79N model, similar to TG-ssTnI, exhibiting increased myofilament sensitization to Ca^{2+} , but without hypertrophy. No measurements similar to those carried out here with the TG-ssTnI myocytes have been done. Thus it remains possible that changes in Ca^{2+} may exacerbate the effects of the localized energy deprivation. In both the TG-ssTnI and TG-I79N model arrhythmias are induced by isoproterenol challenge [18, 57]. It is unlikely though that an energy deprivation is responsible for the arrhythmias in the ssTnI model, which undergoes metabolic remodeling resulting in increased glycolysis compared to controls. We have previously demonstrated that TG-ssTnI hearts are highly resistant to global ischemia with preservation of ATP concentration as a result of increased glycolysis [22]. We think in the case of TG-ssTnI hearts it is highly unlikely that there are localized energy deficits, and that remodeling of Ca^{2+} fluxes is more likely to be responsible. Mutations in cTnT are well documented to alter Ca-fluxes and expression of proteins significantly affecting Ca-fluxes [17, 56]. In the case of the cTnT-R92W mutation compared to NTG controls there is also an increase in NCX expression at 6 month of age, but no correlation with NCX activity [56]. Acute administration of the Ca-sensitizing agent EMD also induces arrhythmias associated with focal energy deficits [57], but no studies have been carried out testing whether chronic administration also induces remodeling of Ca-flux mechanisms.

CONCLUSION

Individuals with FHC generally die young, and we think our data adds to our understanding of the role of increased myofilament Ca-sensitivity. We report that there are differences in Ca^{2+} transport mechanisms between NTG and TG-ssTnI myocytes that are a function of age. The most striking difference is in the NCX that changes from a contribution to Ca^{2+} of 16% in young TG-ssTnI mice to 5% in an adult TG-ssTnI. In both the younger and older groups of TG-ssTnI myocytes, our data also indicate the possibility of a lower SR Ca^{2+} load. A lower SR Ca^{2+} load would be expected to reduce the possible induction of spontaneous release of Ca^{2+} from the SR with arrhythmogenic consequences [58]. This may seem a contradiction to our conclusion inasmuch as a However, previous studies have demonstrated that the increase in myofilament Ca-sensitivity in the ssTnI heart does induce arrhythmias and creates an arrhythmogenic substrate [18]. Our data support the possibility that modifications in Ca^{2+} fluxes in FHC and TG-ssTnI myocytes act in concert with chronically increased myofilament response to Ca^{2+} to generate arrhythmias leading to sudden death.

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HIGHLIGHTS

- Persistent increases in myofilament Ca-sensitivity induce altered Ca-fluxes
- The changes in Ca-fluxes are age dependent.
- A change in NCX activity and expression is prominent and affects relaxation.
- There are also changes in SR Ca² load, which may be functionally significant.
- Alterations induced by increased myofilament Ca-response may induce arrhythmias.

Fig. 1. Superimposed Ca^{2+} transients and shortening traces obtained in TG-ssTnI and NTG young and older adult myocytes

Fluorescence signals (expressed as F/F_0) from Ca^{2+} transients of young myocytes recorded during a twitch (A), caffeine application in NT solution (B) and caffeine in Na- and Ca-free solution (C). Re-lengthening and shortening (expressed as a percentage of the total cell length) are depicted in the lower panel for twitch (D), contracture under caffeine in NT (E) and contracture under caffeine in Na- and Ca-free solution (F). Fluorescence signals (expressed as F/F_0) for older adult myocytes during a twitch (G), contractures induced by caffeine application in NT solution (H) and in Na- and Ca-free solution (I). Shortening traces (expressed as percentage of total cell length) for a twitch (J), CaffNT (K) and Caff00 (L). Note the larger amplitudes and longer duration in older TG-ssTnI myocytes twitch and contractures compare to older NTG, which are opposite the results obtained from the young group of myocytes.

