Exercise training reverses myocardial dysfunction induced by CaMKIIδ_C overexpression by restoring Ca^{2+}-homeostasis

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Short title: CaMKIIδ_C overexpression and exercise training

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Abstract

Aim: Several conditions of heart disease, including heart failure and diabetic cardiomyopathy are associated with up-regulation of cytosolic Ca^{2+}/calmodulin-dependent protein kinase II ($CaMKII\delta_C$) activity. In the heart, $CaMKII\delta_C$ isoform targets several proteins involved in intracellular Ca^{2+} homeostasis. We hypothesized that high intensity endurance training activates mechanisms that enable a rescue of dysfunctional cardiomyocyte Ca^{2+} handling and thereby ameliorate cardiac dysfunction despite continuous and chronic elevated levels of $CaMKII\delta_C$. **Methods:** $CaMKII\delta_C$ transgenic (TG) and wild-type (WT) mice performed aerobic interval exercise training over 6 weeks. Cardiac function was measured by echocardiography in vivo, and cardiomyocyte shortening and intracellular Ca^{2+}-handling in vitro. **Results:** TG mice had reduced global cardiac function, cardiomyocyte shortening (47% reduced compared to WT, $P<0.01$) and impaired Ca^{2+}-homeostasis. Despite no change in the chronic elevated levels of $CaMKII\delta_C$, exercise improved global cardiac function, restored cardiomyocyte shortening, and re-established Ca^{2+}-homeostasis to values not different from WT. The key features to explain restored Ca^{2+}-homeostasis after exercise training were increased I_{CaL} density and flux by 79% and 85%, respectively ($P<0.01$), increased SERCa2a function by 50% ($p<0.01$) and reduced diastolic SR Ca^{2+}-leak by 73% ($P<0.01$), compared to sedentary TG mice. **Conclusion:** Exercise training improves global cardiac function as well as cardiomyocyte function in the presence of a maintained high CaMKII activity. The main mechanisms of exercise-induced improvements in TG $CaMKII\delta_C$ mice are mediated via increased L-type Ca^{2+} channel currents, improved SR Ca^{2+}-handling by restoration of SERCA2a function in addition to reduced diastolic SR Ca^{2+}-leak.

New & Noteworthy: The novel findings in this study is that high intensity endurance training turned the heart failure phenotype in $CaMKII\delta_C$ over-expressing mice towards a more healthy phenotype. We report improved cardiac and cardiomyocyte function and Ca^{2+} handling by reducing diastolic Ca^{2+} leak and restoring SR Ca^{2+} content through compensatory mechanisms of restored SERCA2a function, NCX function and increased L-type Ca^{2+} currents. The present data extend the basis for further understanding of cardiac adaptations to exercise training.

Introduction

In recent years, exercise training has arisen as an important clinical treatment strategy for cardiovascular disease. Exercise training not only reduces cardiovascular risk factors, but several studies also show beneficial effects on cardiac function along with reversal of cellular abnormalities such as hypertrophy and remodeling, and aberrant Ca^{2+} handling and contractile function (7, 15, 20). Furthermore, improvements in maximal oxygen uptake (VO_{2max}) as well as cardiac function are reported more pronounced with high intensity endurance training both in experimental animal models (12) as well as in patients with cardiovascular disease (34, 37). Regulation of the protein kinase Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which occurs in cardiac muscle after exercise training (11, 29) could contribute to these effects since CaMKII regulates several aspects of cardiomyocyte function.

In the heart, the predominant isoform of CaMKII is the cytosolic δ isoform CaMKIIδ_C (6, 30), which targets several proteins involved in intracellular Ca^{2+} homeostasis, including the sarcoplasmic reticulum (SR) Ca^{2+}-release channel (ryanodine receptor, RyR2), the L-type Ca^{2+}-channel (LTCC) and phospholamban (PLN), which regulates SR Ca^{2+}-ATPase (SERCA2a) activity. Several models of heart disease, including heart failure (9, 16) and diabetic cardiomyopathy (29) are associated with upregulation of CaMKII activity. In line with this, overexpression of the $delta_C$ isoform CaMKIIδ (CamKIIδ_c) has been shown to detrimentally alter Ca^{2+}-handling and contractility (19, 25). Especially increased RyR2 Ca^{2+} sensitivity that causes leaky RyR2s has received great attention in the phenotypic changes observed in cardiomyocytes with increased activity of CaMKIIδ_C (1, 5, 22).

We hypothesized that high intensity endurance training could enable restoration of dysbalanced cardiomyocyte Ca^{2+}-homeostasis and thereby ameliorate cardiac dysfunction even in the face of continuous and chronic elevated levels of CaMKIIδ_C.

Material and methods

Animals

Transgenic CaMKIIδ_C mice (TG) with increased CaMKII activity were generated as previously described (40). Briefly, Hemagglutinin (HA)-tagged rat wild-type CaMKIIδ_C cDNA were subcloned

into the SalI site of pBluescript-based TG vector between the 5.5-kb murine α-MHC promoter and a human growth hormone (HGH) polyadenylation sequences. Purified linear transgene fragments were injected into pronuclei of fertilized mouse oocytes. The resultant pups were screened for the presence of the transgene by PCR, using a CaMKII specific primer (5′-TTGAAGGGTGCCATCTTGACA-3′) and a TG vector specific primer (5′-GGTCATGCATGCCTGGAATC-3′). To determine the transgene copy number, Southern blot analysis was performed with EcoRI-digested genomic DNA and a P-labeled 1.7 kb EcoRI-SalI α-MHC fragment as a probe. Founder mice were bred with C57BL/6 or Black Swiss wild-type (WT) mice to generate TG and WT offspring. Three months-old TG mice underwent aerobic interval endurance training (N=12) or remained sedentary (N=12), and were compared to age-matched sedentary WT littermate controls (N=12) aerobic interval endurance trained WT littermate control mice (N=12). 24 hours after the last training session, the mice were sacrificed and cardiomyocytes isolated to examine contractile function, Ca^{2+}-cycling and diastolic SR Ca^{2+}-leak. The Norwegian council for Animal Research approved the study, which was in accordance with the Guide for the Care and Use of Laboratory Animals published by the European Commission Directive 86/609/EEC.

Maximal oxygen uptake (VO_{2max})

The mice warmed up for 20 min at 50-60% of the maximal oxygen uptake (VO_{2max}), whereupon treadmill velocity was increased by 0.03 $m \cdot s^{-1}$ every 2 min until VO_2 reached a plateau despite increased workload. VO_{2max} recordings were obtained by treadmill placed in a closed metabolic chamber according to previous validated methods (10, 35).

Endurance training

The aerobic interval endurance-training program was performed as previously described (13, 35). During training, the mice ran uphill (25°) on a treadmill for 80 min: following 20 min of warm-up at a speed corresponding to 50-60% VO_{2max} the mice performed intervals during a period of 60 min, alternating between 4 min at an exercise intensity corresponding to 85-90% of VO_{2max}, and 2 min active recovery at 50-60%; giving a total of 40 min (10 intervals) at high intensity and a total of 20

min of recovery between intervals. Exercise was performed 5 days per week over 6 weeks; controls were age-matched CaMKIIδ_C TG or WT mice that remained sedentary or exercised. The time frame of the intervention period was chosen on background of previous publications showing a robust change in VO_{2max}, as well as in cardiomyocyte function and calcium handling in experimental animal models (10, 13, 35). In exercising animals, VO_{2max} was measured every second week to adjust band speed in order to maintain the intended intensity throughout the experimental period, whereas in the sedentary group VO_{2max} was measured before and after the experimental period.

