

Inhibition of Elevated Ca^{2+} /Calmodulin-Dependent Protein Kinase II Improves Contractility in Human Failing Myocardium

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Rationale: Heart failure (HF) is known to be associated with increased Ca^{2+} /calmodulin-dependent protein kinase (CaMK)II expression and activity. There is still controversial discussion about the functional role of CaMKII in HF. Moreover, CaMKII inhibition has never been investigated in human myocardium.

Objective: We sought to investigate detailed CaMKII δ expression in end-stage failing human hearts (dilated and ischemic cardiomyopathy) and the functional effects of CaMKII inhibition on contractility.

Methods and Results: Expression analysis revealed that CaMKII δ , both cytosolic δ_C and nuclear δ_B splice variants, were significantly increased in both right and left ventricles from patients with dilated or ischemic cardiomyopathy versus nonfailing. Experiments with isometrically twitching trabeculae revealed significantly improved force frequency relationships in the presence of CaMKII inhibitors (KN-93 and AIP). Increased postrest twitches after CaMKII inhibition indicated an improved sarcoplasmic reticulum (SR) Ca^{2+} loading. This was confirmed in isolated myocytes by a reduced SR Ca^{2+} spark frequency and hence SR Ca^{2+} leak, resulting in increased SR Ca^{2+} load when inhibiting CaMKII. Ryanodine receptor type 2 phosphorylation at Ser2815, which is known to be phosphorylated by CaMKII thereby contributing to SR Ca^{2+} leak, was found to be markedly reduced in KN-93-treated trabeculae. Interestingly, CaMKII inhibition did not influence contractility in nonfailing sheep trabeculae.

Conclusions: The present study shows for the first time that CaMKII inhibition acutely improves contractility in human HF where CaMKII δ expression is increased. The mechanism proposed consists of a reduced SR Ca^{2+} leak and consequently increased SR Ca^{2+} load. Thus, CaMKII inhibition appears to be a possible therapeutic option for patients with HF and merits further investigation. (*Circ Res.* 2010;107:1150-1161.)

Key Words: Ca^{2+} /calmodulin-dependent kinase II ■ heart failure ■ contractility ■ calcium ■ sarcoplasmic reticulum Ca^{2+} leak ■ ryanodine receptor

Heart failure (HF) is accompanied by systolic and diastolic contractile dysfunction caused by abnormalities in intracellular Ca^{2+} handling and structural remodeling. Several targets associated with the remodeling processes have been identified. The sarcoplasmic reticulum (SR) Ca^{2+} -ATPase (SERCA) protein levels have been reported to be downregulated and paralleled by a reduced SR Ca^{2+} uptake capacity in the human failing heart.¹⁻³ In contrast, the sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger protein expression and activity were found to be increased thereby even more effectively competing with the reduced SERCA activity for cytosolic Ca^{2+} -removal.^{3,4} The net effect is an impaired SR Ca^{2+} loading, which leads to smaller intracellular Ca^{2+}

transients and elevated diastolic Ca^{2+} levels in HF.⁵ Thus, impaired contractility with reduced contractile force and diastolic dysfunction are well-accepted determinants in HF.⁶

Intracellular Ca^{2+} homeostasis of cardiac myocytes is also regulated by phosphorylation of several key Ca^{2+} -handling proteins. An important regulatory kinase is the Ca^{2+} /calmodulin-dependent protein kinase (CaMK)II.⁷ It is a serine/threonine protein kinase that modulates several intracellular Ca^{2+} -handling proteins such as SR Ca^{2+} -release channels (ryanodine receptors, RyR2), phospholamban (PLB), and L-type Ca^{2+} channels (LTCCs) but also Na^+ channels.⁷ The predominant cardiac isoform is CaMKII δ , with the splice variant δ_C being primarily cytosolic and δ_B being localized to

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the nucleus.^{8,9} Because CaMKII accelerates relaxation via PLB phosphorylation and increased CaMKII protein levels correlate positively with impaired ejection fraction in the myocardium of patients with HF, it was proposed that elevated CaMKII expression and activity may be a compensatory mechanism to keep hearts from complete failure.⁹ In contrast, CaMKII may be even involved in the pathogenesis of hypertrophy and HF because CaMKII transgenic mice develop severe HF.¹⁰ There is a direct association of CaMKII and the RyR2 increasing diastolic SR Ca²⁺ leak despite (or actually leading to) reduced SR Ca²⁺ load in CaMKII transgenic mice.^{10,11} SR Ca²⁺ leak could be markedly reduced by CaMKII inhibition in this mouse HF model, as well as in rabbit HF models, providing evidence for a direct relation between CaMKII activity and reduced SR Ca²⁺ load caused by increased Ca²⁺ spark frequency.^{10,12,13}

Since Kirchhefer et al reported that CaMKII activity was increased in the left ventricles (LVs) of HF patients with dilated cardiomyopathy (DCM) in 1999,⁹ nothing was published about the role of CaMKII in human failing myocardium. Moreover, the function of CaMKII and its regulatory mechanisms in the failing heart are still a controversial issue. Because all functional studies to date were performed in animal models and knowledge about regional CaMKII expression in human myocardium is rare, it remains unclear whether and how CaMKII inhibition actually alters intracellular Ca²⁺ handling and myocardial contractility. Thus, the present study investigated CaMKII expression in DCM and ischemic cardiomyopathy (ICM) human LV and right ventricle (RV) myocardium. The second objective was to determine the functional effects of CaMKII by pharmacological CaMKII inhibition in HF.

Methods

An expanded Methods section is available in the Online Data Supplement at <http://circres.ahajournals.org>.

Human Myocardial Tissue

Experiments were performed with myocardium from 43 end-stage failing hearts (New York Heart Association heart failure classification IV; ICM, n=25; DCM, n=15; other, n=3) and 12 nonfailing (NF) donor hearts that could not be transplanted for technical reasons. Control subjects had no history of heart disease and had normal LV function.

Large Animal Model

Four sheep were sedated with Telazol (6 mg/kg) and were endotracheally intubated. Anesthesia was maintained with 1% to 2% isoflurane. The hearts were explanted via a left thoracotomy, which was performed through the fourth intercostal space.

Trabeculae Preparation and Experiments

Thin ventricular trabeculae were isolated from the RV of human end-stage failing hearts.^{14–16} For isometric force recordings, trabeculae were superfused with Krebs–Henseleit solution and connected to a force transducer.

Myocyte Isolation

Chunk isolation was performed with LV myocardial slices using collagenase (Worthington type 2; 250 U/mg) and trypsin (25%

Non-standard Abbreviations and Acronyms

CaMK	Ca ²⁺ /calmodulin-dependent protein kinase
DCM	dilated cardiomyopathy
FFR	force frequency relationship
HF	heart failure
I-1	phosphatase inhibitor-1
ICM	ischemic cardiomyopathy
LTCC	L-type Ca ²⁺ channel
LV	left ventricle
NF	nonfailing
PKA	protein kinase A
PLB	phospholamban
PP	protein phosphatase
RV	right ventricle
RyR	ryanodine receptor
SERCA	sarcoplasmic reticulum Ca ²⁺ -ATPase
SR	sarcoplasmic reticulum

trypsin). Only elongated cells with cross-striations and without granulation were selected for experiments.

