

Diastolic dysfunction and arrhythmias caused by overexpression of CaMKII δ_C can be reversed by inhibition of late Na⁺ current

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Abstract Transgenic (TG) Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) δ_C mice develop systolic heart failure (HF). CaMKII regulates intracellular Ca²⁺ handling proteins as well as sarcolemmal Na⁺ channels. We hypothesized that CaMKII also contributes to diastolic dysfunction and arrhythmias via augmentation of the late Na⁺ current (late I_{Na}) in early HF (8-week-old TG mice). Echocardiography revealed severe diastolic dysfunction in addition to decreased systolic ejection fraction. Premature arrhythmogenic contractions (PACs) in isolated isometrically twitching papillary muscles only occurred in TG preparations (5 vs. 0, $P < 0.05$) which could be completely terminated when treated with the late I_{Na} inhibitor ranolazine (Ran, 5 $\mu\text{mol/L}$). Force–frequency relationships revealed significantly reduced twitch force amplitudes in TG papillary muscles. Most importantly, diastolic tension increased with raising frequencies to a greater extent in TG papillary muscles compared to WT specimen (at 10 Hz: 3.7 ± 0.4 vs. 2.5 ± 0.3 mN/mm²; $P < 0.05$). Addition of Ran improved diastolic dysfunction to 2.1 ± 0.2 mN/mm²

(at 10 Hz; $P < 0.05$) without negative inotropic effects. Mechanistically, the late I_{Na} was markedly elevated in myocytes isolated from TG mice and could be completely reversed by Ran. In conclusion, our results show for the first time that TG CaMKII δ_C overexpression induces diastolic dysfunction and arrhythmogenic triggers possibly via an enhanced late I_{Na} . Inhibition of elevated late I_{Na} had beneficial effects on arrhythmias as well as diastolic function in papillary muscles from CaMKII δ_C TG mice. Thus, late I_{Na} inhibition appears to be a promising option for diastolic dysfunction and arrhythmias in HF where CaMKII is found to be increased.

Keywords Heart failure · Arrhythmias · Contractility · Diastolic dysfunction · Excitation–contraction coupling · CaMKII

Introduction

Systolic contractile dysfunction in heart failure (HF) is caused by altered intracellular ion homeostasis and structural remodeling. Several pathomechanisms have been identified. The sarcoplasmic reticulum (SR) Ca²⁺-ATPase (SERCA) protein levels and its activity are reduced in parallel to a diminished SR Ca²⁺-uptake capacity in the failing heart [8, 9, 19, 22]. The functional consequence is an impaired SR Ca²⁺-loading leading to smaller intracellular Ca²⁺ transients and elevated diastolic Ca²⁺ levels [3]. Ca²⁺ homeostasis is further regulated by phosphorylation of several key proteins thereby controlling Ca²⁺- as well as Na⁺-fluxes in hypertrophy [13] and HF [15]. In this context, the Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) is of major importance [15]. The predominant cardiac isoform is CaMKII δ with the splice variant δ_C

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being primarily cytosolic [32]. CaMKII is a serine/threonine protein kinase that modulates several Ca^{2+} -dependent proteins, such as SR Ca^{2+} -release channels (ryanodine receptors, RyR2), phospholamban (PLB) which regulates SERCA and L-type Ca^{2+} -channels.

We and others have recently shown that CaMKII δ also regulates Na^+ channels in myocytes, most likely by association with and phosphorylation of sarcolemmal Na^+ channels [1, 31]. Overexpression of CaMKII δ was associated with an enhanced late I_{Na} that inactivates with much slower kinetics [31]. Although the amplitude of this current is very small compared to peak I_{Na} , the slow inactivation kinetic results in a substantial Na^+ entry during the action potential (AP) leading to a significant prolongation of the AP duration [7, 24].

There is strong evidence for an increased late I_{Na} in ventricular myocytes and its contribution to HF via $[\text{Na}^+]_i$ elevation [5, 18, 20, 29]. Mechanistically, augmentation of the late I_{Na} plays a crucial role in impaired contractility and repolarization of the failing myocardium [17, 29]. We have recently shown that an increased late I_{Na} can substantially elevate intracellular Na^+ and consequently diastolic Ca^{2+} via reverse mode of the Na^+ / Ca^{2+} -exchanger (NCX) leading to diastolic dysfunction in isolated human ventricular end-stage failing myocardium [24].

Furthermore, it is well accepted that an increased late I_{Na} is known to produce arrhythmogenic triggers, such as early afterdepolarizations (EADs) via prolongation of the cardiac AP or delayed afterdepolarizations (DADs) caused by a Na^+ -dependent diastolic Ca^{2+} overload and oscillatory SR Ca^{2+} -release which may activate a transient inward current (I_{Ti}) [14, 17, 23].

We have recently shown that pharmacological inhibition of CaMKII reduces cellular proarrhythmogenic events and decreases arrhythmias in vivo in TG CaMKII δ_{C} mice with severe HF due to reduced spontaneous SR Ca^{2+} release events (Ca^{2+} sparks) [21]. We hypothesized that CaMKII-dependent late I_{Na} augmentation is a novel cause for diastolic dysfunction and arrhythmias in CaMKII TG mice with non-ischemic compensated HF.

Methods

CaMKII δ_{C} transgenic mice

CaMKII δ_{C} transgenic (TG) mice were generated using an α -MHC promoter as previously described [33]. We used 8-week-old CaMKII δ_{C} -TG mice and age- and sex-matched WT littermates. All animal studies were approved by the Ethics Committee of the Medical Faculty of the University of Göttingen.

