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While systolic cardiomyocyte function is preserved, diastolic myocyte function and recovery from acidosis are impaired in CaMKII δ -KO mice

Stefan Neef^{a,1}, Can M. Sag^{a,1}, Maria Daut^a, Henrik Bäumer^a, Clemens Grefe^a, Ali El-Armouche^b, Jaime DeSantiago^c, Laetitia Pereira^c, Donald M. Bers^c, Johannes Backs^d, and Lars S. Maier^{a,*}

Lars S. Maier: lmaier@med.uni-goettingen.de

^aDept. of Cardiology and Pneumology, Georg-August-University, Göttingen, Germany

^bDept. of Pharmacology/Heart Center, Georg-August-University, Göttingen, Germany

^cDept. of Pharmacology, UCD, USA

^dDept. of Cardiology, Angiology and Pneumology, Ruprecht-Karls-University, Heidelberg, Germany

Abstract

Objective—CaMKII contributes to impaired contractility in heart failure by inducing SR Ca²⁺-leak. CaMKII-inhibition in the heart was suggested to be a novel therapeutic principle. Different CaMKII isoforms exist. Specifically targeting CaMKII δ , the dominant isoform in the heart, could be of therapeutic potential without impairing other CaMKII isoforms.

Rationale—We investigated whether cardiomyocyte function is affected by isoform-specific knockout (KO) of CaMKII δ under basal conditions and upon stress, i.e. upon β -adrenergic stimulation and during acidosis.

Results—Systolic cardiac function was largely preserved in the KO *in vivo* (echocardiography) corresponding to unchanged Ca²⁺-transient amplitudes and isolated myocyte contractility *in vitro*. CaMKII activity was dramatically reduced while phosphatase-1 inhibitor-1 was significantly increased. Surprisingly, while diastolic Ca²⁺-elimination was slower in KO most likely due to decreased phospholamban Thr-17 phosphorylation, frequency-dependent acceleration of relaxation was still present. Despite decreased SR Ca²⁺-reuptake at lower frequencies, SR Ca²⁺-content was not diminished, which might be due to reduced diastolic SR Ca²⁺-loss in the KO as a consequence of lower RyR Ser-2815 phosphorylation. Challenging KO myocytes with isoproterenol showed intact inotropic and lusitropic responses. During acidosis, SR Ca²⁺-reuptake and SR Ca²⁺-loading were significantly impaired in KO, resulting in an inability to maintain systolic Ca²⁺-transients during acidosis and impaired recovery.

Conclusions—Inhibition of CaMKII δ appears to be safe under basal physiologic conditions. Specific conditions exist (e.g. during acidosis) under which CaMKII-inhibition might not be

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*Corresponding author at: Abt. Kardiologie und Pneumologie/Herzzentrum, Deutsches Zentrum für Herz-Kreislauf-Forschung, Georg-August-Universität Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany. Tel.: +49 551 39 6372; fax: +49 551 39 14131.

¹Equal contribution.

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helpful or even detrimental. These conditions will have to be more clearly defined before CaMKII inhibition is used therapeutically.

Keywords

CaMKII; Excitation contraction coupling; Acidosis; Calcium handling; SR Ca²⁺-leak; SERCA function

1. Introduction

CaMKII is a multifunctional serine/threonine protein kinase. Its activation is Ca²⁺/calmodulin-dependent [1]. Different CaMKII isoforms (α , β , γ and δ) exist [2]. In the heart, CaMKII δ and γ are expressed with CaMKII δ being the predominant isoform [2,3]. In cardiomyocytes, CaMKII is a critical regulator of ECC, regulating L-type Ca²⁺-channels [4], SERCA activity by PLB phosphorylation [5], RyR function [6–8], as well as Na⁺- [9] and K⁺-currents [10].

CaMKII was suggested to be involved in pathophysiological remodeling of cardiomyocyte Ca²⁺-handling in heart failure [6,8,11–16], ventricular arrhythmogenesis [14,17,18], and atrial fibrillation [7,19]. Consequently, CaMKII-inhibition may be a novel therapeutic principle [8,15,20]. We [12] and others [21] showed that specific KO of CaMKII δ is protective during pressure overload.

CaMKII was also suggested to play important physiological regulatory roles, e.g. in the force–frequency relationship, SR Ca²⁺-release, and adrenergic signaling [8,22,23]. In addition, CaMKII seems to be involved in maintaining intracellular Ca²⁺-transients and myocyte contractility during acidosis [5,24,25], a pathophysiologically relevant situation during cardiac ischemia. Yet, *in vivo* cardiac function in our first report was not impaired under physiological conditions [12] suggesting that ECC must be largely intact. Consequently, to investigate the mechanisms by which loss of CaMKII δ may be compensated with respect to preserving physiological ECC and the possible effects on CaMKII regulatory mechanisms, we now performed extended mechanistic *in vitro* studies under basal conditions and upon stress (i.e. β -adrenergic stimulation and acidosis).

2. Methods

All investigations conformed to the “Guide for the Care and Use of Laboratory Animals” published by the US NIH (publication no. 85-23, revised 1996). For all experiments, investigators were blinded with respect to the genotype. All data are presented as mean $\pm$ SEM. Statistical analyses were performed using Student's t-test for unpaired values, two-way repeated measures (RM) ANOVA (with Holm–Sidak or Fisher LSD as post hoc test) or Fisher's exact test, as appropriate. Values of $p < 0.05$ were considered statistically significant.