Intracellular Ca²⁺ Imaging

Measurement of Ca²⁺ Sparks

Isolated myocytes were incubated with 10 μmol/L Fluo-3AM, which also contained either CaMKII inhibitors or control. Ca²⁺ spark measurements were performed with a laser-scanning confocal microscope. During continuous superfusion of the chamber, spark frequency was measured after loading the SR with Ca²⁺ by repetitive field stimulation. Ca²⁺ spark size was calculated as product of amplitude (F/F₀), duration and width. From this, we inferred the average leak per cell by multiplication of Ca²⁺ spark size with the mean spark frequency of the respective cell.

Determination of SR Ca²⁺ Content

Myocytes were field stimulated at 1 Hz, and SR Ca²⁺ content was assessed by caffeine-induced Ca²⁺ transients. These amplitudes were used as a measure for SR Ca²⁺ content.¹⁶

Western Blots

Ventricular myocardium was homogenized. Protein concentration was determined by BCA assay (Pierce Biotechnology). Denatured tissue was subjected to Western blotting using different antibodies. Chemiluminescent detection was performed with Immobilon Western (Millipore). Phosphorylation values were normalized to protein expression.

Data Analysis and Statistics

Force values were normalized to the cross-sectional area of the trabeculae (width×thickness×π/4) and expressed in mN/mm². All data are expressed as means±SEM. Student's *t* test or 2-way repeated-measures ANOVA with Holm–Sidak tests were used to test for significance. A value of *P*<0.05 was considered significant.

Results

CaMKII Expression in Human Heart Failure

To further extend the knowledge about CaMKIIδ expression and distribution between LV and RV, we performed expres-

CaMKII δ expression in DCM and ICM

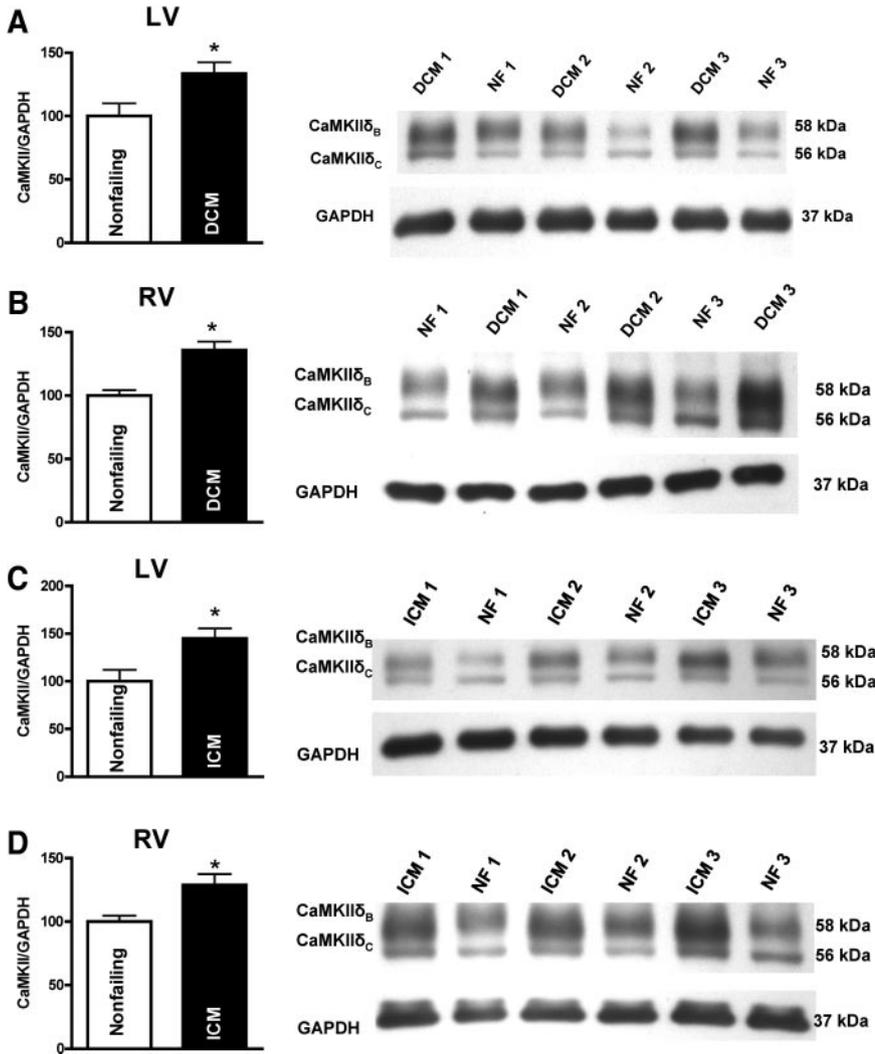


Figure 1. Western blots showing increased CaMKII δ expression in human heart failure. **A**, CaMKII δ expression levels are significantly increased in LV myocardium from patients with DCM vs NF (n=10 vs 8). **B**, Similar differences were found in the RV (n=8 vs 6). **C**, In LV myocardium from patients with ICM, CaMKII δ expression levels were also markedly increased vs NF myocardium (n=12 each). **D**, CaMKII δ levels in RV were significantly increased in ICM myocardium compared with NF control (n=6 each). *P<0.05 vs NF.

sion analysis in human end-stage myocardium from hearts with DCM and ICM compared with NF (LV and RV, respectively) donor hearts. Figure 1A shows a typical Western blot of CaMKII δ . The average LV CaMKII δ expression in DCM was elevated by $33.5 \pm 8.9\%$ (n=10) versus NF controls (n=8, P<0.05) when normalized to GAPDH). Similar results were obtained in DCM with RV CaMKII δ being increased by $35.8 \pm 6.8\%$ (n=8) compared with NF (n=6, P<0.05; Figure 1B).

Furthermore, we show that ICM is also associated with significantly upregulated CaMKII δ expression in both heart chambers (Figure 1C and 1D). LV CaMKII δ expression was increased by $45.1 \pm 12.9\%$ (n=12 each, P<0.05) compared with NF and by $28.9 \pm 9.5\%$ in RV myocardium versus NF (n=6 each, P<0.05). Of note, in DCM, as well as in ICM, a similar elevation of both splice variants CaMKII δ_C and CaMKII δ_B was found (Table 1) independent of LV or RV tissue. Finally, we also investigated the CaMKII γ isoform and found an increase of $43.1 \pm 12.7\%$ in LV failing myocardium (P<0.05, n=9 versus 8; data not shown).

Effects of CaMKII Inhibition on Contractility

Functional experiments were always performed using paired isolated trabeculae of the same area of the same heart. Twitch force was not different at baseline conditions (1 Hz) before incubation with the compounds being 6.6 ± 1.6 mN/mm² for the KN-92 control group and 6.8 ± 1.1 mN/mm² for those that were incubated with KN-93 (n=14 trabeculae of 9 hearts each, P=0.9).

Because it is known that CaMKII activity increases with higher frequencies, we obtained force frequency relationships

Table 1. Detailed Protein Expression Values of CaMKII and Its Splice Variants CaMKII δ_C (Cytosolic) and δ_B (Nuclear) in Human Failing Myocardium From DCM and ICM Origins

	DCM		ICM	
	LV	RV	LV	RV
Total	133.5±8.9*	135.8±6.8*	145.1±12.9*	128.9±9.5*
δ_C	151.3±11.7*	127.4±9.2*	146.5±12.8*	151.7±9.9*
δ_B	130.3±5.7*	134.4±5.3*	144.3±18.7*	131.8±9.9*

Values are normalized to GAPDH and finally to NF myocardium, which was also normalized to GAPDH before. *P<0.05 vs nontailing.