Transthoracic echocardiography

Transthoracic echocardiography was performed using a Vevo2100 (VisualSonics, Toronto, Canada) system with a 30 MHz center frequency transducer. Briefly, animals were anesthetized with 3% isoflurane, and temperature-, respiration-, and ECG-controlled anesthesia was maintained with 1.5% isoflurane. Two-dimensional cine loops with frame rates of >200 frames/s of a long-axis view and a short-axis view at mid-level of the papillary muscles as well as M-mode loops of the short-axis view were recorded. Thicknesses of the anterior myocardial wall (AWTh), the posterior myocardial wall (PWTh), the inner diameter of the left ventricle (LVID) and the area of the left ventricular cavity (Area) were measured in systole and diastole from the short-axis view according to standard procedures [4]. Maximal left ventricular length was measured from the long-axis view. Systolic and diastolic left ventricular volumes were calculated using the area-length method. Maximal diastolic radial velocity of the anterior wall was analyzed using the VevoStrain software (VisualSonics, Toronto, Canada). Measurements were obtained by an examiner blinded to the genotype of the animals.

Mouse intact papillary muscle preparation

TG mice were anesthetized using isoflurane. Hearts were rapidly excised and retrogradely perfused with a modified Krebs–Henseleit buffer solution containing (in mmol/L) Na^+ 140.5, K^+ 5.1, Mg^{2+} 1.2, Ca^{2+} 0.25, Cl^- 124.9, SO_4^{2-} 1.2, PO_4^{3-} 2.0, HCO_3^- 20, glucose 10, and butanedione monoxime (BDM) 20, equilibrated with carbogen (95% O_2 , 5% CO_2 , pH 7.4). Intact papillary muscles were isolated from the RV wall using a stereoscopic microscope in a dissection chamber. Cross-sectional dimensions were similar in WT as well as TG mice (width \times thickness \times $\pi/4$, $0.22 \pm 0.01 \text{ mm}^2$ for TG and $0.16 \pm 0.07 \text{ mm}^2$ for WT, $P = 0.3$).

Characterization of contractile phenotype

For isometric force recordings, papillary muscles were mounted in an organ chamber and connected to the force transducer (Scientific Instruments, Heidelberg, Germany). Papillary muscles were superfused with Krebs–Henseleit solution (in mmol/L: NaCl_2 116, KCL 5, NaH_2PO_4 2, MgCl_2 1.2, Na_2SO_4 1.2, NaHCO_3 20, CaCl_2 initially 0.25; end concentration 1.25, glucose 10) that was also oxygenated with 95% O_2 and 5% CO_2 (37°C). Isometric contractions were elicited using electrical field stimulation with a basal stimulation frequency of 4 Hz (voltage 25% above threshold, normally 3–5 V, stimulator, Scientific

Instruments, Heidelberg, Germany). Ca^{2+} (0.25 mmol/L) was added every 2 min after a 30 min washout phase until the final concentration of 1.25 mmol/L was reached in order to prevent a Ca^{2+} -induced contracture. After an equilibration period of 20 min, the muscles were gradually stretched until the maximum steady-state twitch force was achieved and 5 $\mu\text{mol/L}$ ranolazine (Ran, Gilead, Palo Alto, USA) or vehicle was added to the bath solution. After having characterized the basal effect of late I_{Na} inhibition, force–frequency relationships were obtained using increasingly higher stimulation rates of 2, 4, 6, 8, 10, and then back to 4 Hz. To measure SR Ca^{2+} -load, post-rest behavior was assessed by using increasing rest intervals of 5 and 10 s between beats at a basal stimulation frequency of 4 Hz [24, 26]. Short periods of rest increase force of contraction of the first beat upon restimulation which is considered to be dependent on SR Ca^{2+} -uptake and release [25]. In addition, persistent premature arrhythmogenic contractions (PACs) were assessed [26]. PACs had to be stable and to persist continuously over 5 min before the effect of Ran was evaluated [26].

Patch-clamp experiments

Ruptured-patch whole-cell voltage-clamp was used to measure I_{Na} as described previously [24, 26]. Microelectrodes (2–3 M Ω) were filled with (mmol/L) 40 CsCl, 80 Cs-glutamate, 10 NaCl, 0.92 MgCl₂, 5 Mg-ATP, 0.3 Li-GTP, 10 HEPES, 0.03 niflumic acid, 0.02 nifedipine, 0.004 strophanthidin, 5 BAPTA (tetraesium salt), 1 5,5'-dibromo BAPTA (tetrapotassium salt), 1.49 CaCl₂ (free $[\text{Ca}^{2+}]_i = 100 \text{ nmol/L}$; pH 7.2, CsOH). The bath solution contained (mmol/L) 130 NaCl, 10 tetraethylammonium chloride, 4 CsCl, 1 MgCl₂, 10 glucose, 10 HEPES, (pH 7.4, NaOH). Myocytes were mounted on the stage of a microscope (Nikon Eclipse TE2000-U, Düsseldorf, Germany). Fast capacitance which is generated largely by the pipette itself was compensated in cell-attached configuration. Liquid junction potentials (3–6 mV) were corrected. Membrane capacitance was compensated after patch rupture, access resistance was typically <7 M Ω . All recordings were started 5 min after rupture. Signals were filtered with 2.9 and 10 kHz Bessel filters and recorded with an EPC10 amplifier (HEKA Elektronik Dr. Schulze GmbH, Lambrecht/Pfalz, Germany). Myocytes were held at –120 mV and late I_{Na} was elicited using 250 ms depolarizing pulses to –30 mV. Each pulse was preceded by a 5 ms pre-pulse to +50 mV in order to optimize voltage control. The measured currents were normalized to the membrane capacitance. I_{Na} decay (first 200 ms) was fitted using a double exponential function $y(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + y_0$. All patch-clamp experiments were conducted at room temperature.