2.1. Generation of isoform-specific CaMKII δ -KO

The exact process of genetic ablation of CaMKII δ was previously described [12]. Briefly, a ubiquitous KO was achieved by recombining LoxP sites flanking exons 1 and 2 of the CaMKII δ locus in the germline.

2.2. Echocardiography

After anesthetization with avertin (0.01 ml/g i.p.), mice were investigated by 2D guided M-mode echocardiography (VS-VEVO 660/230, Visualsonics). In addition to ventricular

dimensions measured using the standard leading edge convention, left ventricular fractional shortening and mass were calculated.

2.3. Isolation of cardiomyocytes

Myocytes were isolated as previously described [12,14]. Briefly, hearts were Langendorff-perfused with nominally Ca^{2+} -free solution containing (in mM) NaCl 113, KCl 4.7, KH_2PO_4 0.6, $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ 0.6, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 1.2, NaHCO_3 12, KHCO_3 10, HEPES 10, taurine 30, BDM 10, glucose 5.5, phenol-red 0.032 for 4 min at 37 °C (pH 7.4). Then, 7.5 mg/ml liberase 1 (Roche), trypsin 0.6%, and 0.125 mM CaCl_2 were added to the perfusion solution. Ventricular tissue was collected in buffer supplemented with 5% bovine calf serum and dispersed by cutting and pipetting. Ca^{2+} was reintroduced stepwise to 0.8 mM. For measurements, cells were freshly plated onto chambers coated with laminin.

2.4. Experimental solutions

NT was used consisting of (in mM) 140 NaCl, 4 KCl, 5 HEPES, 1 MgCl_2 , 10 glucose, 1 CaCl_2 (pH 7.4, 35 °C with NaOH). Fluo-3 and Fura-2 were diluted in NT with Pluronic F-127 0.2 mg/ml.

2.5. Epifluorescence experiments

Ca^{2+} -epifluorescence and sarcomere length were measured as reported previously [12,14] at 35 °C. Myocytes were loaded with Fluo-3/AM (10 μM) for 20 min. Excitation was at 480 ± 15 nm, emission was at 535 ± 20 nm, and F/F_0 was calculated. In parallel, myocyte contraction was investigated using a sarcomere length detection system (IonOptix). Myocytes were field-stimulated at 1, 2 and 4 Hz until steady-state was achieved. For more correct Ca^{2+} -transient decline the time constant τ from the last Ca^{2+} -transient before a 3 s pause was assessed. SR Ca^{2+} -content was estimated by application of 10 mM caffeine (1 Hz) and τ of the caffeine-induced transient was calculated to estimate NCX-function. For semiquantitative assessment of cytosolic Ca^{2+} -levels, cells were loaded with 10 μM Fura-2/AM for 15 min. Fura-2 fluorescence ratio was calculated by division of the signal obtained at 340 nm by that at 380 nm.

2.6. Iso experiments

To investigate adrenergic response, cells were treated with 4 nM Iso.

2.7. Ca^{2+} -spark measurements

Myocytes were superfused with NT containing 3 mM Ca^{2+} (21 °C, pH 7.4) after incubation with Fluo-4/AM (10 μM , 15 min). Using a laser-scanning confocal microscope (LSM5, Zeiss), Fluo-4 was excited at 488 nm and emission was collected through a 505 nm long-pass filter. Fluorescence images were recorded in line-scan mode with 512 pixels per 38.4 μm wide scanline. Following 1 Hz stimulation, Ca^{2+} -spark frequency (CaSpF) was measured at resting conditions and normalized to myocyte width and scanning interval (1/100 $\mu\text{m/s}$). Spark fluorescence was averaged over a 30 pixel (2.25 μm) wide window, meaning that spark width is represented in the averaged signal. Ca^{2+} -spark amplitude (F/F_0) and decay characteristics were determined for each spark. Diastolic SR Ca^{2+} -leak for sparking cells was calculated as the product of frequency, amplitude and time to 50% decay ($\text{rt}_{50\text{CaSp}}$). Total diastolic SR Ca^{2+} -leak is based on all myocytes investigated and consequently also includes cells with functional Ca^{2+} -transients that had no Ca^{2+} -sparks during the scan interval.

2.8. Acidosis experiments

Acidosis was induced by switching superfusion from pH 7.4 to NT with a pH of 6.75 to mimic intracellular ischemia with values of even lower than 6.5 [25,26]. Myocytes were monitored during early (~1–4 min) and late acidosis (~5–10 min) [5]. CaMKII was inhibited using KN-93 (1 μ M) vs. its inactive analogue KN-92 (1 μ M).