Cardiomyocytes shortening and Ca^{2+}-cycling

At the end of the exercise-training period the heart was removed during 3% Isoflurane anestesia and immediately transferred for cardiomyocyte cell isolation by retrograde Langendorff perfusion and collagenase type II (Worthington, UK) as earlier described (40). Isolated cardiomyocytes were loaded with Fura-2/AM for detection of Ca^{2+}-handling properties (2 µmol/L, Molecular Probes, Eugene, OR). To ensure similar loading of the cardiomyocytes we incubated the cells for exactly 30 minutes and all cells were allowed at least 10 minutes in normal HEPES solution before any recordings. Cardiomyocytes were stimulated by bipolar electrical pulses with increasing frequencies 1-3 Hz on an inverted epifluorescence microscope (Nikon TE-2000E, Tokyo, Japan), whereupon cell shortening was recorded by video-based myocyte sarcomere spacing (SarcLen™, IonOptix, Milton, MA) and intracellular Ca^{2+}-concentration ($[Ca^{2+}]_i$) was measured by fluorescence after excitation by alternating 340 and 380 nm wavelengths ($F^{340/380}$ ratio) (Optoscan, Cairn Research, Kent, UK). During the stimulation protocol, cells were continuously perfused with normal physiological HEPES based solution (1.8 mmol/L Ca^{2+}, 37°C). In a subset of experiments, H-89 (3 µmol/L for 1 hour, Sigma, St. Louis, USA) to block protein kinase A (PKA), or autocamtide-2-related inhibitory peptide (AIP, 1 µmol/L for 1 hour, Sigma, St.Louis, USA) to block CaMKIIδ_C, were added to the solutions. Cell size was measured in cardiomyocytes not introduced to FURA2-AM with a graticule on the microscope and volume was calculated with the formula: cell area (length x cell midpoint width) μm^2 x 0.00759ρL/μm^2, as previously established by 2D light and 3D confocal microscopy (26).

Diastolic Ca^{2+}-leak

A method similar to that established by Shannon *et al*.(27) was used to determine diastolic Ca^{2+}- leak from the SR. To bring the cellular Ca^{2+}-content to a steady state, we stimulated the cardiomyocytes electrically at 1 Hz in normal HEPES based 1.8 mmol/L Ca^{2+}-solution for 30-60 seconds. After the last electric stimulus, we rapidly switched the perfusion to a $0Na^{+}/0Ca^{2+}$ containing solution and measured diastolic Ca^{2+} concentration in quiescent non-stimulated cardiomyocytes (one minute) ± Tetracaine (1 mmol/L). The $0Na^{+}/0Ca^{2+}$ solution prevents the Na^{+} - Ca^{2+} exchange, which is the primary Ca^{2+}-influx and efflux mechanism at rest. Tetracaine blocks the Ca^{2+}-leak over the RyR (21, 27). The quantitative difference between diastolic Ca^{2+}-concentration with and without tetracaine determine leak. After the one-minute period in $0Na^{+}/0Ca^{2+}$ ± tetracaine solution, we added caffeine (10 mmol/L) to assess SR Ca^{2+}-content. Diastolic Ca^{2+}-leak is presented as diastolic $[Ca^{2+}]_i$ in relation to total SR Ca^{2+}-content. In a subset of experiments, H-89 (3 µmol/L for 1 hour) to block PKA or AIP (1 µmol/L for 1 hour) to block CaMKII, were added to the solutions.

Ca^{2+} waves

Cardiomyocytes loaded with Fluo-3/AM (10 µmol/L, Molecular Probes) were used to determine frequency of Ca^{2+} waves by confocal line scan (Pascal, Carl Zeiss, Jena, Germany)

Voltage clamp

Single isolated mouse cardiomyocytes were superfused with a HEPES-buffered Krebs-Henseleit solution containing (mM): NaCl (140), KCl (4), HEPES (5), $MgCl_2$ (1), $CaCl_2$ (1.8), glucose (11.1), 4-aminopyridine (5mM, to block K^{+} currents), niflumic acid (0.1mM, to block Ca^{2+}-activated Cl^{-} currents), and Tetrodotoxin (5µM, to block I_{Na}), pH 7.4 with NaOH (37ºC) in a chamber mounted on the stage of an inverted microscope. Microelectrode pipettes were filled with an intracellular solution of composition (mM): KCl (20), K aspartate (100), tetraethylammonium chloride (TEA-Cl, 20), HEPES (10), $MgCl_2$ (4.5), disodium ATP (4), disodium creatine phosphate (1), EGTA (0.01), pH 7.25 with KOH. *I_{CaL} protocol:* Voltage clamp was achieved via whole cell ruptured patch technique using an Axoclamp 2B amplifier (Axon Instruments, CA, USA) in discontinuous (switch clamp) mode.

Pipette resistance was ~6 MΩ. Whole cell patch clamp was performed on single isolated mouse cardiomyocytes. The cell was clamped at -80 mV and the voltage stepped to -40 mV for 50 ms, before stepping to 0 mV for 150 ms. The protocol was repeated at 2Hz for 90s. The last 10 L-Type Ca^{2+} current recordings were averaged and analyzed.

Western blot analyses

Cardiac tissue was homogenized in Tris buffer containing (mmol/L): 20 Tris-HCl, 200 NaCl, 20 NaF, 1 Na_3VO_4, 1 dithiothreipol, 1% Triton X-100 (pH 7.4), PhosSTOP (Roche Diagnostics, Grenzach-Wyhlen, Germany), and complete protease inhibitor cocktail (Roche Diagnostics, Grenzach-Wyhlen, Germany). Protein concentration was determined by bicinchoninic acid assay (Thermo Fisher Scientific Inc., Rockford, USA). Denatured tissue homogenates (30 min at 37°C or 5 min at 95°C, 2% beta-mercaptoethanol) were used for Western blotting (8%-15% sodium dodecylsulfate-polyacrylamide gel) using anti-CaMKIIδ (1:15000, gift from D. M. Bers, University of California, Davis, USA), anti-phospho-CaMKII (1:1000, Thermo Fisher Scientific Inc., Rockford, USA), anti-RYR2 (1:10000, Sigma, St.Louis, USA), Anti-RYR2 Phospho Serine-2814 (1:5000, Badrilla, Leeds, UK), anti-glyceraldehyde-3-phosphate dehydrogenase (1:20000, Biotrend Chemikalien, Köln, Germany) as primary, and horseradish peroxidase conjugated donkey anti-rabbit and sheep anti-mouse immunoglobulin G (1:10,000, Amersham Biosciences, Freiburg, Germany) as secondary antibodies. Chemiluminescent detection was performed with Millipore Immobilion Western (Millipore, Billerica, USA). For SERCA2a and L-type Ca^{2+}channel determination, primary antibodies were anti-SERCA2a (1:2000, Badrilla, Leeds, UK), and for L-type Ca^{2+} channel the primary antibody was anti-CACNA1C (1:350, Abcam, Cambridge, UK) and anti-GAPDH (1:2000, ThermoFisher MA5-15738). 50µg protein was separated on Bis-Tris SDS-PAGE ready gels and transferred to PVDF membranes (Thermo Fisher Scientific Inc., Rockford, USA). Secondary antibodies used were IRDye 800CW goat anti-mouse (1:10000, Li-Cor Biotec, Nebraska, USA) and IRDye 680LT donkey anti-rabbit (1:30000, Li-Cor Biotec). Protein bands were visualized using an Odyssey fluorescence imaging system and band intensities quantified using Li-Cor Image Studio 3.1 (Li-Cor Biotec).

Statistical analysis

Data are shown as mean±SD, except where indicated. One-way ANOVA with Bonferroni post-hoc test adjusted for multiple comparisons was used to identify the statistical differences between the groups and Mann-Whitney U was used when appropriate. $P<0.05$ was considered statistically significant.