Western blots

Myocardium was homogenized in Tris buffer containing in mmol/L: 20 Tris-HCl, pH 7.4, 200 NaCl, 20 NaF, 1 Na₃VO₄, 1 DTT, 1% Triton X-100 and complete protease inhibitor cocktail (Roche Diagnostics, Grenzach-Wyhlen, Germany). Protein concentration was determined by BCA assay (Pierce Biotechnology, Rockford, USA). For NCX, denatured cell lysates and tissue homogenates (on ice in 2% beta-mercaptoethanol) were subjected to Western blotting (8% SDS-polyacrylamide gels) using anti-NCX (1:5000; Swant, Bellinzona, Switzerland), anti-GAPDH (1:40,000, Biotrend Chemikalien GmbH, Köln, Germany) as primary and an HRP-conjugated donkey anti-rabbit and sheep anti-mouse IgG (1:10,000; Amersham Biosciences, Freiburg, Germany) as secondary antibody. SERCA blots were performed using denatured cell lysates and tissue homogenates (on ice in 2% beta-mercaptoethanol) which were subjected to Western blotting (8% SDS-polyacrylamide gels) using anti-SERCA2 (1:20,000; Affinity BioReagents, Rockford, USA) and anti-GAPDH (1:40,000, Biotrend Chemikalien GmbH, Köln, Germany) as primary and a sheep anti-mouse IgG (1:10,000; Amersham Biosciences, Freiburg, Germany) as secondary antibody. Chemiluminescent detection was done with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, USA).

Data analysis and statistics

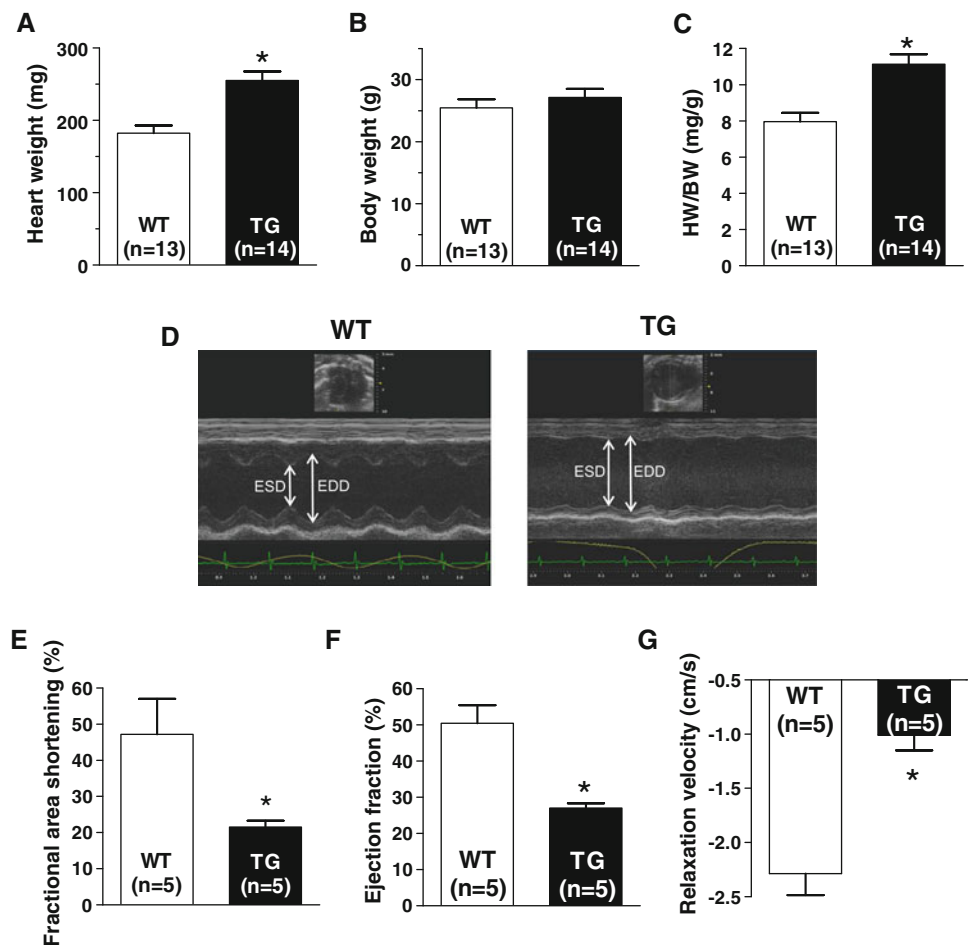
Force values were normalized to the cross-sectional area of each muscle (width \times thickness \times $\pi/4$) and expressed in mN/mm². All data are shown as mean \pm S.E.M. Student's *t* test, 2-way or 1-way repeated measures ANOVA with post hoc tests or Fisher's exact test were used to test for significance. A two-sided *P* value of <0.05 was considered significant.