2.9. Western blot

Hearts were homogenized in Tris buffer containing (in mM) Tris–HCl 20, NaCl 200, NaF 20, Na₃VO₄ 1, DTT 1, 1% Triton X-100 (pH 7.4) and complete protease-inhibitor cocktail (Roche). Protein concentration was determined by BCA assay (Pierce Biotechnology). Denatured tissue homogenates were subjected to Western blotting (4–15% gradient and 10% SDS-polyacrylamide gels) using anti-phospho-CaMKII (1:1,000, Thermo Fisher Scientific), anti-RyR2 (1:10,000, Sigma-Aldrich), anti-phospho-RyR2-2809 (1:5,000, Badrilla), anti-phospho-RyR2-2815 (1:5,000, Badrilla), anti-SERCA2a (1:20,000, Affinity Bioreagents), anti-PLB (1:10,000, Millipore), anti-phospho-Ser-16 (1:10,000, Badrilla), anti-phospho-Thr-17 (1:10,000, Badrilla), anti-NCX (1:5,000, Swant), anti-I-1 (1:2,000, Novus Biologicals), anti-CSQ (1:20,000, Affinity Bioreagents), and anti-GAPDH (1:20,000, Biotrend) antibodies. Chemiluminescent detection was done with SuperSignal West Pico Substrate (Pierce). Expression/phosphorylation levels are given as percent of WT levels and are either normalized to GAPDH or the respective reference protein.

2.10. CaMKII activity assay

Ventricular tissue lysates were prepared using extraction buffer containing (in mM) Tris–HCl 20, EDTA 2, EGTA 2, DTT 2 as well as complete protease-inhibitor cocktail (Roche). CaMKII activity was then measured using the SignaTECT CaMKII assay system (Promega). Briefly, lysates were incubated with reaction buffer (250 mM Tris–HCl, 50 mM MgCl₂, 2.5 mM DTT) and either activation buffer (5 mM CaCl₂, 5 μ M calmodulin, 0.1 mg/ml bovine serum albumin) or control buffer (5 mM EGTA) also containing the bionitylated peptide substrate as well as 0.5 mM ATP and 0.5 μ Ci [γ -³²P]ATP. Reactions were allowed for 3 min at 30 °C before being terminated by adding 7.5 M guanidine-HCl. Equal amounts of samples were blotted onto the kit's SAM2 biotin capture membrane and washed with 2 M NaCl and 1% H₃PO₄. Kinase activity was assessed by scintillation counting.

3. Results

3.1. Preserved baseline characteristics in CaMKII δ -KO vs. WT mice

KO mice gained normal weight (male/female KO 25.2 $\pm$ 0.8/20.4 $\pm$ 0.9 g, n = 11/11 vs. male/female WT 27.1 $\pm$ 0.9/20.4 $\pm$ 2.0 g, n = 11/7) at 11 weeks of age. In the age-matched animals (KO 11.2 $\pm$ 0.2 weeks vs. WT 11.1 $\pm$ 0.5 weeks), heart weight/body weight was not different in KO compared to WT (Fig. 1A).

Echocardiography showed no differences between KO and WT animals with respect to cardiac fractional shortening *in vivo* (Fig. 1B) or septum and posterior wall thickness (Figs. 1C and D). Calculated left ventricular mass per body weight was also not different (5.35 $\pm$ 0.20 mg/g in KO vs. 5.24 $\pm$ 0.30 mg/g in WT). Enddiastolic (3.47 $\pm$ 0.07 mm vs. 3.33 $\pm$ 0.14 mm in WT) and endsystolic diameters (1.80 $\pm$ 0.09 mm vs. 1.67 $\pm$ 0.11 mm in WT) were also unaltered.

3.2. Total CaMKII activity and I-1 expression in CaMKII δ -KO mice

CaMKII activity in KO mice was reduced to 12.9 $\pm$ 3.9% compared to WT (p < 0.05; Fig. 1E). To further investigate possible compensatory mechanisms, we studied expression levels

of I-1 and found this to be strongly increased to $190 \pm 21\%$ compared to WT ($p < 0.05$; Fig. 1F).

3.3. Single cell systolic function is not impaired under physiologic conditions

Ca^{2+} -transient amplitudes at increasing stimulation rates were not impaired in KO cardiomyocytes (Fig. 2A). At 1 Hz stimulation frequency, Ca^{2+} -transients ($n = 60$) were even slightly higher than in WT ($n = 57$) as previously described by us [12], while at increasing stimulation frequencies there were no more differences (Fig. 2B). Systolic fractional SR Ca^{2+} -release during baseline stimulation (calculated as ratio of electrically stimulated Ca^{2+} -transients through caffeine induced Ca^{2+} -transients) showed no difference in the KO ($69.0 \pm 1.5\%$, $n = 54$ vs. $67.2 \pm 1.8\%$ in WT, $n = 45$). Single cell contractility (twitch amplitude) was slightly higher by trend, but not significantly altered at any stimulation frequency (Fig. 2C), corresponding to unaltered echocardiographic *in vivo* contractility.