Results

Total CaMKIIδ protein expression was increased seven-fold in TG mice compared to WT, whereas CaMKII phosphorylation at the auto-activation site threonine-286 increased two-fold. Exercise did not modify either of these parameters (Figure 1A-C). However, despite no effect of exercise training on regulation of these proteins, we observed that the TG mice adapted to high intensity exercise training such that parameters of several aspects of in vivo cardiac and ex vivo cardiomyocyte function improved or restored to levels comparable to basal levels (WT untrained). Moreover, the training response with regards to aerobic capacity and cardiac and cardiomyocyte function followed the same pattern as seen after exercise training in the WT group. Exercise was well tolerated in all groups and we did not observe any adverse effects in any of the animals. No mortality was observed during the experimental period.

Aerobic capacity, cardiac function and response to exercise training

The increased expression of CaMKIIδ_C led to a significant reduction in aerobic capacity as maximal oxygen uptake (VO_{2max}) in sedentary TG mice was 75% to that of WT mice. However, six weeks of exercise training restored VO_{2max} in TG mice to levels similar to WT mice (Figure 1D). As aerobic capacity is closely related to cardiac pump function, we measured cardiac parameters by echocardiography. Cardiac output, stroke volume, and ejection fraction were significantly reduced in sedentary TG mice, suggesting cardiac dysfunction, whereas parameters of left ventricle (LV) lumen dimensions indicated dilation (Table 1). Exercise training improved cardiac output, stroke volume, and ejection fraction significantly ($p<0.01$, Table 1). Hence, deficits in both aerobic capacity and global

cardiac function were improved by exercise training in TG mice. Similar effects were seen after exercise in WT mice.

C*ardiomyocyte size and contractility*

We found significantly larger cardiomyocyte size in TG mice compared to WT mice; exercise training reduced the volume significantly (Figure 1E), indicating a reversal of the pathologic hypertrophy. In the WT exercise group, we observed the opposite scenario with increased cardiomyocyte size, indicating a physiologic hypertrophy that commonly is observed after exercise in healthy individuals. Cardiomyocyte contractility, measured as fractional shortening, was reduced by ~47% in TG mice compared to WT mice, whereas exercise training fully restored cardiomyocyte fractional shortening (Figure 2A&B). Also, time to 50% re-lengthening was prolonged in isotonically contracting cardiomyocytes from TG mice, but exercise training normalized this (Figure 2C).

L-type Ca^{2+} current (I_{CaL})

Since transmembrane Ca^{2+} -flux initiates cardiomyocyte excitation-contraction coupling and contractility, we examined the I_{CaL}. Exercise training in TG mice increased the I_{CaL} density and flux significantly by 79% and 85%, respectively ($p<0.01$, Figure 3). Similar alterations were observed in exercised WT mice. The increased L-type Ca^{2+} channel current after exercise training was at least partly explained by the significantly increased protein expression in exercised TG mice compared to TG sedentary ($p<0.05$, Figure 3).

Ca^{2+} transients and SR Ca^{2+} content

The Ca^{2+}-transient amplitude was ~58% lower in TG mice compared to WT mice, but this difference was absent after exercise training, indicating that the Ca^{2+}-transient amplitude was corrected by exercise training (Figure 4A&B). This increase in Ca^{2+}-transient amplitude in response to exercise training was comparable to the effect observed in WT mice. Reduced Ca^{2+}-transient amplitude in TG has been suggested to result from reduced SR Ca^{2+} content compared to that observed in

cardiomyocytes from WT mice (19, 25). We confirmed that caffeine-evoked SR Ca^{2+} content was reduced in TG compared to WT; exercise training restored the SR Ca^{2+}-content to sedentary WT levels (Figure 4C).

Diastolic Ca^{2+}-control

Diastolic Ca^{2+} levels during twitch contractions were lower in TG mice compared to WT mice, whereas exercise training restored diastolic Ca^{2+} to levels comparable to WT mice (Figure 5A).

Time to 50% Ca^{2+}-transient decay was significantly prolonged in TG mice compared to WT, whereas exercise training abolished this difference (Figure 5B). To further analyze the characteristics of diastolic Ca^{2+} handling, we examined the rate constants of cytoplasmic Ca^{2+} removal (Figure 5C). During a normal twitch-induced Ca^{2+}-transient, Ca^{2+} is removed by the SERCA2a, NCX, and the plasma membrane Ca^{2+} ATPase (PMCA), and the rate constant of Ca^{2+} decline in this situation (K_{tw}) can be described as the sum of the rate constants associated with each efflux mechanism. During caffeine-induced Ca^{2+}-transients, the contribution from SERCA2a is abolished, and the decay rate constant thus depends only upon NCX and PMCA. To derive the rate constant of NCX (K_{NCX}), the rate constant of Ca^{2+} removal during caffeine-induced Ca^{2+} transients in a solution containing 0 Na^{+} and 0 Ca^{2+} was measured and subtracted from the rate constant in the presence of these ions (3). First, the rate constant attributed to PMCA was negligible small and there were no differences between groups. The rate constant of Ca^{2+} removal during a caffeine-induced Ca^{2+} transient (SERCA2a contribution thus abolished) was significantly higher in TG mice, indicating an increased NCX function (Figure 5D). To quantify the contribution from SERCA2a, a simple model was used based on the following assumptions: SERCA2a transport rate is $K_{SERCA2a} = K_{TW} - K_{NCX}$, and the relative contribution by SERCA2a is $K_{SERCA2a}/K_{TW}$. Thus, for WT mice the $K_{tw} = 0.91\ s^{-1}$, $K_{NCX} = 0.06\ s^{-1}$ and $K_{SERCA2a} = 0.85\ s^{-1}$, and 93% of the total Ca^{2+} removal was attributed to SERCA2a (Figure 5E). In TG mice, K_{tw} ($0.58\ s^{-1}$) was reduced and K_{NCX} ($0.09\ s^{-1}$) was increased, resulting in a $K_{SERCA2a}$ of $0.49\ s^{-1}$.

This implies that SERCA2a was responsible for 84% of the total Ca^{2+} removal, which was reduced by 42% when compared to WT mice (from $0.85\ s^{-1}$ to $0.49\ s^{-1}$). In contrast, NCX function was increased by ~50% (from $0.06\ s^{-1}$ to $0.09\ s^{-1}$) in the TG group. After exercise training in TG mice, K_{tw}

= 0.8 s^{-1}, K_{NCX}=0.06 s^{-1} and K_{SERCA}=0.74 s^{-1}, which indicates that both SERCA2a and NCX functions were restored to normal levels (Figure 5C-E). At the protein level SERCA2a was 26% lower in TG mice compared to WT. SERCA2a protein expressions was 28% higher in exercised TG mice (Figure 5F, NS) compared to sedentary TG, which is in agreement with functional SERCA2a data from isolated cardiomyocytes.

Diastolic SR Ca^{2+}-leak

In TG mice, the diastolic SR Ca^{2+}-leak was higher (19±3% of total SR Ca^{2+} in TG vs. 3±2% in WT, P<0.01, Figure 6A), which associated with a significant reduction in the total SR Ca^{2+}-content compared to WT mice. Exercise training normalized SR Ca^{2+}-leak to levels comparable to WT mice. The increased Ca^{2+}-leak in TG mice was related to the overexpression of CaMKIIδ_C, since inhibition of CaMKIIδ_C by autocamtide 2-inhibitory peptide (AIP) reduced the leak to levels of WT mice (Figure 6B). To control for a PKA-related effect on Ca^{2+}-leak , separate cells were incubated with H-89, but under these conditions no effect on SR Ca^{2+}-leak was observed (Figure 6B). None of the CaMKII or PKA inhibitors had any effect on Ca^{2+}-leak in sedentary WT, exercise trained WT mice or exercise trained TG mice; however, in these groups, the baseline Ca^{2+} leak was already minimal (Figure 6A). In line with this, Ca^{2+} wave frequency was increased in TG mice compared to WT mice, but exercise training reduced the wave generation to WT levels (Figure 6C).