(FFR) in trabeculae stimulated at frequencies that varied between 0.5 to 3 Hz (Figure 2A and 2B).¹⁶ Whereas basal contractility at 0.5 Hz was unchanged in CaMKII inhibited trabeculae (4.6 ± 0.9 versus 4.2 ± 0.8 mN/mm² for KN-93, $P=0.78$), twitch force amplitude was largely increased at higher stimulation frequencies. Figure 2A and 2B shows original registrations of trabeculae stimulated at increasing frequencies in the presence of KN-92 or KN-93. The rather negative FFR in KN-92 control trabeculae changed to a positive FFR in trabeculae in KN-93-treated trabeculae. Twitch force amplitude increased by $92 \pm 20\%$ for KN-93 and only by $10 \pm 13\%$ for KN-92 at 2 Hz and by $101 \pm 33\%$ versus $-4 \pm 15\%$ at 3 Hz (both frequencies $P < 0.05$ and ANOVA $P < 0.05$ compared with KN-92, $n=14/9$ each; Figure 2C). Of note, the significant increase in force amplitude of KN-93-treated trabeculae was also significant regarding peak force values (data not shown). Also, to exclude unspecific effects of KN-92 (eg, on LTCC), we performed FFR experiments without any drug showing very similar results as compared with KN-92 (data not shown).

In contrast, the beneficial effect of CaMKII inhibition was not associated with significant alterations of diastolic tension at increasing frequencies (Figure 2D).

Similarly, we did not find any influence of CaMKII inhibition on relaxation kinetics of the trabeculae with frequency-dependent acceleration of relaxation being found in both groups to a similar extent (Figure 2E).

To confirm our findings, we additionally performed FFRs using AIP and did find comparable effects (Figure 2F). Twitch force amplitude increased by $52 \pm 14\%$ for AIP and only by $9 \pm 11\%$ for controls at 2 Hz (ANOVA $P < 0.05$, $n=9$ each; Figure 2G), whereas diastolic tension and relaxation time were not statistically changed (Figure 2H and 2I). Control twitch force values of this series are very similar to those that were observed in the presence of KN-92.

Because fresh human NF myocardium is difficult to obtain, we performed experiments with NF myocardium from a large animal. Interestingly we did not see a positive inotropic effect in sheep trabeculae in the presence of KN-93 compared with KN-92 (Figure 3A). Twitch force amplitude relative to 0.5 at 2 Hz was $205 \pm 48\%$ for KN-93 and $171 \pm 32\%$ for KN-92 (ANOVA $P=0.67$, $n=6$ each; Figure 2B), and diastolic tension was unchanged (Figure 3C). Figure 3D shows that there was a trend toward a slower relaxation time in the presence of KN-93 that however did not reach statistical significance.

SR Ca²⁺ Load Is Increased Because of Inhibition of CaMKII

Periods of 10, 30, and 120 seconds of rest were evaluated and the first twitch was normalized to the last twitch before rest. Original registrations in Figure 4A show that postrest behavior was significantly improved in the presence of KN-93. This effect was more pronounced after long rest intervals. The first twitches after 120 seconds of rest normalized to the last twitches before rest were more than doubled, with an average of 2.5 ± 0.4 in specimen treated

with KN-93 versus 1.5 ± 0.3 in the presence of KN-92 ($n=12/7$ each, $P < 0.05$; Figure 4B).

This effect may be attributable to an increased SR Ca²⁺ load. Therefore, we directly measured SR Ca²⁺ content. In the representative original tracings (Figure 4C), KN-93 clearly increased the amplitude of the caffeine-induced Ca²⁺ transient. The F/F_0 was 2.1 ± 0.3 for KN-93 compared with 1.3 ± 0.1 for KN-92 ($n=5/3$ versus $n=9/3$ cells, $P < 0.05$; Figure 4D). These results indicate that improved contractility of multicellular human failing myocardium may be caused by an improved SR Ca²⁺ content during CaMKII inhibition.

CaMKII Inhibition Reduces SR Ca²⁺ Leak in Human Heart Failure

To investigate whether the increased SR Ca²⁺ content in the presence of CaMKII inhibition may result from a reduced SR Ca²⁺ leak we also measured SR Ca²⁺ spark frequency. The original line scans in Figure 5A exhibit that Ca²⁺ spark frequency was strongly decreased in myocytes that were CaMKII-inhibited. The mean Ca²⁺ spark frequency was 212 ± 27 pL⁻¹ · sec⁻¹ in KN-92 and 137 ± 16 pL⁻¹ · sec⁻¹ in the presence of KN-93 ($n=31/7$ versus $n=28/7$ cells, $P < 0.05$; Figure 5B). Moreover, experiments presented in Figure 5C revealed that the Ca²⁺ spark duration was also slightly reduced in the presence of KN-93 (71.7 ± 1.2 versus 68.0 ± 1.3 ms for KN-92, $P < 0.05$). This leads to a significant decrease in total calculated SR Ca²⁺ leak in CaMKII-inhibited myocytes by 30% ($P < 0.05$; Figure 5D). Experiments were repeated using another CaMKII inhibitor AIP (55 myocytes versus 51 serving as controls). A total of 47% of the measured myocytes developed SR Ca²⁺ sparks under control conditions and only 29% in AIP-treated cells (1 μmol/L, $P=0.06$). We also found comparable results to our KN-93 findings such as a depressed SR Ca²⁺ spark frequency from 300 ± 42 pL⁻¹ · sec⁻¹ (controls) to 120 ± 20 pL⁻¹ · sec⁻¹ in AIP-treated myocytes ($n=23$ versus 16, $P < 0.05$; Figure 5E and 5F). The total calculated SR Ca²⁺ leak was significantly reduced by 39% in the presence of AIP (Figure 5H). Detailed SR Ca²⁺ spark parameters are presented in Table 2.

Altered Phosphorylation Status During CaMKII Inhibition

To verify the efficacy of the used CaMKII inhibitors, we performed phosphorylation analysis in CaMKII-inhibited trabeculae. A FFR was performed in the presence of KN-93 versus KN-92 or AIP versus control and specimen were immediately frozen away. As presented in Figure 6A, KN-93 led to a downphosphorylation of CaMKII at Thr286 of $65.8 \pm 11.2\%$ ($P < 0.05$, $n=7$ each) and in AIP-treated homogenated specimen of $83.1 \pm 8.2\%$ ($P < 0.05$, $n=4$ each; Figure 6B).

Because relaxation kinetics were unchanged during CaMKII inhibition, we also investigated CaMKII effects on PLB. The CaMKII phosphorylation site at Thr17 of PLB (monomer) was not significantly altered after treatment with KN-93, as presented in Figure 6C ($P=0.48$, $n=8$ each).