3.4. Single cell diastolic function is impaired upon CaMKII δ -KO

Decay of the Ca^{2+} -transient corresponds to cytosolic Ca^{2+} -elimination during diastole, which is largely due to SR Ca^{2+} -reuptake via PLB-regulated SERCA in mice [26]. Ca^{2+} -transient decay was slightly, but significantly slower in KO myocytes ($n = 60$ vs. $n = 43$ in WT, Figs. 3A and B). Relaxation of the twitch, while also dependent on the mechanical properties of the myocyte, strongly depends on cytosolic Ca^{2+} -decay. Consequently, twitch relaxation was also slower in KO (Fig. 3C). The reason for this appears to be reduced PLB phosphorylation at the CaMKII-specific Thr-17 site (Fig. 3D), while phosphorylation at the PKA-specific Ser-16 site (Fig. 3E), and PLB protein expression were unaltered (Fig. 3F). SERCA/PLB expression ratio (Fig. 3G) was significantly higher in KO mice, presumably compensating reduced PLB-phosphorylation with respect to SR Ca^{2+} -reuptake. FDAR as assessed by the FDAR-ratio (τ of the Ca^{2+} -transient at 4 Hz divided by τ at 1 Hz) was completely uncompromised with $75.5 \pm 1.2\%$ in the KO vs. $76.1 \pm 0.9\%$ in the WT (Fig. 3B).

3.5. SR Ca^{2+} -content is not altered in the face of decreased SR Ca^{2+} -leak

Despite this impaired diastolic SR Ca^{2+} -reuptake, SR Ca^{2+} -content was largely unaltered in the KO ($p = 0.11$, Figs. 4A and B). We therefore assessed spontaneous SR Ca^{2+} -sparks as a measure for SR Ca^{2+} -leak (Fig. 4C). The percentage of sparking cells of all measured myocytes was significantly decreased in KO ($p < 0.05$, Fig. 4D), while Ca^{2+} -spark frequency in these sparking cells was slightly, but not significantly, lower in the KO with 1.49 ± 0.13 sparks/100 $\mu\text{m/s}$ ($n = 55$ sparking cells) compared to 1.66 ± 0.11 sparks/100 $\mu\text{m/s}$ in WT ($n = 148$ sparking myocytes, $p = 0.37$). Ca^{2+} -spark amplitude was not altered with 1.51 ± 0.02 F/F_0 ($n = 98$ sparks) in KO vs. 1.52 ± 0.02 F/F_0 ($n = 128$ sparks) in WT, and also time to 50% decay of the sparks was unaltered with $\text{rt50}_{\text{CaSp}} 23.43 \pm 1.09$ ms in KO and 22.00 ± 0.85 ms in WT sparks. In consequence, calculated total SR Ca^{2+} -leak across all myocytes investigated (i.e. pooling those with and without sparks during the registration period) was clearly lower in the KO with 91.7 ± 4.7 $\text{F/F}_0/\text{m}$ compared to 156.6 ± 6.5 $\text{F/F}_0/\text{m}$ in the WT (Fig. 4E), while SR Ca^{2+} -leak across only those cells that produced Ca^{2+} -sparks, showed no difference with 532.2 ± 27.1 $\text{F/F}_0/\text{m}$ in KO compared to 554.6 ± 22.9 $\text{F/F}_0/\text{m}$ in WT ($p = 0.53$).

Underlying this reduced SR Ca^{2+} -leak, we found reduced RyR phosphorylation at the CaMKII-specific Ser-2815 site (Fig. 4F), while phosphorylation at Ser-2809 (Fig. 4G), which is mainly a target of PKA, was not significantly decreased in the presence of unaltered RyR expression (KO $139.8 \pm 10.0\%$ vs. WT $100.0 \pm 19.3\%$).

Despite decreased SR Ca^{2+} -leak, diastolic free Ca^{2+} -levels were significantly higher in KO ($n = 29$) compared to WT myocytes ($n = 23$, Fig. 4H). This correlated with significantly shorter diastolic sarcomere lengths in KO vs. WT (Fig. 4I, KO $n = 43$ –57, WT $n = 39$ –60). These observations suggest that other mechanisms apart from SR Ca^{2+} -leak must influence diastolic Ca^{2+} (and dominate over SR Ca^{2+} -leak in this regard under physiologic conditions). Consequently, we investigated Ca^{2+} -extrusion by NCX and found this to be significantly impaired in the KO (Fig. 4J). However, it appears that the mechanism for this was not a reduction of NCX expression, since this was not significantly altered in the KO ($112.8 \pm 8.0\%$ vs. WT $100 \pm 6.8\%$, $n = 4$ vs. $n = 7$).

3.6. Inotropic and lusitropic responses to adrenergic stimulation are intact

Upon adrenergic stimulation using Iso, both WT and KO showed significant inotropic responses and KO myocytes did not differ significantly from WT with respect to Ca^{2+} -transient amplitudes (Fig. 5A, KO $n = 46$, WT $n = 36$; data from $n = 17$ vs. $n = 11$ wash-in experiments shown in Fig. 5B) or contractility (Fig. 5C).

Lusitropic response to Iso as demonstrated in WT myocytes with clearly faster relaxation and cytosolic Ca^{2+} -elimination was also present and strong in the KO (Fig. 5D, KO $n = 17$, WT $n = 11$). However, the slightly slower relaxation kinetics in the KO were also present under Iso stimulation and in fact were even slightly more pronounced in so far as post-hoc test now also showed significance for 4 Hz stimulation (Fig. 5E, KO $n = 46$, WT $n = 36$). Myocyte twitch relaxation under Iso was also slower in the KO for all frequencies investigated (Fig. 5F).