Results

Cardiac phenotype of transgenic CaMKII δ_C mice

In CaMKII δ_C TG mice the heart weight/body weight ratio was increased by ~40% as compared to WT (Fig. 1a–c, *n* = 13 vs. 14, *P* < 0.05) indicating an early stage of hypertrophy (8-week-old mice) since this value can increase to more than twofold in 3-month-old mice [16]. Figure 1d shows original M-mode echocardiographic recordings with ventricular systolic dysfunction in a TG as compared to a WT mouse. TG mice have a reduced fractional area shortening (Fig. 1e) as well as a reduced ejection fraction of $27 \pm 1\%$ compared to $50 \pm 5\%$ in WT mice (Fig. 1f, *n* = 5 each, *P* < 0.05). In order to evaluate diastolic function the

Fig. 1 Cardiac phenotype characteristics. **a** Heart weight of TG CaMKII δ_C mice was increased compared to WT ($n = 14$ vs. 13, $P < 0.05$), while **b** body weight remained unchanged. **c** Mean values of heart weight/body weight ratio (HW/BW). **d** Representative echocardiographic recordings of a WT and TG CaMKII δ_C mouse. Mean values of **e** fractional area shortening and **f** ejection fraction ($n = 5$ each, $P < 0.05$). **g** A reduced relaxation velocity in TG CaMKII δ_C mice indicates diastolic dysfunction ($P < 0.05$)



relaxation velocity was also determined. TG CaMKII δ_C mice had markedly impaired relaxation velocities of 1.0 ± 0.4 cm/s compared to 2.3 ± 0.2 cm/s in WT mice (Fig. 1g, $P < 0.05$). Therefore, these mice suffer from diastolic in addition to systolic dysfunction.

Changes in Ca²⁺ handling proteins

We investigated SERCA and NCX protein abundance in WT and CaMKII δ_C TG left ventricular myocardium to assess their possible involvement in the alterations of diastolic left ventricular performance. We found that SERCA protein levels were reduced to $74 \pm 10\%$ in TG CaMKII δ_C mice (Fig. 2a, $n = 7$ vs. 6 WT, $P < 0.05$), while NCX protein abundance was unaltered (Fig. 2b). Here, it is important to note that NCX protein levels in 3-month-old TG CaMKII δ_C mice, a time where these mice develop overt HF, are dramatically increased [16].

Premature contractions in isolated papillary muscles

While muscles from WT mice did not develop PACs [26] we found PACs in all investigated TG muscles ($n = 5$;

$P < 0.05$, Fisher's test, Fig. 3a, b). Addition of vehicle solution did not terminate PACs, whereas these were inhibited by adding $5 \mu\text{mol/L}$ Ran to the bath solution (Fig. 3c, d, $P < 0.05$, Fisher's test). These antiarrhythmic effects of Ran were observed within 2 min of exposure to the drug. Thereafter, the papillary muscles remained stable and generated a regular rhythmic stimulation-dependent response.

Basal contractility

In another series of experiments the basal effect of $5 \mu\text{mol/L}$ Ran was investigated in TG muscles stimulated at 4 Hz. After an incubation period of 15 min twitch force amplitude was $81.1 \pm 6.3\%$ (normalized to baseline) in vehicle-treated specimen as compared to $69.3 \pm 6.7\%$ ($n = 9$ each, $P = 0.22$, data not shown) in the presence of $5 \mu\text{mol/L}$ Ran. While force amplitude was unchanged we found a reduced diastolic tension in Ran-treated muscles to $87.0 \pm 2.8\%$ as compared to vehicle with $99.6 \pm 5.5\%$ ($P < 0.05$, Fig. 4a).

Post-rest behavior of papillary muscles was determined at rest intervals of 5 and 10 s to biomechanically assess SR

Fig. 2 Protein analysis of SERCA and NCX. **a** Mean values of SERCA protein levels indicate a downregulation in TG CaMKII δ c mice ($n = 6$ vs. 7 WT, $P < 0.05$). **b** There was no difference in NCX protein expression between WT and TG CaMKII δ c ($n = 7$ vs. 6)