Finally, we examined the mechanism of reduced diastolic SR Ca^{2+} leak by analyzing protein phosphorylation of RyR2 at the CaMKII-specific residue Serine-2814. We found that the phosphorylation was increased by over 100% in sedentary TG mice compared to WT mice ($p<0.05$) (Figure 6D) and that this increase remained despite normalization of the SR Ca^{2+} leak. The Serine-2814 phosphorylation status was neither changed by exercise training in WT mice.

Discussion

The present study demonstrates for the first time that exercise training suppresses the detrimental cardiac-based effects of transgenic CaMKIIδ_C overexpression in vivo and in vitro without significantly changing the CaMKIIδ_C expression level or its phosphorylation. After exercise training the following

aspects of cardiac function were improved or restored to levels similar to that observed in the WT (untrained) animals: (1) global cardiac function in vivo and cardiomyocyte contractility; (2) I_{CaL}; (3) diastolic Ca^{2+} levels and twitch Ca^{2+} transient amplitude; (4) propensity for spontaneous SR Ca^{2+} release; (5) SR Ca^{2+} content; (6) SERCA2a mediated SR Ca^{2+} uptake and; (7) Ca^{2+} efflux by NCX.

Cardiomyocyte function and Ca^{2+} transients

This study show that overexpression of CaMKIIδ_C leads to cardiac dysfunction reminiscent of heart failure, with depressed Ca^{2+} cycling, cardiomyocyte malfunction and increased diastolic SR Ca^{2+} leak. The data confirm as such previous findings in this model (19, 25, 40), with a functionally detrimental effect of chronically increased CaMKII signaling. The prolonged time to Ca^{2+} removal was mainly due to the ~42% reduction in SERCA2a function in TG mice. NCX function was increased by ~48%, which would favor Ca^{2+} extrusion across the sarcolemma and a reduction of diastolic Ca^{2+}-concentration (19). This is not unexpected since commonly reduced SERCA2 activity is accompanied by increased NCX activity in models of cardiac pathology (8, 18, 23). Increased activity of CaMKIIδ_C would normally be expected to chronically enhance SERCA2a function by augmenting phosphorylation of threonine-17 PLN (40), but as previously reported, SERCA2a expression is reduced in the TG model (19, 40), an effect that dominates over the stimulation of SERCA2a activity from enhanced CaMK phosphorylation. As previously reported in CaMKIIδ_C TG mice (39), SR Ca^{2+} content is reduced, this can be linked to the reduced SERCA2a activity and the NCX-linked reduction of diastolic Ca^{2+} levels, both of which will reduce SERCA2a activity and subsequent SR Ca^{2+} content. Therefore, the exercise training effect in TG mice, with reduced extrusion of Ca^{2+} across the plasma membrane via the NCX combined with increased L-type Ca^{2+} currents would in combination with the increased SERCA2a activity enable more SR Ca^{2+} loading and explain the restored Ca^{2+} homeostasis observed after exercise training.

SR Ca^{2+} leak

Increased diastolic SR Ca^{2+} leak via the RyR2 and increased spontaneous Ca^{2+} wave generation observed in TG mice has previously been linked to reduced Ca^{2+} transient amplitude and

330 reduced SR Ca^{2+} content, *i.e.* changes that would limit contractility (2, 33). A recent study of the same
331 TG mice found a higher frequency of delayed afterdepolarizations and increased propensity to
332 arrhythmias as a result of increased SR Ca^{2+} leak (25). The increased SR Ca^{2+} leak is believed to
333 result from the increased activity of CaMKII leading to hyper-phosphorylation of the RyR2 at Serine-
334 2814. This would increase the RyR2 sensitivity to Ca^{2+} and thereby increase the open probability of
335 RyR2 (1, 19, 25). The data from the present study showing AIP to abolish the high SR Ca^{2+} leak
336 observed in sedentary TG mice support this concept. However, despite compelling evidence
337 considering RyR Serine-2814 phosphorylation to be causal in SR Ca^{2+} leak, the exercise training-
338 induced reduction in SR Ca^{2+} leak was not due to a reduction in overall CaMKII activity or
339 phosphorylation status of the RyR at the serine-2814. Changes in antioxidant enzymes activity and
340 oxidative stress following the exercise training period could possibly alter the activation state of
341 CaMKII, as oxidation of CaMKII increases its activity and consequently causes more leaky RyR
342 channels (32). Our data identifying no exercise-induced changes in the phosphorylation status of either
343 the threonine-286 site of CaMKII or the serine-2814 site of RyR2 does, however, indicate that it is
344 unlikely that oxidation of CaMKII could be a central player in modulating the exercise-induced
345 reduction in RyR2-associated SR Ca^{2+} leak, at least in this model of continuous TG overexpression of
346 CaMKIIδ_C. Further analyses are therefore needed to determine the compensatory mechanisms by
347 exercise that counteracts the chronic high levels of CamKII and serine-2814 phosphorylation upon SR
348 Ca^{2+}-leak in these TG mice.
349 A link between increased RyR2-mediated SR Ca^{2+} leak and increased propensity for
350 arrhythmias has received attention lately, especially in heart failure (4, 23, 28, 31, 38), and novel Ca^{2+}
351 release channel-stabilizing drugs have been proposed on this basis (17). The finding that exercise
352 training reduces diastolic SR Ca^{2+} leak is interesting since it ameliorates a deleterious defect in failing
353 hearts through a physiological adaptation mechanism, and may therefore provide an alternative route
354 to the same outcome. This mechanism has also been suggested to be activated by exercise training in
355 the post-myocardial infarction heart failure model (14). It is also important to note that exercise
356 training reverses the increased NCX activity. Thus, these effects suggest that exercise training may
357 have the potential to reduce delayed afterdepolarizations that potentially trigger ventricular

arrhythmias, by synergistically improving diastolic intracellular Ca^{2+} homeostasis via reduced spontaneous SR Ca^{2+} release and reduced NCX activity. The data on reduced frequency of spontaneous Ca^{2+} waves after exercise training in TG CaMKIIδ_C mice does indeed support reduced potential for triggering of ventricular arrhythmias.