3.7. Recovery from acidosis requires CaMKII δ

CaMKII activity is increased during acidosis where it is crucial in maintaining SR Ca^{2+} -reuptake through PLB phosphorylation [25]. Since CaMKII activity is decreased and PLB phosphorylation reduced in the KO, we speculated that SR Ca^{2+} -loading and resulting Ca^{2+} -transients might be impaired during acidosis.

CaMKII δ -KO ($n = 41$) and WT control myocytes ($n = 49$) showed decreased Ca^{2+} -transient amplitudes during early acidosis (Figs. 6A and B, $p < 0.05$ vs. control, respectively). While WT myocytes maintained their Ca^{2+} -transient amplitudes during late acidosis compared to non-acidic conditions, Ca^{2+} -transients in KO cells decreased even further in late acidosis to ~64% of control conditions (Figs. 6A and B, $p < 0.05$ between genotypes). This was associated with preserved relaxation properties in WT whereas KO myocytes showed progressively impaired relaxation, indicating decreased SR Ca^{2+} -reuptake (Figs. 6A and C, $p < 0.05$ between genotypes).

In line with this, diastolic F_0 as an approximate measure for diastolic $[\text{Ca}^{2+}]_i$ also showed divergent results between groups with a relative decrease of F_0 in WT compared to an increase in KO myocytes (Fig. 7B, $p < 0.05$ between genotypes during late acidosis). Importantly, WT myocytes ($n = 19$) showed an increase in SR Ca^{2+} -content during acidosis, whereas it dramatically decreased in the KO group ($n = 16$) (Figs. 7A and C, $p < 0.05$). We further confirmed the recovery to be a CaMKII-dependent effect by showing that recovery occurred in only ~19% of WT myocytes treated with CaMKII-inhibitor KN-93 while recovery occurred in all myocytes treated with control substance KN-92 ($p < 0.05$, Figs. 7D and E).

4. Discussion

In the present study we investigated whether loss of CaMKII δ affects basal cardiac function. Our results show that systolic function *in vivo* and *in vitro* is not compromised and that the

response to β -adrenergic stimulation *in vitro* is not diminished. SR Ca^{2+} -leak seems to be reduced although SR Ca^{2+} -load under basal conditions is largely unaltered. In contrast, diastolic function and SR Ca^{2+} -reuptake appear to be compromised, leading to impaired recovery during acidosis. These functional data are supported by altered CaMKII-dependent phosphorylation status of RyR and PLB and suggest that targeted inhibition of CaMKII δ is possible without fundamentally altering basal ECC. This observation may be due to the fact that remaining CaMKII activity is sufficient to maintain physiologic regulatory roles of CaMKII under basal conditions, but not during pathophysiologic acidosis.

4.1. CaMKII δ -KO myocytes have normal systolic but impaired diastolic function

Our data show normal systolic function both *in vitro* and *in vivo* under basal conditions and upon Iso-stress. One important point may be that relatively low basal CaMKII activity may contribute to the modest baseline phenotype. Likewise, sympathetic inotropy and lusitropy may not require strong CaMKII activation. For comparison, some parallel baseline studies were also done using mice from a different CaMKII δ -KO line [21] and are described in the Supplement. That line exhibited similar WT vs. KO differences in Ca^{2+} -handling to the mice used here. However, diastolic function as assessed by diastolic Ca^{2+} -elimination and relaxation of isolated myocytes, is impaired. The molecular basis for this appears to be reduced PLB phosphorylation at the CaMKII-specific Thr-17 site, while there is no change in PKA-dependent PLB phosphorylation. Our results also show a higher SERCA/PLB ratio in the KO. However, this potential compensatory mechanism is not sufficient due to the slowed relaxation found in our study and we therefore conclude that the effect of much lower PLB phosphorylation dominates functionally. Of note, SERCA expression has vice versa been found to be lower in CaMKII δ C-overexpressing mice [6] and is a hallmark of heart failure allowing for the exciting possibility that CaMKII might be directly involved in SERCA regulation by excitation-transcription coupling.

Yet, invasive hemodynamic measurements in another mouse model of CaMKII δ -KO had not shown alterations in relaxation [21]. But in contrast to our experiments these *in vivo* measurements were performed in the presence of hemodynamic preload, which will facilitate relaxation. In addition, heart rate *in vivo* is high in mice and the effects observed by us were strongest at lower stimulation frequencies. So while our data point to a potential impact of CaMKII δ -KO on diastolic function in single cardiomyocytes, it seems that this apparently does not necessarily translate into detrimental hemodynamic consequences *in vivo* which may be a limitation of the present study.

Of note, the slower twitch relaxation in the KO appears to be slightly more pronounced than the slower Ca^{2+} -transient decay. Conversely, while Ca^{2+} -transient amplitudes are slightly increased in the KO, we found single cell contractility to be increased to a lesser extent and not significantly so. These differences in extent of $[\text{Ca}^{2+}]_i$ vs. contraction might be due to the highly nonlinear nature of myofilament Ca^{2+} sensitivity or changes in myofilament function in the KO mice.