Effects of CaMKII Inhibition on contractility I

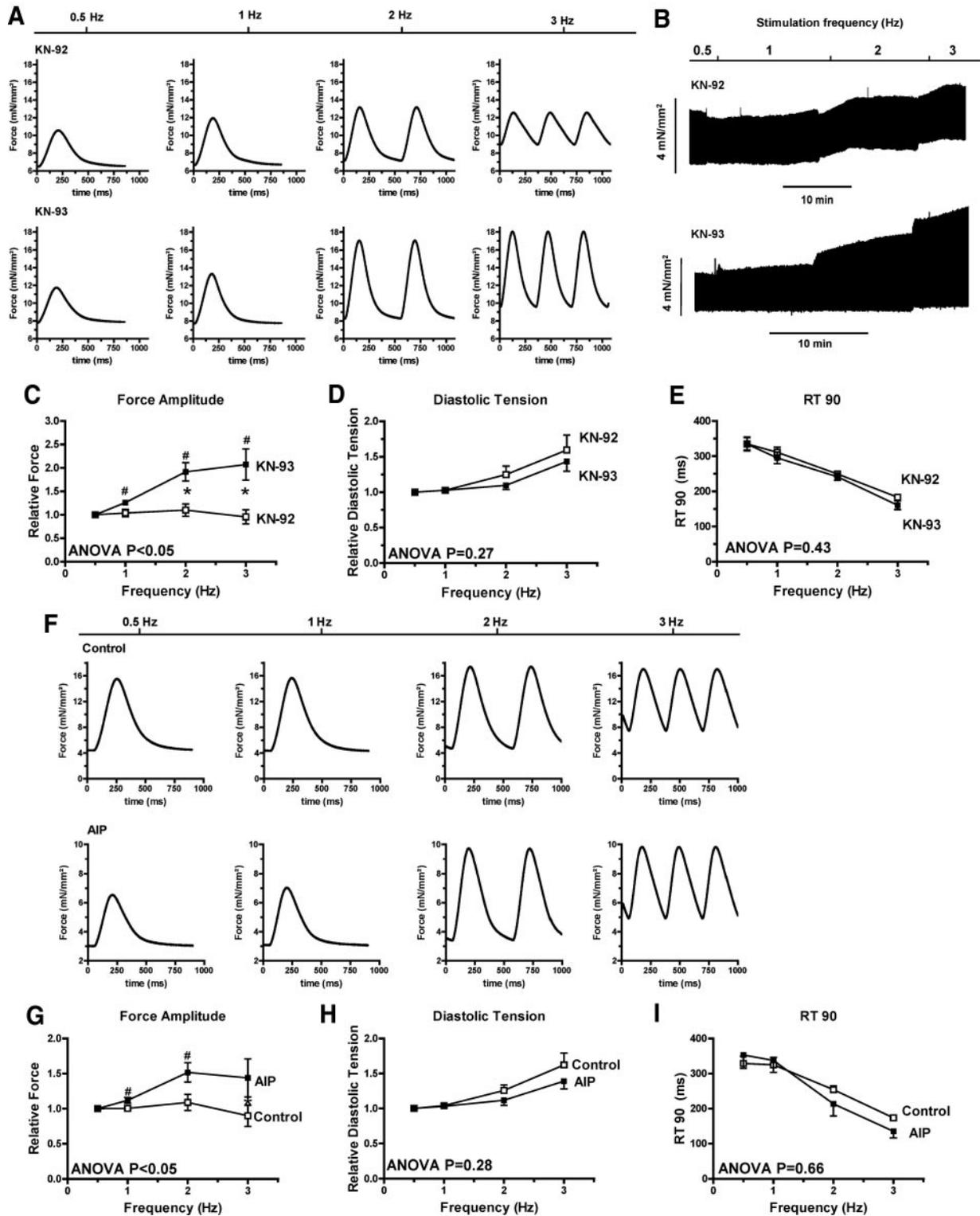


Figure 2. Influence of CaMKII inhibition on contractility of trabeculae during increasing stimulation frequencies. **A**, Representative single twitches in the presence of KN-92 or KN-93. **B**, An original registration with a slower writing speed nicely shows the positive inotropic effect in the presence of KN-93 but not KN-92. **C**, Mean force amplitudes normalized to the lowest frequency (0.5 Hz absolute values 4.6 ± 0.9 vs 4.2 ± 0.8 mN/mm² for KN-93), showing significantly increased force amplitudes at 2 and 3 Hz in the presence of KN-93 vs KN-92 ($n = 14/9$ each). **D**, Normalized diastolic tension was not significantly different between both groups during increasing frequencies. **E**, Time to 90% relaxation was not changed between KN-93 and KN-92. **F**, Original tracings exhibit a positive inotropic effect in the presence of another CaMKII-inhibitor AIP compared with control. **G**, Mean force amplitudes normalized to the lowest frequency showing significantly increased force amplitudes in the presence of AIP compared with control ($n = 9$ each). **H and I**, Averaged values of diastolic tension (**H**) and time to 90% of relaxation (**I**) in the presence of AIP. * $P < 0.05$ vs KN-92 or control (repeated-measures ANOVA and post hoc test); # $P < 0.05$ vs baseline.

Effects of CaMKII Inhibition on contractility II

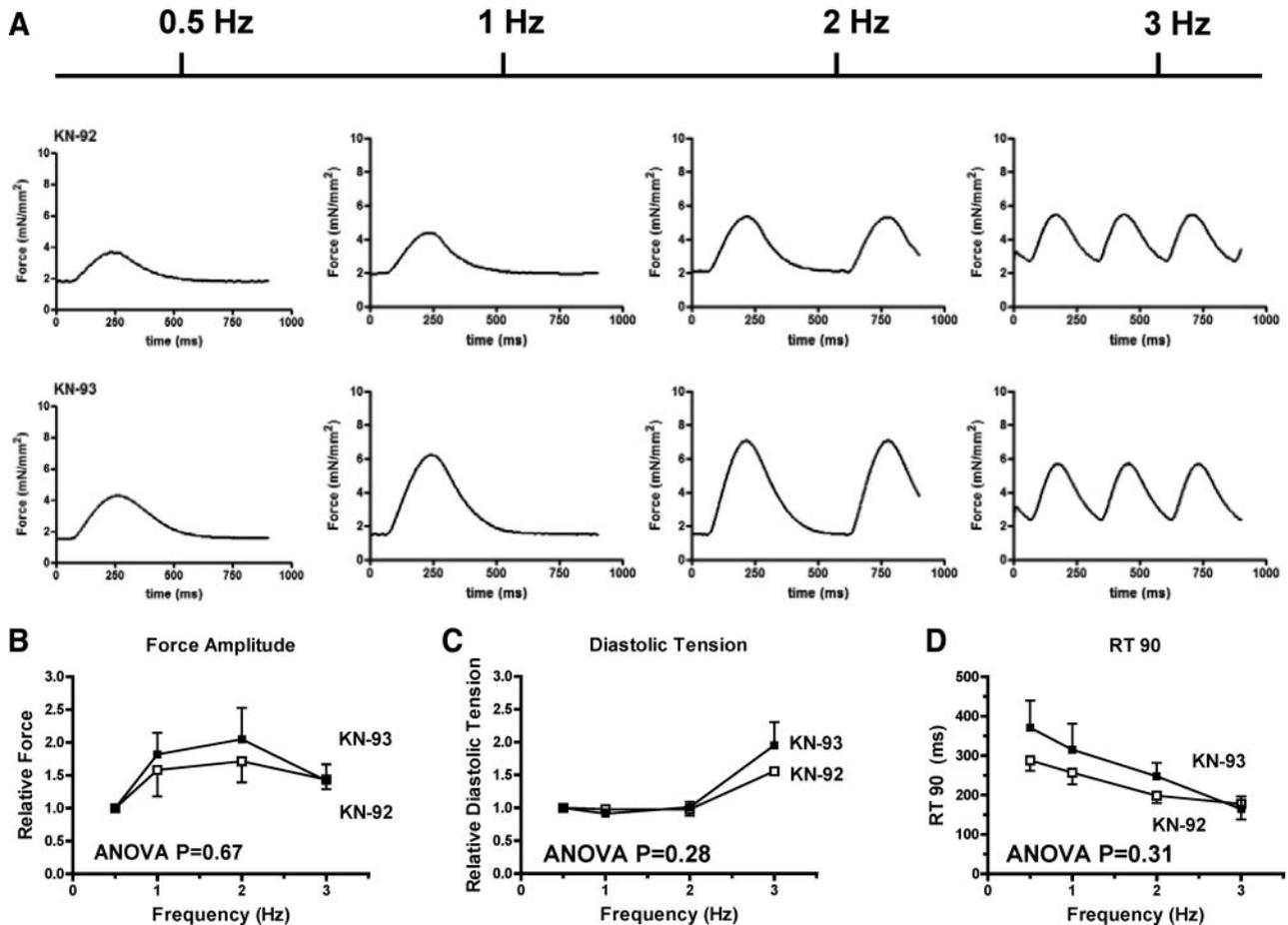


Figure 3. Influence of CaMKII inhibition on contractility of NF sheep trabeculae during increasing stimulation frequencies. **A**, Original tracings show no difference of contractile force between KN-93 or KN-92. **B through D**, CaMKII inhibition did not affect force amplitudes, diastolic tension, or relaxation time in NF myocardium treated with KN-93 compared with KN-92 (n=6 each).