Functional cardiac and cardiomyocyte properties

VO_{2max} is regarded as the best indicator of cardio-respiratory endurance, where cardiac output is a key determinant of VO_{2max} as it set the upper limit for O_2- supply to working muscles (24). Chronic overexpression of CaMKIIδ_C has previously been shown to cause a significant depression of cardiac function and remodeling of the heart, similar to observations in heart failure (19, 40), our findings of significantly reduced VO_{2max} in TG mice was therefore in agreement with our hypothesis. Reduced cardiac function in the TG CaMKIIδ_C overexpression model has previously been explained by pathological remodeling of the heart and breakdown of normal Ca^{2+}-handling via phosphorylation of Ca^{2+} regulatory proteins (19, 40), which was confirmed in the present study. The improvements observed in VO_{2max} after exercise training are furthermore in line with improvements in cardiomyocyte functional properties as well as improvements observed in stroke volume and cardiac output. In addition to restoring cardiomyocyte contractility, exercise training also reduced the pathological cellular hypertrophy in TG mice, although it did not completely normalize cell size. Improvements in cardiomyocyte function followed the same pattern as changes in Ca^{2+} cycling and are consistent with previous studies using the same exercise training model in animals with post-myocardial infarction heart failure (36) and diabetic cardiomyopathy (29). LV ejection fraction increased from ~20% to 30%, which has an important clinical value. However, the improvements of in vivo cardiac function measured by echocardiography are less pronounced compared to findings in isolated cardiomyocytes. This may suggest that structural remodeling in the TG mice with continuously activated CaMKII mice cannot be completely normalized by exercise training under the current conditions. The comparisons between single cell contractility and that of the whole heart are made complex because of the additional factors that apply to the intact myocardium including: (1) isometric and isotonic components to the contractile event in whole heart (only isotonic in single cell), (2) Interstitial fibrosis

386 in whole hearts and (3) changes in system peripheral resistance. Our data reflect the physiological
387 relevance of in vivo measurements in addition to in vitro assessments of isolated cardiomyocytes
388 contracting in non-isometric conditions. Further work is required to investigate the basis of the
389 differences between whole heart and single cell contractility parameters
390
391 *Conclusions*
392 Exercise training improved in vivo cardiac function, restored cardiomyocyte function, plasma
393 membrane and sarcolemmal and intracellular Ca^{2+} fluxes and abolished the abnormally high diastolic
394 SR Ca^{2+} leak in mice with TG overexpression of CaMKIIδ_C. Thus, despite a continuous background of
395 abnormally high CaMKIIδ_C, exercise training triggers mechanisms such as improved L-type Ca^{2+}
396 channels, SR Ca^{2+}-handling by restoration of SERCa2a function in addition to reduced diastolic SR
397 Ca^{2+}-leak thereby restoring cardiomyocyte Ca^{2+}-homeostasis.
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Figure legends:

Figure 1

A, CaMKII total protein levels and B, phosphorylated CaMKII at Threonine-286. Protein measurements are presented as mean ± SEM (number of animals each group (N=4). C, Examples of western blots of protein regulation. D, Maximal oxygen uptake was measured in all animals included in the study. (VO_{2max}) was reduced in transgenic (TG) CaMKIIδ_C overexpressing mice (N=12) compared to WT sedentary (N=12); exercise increased VO_{2max} in both TG (N=12) and WT (N=12). E, Cardiomyocyte volume was significantly larger in TG mice (N=5) compared to WT (N=5); exercise reduced cell volume in TG (N=5), but increased cell volume in WT (N=5). Data in D and E are presented as mean ± SD. † $P<0.01$ vs. WT sedentary, * $P<0.05$ vs. sedentary WT, # $P<0.05$ vs. sedentary TG.

Figure 2

A, representative sample tracings of cardiomyocyte fractional shortening from sedentary and exercise trained transgenic (TG) CaMKIIδ_C overexpressing mice, and sedentary and exercised WT mice. B, fractional shortening was significantly reduced in TG, whereas exercise training in TG restored this to WT levels. C, time to 50% relengthening was longer in TG and restored after exercise training, with a comparable response to that of exercise training in WT. ** $P<0.01$ vs. other groups. There were no significant differences between exercise trained TG and WT mice. n=25-30 cells per group)

Figure 3

A, Ca^{2+} flux through I_{CaL} was reduced in sedentary TG compared to trained TG. B, Representative L-type Ca^{2+} current (I_{CaL}) recordings from sedentary transgenic (TG) CaMKIIδ_C overexpressing mice (red), trained TG (blue), sedentary WT (green), and trained WT (black). C, I_{CaL} density was reduced in sedentary TG compared to trained TG. C, WT sedentary: n=14 cells; WT exercise: 14 cells; TG sedentary: 19 cells; exercise TG: n=14 cells. D, Protein expression on L-type Ca^{2+}-channel was significantly increased after exercise training in TG mice (number of mice in each group, N=4). Data are presented as mean ± SEM. * $P<0.05$ vs. trained TG. # $P<0.5$ between exercise trained WT vs. Sedentary WT.

Figure 4

A, representative traces of Ca^{2+}-transients by Fura-2/AM ratio ($F^{340/380}$) recordings. B, twitch-stimulated Ca^{2+}- transient amplitude (Fura-2/AM ratio $F^{340/380}$) was reduced in transgenic (TG) CaMKIIδ_C overexpressing mice compared to WT. Exercise training increased the Ca^{2+}- transient amplitude in both TG and WT; in TG to levels comparable to WT mice. C, caffeine-evoked Ca^{2+}-transient amplitude (SR Ca^{2+}-content) was reduced in TG mice compared to WT. Exercise training increased the SR Ca^{2+} content in both TG and WT; in TG to levels comparable to sedentary WT. ** $P<0.01$ vs. other groups, *$P<0.05$ vs. other groups. There were no significant differences between exercise trained TG and sedentary WT mice. Cells in each group (n=25-30)

Figure 5

A, diastolic Ca^{2+}- levels were lower in sedentary transgenic (TG) CaMKIIδ_C overexpressing mice, but this was raised to sedentary WT levels by exercise training; exercise training had, however, no effect in WT. B, time to 50% Ca^{2+}-decay was prolonged in TG mice compared to WT, but reduced by exercise training to WT levels; exercise training also reduced time to 50% Ca^{2+}-decay in WT. C, example traces of Ca^{2+}-transients evoked by twitch-stimulations and Caffeine-stimulations. D, calculated NCX rate constant of Ca^{2+} removal in; the NCX rate was increased in TG whereas exercise training normalized the rate; exercise training had no effect in WT. E, calculated SERCA2a

rate constant of Ca^{2+} removal; SERCA2a rate was reduced in TG mice compared to WT, whereas exercise training increased rate in both TG and WT.** P<0.01 vs. other groups, *P<0.05 vs. WT. Cells in each group (n=25-30). F, Protein expression of SERCA2a (protein-expressions are presented as mean ± SEM, (number of mice in each group, N=4, No significant differences was observed between groups)

Figure 6

A, diastolic SR RyR Ca^{2+} leak in normal HEPES 1.8 Ca^{2+} solution in sedentary and exercise trained transgenic (TG) CaMKIIδ_C overexpressing mice and WT mice; and B, RyR Ca^{2+} leak after incubation by AIP (to inhibit CaMKII) and H-89 (to inhibit PKA) in sedentary TG mice. Note that exercise training reduced the Ca^{2+} leak to levels found in WT mice, and inhibiting CaMKII with AIP abolished Ca^{2+} leak. PKA inhibition by H-89 had no significant effect on reducing Ca^{2+} leak. No significant effects of H-89 or AIP were seen in any of the other groups. C, frequency of spontaneous Ca^{2+} waves was higher in sedentary TG compared to WT; exercise training reduced Ca^{2+} wave frequency to WT levels. Number of animals in each group for cardiomyocyte data (N=5), number of cells in each group (n=25-30). D, phosphorylation of Serine-2814 residues at RyR2; example blots in inset (protein-expressions are presented as mean ± SEM, (number of rats in each group, N=4). ** $P<0.01$ vs. other groups and *$P<0.05$ vs. other groups. # $P<0.05$ between TG and WT sedentary.