4.2. FDAR is not impaired in CaMKII δ -KO

CaMKII was suggested to be central for FDAR. While there have been reports of impaired or absent FDAR upon CaMKII inhibition [27,28] these reports were challenged by others [29,30] and temporal dissociation between FDAR and CaMKII-activation has been shown [31]. Our data demonstrate that FDAR is not compromised by loss of CaMKII δ (see also Supplemental Fig. II), suggesting that increasingly faster Ca^{2+} -reuptake upon higher frequencies might be due to a CaMKII δ -independent mechanism. Although CaMKII activity is decreased to 13% in the CaMKII δ -KO (Fig. 1E), we cannot rule out the possibility that

FDAR is mediated by another CaMKII isoform (e.g. CaMKII γ which may compensate for loss of CaMKII δ in FDAR).

Our data show that NCX function is slightly decreased in the CaMKII δ -KO while enhanced NCX function was found in CaMKII δ_C overexpressing mice [6]. In part, this reduced NCX function in the KO might contribute to slower Ca²⁺-elimination at low stimulation frequencies. Given the greater contribution of SERCA (~90%) to diastolic Ca²⁺-elimination as compared to NCX (~8%) [26], as well as the reduced NCX function observed, we believe that effects of PLB phosphorylation dominate over NCX effects with respect to diastolic Ca²⁺-decay. The reason for reduced NCX activity in our KO mouse is less clear, since NCX protein expression is unaltered. It is possible that modifications of NCX or a slight increase in [Na⁺]_i [32] might be the reason for this apparent discrepancy. However, our results do not distinguish whether such changes might be directly due to CaMKII δ or are rather secondary effects of the KO.

4.3. Reduced SR Ca²⁺-leak, but unaltered SR Ca²⁺-content and increased diastolic Ca²⁺ in CaMKII δ -KO

Due to spontaneous SR Ca²⁺-release events occurring less frequently in KO-myocytes, we found total SR Ca²⁺-leak to be reduced under basal conditions (at least when also including cells with no sparks but regular Ca²⁺-transients). Our Western blot data indicate that reduced RyR phosphorylation, most importantly at the CaMKII-specific Ser-2815 site, may be the reason for this reduced SR Ca²⁺-leak. Diastolic SR Ca²⁺-leak is not only suggested to reduce SR Ca²⁺-content [6] and impair contractility [15] but also may be an important arrhythmogenic mechanism [14]. Despite lower SR Ca²⁺-leak, SR Ca²⁺-content was unaltered. We assume that this is because SERCA function is also lower, so that decreased SR Ca²⁺-loss might have been counter-balanced by decreased SR Ca²⁺-reuptake. It has been reported in another mouse model that reduced Ca²⁺-spark frequency in CaMKII δ -KO upon TAC also did not lead to higher SR Ca²⁺-content [21] and we had also observed reduced SERCA function in KO vs. WT in our TAC model [12]. This is also interesting in so far as it had been speculated that preservation of SR Ca²⁺-content by reduction of SR Ca²⁺-leak could be a major protective mechanism in CaMKII-inhibition or -KO with respect to contractility. Apart from SR Ca²⁺-content, lower SR Ca²⁺-leak might also be protective with respect to arrhythmogenesis [14]. In any case, reduced CaMKII-dependent phosphorylation of the RyR can be assumed to be the cause for reduced SR Ca²⁺-leak in the KO, since the other explanation for reduced sparks – a lower SR Ca²⁺-load – can be excluded.

It is not clear why there are differences in the percentage of sparking myocytes, but this may simply be a statistical manifestation of the low spark rate (most myocytes had only 1–2 observed sparks during the scan interval). Thus the lower overall Ca²⁺ spark frequency and greater number of spark-free KO myocytes likely reflect a real decrease in diastolic SR Ca²⁺ leak. Notably, part of SR Ca²⁺ leak is not detectable as Ca²⁺ sparks [33], but such spark-independent SR Ca²⁺ leak was not assessed in these mice. However, we performed some parallel studies in another CaMKII δ -KO mouse line [21] which included measurement of total SR Ca²⁺ -leak using the Shannon tetracaine induced shift method over a range of SR Ca²⁺ -loads [15]. Supplemental Fig. IIIC shows that CaMKII δ -KO vs. WT mice showed lower leak at a given SR Ca²⁺ -load. In those CaMKII δ -KO mice we also saw lower Ca²⁺ -spark frequency when normalized to SR Ca²⁺ -load (Supplement Fig. IIIB).

Interestingly, diastolic sarcomere length is shortened and diastolic free Ca²⁺-levels are increased in the KO. One typical cause of elevated diastolic Ca²⁺-levels (as found in heart failure) is increased SR Ca²⁺-leak. However, this is not the case in the current study. We rather believe that a combination of increased Ca²⁺-influx and reduced Ca²⁺-extrusion from the cytoplasm may be the reason for elevated Ca²⁺-levels: Our data show a reduction of

NCX-activity, while there is also significantly increased L-type Ca^{2+} -current associated with increased $\text{Ca}_v1.2$ -expression [34]. Conversely, $\text{Ca}_v1.2$ -expression is decreased by CaMKII overexpression [35]. This combination of increased Ca^{2+} -influx and an impaired ability to eliminate Ca^{2+} by reduced Ca^{2+} -extrusion (via NCX) and reduced SR Ca^{2+} -reuptake might have caused a shift towards slightly higher Ca^{2+} -levels in the cytosol in the KO. However, other Ca^{2+} -handling pathways and buffering entities like sarcolemmal Ca^{2+} -ATPase, mitochondria and myofilaments might also be involved. Also, a small increase in $[\text{Na}^+]_i$ could elevate diastolic $[\text{Ca}^{2+}]_i$ via effects on NCX function.