Fig. 2. Ca^{2+} -transients obtained in young and older adult TG-ssTnI and NTG myocytes
Panels A-C illustrate twitch, CaffNT and Caff00 contracture amplitudes for younger myocytes. Panels D-F show the corresponding relaxation times (τ) for younger myocytes. TG-ssTnI myocytes show bigger (although not significantly different) twitch amplitude (twitch $F/F_0 = 0.80 \pm 0.06$ vs 0.56 ± 0.06 NTG). SR Ca^{2+} content (inferred by the amplitude of caffeine contractures) in young TG-ssTnI cells was not significantly different compared to young NTG myocytes (CaffNT $F/F_0 = 2.40 \pm 0.52$ vs 3.18 ± 0.60 , Caff00 $F/F_0 = 2.70 \pm 0.55$ vs 3.45 ± 0.40 TG-ssTnI and NTG, respectively). Kinetics of twitch relaxation, as previously shown by Fentzke et al. (19) is relatively slow in TG-ssTnI (twitch $\tau = 367 \pm 39$ vs 301 ± 21 ms in NTG, but not significantly different. Both caffeine contractures are significantly faster in young TG-ssTnI (CaffNT $\tau = 2.18 \pm 0.23$ vs 4.3 ± 0.33 s, $p < 0.05$, Caff00 $\tau = 11.94 \pm 1.9$ vs 18.0 ± 1.6 s, $p < 0.05$ TG-ssTnI and NTG, respectively). During a twitch, Ca^{2+} transient amplitude follow the same tendency observed in the younger group, e.g. bigger

amplitude but not significantly different between TG-ssTnI and NTG ($F/F_0 = 1.02 \pm 0.17$. TG-ssTnI vs 0.71 ± 0.11 NTG). Caffeine-induced increased fluorescence levels for the TG-ssTnI group (CaffNT $F/F_0 = 3.37 \pm 0.68$ vs 2.27 ± 0.53 , Caff00 $F/F_0 = 3.63 \pm 0.73$ vs 2.80 ± 0.72 were not significantly different from NTG (G-I). Relaxation kinetics (J-L) exhibit a slower recovery statistically significant for both caffeine contractures (twitch $\tau = 390 \pm 26$ vs 362 ± 32 ms, $p > 0.05$, CaffNT $\tau = 4.99 \pm 0.54$ vs 2.95 ± 0.33 s, $p < 0.05$, Caff00 $\tau = 17.12 \pm 1.5$ vs 12.01 ± 1.3 s, $p < 0.05$, TG-ssTnI and NTG, respectively). * indicates $p < 0.05$ between TG and WT.

Young Myocytes

Old Myocytes

Fig. 3. Shortening and contracture data obtained in younger and older adult TG-ssTnI and NTG myocytes

Panels A–C illustrate differences in twitch, CaffNT and Caff00 amplitudes in young mice. Panels D–F show the kinetics of relaxation expressed as $t_{1/2}$ (D–F). Twitch amplitude is significantly bigger in TG-ssTnI myocytes (7.6 ± 0.4 vs $4.8 \pm 0.3\%$ NTG, $p < 0.05$). Caffeine contractures in NT and Na and Ca-free solutions exhibit smaller amplitudes in younger TG-ssTnI myocytes, but this difference is not significant between the two groups (CaffNT: 19.3 ± 2.9 vs $24.6 \pm 2.3\%$, Caff00: 22.1 ± 2.5 vs $27.0 \pm 3.2\%$, n.s TG-ssTnI vs NTG respectively). Twitch relaxation is slow in TG-ssTnI ($t_{1/2} = 245 \pm 8.6$ vs 206 ± 23 ms NTG), but both caffeine contractures exhibit a significant reduction (CaffNT $t_{1/2} = 2.53 \pm 0.7$ vs 12.3 ± 2.3 s, $p < 0.01$, Caff00 $t_{1/2} = 13.21 \pm 2.01$ vs 25.02 ± 2.9 s, $p < 0.05$, TG-ssTnI vs NTG respectively). The fast re-lengthening of TG-ssTnI cells indicates a bigger relative

contribution of the NCX and the slow mechanisms to the relaxation compared to the younger NTG myocytes. Twitch amplitude is significantly bigger in older adult TG-ssTnI mice compared with older NTG myocytes (8.7 ± 0.5 vs $6.1 \pm 0.48\%$, $p < 0.05$) (G–L). Caffeine applications produced bigger contractures in older TG-ssTnI cells significantly only in the case of caffeine application in NT (CaffNT: 25 ± 2.0 vs $19.0 \pm 1.9\%$, $p < 0.05$. Caff00: 27.0 ± 4.1 vs 22.1 ± 3.0 , $P > 0.05$, TG-ssTnI and NTG, respectively). Half-time to relaxation values ($t_{1/2}$) illustrate a slower twitch for TG-ssTnI (twitch $t_{1/2} = 252 \pm 26$ vs 228 ± 23 ms NTG, $P > 0.05$) and long lasting caffeine contractures (CaffNT $t_{1/2} = 20.04 \pm 2.12$ vs 7.5 ± 1.3 s $p < 0.01$, Caff00 $t_{1/2} = 30.0 \pm 3.5$ vs 23.1 ± 2.5 s, $P > 0.05$, TG-ssTnI and NTG respectively). * indicates $p < 0.05$ between TG and WT.