Similar results were obtained by investigation of a proposed CaMKII phosphorylation site at the LTCC β_{2a} subunit (Thr498).¹⁷ The common p-CaMKII (Thr286) antibody was shown to detect CaMKII-dependent phosphorylation of the LTCC β_{2a} subunit.¹⁷ In the same trabeculae where CaMKII phosphorylation was significantly depressed after treatment with KN-93, we found an unchanged Thr498 phosphorylation status between KN-93- and KN-92-treated trabeculae (n=4 each; Figure 6D).

Finally, we were interested whether the observed effects on SR Ca^{2+} leak are mediated via modulation of the RyR2 phosphorylation status. CaMKII is considered to phosphorylate Ser2815¹⁸ rather than Ser2809, which may be also phosphorylated by protein kinase (PK)A.^{19–21} In CaMKII-inhibited homogenates versus controls, RyR2 phosphorylation (normalized to RyR2 protein levels) was increased at both Ser2809 and Ser2815 sites. Figure 6E shows that CaMKII inhibition lead to a $42.6 \pm 9.8\%$ (n=5/3 each, $P < 0.05$) decrease of RyR2 phosphorylation at Ser2809 and a more pronounced effect at the CaMKII specific binding site Ser2815 of $74.0 \pm 11.3\%$ (n=3/2 each, $P < 0.05$; Figure 6F). Thus, our results suggest that decreased SR Ca^{2+} leak resulting from CaMKII inhibition may be attributable to reduced RyR2 phosphorylation.

Discussion

This study demonstrates for the first time that inhibition of elevated CaMKII levels in human end-stage failing but not in sheep NF myocardium significantly improves cardiac contractility. CaMKII δ_B and δ_C protein levels were significantly increased in ICM and DCM in both LV as well as RV myocardium. CaMKII-inhibitor-dependent positive inotropic effects that occurred during FFR were elucidated by performing postrest experiments, caffeine measurements, and determination of SR Ca^{2+} leak. SR Ca^{2+} load was significantly improved most likely because of a reduced SR Ca^{2+} leak during CaMKII inhibition. The mechanism responsible for this may be the reduced RyR2 phosphorylation at Ser2809 and Ser2815.

CaMKII in the Failing Heart

During excitation–contraction coupling, CaMKII phosphorylates several Ca^{2+} -handling proteins. Thus, CaMKII can substantially modulate Ca^{2+} influx and SR Ca^{2+} release and uptake. After PLB phosphorylation via CaMKII or PKA, SERCA activity and SR Ca^{2+} uptake can be enhanced, leading to faster relaxation of the myocytes. Because of the increased CaMKII expression in human failing myocardi-

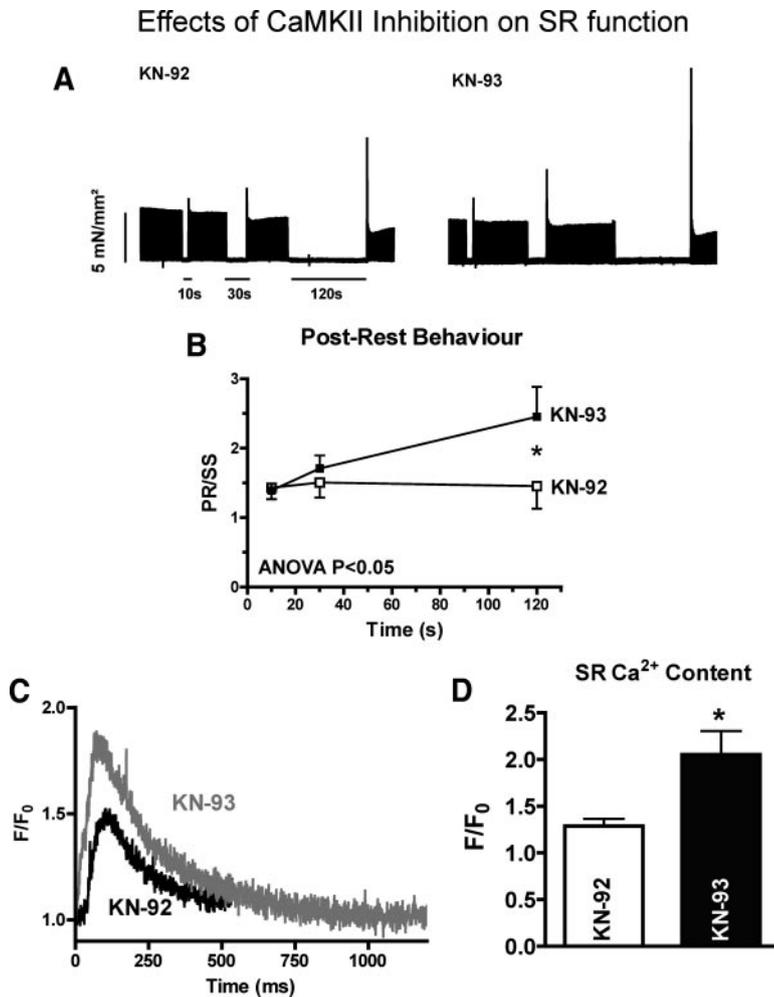


Figure 4. Inhibition of CaMKII modulates the postrest behavior of trabeculae from human failing hearts. **A**, Original tracings of twitch force at increasing rest intervals. The magnitude of the first beat after rest is considered to reflect the relation between SR Ca²⁺ uptake and loss during the rest interval. Inhibition of CaMKII clearly increased the amplitude of the first twitch after the rest interval. **B**, Mean values of postrest behavior which is plotted as the relation of the first twitch after rest normalized to the last before rest (PR/SS), indicating improved postrest behavior resulting from CaMKII inhibition using KN-93 (n=12/7 each; $P < 0.05$). **C**, Original caffeine-induced SR Ca²⁺ transients showing increased SR Ca²⁺ content in the presence of KN-93. **D**, Mean values of significantly increased SR Ca²⁺ content during treatment with KN-93 vs KN-92 (n=5 vs 9). * $P < 0.05$ vs KN-92.

um it has been proposed that elevated CaMKII in HF plays a compensatory role to keep myocardium from complete failure.^{9,22}

However, in recent years evidence arose that increased levels of CaMKII may have actually adverse effects as it was shown that CaMKII overexpression in a transgenic mouse model leads to cardiac hypertrophy and severe DCM.^{10,11} Moreover, a rabbit model of nonischemic HF indicated that CaMKII-dependent phosphorylation of the Ryr2 is involved in enhanced SR Ca²⁺ leak and reduced SR Ca²⁺ load and thus may contribute to arrhythmias and contractile dysfunction in the failing heart.¹³ We have recently shown that in human atrial fibrillation where CaMKII levels are also elevated, CaMKII increases SR Ca²⁺ leak, potentially contributing to arrhythmias.²³

The results of the present study show that increased CaMKII in human HF is not limited to LV from patients with DCM as it was initially shown.^{9,22} In fact, we found that CaMKII is also increased in ischemic origins and also in the RV of ICM as well as DCM. Thus, it appears that in the majority of end-stage HF etiologies CaMKII increases independently of the genesis, which might be a general phenomenon and therefore important for further *in vivo* investigations.