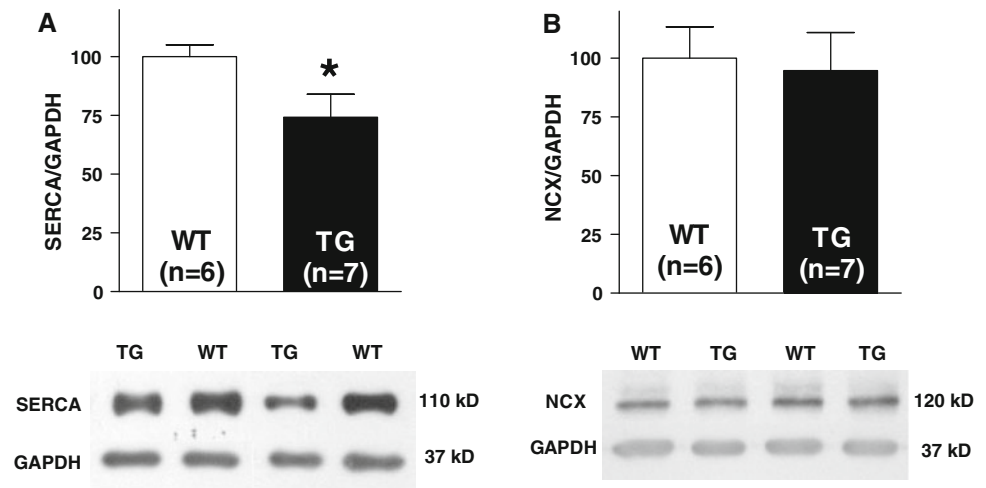
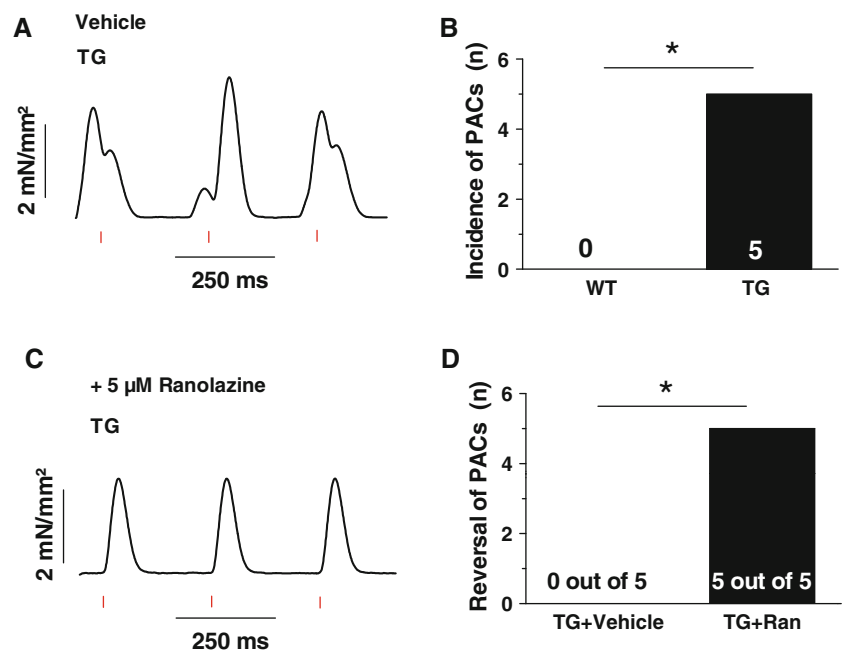


Fig. 3 Spontaneous premature arrhythmogenic contractions (PACs). **a** Original tracings showing PACs in a TG CaMKII δ c muscle treated with vehicle control. The red marks indicate regular stimulation pulses (4 Hz). **b** Mean values of PACs in WT and CaMKII δ c muscles ($n = 0$ vs. 5, $P < 0.05$, Fisher's test). **c** Addition of 5 μ mol/L ranolazine (Ran) reversed the PACs in the same preparation back to a stimulation-dependent rhythm. **d** All PACs in CaMKII δ c muscles could be reversed by Ran addition ($P < 0.05$, Fisher's test)



Ca²⁺ content [24, 26]. Figure 4b summarizes these results indicating that SR function is impaired in specimen from TG mice while addition of Ran had no further effect on post-rest behavior. At 10 s of rest the first twitch after rest divided by the last before rest (pr/ss) was 2.7 ± 0.5 in WT muscles and was reduced in specimen from TG mice to 1.6 ± 0.2 (Fig. 4b, $n = 7$ each, $P < 0.05$). Ran did not affect post-rest behavior in TG papillary muscle (Fig. 4b).

Contractile response at increasing stimulation frequencies

In order to determine the effect of Ran on contractile function, we obtained force–frequency relationships in muscles stimulated at frequencies up to 10 Hz (Fig. 5a). TG papillary muscles presented with lower baseline force

compared to WT and a negative staircase phenomenon (RM-ANOVA $P < 0.05$, WT vs. TG: $n = 10$ vs. 11; Fig. 5b). In a second series of experiments, TG papillary muscles were treated with 5 μ mol/L Ran. This intervention had no effect on twitch force amplitude in TG muscles ($n = 11$, RM-ANOVA $P = 0.6$; Fig. 5b).

In contrast, the TG muscles developed a constant increase in diastolic tension up to 3.7 ± 0.4 mN/mm² compared to WT specimen with 2.5 ± 0.2 mN/mm² ($n = 11$ vs. 10, RM-ANOVA $P < 0.05$), which we interpret as an ex vivo presentation of diastolic dysfunction. This effect was more pronounced at higher stimulation frequencies. Ran normalized diastolic tension markedly to 2.3 ± 0.3 mN/mm² ($n = 11$, RM-ANOVA $P < 0.05$). Of note, there was no significant reduction of diastolic tension in WT specimen in the presence of Ran (data not shown).