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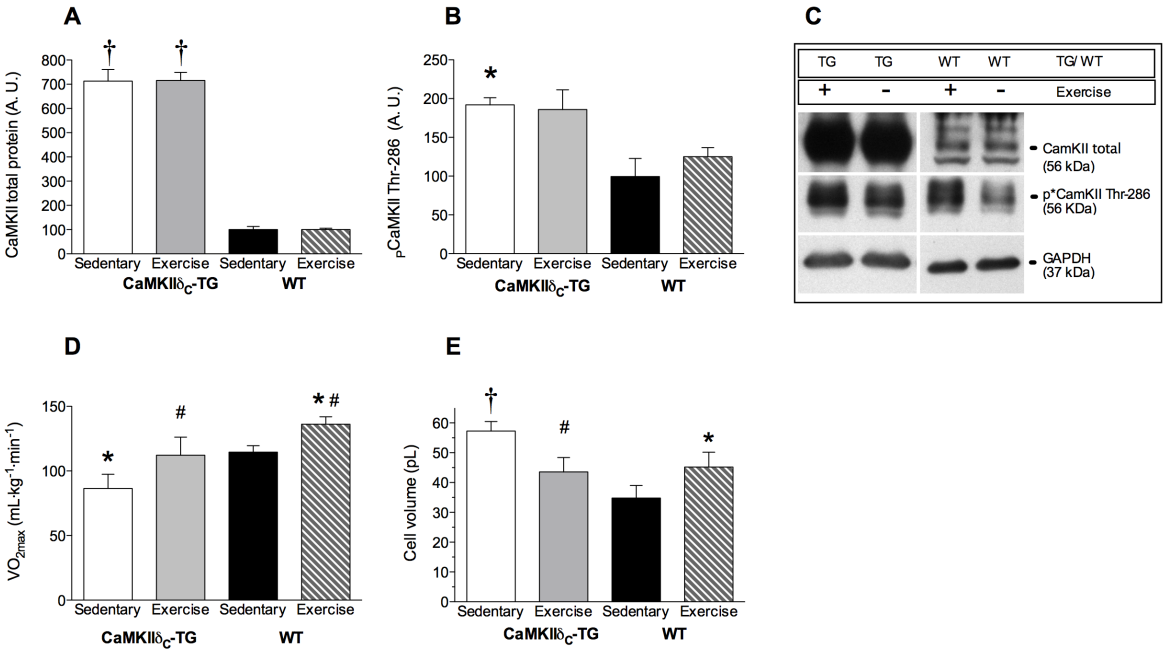
A
CaMKII total protein (A. U.)
800
700
600
500
400
300
200
100
0
Sedentary
Exercise
Sedentary
Exercise
CaMKIIδC-TG
WT
B
pCaMKII Thr-286 (A. U.)
250
200
150
100
50
0
Sedentary
Exercise
Sedentary
Exercise
CaMKIIδC-TG
WT
C
TG
TG
WT
WT
TG/ WT
+
-
+
-
Exercise
CamKII total
(56 kDa)
p*CamKII Thr-286
(56 KDa)
GAPDH
(37 kDa)
D
VO2max (mL·kg-1·min-1)
150
100
50
0
Sedentary
Exercise
Sedentary
Exercise
CaMKIIδC-TG
WT
E
Cell volume (pL)
60
50
40
30
20
10
0
Sedentary
Exercise
Sedentary
Exercise
CaMKIIδC-TG
WT

Figure 1.

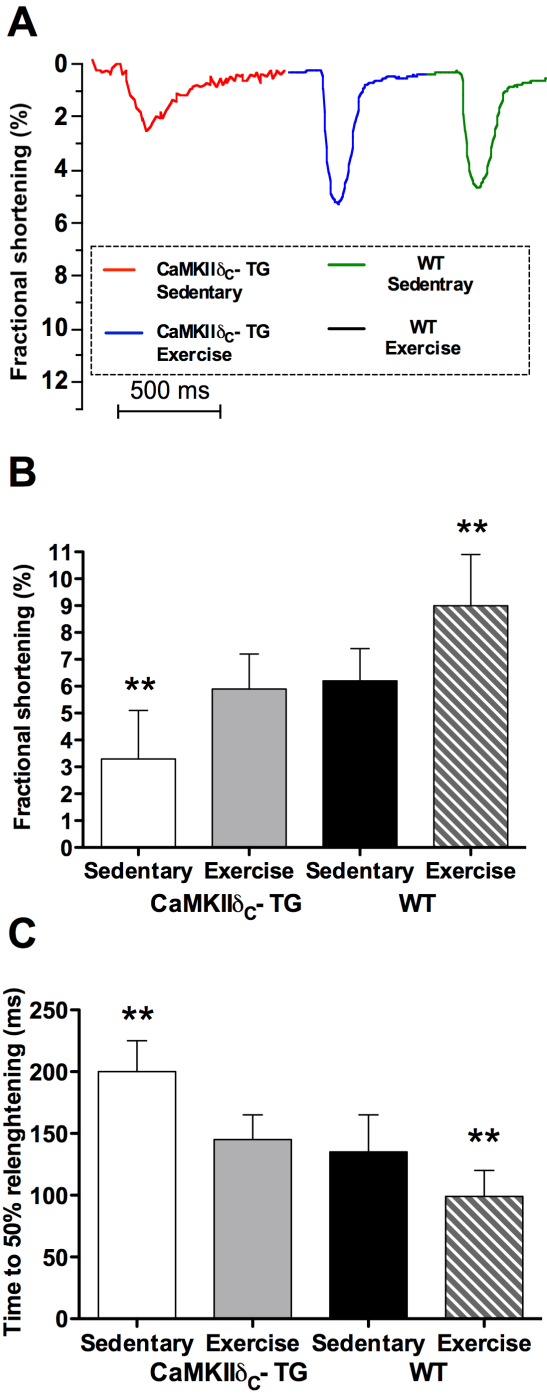
A
Fractional shortening (%)
0
2
4
6
8
10
12
CaMKIIδC- TG Sedentary
WT Sedentray
CaMKIIδC- TG Exercise
WT Exercise
500 ms
B
Fractional shortening (%)
11
10
9
8
7
6
5
4
3
2
1
0
**
**
Sedentary Exercise Sedentary Exercise
CaMKIIδC- TG
WT
C
Time to 50% relenghtening (ms)
250
200
150
100
50
0
**
**
Sedentary Exercise Sedentary Exercise
CaMKIIδC- TG
WT

Figure 2.