CaMKII Inhibition in Human Failing Myocardium

Here, we demonstrate that inhibition of CaMKII increases contractility in the human failing myocardium with a more pronounced effect at higher stimulation-frequencies. This effect was not observed in NF sheep trabeculae. Postrest potentiation together with caffeine experiments revealed that this observation can be explained by an increased SR Ca²⁺ load in the presence of CaMKII inhibitors in human failing myocardium.

To understand the mechanism behind this finding we investigated SR Ca²⁺ leak in freshly isolated myocytes. SR Ca²⁺ leak together with reduced SERCA activity is considered to be a major contributor to contractile dysfunction in HF by losing Ca²⁺ from the SR and consequently from the intracellular milieu probably via increased Na⁺/Ca²⁺ exchanger.²⁴ As a measure for SR Ca²⁺ leak we found that SR Ca²⁺ spark frequency is highly sensitive to KN-93 although the reduced total SR Ca²⁺ leak is more driven by the markedly diminished Ca²⁺ spark frequency than by effects on spark duration as confirmed by experiments using AIP. This finding is in agreement with previous reports showing transgenic overexpression of CaMKII being associated with reduced SR Ca²⁺ load caused by increased SR Ca²⁺ leak.¹⁰ Most importantly, these effects could be reversed by KN-93 in our study. Obviously, SR

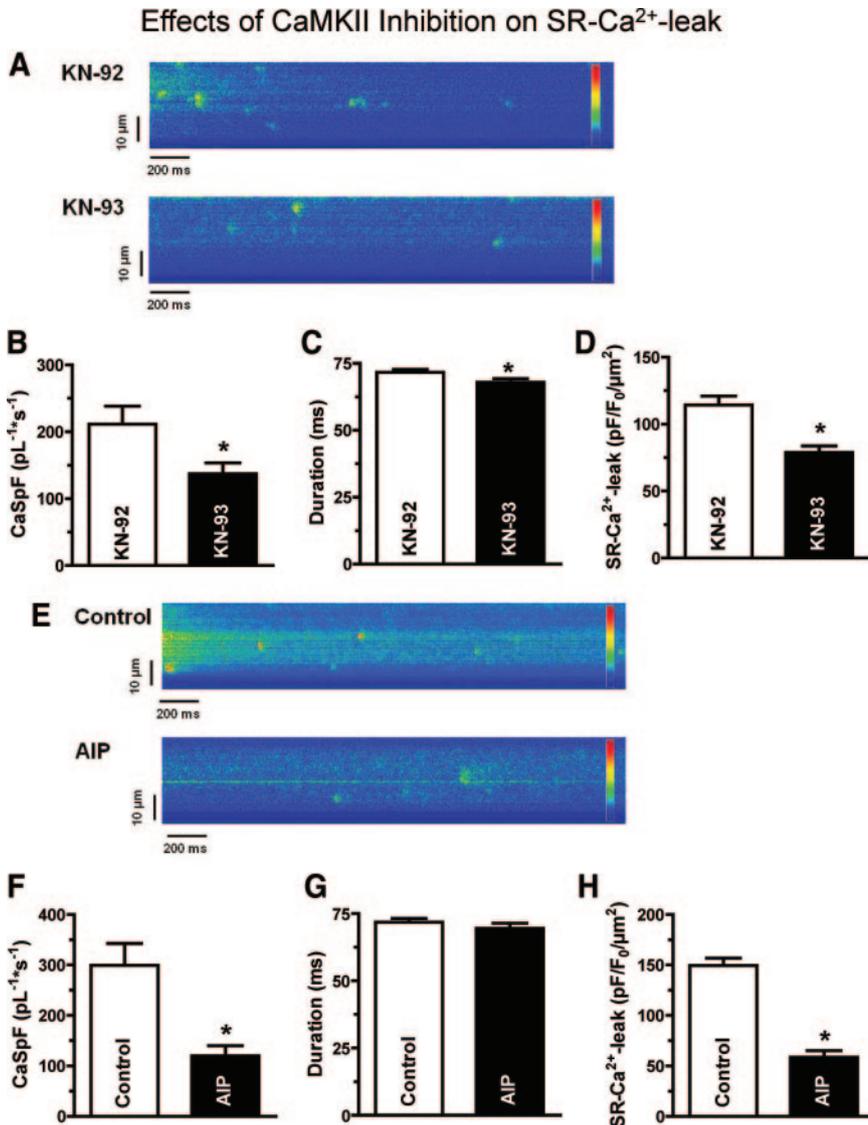


Figure 5. Spontaneous SR Ca²⁺ release events (Ca²⁺ sparks) in isolated human failing myocytes. **A**, Original line scans show decreased SR Ca²⁺ spark frequency in a myocyte treated with KN-93 compared with a myocyte in the presence of KN-92. **B**, SR Ca²⁺ spark frequency was significantly decreased in the presence of KN-93 (n=28/7) vs KN-92 (n=31/7). **C**, Mean SR Ca²⁺ spark duration (KN-93 n=63 vs KN-92 n=107). **D**, Calculated total SR Ca²⁺ leak could be significantly reduced by inhibition of CaMKII. **E**, Line scans in the presence of AIP also decreased the SR Ca²⁺ spark frequency. **F**, SR Ca²⁺ spark frequency could be significantly decreased in the presence of AIP (n=16) vs control (n=23). **G**, Mean SR Ca²⁺ spark duration (AIP, n=31; vs control, n=105). **H**, Calculated total SR Ca²⁺ leak could be significantly reduced by inhibition of CaMKII. *P<0.05 vs KN-92 or AIP.

Ca²⁺ leak can be reduced independent of the species, cause, or amount of CaMKII expression because overexpression in the latter transgenic mouse model was more than 10-fold compared with control conditions and in the present study CaMKII expression was increased by only 30% to 40%. Also, Ai et al investigated effects of CaMKII on SR Ca²⁺-function in a rabbit model of nonischemic HF.¹³ In this model, CaMKII was found to hyperphosphorylate the RyR2 leading to increased SR Ca²⁺ leak and reduced SR Ca²⁺ load. By inhibiting CaMKII using

KN-93, these authors showed that SR Ca²⁺- content as well as Ca²⁺ transients could be increased.¹³

In the present study we attribute the reduced SR Ca²⁺ leak in the presence of KN-93 to a reduced phosphorylation of the RyR2 at the CaMKII specific binding site (Ser2815) and to a lesser extent, at Ser2809 which can also be phosphorylated by PKA. Wehrens et al performed site-directed mutagenesis and identified an exclusive CaMKII-specific binding site at Ser2815 on recombinant RyR2.¹⁸ However, previous studies have suggested that