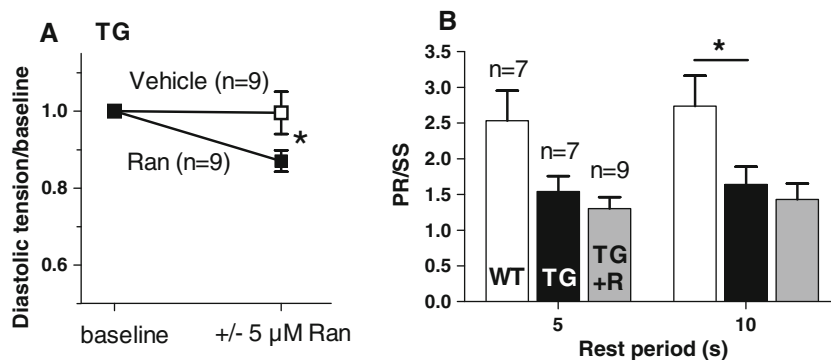
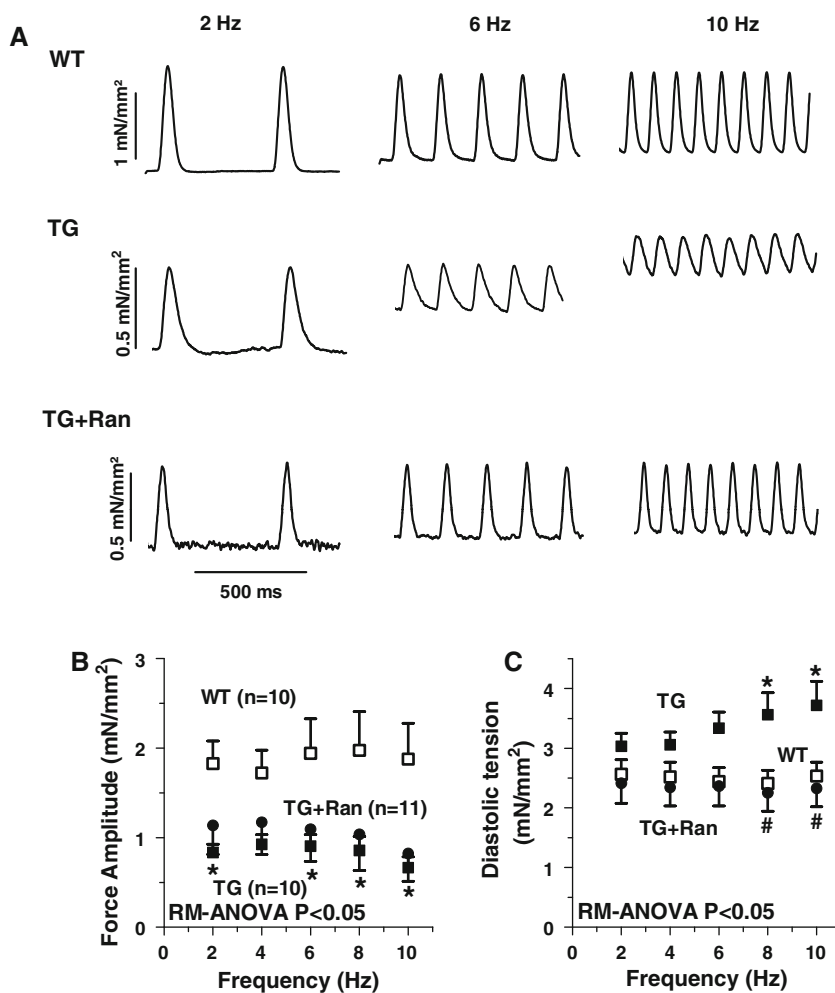


Fig. 4 a Basal effects of 5 μmol/L ranolazine (Ran) on diastolic tension of TG CaMKIIδc muscles ($n = 9$ each, $P < 0.05$). **b** Mean values of post-rest behavior (ratio of first twitch after rest normalized to the last one before rest, PR/SS) indicating impaired post-rest

behavior in TG CaMKIIδc mice (at 10 s of rest: $n = 7$ each, $P < 0.05$). Ran did not cause contractile differences compared to vehicle treated specimen ($n = 7$ vs. 9)

Fig. 5 Force–frequency relationships. **a** Original tracings of muscles isolated from WT and CaMKIIδc TG mice without and with 5 μmol/L ranolazine (Ran) at increasing frequencies. While the WT muscle did not develop an increase in diastolic tension, the CaMKIIδc TG muscle exhibit a marked diastolic dysfunction that could be reduced Ran. **b** Mean data of twitch force amplitude of WT and CaMKIIδc TG muscles with and without Ran ($n = 10$ vs. 11, WT vs. TG, $P < 0.05$). **c** Average values showing increases in diastolic tension in CaMKIIδc TG muscles that could be reduced in the presence of Ran ($P < 0.05$)



Measurements of the late Na⁺ current

The slow time constant (τ_2) of the double exponential function which largely corresponds to late I_{Na} was prolonged in TG compared to WT myocytes at 0.5 Hz (81.9 ± 8.3 vs. 37.6 ± 3.6 ms, $n = 11$ vs. 6, $P < 0.05$,

Figs. 6a, b, Table 1). Interestingly, this prolongation of the slow, second inactivation phase in TG could be reversed in the presence of Ran (38.8 ± 4.1 ms, $n = 7$, $P < 0.05$ vs. vehicle), which is in accordance with substantial late I_{Na} inhibition. In contrast, there was no significant prolongation of fast I_{Na} decay in TG myocytes vs. WT littermates

Fig. 6 a Original late I_{Na} tracings at 1 Hz in WT and CaMKII δ_C TG myocytes in the presence and absence of 5 μ mol/L ranolazine (Ran). The voltage protocol is shown in the inset. **b** Na^+ current decay was fitted with a double exponential equation. The time constant of the late decay phase was markedly prolonged in myocytes from CaMKII δ_C TG mice vs. WT ($n = 11$ vs. 6, $P < 0.05$). Addition of Ran shortened this time constant in myocytes from CaMKII δ_C TG mice consistent with substantial late I_{Na} inhibition ($n = 7$, $P < 0.05$)