The discrepancy between the large effects on diastolic Ca^{2+} as compared to the small changes in diastolic sarcomere length might be because some of the diastolic $[\text{Ca}^{2+}]_i$ rise would be below the threshold for myofilament activation. Altered myofilament properties or the use of mechanically unloaded myocytes could also contribute to that difference.

4.4. Stress response: preserved response to β -adrenergic stress but impaired SR Ca^{2+} -reuptake during acidosis

Our data demonstrate preserved inotropic and lusitropic effects of β -adrenergic stimulation in the KO (see also [21]). In contrast, CaMKII δ deficiency became functionally relevant during experimental acidosis, which may be of clinical relevance during myocardial infarction, ischemia-reperfusion, and even an acute increase in afterload that may cause subendocardial ischemia, which is likely accompanied by pH reduction. During the early phase of acidosis, low extracellular pH leads to an increased $[\text{H}^+]_i$ which drives the Na^+/H^+ -exchanger to extrude H^+ from the cell in exchange with Na^+ . This causes an increase in $[\text{Na}^+]_i$. Na^+ then gets exchanged for Ca^{2+} via the reverse mode of NCX so that diastolic $[\text{Ca}^{2+}]_i$ rises [26]. Regarding our present study, it seems as if this early, NCX-dependent mechanism is not affected in the KO as diastolic Ca^{2+} rose in parallel to WT cells during *early* acidosis. During *late* acidosis, increased $[\text{Ca}^{2+}]_i$ is known to activate CaMKII [25]. In healthy cardiac myocytes, CaMKII phosphorylates PLB at Thr-17 thereby partially reactivating depressed SR Ca^{2+} -reuptake, which leads to an increase in SR Ca^{2+} -content. Increased SR Ca^{2+} -content ultimately causes the SR to release more Ca^{2+} per systole, so that progressively increasing Ca^{2+} -transients help to partially overcome depressed myocyte contractility, a mechanism by which the cell can temporarily cope with clinically relevant situations such as myocardial ischemia. Furthermore, CaMKII-dependent enhanced SR Ca^{2+} -filling contributes to keep diastolic Ca^{2+} -levels within the physiological range. And indeed, all of these protective mechanisms [25,26] were present in control cells in the current study.

In contrast, in the CaMKII δ deficient cells, there was a further *increase* in diastolic Ca^{2+} during late acidosis in the face of progressively *slowed* Ca^{2+} -transients, which was in clear contrast to the partially reaccelerating Ca^{2+} -transient decay in control cells. We believe that this most likely results from reduced CaMKII-dependent PLB phosphorylation in KO cells. Consequently, SR Ca^{2+} -content progressively decreased during acidosis, which resulted in decreased Ca^{2+} -transients and increased diastolic $[\text{Ca}^{2+}]_i$ in KO cells. This inability of CaMKII δ deficient cells to maintain contractile force could be clinically problematic in a pathophysiological setting such as acidosis or ischemia. Indeed, during myocardial ischemia, extracellular pH can fall well below 7.0 within minutes, reaching intracellular pH values of ~ 6.5 [26]. Our current experimental model may be of relevance to that situation.

Our acidosis data seems to be in line with the generally impaired diastolic function observed in KO myocytes, only that under these specific stress conditions, affected diastolic function (SR Ca^{2+} -reuptake) became functionally relevant also for systolic function: It therefore appears that this pathophysiological constellation reveals a necessity for CaMKII δ activity for cardiomyocyte function and unmasks a crucial role for CaMKII δ in preserving sufficient

SR Ca^{2+} -loading by facilitation of SERCA-activity during acidosis. Specifically, there is impaired systolic function (decreased Ca^{2+} -transients) in the KO during acidosis due to the generally impaired diastolic function in this genotype (reduced recruitment of SERCA activity in acidosis) and an inability to recruit sufficient additional SERCA activity without CaMKII δ . This hypothesis is further confirmed by our finding that pharmacological CaMKII-inhibition prevented recovery of Ca^{2+} -transients from acidosis in WT animals. It appears that the CaMKII/PLB-pathway is critical for recovery from acidosis and that loss of CaMKII δ cannot be compensated in this respect (e.g. by CaMKII γ or enhanced SERCA expression).

4.5. Compensatory upregulation of I-1

While our CaMKII-activity assay showed a clear reduction of CaMKII-activity, phosphorylation levels of CaMKII-targets PLB and RyR were also reduced, but to a lesser extent. We found upregulation of I-1, which is an important inhibitor of protein phosphatase-1 activity, this way also regulating CaMKII-dependent phosphorylation of PLB [36] and RyR [37]. This mechanism may represent a novel link between Ca^{2+} - and I-1 mediated β -adrenergic signaling.