Table 2. Detailed Mean Values of SR Ca²⁺ Spark Parameters

	Frequency (pL ⁻¹ ·s ⁻¹)	TTP (ms)	Amplitude (F/F ₀)	Duration (ms)	Size (F/F ₀ ·ms·μm)	SR Ca ²⁺ Leak (pF/F ₀ ·μm ⁻²)	RT50 (ms)	RT90 (ms)
KN-92	211.59±26.77	11.96±0.39	1.70±0.02	71.68±1.12	369.22±10.97	114.27±6.71	18.55±0.50	44.22±0.84
KN-93	137.26±16.31*	10.48±0.45*	1.65±0.03	67.96±1.35*	338.15±13.40	60.16±3.72*	17.02±0.66	42.59±0.99
Control	299.55±43.18	10.89±0.29	1.68±0.02	71.78±1.41	371.38±17.79	149.66±7.15	16.64±0.43	42.54±0.80
AIP	119.79±20.10*	10.86±0.50	1.65±0.05	69.52±1.85	376.89±19.64	58.81±6.23*	16.37±0.60	41.42±1.24

Cardiomyocytes were either treated with KN-92 vs KN-93 or AIP vs control. RT50 indicates time from peak to 50% of relaxation; RT90, time from peak to 90% of relaxation; TTP, time to peak. *P<0.05 vs the corresponding control group (KN-92 or control).

Phosphorylation status during CaMKII Inhibition

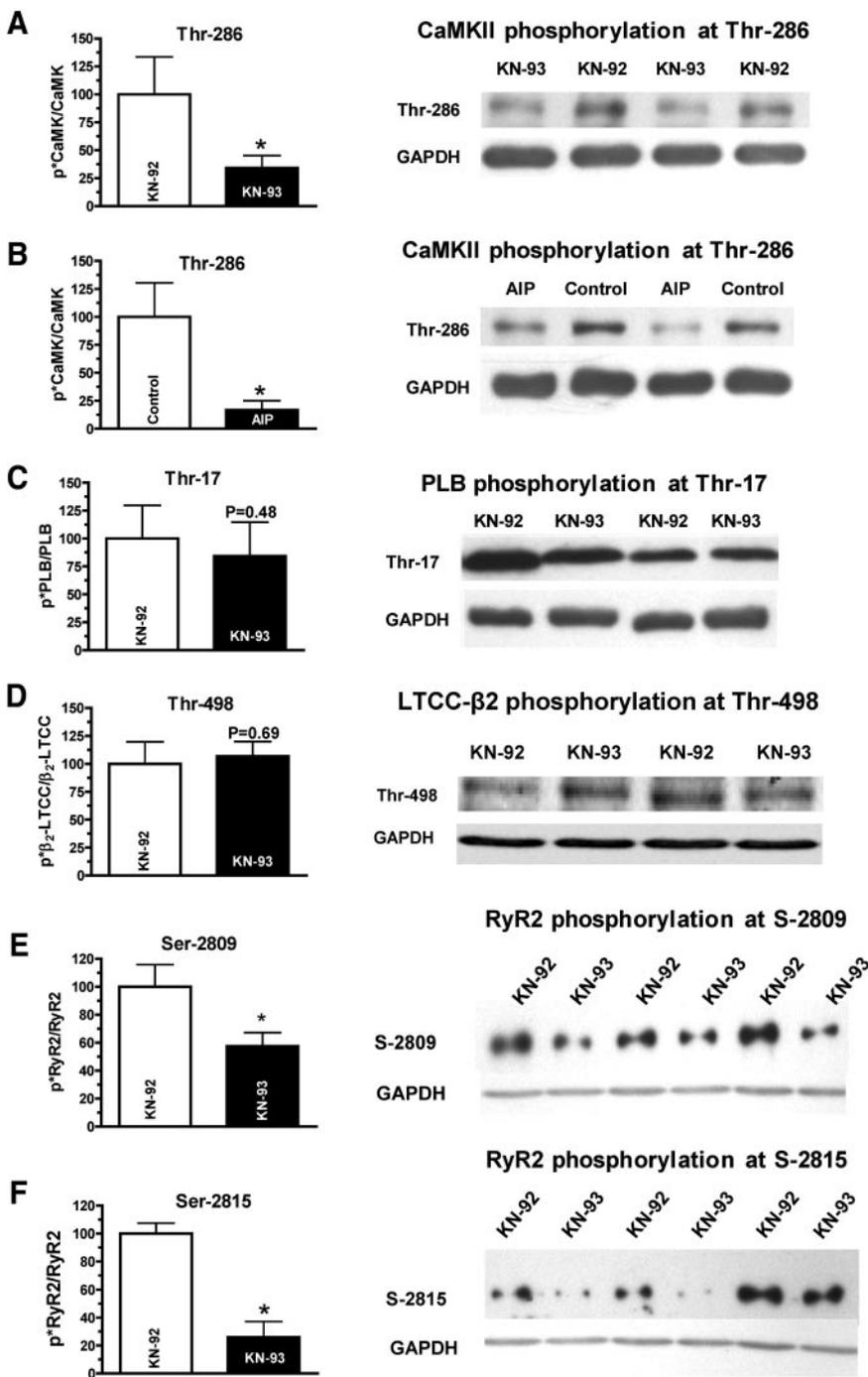


Figure 6. Western blots of homogenated trabeculae that were treated with either KN-92 vs KN-93 or control vs AIP. **A**, Mean values and original Western blot showing significant CaMKII inhibition at Thr286 in KN-93-treated homogenates ($P < 0.05$, $n = 7$ each). **B**, Mean values and original Western blot indicating a potent CaMKII inhibition at Thr286 in AIP-treated homogenates ($P < 0.05$, $n = 4$ each). **C**, Mean values of PLB (monomer) phosphorylation at Thr17 were unaffected by KN-93 ($n = 8$ each). **D**, KN-93 did not lead to a dephosphorylation of the proposed CaMKII-dependent phosphorylation site of the β_{2a} subunit of LTCC ($n = 4$ each). **E**, The RyR2 binding site for PKA and CaMKII showed a significant reduction of phosphorylation (normalized to RyR2) at Ser2809 ($n = 5/3$ each) in the CaMKII inhibition group. **F**, Phosphorylation of the CaMKII-specific binding site ($n = 3/2$ each). * $P < 0.05$ vs KN-92 or control.

Ser2809 may be the phosphorylation site of both PKA and CaMKII based on sequencing of tryptic phosphopeptides^{20,25} or a phosphoepitope-specific antibody.