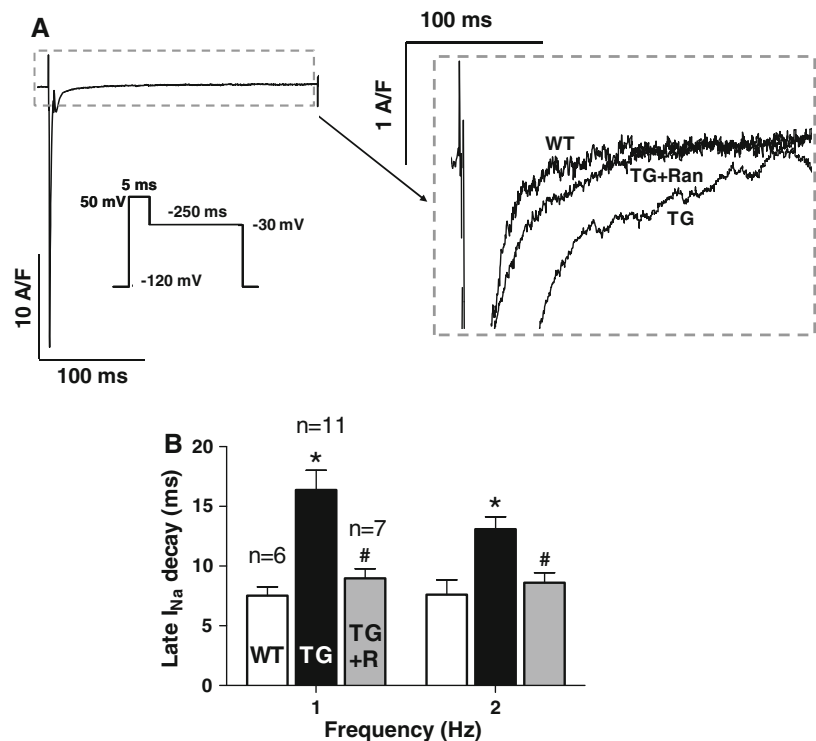


Table 1 Fit parameters (ms) for I_{Na} decay using a double exponential function $y(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + y_0$

Group	τ_1	τ_2
WT	3.7 ± 0.7	$37.6 \pm 3.6^*$
TG	6.1 ± 1.0	81.9 ± 8.3
TG + Ran	5.0 ± 0.8	$38.8 \pm 4.1^*$

I_{Na} decay (first 200 ms) was fit for registrations at 0.5 Hz. Time constant for slow I_{Na} decay τ_2 corresponding to the late phase of I_{Na} decay (i.e. late I_{Na}) was increased in TG vs. both WT and TG + Ran. In contrast, fast I_{Na} decay represented by τ_1 was not significantly changed

* t Test and 1-way ANOVA with Dunnett's post-test $P < 0.05$ vs. TG

(Table 1). Moreover, Ran also did not influence fast I_{Na} decay kinetics.

Discussion

Here, we show for the first time that 8-week-old TG CaMKII δ_C mice develop non-ischemic diastolic dysfunction in addition to systolic HF. In these mice, late I_{Na} was markedly higher as compared to WT. Inhibition of late I_{Na} in vitro using Ran led to a significant improvement of diastolic function without negative inotropic effects. Arrhythmogenic events can be found in multicellular preparations of TG CaMKII δ_C mice even in the absence of isoprenaline. Moreover, Ran shows antiarrhythmic effects even in the absence of NCX upregulation. In summary, diastolic

dysfunction and arrhythmias share a similar molecular mechanism by means of increased diastolic Na^+ . Both clinical problems may be approached by inhibiting late I_{Na} .

Cardiac overexpression of CaMKII δ_C

CaMKII δ_C overexpression was shown to be associated with HF [32] and arrhythmias in vivo [21, 31]. CaMKII activity is increased by ~ 3 -fold in TG CaMKII δ_C mice [33] which is similar to the increased CaMKII activity observed in failing human hearts [11, 12].

To reduce complex remodeling caused by advanced HF we investigated younger mice (8 weeks old); this is in clear contrast to earlier studies [21, 27, 31]. Interestingly, although these mice are younger compared to those of other reports, the systolic contractile function of the myocardium is already severely depressed as evident by echocardiography. Muscle experiments in vitro revealed impaired contractile force and post-rest behavior. We found a decreased expression of SERCA protein as a typical change that occurs in the failing myocardium contributing to decreased SR Ca^{2+} content and SR Ca^{2+} release. Therefore, the TG CaMKII δ_C mouse can be regarded as a model of elevated CaMKII activity, but also as a pathophysiological relevant model of non-ischemic HF.

CaMKII δ_C , late I_{Na} , and arrhythmias

In the present work, PACs only occurred in papillary muscles isolated from TG CaMKII δ_C mice, whereas no

arrhythmias were observed in WT specimens. These results are generally in line with our previous findings showing that isoprenaline-induced EADs and DADs are associated with CaMKII δ_C overexpression [21]. Inhibition of CaMKII in the previous report depressed EADs and DADs. Moreover, the current data add important information because even without addition of isoprenaline arrhythmogenic events can be found in muscles in vitro from CaMKII δ_C TG mice.

It was suggested previously that the SR Ca²⁺-leak would play an important role in this pathology [21]. Indeed, cardiomyocytes isolated from CaMKII δ_C mice have an increased open probability of the RyR leading to increased SR Ca²⁺-sparks and Ca²⁺-oscillation which may trigger I_{Ti} and arrhythmias [16, 21, 31].