Fig. 4. Relative contributions to relaxation in younger and older adult myocytes

Based on the Ca^{2+} records the contribution of the three major Ca^{2+} removal mechanisms were estimated as explained in *Methods*. In the younger TG-ssTnI myocyte population the SR Ca^{2+} -ATPase exhibits a significantly smaller contribution compared to younger NTG myocytes ($81.12 \pm 2.8\%$ vs $92.7 \pm 1.02\%$, $p < 0.05$). In the older adult population this contribution is significantly different ($92.65 \pm 0.63\%$ TG-ssTnI, $90.9 \pm 0.98\%$ NTG). The NCX participation exhibits a significant increase in the younger TG-ssTnI compared to young NTG ($16.02 \pm 2.75\%$ TG-ssTnI, $6.9 \pm 0.9\%$ NTG, $p < 0.01$). This difference is lost in the adult population where both groups exhibit a similar contribution ($4.87 \pm 0.6\%$ TG-ssTnI vs $5.5 \pm 0.7\%$ NTG). The slow mechanisms also exhibit a bigger contribution in the younger TG-ssTnI myocytes compared to younger NTG ($3.5 \pm 0.42\%$ vs $1.4 \pm 0.09\%$, $p < 0.05$). In the adult population the participation of the slow mechanism in TG-ssTnI mice decreases to $2.5 \pm 0.35\%$, which is not different from the NTG ($3.4 \pm 0.32\%$).

Fig. 5. Comparison of the effects of caffeine on Ca^{2+} -sensitivity of force development in myofilaments of TG-ssTnI and NTG hearts

Data are normalized to maximum tension achieved before caffeine treatment in the NTG and TG-ssTnI myofilaments. Caffeine did not affect maximum tension in either case. Ca-response is indicated by pCa₅₀. Results demonstrate that the effect of caffeine to increase the response to Ca^{2+} (differences in pCa₅₀) is the same in myofilaments from NTG controls and TG-ssTnI hearts. * indicates $p < 0.05$ for pCa₅₀ values in the presence of caffeine compared to controls.

Fig. 6. Gene expression analysis of young and adult TG-ssTnI and NTG hearts

Quantitative RT-PCR was carried out on 6 genes: proteins involved in cardiac Ca^{2+} -relaxation (A. NCX, B. SERCA, and C. alpha subunit of the L-type Ca^{2+} channel) and sarcomeric protein isoforms (D. skeletal alpha-actin, E. alpha-myosin heavy chain and F. beta- myosin heavy chain). All results were normalized to GAPDH expression. * indicates $p < 0.05$ and # indicates $p < 0.01$, $n = 5$.

Fig. 7. Quantification of the protein expression levels of NCX (A) and SERCA2a (B) in young and adult TG-ssTnl and NTG hearts

Western blots were carried out to determine the relative amount of NCX and SERCA2a protein in the different hearts. * indicates $p < 0.05$

Table I

Ca²⁺ transients, shortening and caffeine contracture amplitudes

	Twitch		CafNT		Caf00	
	[Caf] (F/F ₀)	Shortening (%)	[Caf] (F/F ₀)	Shortening (%)	[Caf] (F/F ₀)	Shortening (%)
Young						
TG-ssTnI	0.80±0.06	7.6±0.4	2.40±0.52	19.3±2.9	2.70±0.55	22.1±2.5
NTG-cTnI	0.56±0.06	4.8±0.3 *	3.18±0.60	24.6±2.3 *	3.45±0.40	27.0±3.2
Adult						
TG-ssTnI	1.02±0.17	8.7±0.5	3.37±0.68	25.0±2.0	3.63±0.73	27.0±4.1
NTG	0.71±0.11	6.1±0.48 *	2.27±0.53	19.0±1.9 *	2.80±0.72	22.1±3.0

* p<0.05 (NTG relative to TG-ssTnI).

Table II

Relative contributions to relaxation in young and older adult myocytes.

	SR Ca^{2+} -ATPase (%)		NCX (%)		Slow Mechanisms (%)	
	Young	Adult	Young	Adult	Young	Adult
TG-ssTnI	81.12±2.8	92.65±0.63	16.02±2.75	4.87±0.6	3.35±0.42	2.5±0.35
NTG	92.70±1.02 *	90.9±0.98	6.9±0.9 **	5.5±0.7	1.4±0.09 *	3.4±0.32

* p<0.05 (NTG relative to TG-ssTnI),

** p<0.01 (NTG relative to TG-ssTnI).