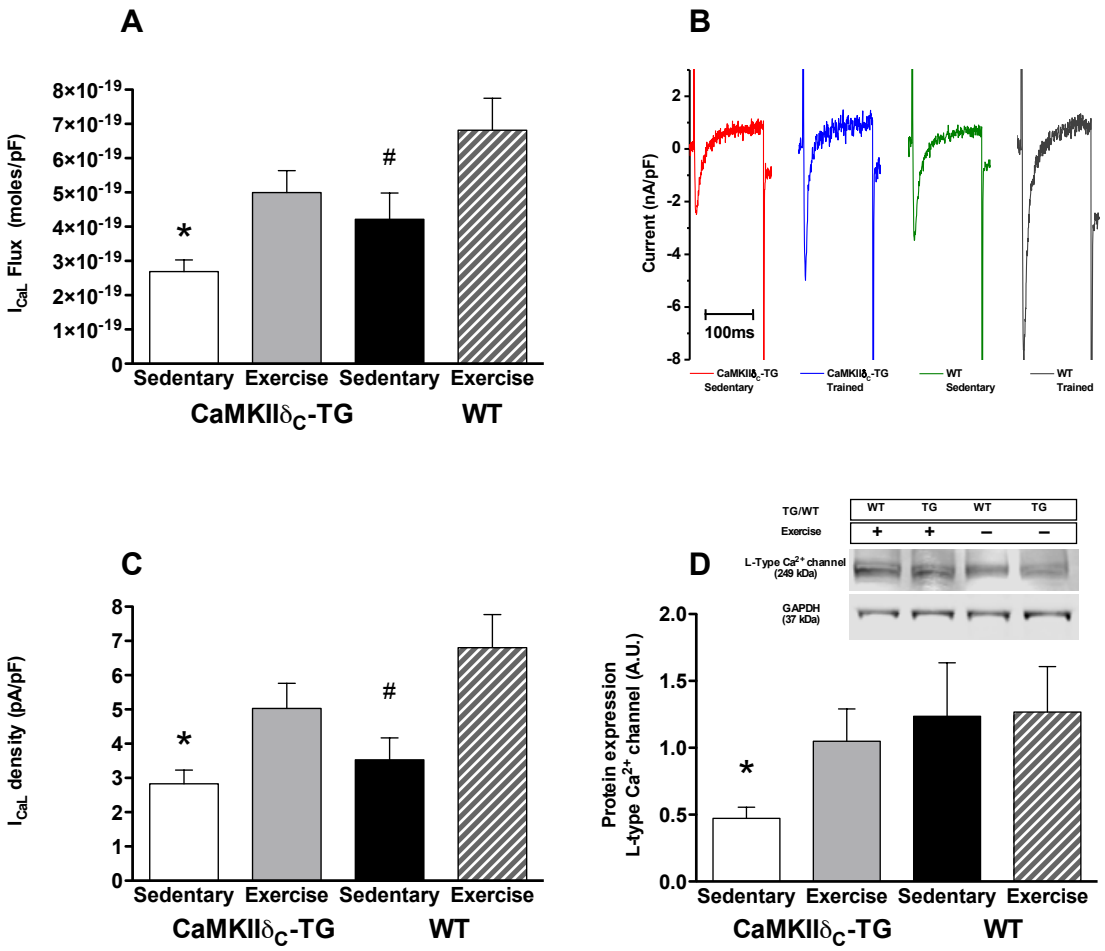
A
B
C
D
Sedentary
Exercise
CaMKIIδC-TG
WT
100ms
Trained
TG/WT
Exercise
L-Type Ca2+ channel (249 kDa)
GAPDH (37 kDa)
Protein expression L-type Ca2+ channel (A.U.)
Current (nA/pF)

Figure 3.

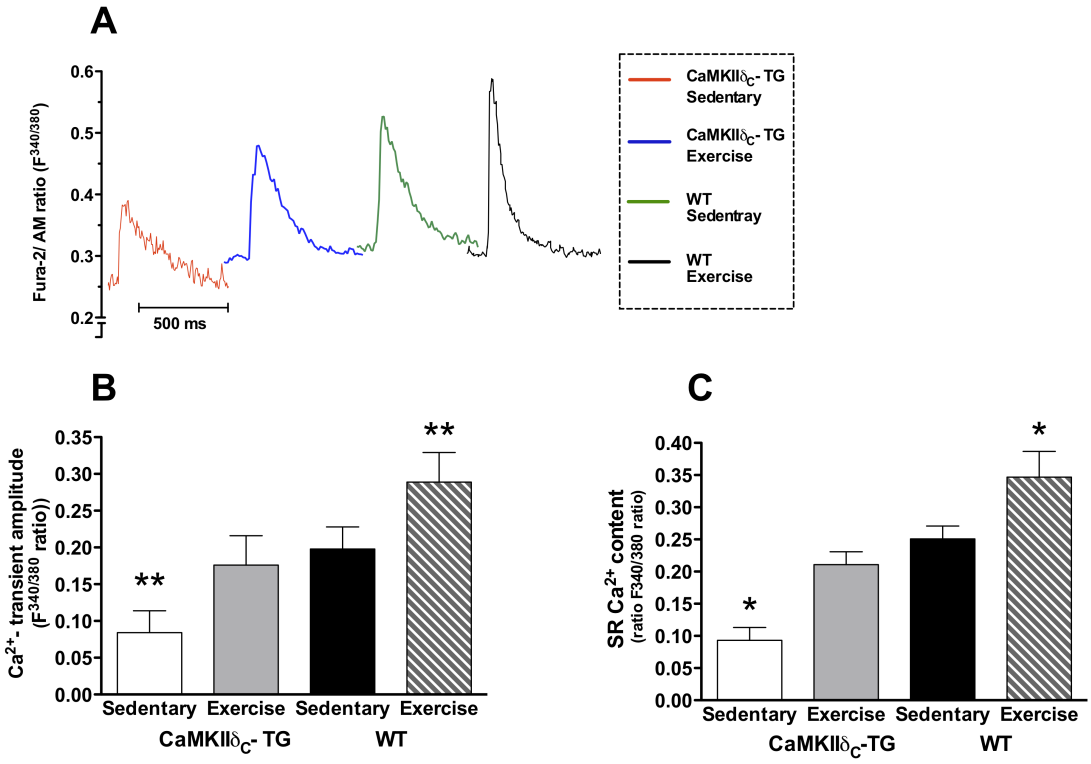
A
Fura-2/ AM ratio (F340/380)
0.6
0.5
0.4
0.3
0.2
500 ms
CaMKIIδc- TG Sedentary
CaMKIIδc- TG Exercise
WT Sedentray
WT Exercise
B
Ca2+- transient amplitude (F340/380 ratio))
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
**
**
Sedentary
Exercise
Sedentary
Exercise
CaMKIIδc- TG
WT
C
SR Ca2+ content (ratio F340/380 ratio)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
*
Sedentary
Exercise
Sedentary
Exercise
CaMKIIδc-TG
WT

Figure 4.

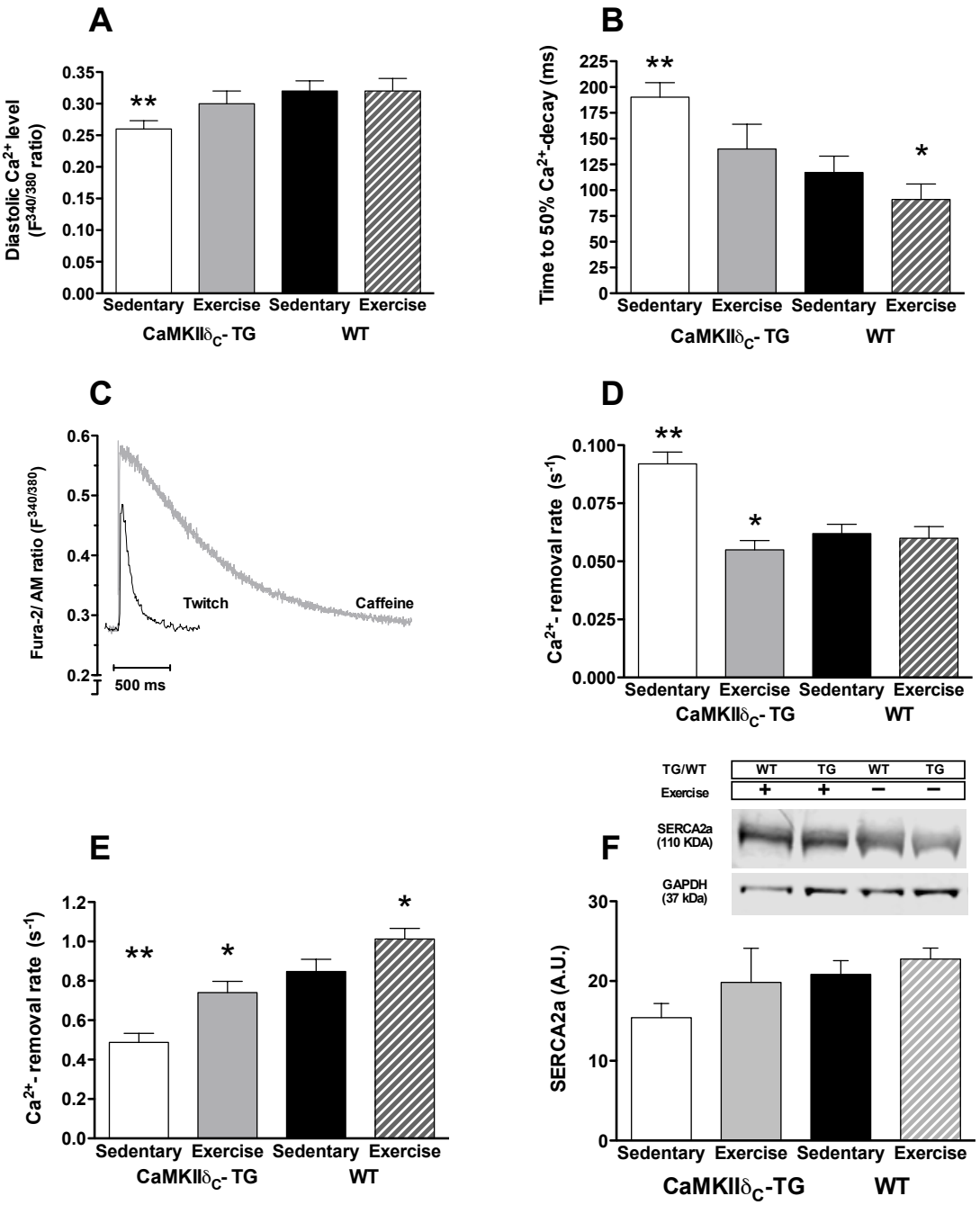
A
Diastolic Ca2+ level (F340/380 ratio)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
**
Sedentary Exercise Sedentary Exercise
CaMKIIδC- TG
WT
B
Time to 50% Ca2+-decay (ms)
225
200
175
150
125
100
75
50
25
0
**
*
Sedentary Exercise Sedentary Exercise
CaMKIIδC- TG
WT
C
Fura-2/ AM ratio (F340/380)
0.6
0.5
0.4
0.3
0.2
Twitch
Caffeine
500 ms
D
Ca2+- removal rate (s-1)
0.100
0.075
0.050
0.025
0.000
**
*
Sedentary Exercise Sedentary Exercise
CaMKIIδC- TG
WT
E
Ca2+- removal rate (s-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
*
Sedentary Exercise Sedentary Exercise
CaMKIIδC- TG
WT
F
TG/WT
WT TG WT TG
Exercise
+ + − −
SERCA2a (110 KDA)
GAPDH (37 kDa)
SERCA2a (A.U.)
30
20
10
0
Sedentary Exercise Sedentary Exercise
CaMKIIδC-TG
WT