However, in addition to SR Ca²⁺ leak, prolonged APs [17] are also observed in these mice which may increase the propensity for EADs. Late I_{Na} is known to prolong the AP and thereby likely contribute to EADs [23]. Since CaMKII was shown to regulate Na⁺ channels, it is possible that this mechanism is also crucially involved in the arrhythmogenesis observed. Hence, antiarrhythmic effects of CaMKII inhibition may actually be mediated largely through late I_{Na} .

Therefore, we tested whether inhibition of late I_{Na} using Ran would lead to a similar reduction of the arrhythmic propensity as described previously [22]. Ran has been shown to selectively inhibit late I_{Na} vs. peak I_{Na} (especially at concentrations of 5 μ mol/L) [2] and we now show indeed that Ran reduces late I_{Na} in TG myocytes. This is in contrast to another study where CaMKII inhibition could not reduce intracellular Na⁺ [32] (through late I_{Na}) and may be explained by the fact that in the current report young mice were used in which only a functional upregulation of late I_{Na} may occur. In contrast in older mice, several other mechanisms may be involved, such as altered expression/trafficking of Na⁺ channels, involvement of other kinases activated during congestive HF, as well as less altered local phosphatase binding/expression at the Na⁺ channel (as reported for RyR).

Nevertheless, we found a strong effect of Ran on PACs in CaMKII δ_C specimen. This effect commonly occurred within minutes and kept the preparation stable in a stimulation-dependent rhythm. These results also indicate that the late I_{Na} may play a crucial role in the arrhythmogenesis in failing hearts and that blockade of late I_{Na} appears useful to control HF associated arrhythmias.

Inhibition of late I_{Na} ameliorates diastolic dysfunction

Elevated diastolic Ca²⁺ as a consequence of reverse NCX activity in Na⁺-overloaded cardiomyocytes may on the one hand cause arrhythmias and on the other hand also impair

diastolic contractile performance of the heart. Echocardiographic analyses confirmed diastolic dysfunction in TG CaMKII δ_C mice. Moreover, in isometrically twitching preparations we found a frequency-dependent increase in diastolic tension compared to WT specimen which may be an additional indication for increased diastolic Ca²⁺. This may be at least partly explained by the finding that SERCA protein is downregulated while NCX is still unchanged in TG hearts isolated from 8-week-old mice, whereas older mice (e.g. 3 months) exhibit increased NCX expression levels [16]. Another possibility for elevated diastolic Ca²⁺ levels (and thus altered mechanical function) may be a slight albeit not significant downregulation of calsequestrin protein expression (data not shown). Calsequestrin is a low-affinity, high-capacity Ca²⁺-binding protein in the SR which is often used as a housekeeping gene. Therefore, we preferred to normalize our Western blot results to GAPDH which is unaltered. Nevertheless, we acknowledge that even slight alterations of calsequestrin expression and hence impaired SR Ca²⁺ storage capacity might be a limitation with respect to the interpretation of our study but the calsequestrin downregulation does not reach the extent as shown in another study with a clear and significant 20% downregulation (vs. WT) when CaMKII δ_C transgenic mice were crossbred with RyR2R4496C mutated mice [6]. It is important to note that to date neither multicellular tissue nor cardiomyocytes isolated from TG CaMKII δ_C mice have been investigated at physiological heart rates (up to 10 Hz). Here, we show that diastolic tension increased at 6 Hz and above.

Wagner et al. have previously shown that overexpression of CaMKII δ_C results in an increase of $[Na]_i$ that could be significantly reduced by CaMKII-inhibitors in acute CaMKII δ_C overexpressing myocytes [31]. However, a similar increase of $[Na]_i$ could not be reversed by CaMKII inhibition in TG CaMKII δ_C mice at 3 months of age suggesting that there may be other sources of Na⁺ entry or CaMKII-independent mechanism involved that may have to do with the remodeling processes during HF progression. However, since late I_{Na} inhibition can shorten the AP, a reduced reverse mode NCX activity may be the consequence leading to less Ca²⁺ overload and less diastolic dysfunction.

In the present work, we were interested whether inhibition of elevated late I_{Na} in TG myocytes might improve contractile function. Indeed, we found evidence for a reduced diastolic dysfunction in the presence of Ran. This effect could be observed under several conditions. Interestingly, this effect was not paralleled by any negative inotropy. The findings of our study are in agreement with our previous report showing improved diastolic function due to inhibition of late I_{Na} [24, 26, 28]. Most importantly, in isolated human failing myocardium CaMKII and late I_{Na} are markedly elevated [11, 12, 18, 31].

Our results indicate that the late I_{Na} plays a crucial role in determining diastolic function. Inhibition of late I_{Na} may counteract the CaMKII effect that induces elevation of this current leading to Na^+ overload of the cell. Moreover, the fact that the Ran effect was pronounced at higher frequencies supports that concept that AP shortening may be an additional mechanism of Ran-dependent improvement of diastolic function. Since diastolic dysfunction can be induced by structural as well as electrical remodeling [10], the sensitivity of TG myocardium to Ran and the acute effect observed in our experiments shows that diastolic function is strongly related to electrical remodeling. However, there are also types of HF showing diastolic dysfunction which depends mainly on fibrosis [30].

Conclusions

Chronic overexpression of CaMKII δ_C leads to diastolic and systolic dysfunction as well as an increased propensity for arrhythmias. Inhibition of an increased late I_{Na} in TG myocardium was effective in treating diastolic dysfunction and arrhythmias. Hence the relation between elevated CaMKII activity and increased late I_{Na} in HF may be of therapeutic interest and merits further investigation.

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