4.6. Summary/perspective

In summary, basal systolic cardiomyocyte function under physiologic conditions does not require CaMKII δ . As a caveat however, CaMKII δ might have important functions under certain pathophysiologic conditions, since the ability of the cells to recover from acidosis was clearly impaired. We suggest that this is due to reduction of SERCA function recruitment by PLB phosphorylation in acidosis upon loss of CaMKII δ . Also, our single cell data suggest that there might be latent impairment of diastolic function already under basal conditions, yet this is not apparent in vivo measurements. Consequently, while therapeutic inhibition of CaMKII δ still appears to be a promising approach, caution is warranted with respect to acidosis, where inhibition of CaMKII might be even detrimental.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
ECC	excitation–contraction coupling
FDAR	frequency-dependent acceleration of relaxation
I-1	phosphatase-1 inhibitor-1
Iso	isoproterenol

KO	CaMKII δ -KO
NCX	Na ⁺ /Ca ²⁺ -exchanger
NT	normale Tyrode's solution
PLB	phospholamban
RyR	ryanodine receptor type 2 (SR Ca ²⁺ -release channel)
SERCA	sarcoplasmic reticulum Ca ²⁺ -ATPase
SR	sarcoplasmic reticulum
TAC	transversal aortic constriction
WT	wild-type

Fig. 1.

Unaltered biometric data and in vivo echocardiography in the KO: heart weight to body weight ratio (A), fractional shortening (B), septum (C), and posterior wall thickness (D). Significant reduction of total CaMKII activity in the KO (E), but increased I-1 protein expression (F).

Fig. 2.

Isolated cardiomyocyte systolic function is not impaired under physiological conditions: as illustrated in original registrations (A), mean Ca²⁺-transient amplitudes (B) are not diminished in KO myocytes resulting in unimpaired cell contractility (C). Note that Ca²⁺-transient amplitude at 1 Hz was previously reported and was included here for comparison [12].

Fig. 3.

Impaired diastolic function due to decreased PLB Thr-17 phosphorylation: original Ca^{2+} -transients at 1 Hz (A) illustrate slower diastolic Ca^{2+} -elimination in KO myocytes. Mean data for Ca^{2+} -decay (τ , B) and twitch relaxation ($\text{rt}_{50\%}$, C) underline impaired diastolic function. # indicates $p < 0.05$ vs. WT in Holm–Sidak post-hoc test. Western blots show significant less PLB Thr-17 phosphorylation (D), but not at Ser-16 (E), unchanged PLB expression (F), but increased SERCA/PLB ratio (G).

Fig. 4.

Despite decreased SR Ca^{2+} -leak due to reduced RyR Ser-2815 phosphorylation, SR Ca^{2+} -content is unaltered: Original registrations of caffeine-induced transients (A) as a measure of SR Ca^{2+} -content which was unaltered (B). SR Ca^{2+} -leak as assessed by Ca^{2+} -sparks (C) is significantly lower in KO myocytes due to less cells showing Ca^{2+} -sparks (D and E). Western blots show significantly reduced RyR Ser-2815 phosphorylation (F) but not at Ser-2809 (G). Fura-2 measurements show higher diastolic $[Ca^{2+}]$ (H) and consequently shorter diastolic sarcomere length (I) in KO myocytes. NCX function, as approximated by caffeine-induced Ca^{2+} -transients (τ , J) is slightly impaired in KO myocytes.

Fig. 5.

Inotropic and lusitropic responses to adrenergic stimulation are intact: mean data for amplitudes (F/F_0 , B) and $\text{rt}_{50\text{Ca}}$ of Ca^{2+} -transients (D) from Iso wash-in experiments, demonstrate preserved inotropic and lusitropic response in KO myocytes. This is also evident at different stimulation frequencies from mean data for Ca^{2+} -transient amplitude (A), twitch amplitudes (C), Ca^{2+} -transient decay ($\text{rt } 50\%$, E) and twitch relaxation ($\text{rt } 50\%$, F).

Fig. 6.

Ca^{2+} -transients and SR Ca^{2+} -reuptake are impaired during acidosis: Representative Ca^{2+} -transients in WT and KO myocytes (A). While Ca^{2+} -transient amplitude increased in WT myocytes during late acidosis, it further decreased in KO myocytes. Mean data for Ca^{2+} -transient amplitudes (F/F_0 , B) and decay (τ_{Ca} , C). * $p < 0.05$ vs. previous phase, # $p < 0.05$ between genotypes using Fisher LSD post-hoc test. RM-ANOVA indicates two-way ANOVA testing for repeated measures.

Fig. 7.

SR Ca^{2+} -content during acidosis is impaired due to diastolic Ca^{2+} -overload: Representative Ca^{2+} -traces show impaired SR Ca^{2+} -loading in KO myocytes as compared to WT myocytes as acidosis progresses (A). Mean data for diastolic Ca^{2+} (B) and SR Ca^{2+} -content (C).

Abolished recovery of Ca^{2+} -transients (D) during late acidosis during CaMKII-inhibition with KN-93 in WT cells. Recovery incidence in WT myocytes (E). $\$p < 0.05$ vs. control conditions (Ctrl), $*p < 0.05$ vs. previous phase, $\#p < 0.05$ between genotypes using Fisher-LSD post-hoc test.