Figure 5.

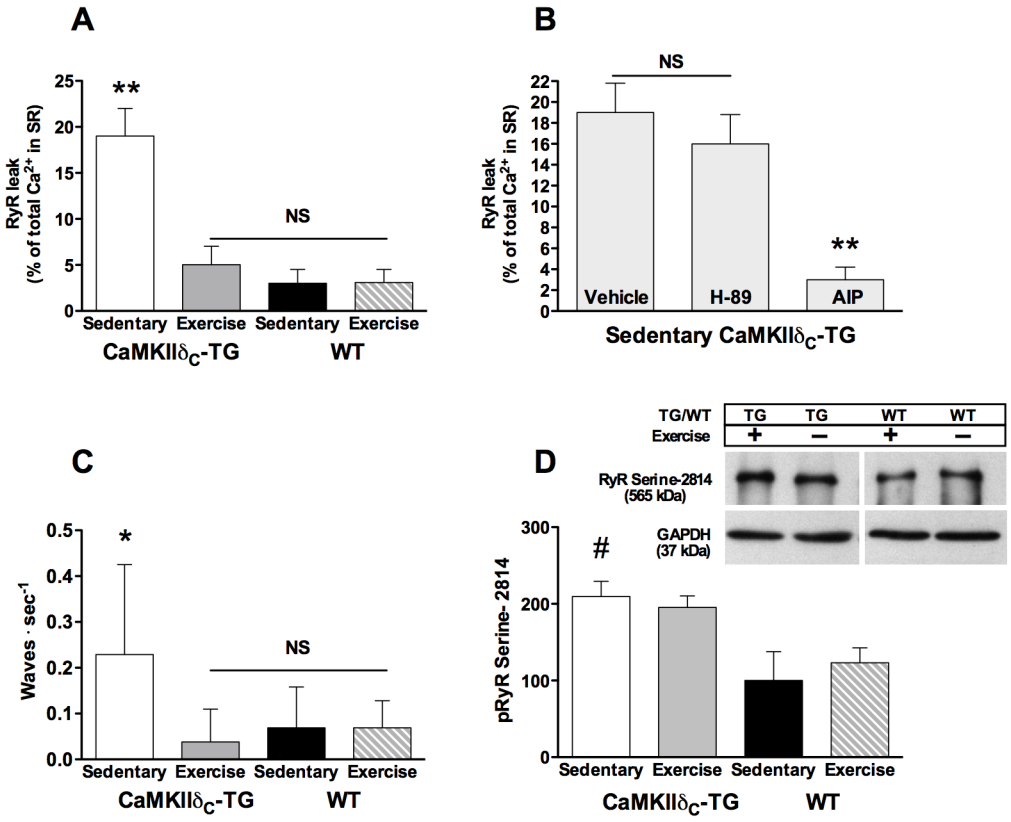
A
RyR leak
(% of total Ca2+ in SR)
**
NS
Sedentary Exercise Sedentary Exercise
CaMKIIδC-TG
WT
B
RyR leak
(% of total Ca2+ in SR)
NS
**
Vehicle
H-89
AIP
Sedentary CaMKIIδC-TG
C
Waves · sec-1
*
NS
Sedentary Exercise Sedentary Exercise
CaMKIIδC-TG
WT
D
TG/WT
TG TG WT WT
Exercise
+ – + –
RyR Serine-2814
(565 kDa)
GAPDH
(37 kDa)
pRyR Serine- 2814
#
Sedentary Exercise Sedentary Exercise
CaMKIIδC-TG
WT

Figure 6.

Table 1. Global cardiac left ventricle (LV) function (echocardiography)

	CaMKIIδ$_C$ TG		Wild type	
	Sedentary	**Exercise**	**Sedentary**	**Exercise**
LV Cardiac output (ml/min)	12.3 ± 2.8 #	17.6 ± 1.1 *	19.0 ± 1.2 *	23.0 ± 3.0 #*
LV Stroke volume (µl)	25.2 ± 4.6 #	35.2 ± 1.8 *	35.5 ± 2.6 *	42.2 ± 4.9 §*
LV Ejection fraction (%)	19.4 ± 3.0 #	29.7 ± 5.8 *#	50.7 ± 3.7 *	64.5 ± 4.5 §*
LV Fractional shortening (%)	8.9 ± 1.4 #	14.0 ± 3.0 *#	25.5 ± 2.2 *	34.8 ± 3.4 §*
LV Diameter; end systole (mm)	4.7 ± 0.2 #	4.3 ± 0.4 #	3.0 ± 0.2 *	2.5 ± 0.2 §*
LV Diameter; end diastole (mm)	5.2 ± 0.2 #	5.0 ± 0.3 #	4.0 ± 0.2 *	3.9 ± 0.2 *
LV Volume; end systole (µl)	105 ± 12.4 #	86.1 ± 17.7 #	35.1 ± 5.8 *	23.5 ± 4.5 §*
LV Volume; end diastole (µl)	130.2 ± 14.0 #	121.3 ± 16.1 #	70.6 ± 7.6 *	65.6 ± 8.0*

Data are mean±SD. CaMKII, Ca^{2+}/calmodulin-dependent kinase II. Difference from sedentary CaMKIIδC TG; * P<0.01. Difference from sedentary WT; # P<0.01, § P<0.05.