Interestingly, we found that inhibition of CaMKII lacks effects on relaxation kinetics in our experiments. Accordingly, Western blot analysis of PLB revealed unchanged phosphorylation status at Thr17 after treatment with KN-93. Until recently, there was a general agreement that CaMKII phosphorylates PLB at Thr17 leading to improved frequency-dependent acceleration of relaxation.²⁶ Because of this, our finding with respect to unchanged relaxation

and CaMKII-mediated PLB phosphorylation is in part surprising, although it has been previously observed that acute CaMKII overexpression neither changed relaxation of cell shortening nor the Ca^{2+} transient decay in rabbit myocytes.²⁷ One possible explanation for CaMKII inhibition leading to marked dephosphorylation of the RyR2, without changes in LTCC or PLB phosphorylation and relaxation in human failing hearts, may be that compartmentalization of protein phosphatase (PP)1 and/or protein phosphatase inhibitor-1 (I-1) plays a crucial role in the phosphorylation/dephosphorylation balance of the cell. It

has been proposed that differential anchoring of PP1 to various compartments can explain differences between RyR2 and PLB phosphorylation in HF.¹⁹ Moreover, it was shown that SR-associated PP1 activity is increased 2.5-fold, suggesting that I-1 preferentially localizes to the SR.²⁸ El-Armouche et al found a good correlation between PLB and I-1 phosphorylation in human HF supporting the notification of a causal relationship and arguing for preferential affection of PLB (at the free SR) compared with the RyR2 located at the junctional SR.²⁹ Taken together, CaMKII-induced phosphorylation effects via PLB may be ameliorated, whereas phosphorylation of the RyR2 may be excessive in human heart failure. Thus, inhibition of CaMKII rather exerts effects on SR Ca²⁺ release in HF similar to our finding of an improved SR Ca²⁺ leak in human atrial fibrillating myocardium.²³ Even if there might be slight effects on relaxation which may not be detectable with our techniques the SR Ca²⁺ leak would counteract small increases of faster Ca²⁺ uptake. In any case, it seems that the major detrimental effect of CaMKII in HF and consequently the most important target emerging from our study is CaMKII-mediated SR Ca²⁺ leak, which greatly outbalanced possible detrimental effects attributable to reduced SERCA-activity on CaMKII inhibition.

Recently, it was reported that knocking out the CaMKII-S2814 phosphorylation site on the RyR in a mouse model decreases the positive inotropic response to an increase of stimulation frequency which is just the opposite of what we have found.³⁰ This finding is in sharp contrast to our results but could be explained by different excitation–contraction coupling of species and possible allosteric effects on the CaMKII-S2814 knockout model.

Because CaMKII is known to phosphorylate LTCCs, we investigated one of the proposed CaMKII phosphorylation sites of the β_{2a} subunit at Thr498.¹⁷ Whereas CaMKII phosphorylation was found to be markedly depressed in KN-93–treated trabeculae, we did not find any changes at the LTCC β_{2a} subunit. Although not intensively studied, this finding (if Thr498 is the corresponding phosphorylation site in human myocardium) could explain why the positive inotropic effect caused by a reduced SR Ca²⁺ leak may not be counteracted by CaMKII-dependent LTCC modulation. Nevertheless, the LTCC β_{2a} subunit phosphorylation site of CaMKII has not been studied in human myocardium and thus requires further intensive research.

Another finding of our study is an unchanged diastolic tension during CaMKII inhibition, although diastolic tension increases with raising frequencies. This might be largely attributed to a strong correlation between fibrosis and diastolic dysfunction in the human heart,³¹ which cannot be influenced by acute ionic modulation.

Limitations of the Study

It must be stated that evaluation of CaMKII effects was acute and pharmacologically performed. Therefore, chronic effects of CaMKII inhibition (eg, on target protein expression or transcription) cannot be ruled out.

KN-93 has been reported to exert unspecific effects (eg, on LTCC).³² However, this may be disregarded from our results, because this effect of KN-93 on I_{Ca} is shared by its inactive analog KN-92³² and we did not observe phosphorylation changes at the proposed binding site of CaMKII at the β_{2a} subunit of LTCC¹⁷ between KN-93– and KN-92–treated trabeculae. Moreover, we recently found similar effects of KN-93 and KN-92 on I_{Ca} in human atrial myocytes.²³ In particular, we confirmed our findings by using AIP in trabeculae, as well as in isolated cardiomyocytes. AIP is known to be CaMKII-specific.³² Additionally, effectiveness of CaMKII inhibition was confirmed by performing tissue phosphorylation analysis after treatment with KN-93, as well as AIP. It has to be stated that the phospho-CaMKII antibody may not distinguish between different CaMKII isoforms. Because CaMKII γ was also found to be upregulated in human HF, we attribute our functional findings to inhibition of CaMKII without any specifications of the isoform. In the present work, CaMKII expression was investigated without evaluation of CaMKII phosphorylation, which has been previously done in human failing hearts.⁹ This decision is based on the fact that reasonable phosphorylation analysis requires shock freezing of tissue within minutes. This may not be maintained after heart explantation especially for NF hearts, which also serve as valve donors and are dissected over some period of time.

Paired RV trabeculae were used in the present study, because LV endocardium consists of a large amount of fibrosis and thus may not be representative of the mean mass of wall myocardium, at least for the functional experiments. One further advantage in using RV trabeculae is that LV myocardium is largely spared from infarction and previous ischemia. To allow the assessment of CaMKII inhibition in RV preparations, CaMKII expression was investigated in the presented study, and no differences were found between CaMKII expression in the RV as compared with the LV. This may be caused by the fact that end-stage failing hearts often undergo severe pump failure of both ventricles.

During CaMKII inhibition, we found a reduced phosphorylation of Thr286 of $\approx 70\%$ to 80% . Thus, our model represents no ablation of CaMKII because residual active CaMKII can still phosphorylate target proteins. Finally, we found a reduced SR Ca²⁺ leak as the potential mechanism causing positive inotropic effects in the presence of CaMK inhibitors. However, another mechanism that may contribute to these effects, either by CaMKII-dependent mechanisms or by the inhibitors that were used, cannot be completely ruled out.

Summary and Conclusion

In summary, the present study shows for the first time that CaMKII inhibition improves contractile function in human end-stage failing myocardium, where CaMKII δ expression is increased. CaMKII-dependent RyR2 phosphorylation leads to increased SR Ca²⁺ leak, depleting the SR of Ca²⁺. CaMKII inhibition reduces this Ca²⁺ leak, resulting in positive inotropic effects observed in our functional experiments. Because CaMKII also plays a role in structural remodeling,^{33,34} CaMKII inhibition should be further clini-

cally investigated with respect to providing novel strategies and therapies in human HF.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Heart failure is associated with increased Ca^{2+} /calmodulin-dependent protein kinase (CaMK)II expression and activity.
- CaMKII-dependent SR Ca^{2+} leak decreases sarcoplasmic reticulum (SR) Ca^{2+} content in animal models of heart failure.
- CaMKII inhibition decreases SR Ca^{2+} leak in animal models of heart failure.

What New Information Does This Article Contribute?

- In isolated human failing myocardium, CaMKII leads to SR Ca^{2+} leak and decreases SR Ca^{2+} content.
- CaMKII inhibition reduces increased SR Ca^{2+} leak and restores SR Ca^{2+} content.
- CaMKII inhibition improves cardiac contractility and may therefore be a novel therapeutic strategy.

It has been shown that CaMKII expression and activity is upregulated in heart failure contributing to electric, structural,

and functional remodeling. Although CaMKII-dependent increased SR Ca^{2+} leak (Ca^{2+} sparks) has previously been reported in animal models of heart failure as a possible important pathophysiological mechanism and therapeutic target, its role in the human failing heart has not been investigated. In this study of failing human myocardium, we show that SR Ca^{2+} leak occurs and is attributable to CaMKII-dependent hyperphosphorylation of the SR Ca^{2+} -release channel (RyR2) and that it leads to elevation of spontaneous diastolic SR Ca^{2+} -release events from the SR. CaMKII inhibition can reduce both RyR2 phosphorylation and SR Ca^{2+} leak, leading to a normalization of SR Ca^{2+} content and, most importantly, to positive inotropic effects during increasing stimulation frequencies. Our results suggest that CaMKII inhibition could be beneficial with respect to heart failure, thus offering a novel therapeutic approach for this disease.

Inhibition of Elevated Ca^{2+} /Calmodulin-Dependent Protein Kinase II Improves Contractility in Human Failing Myocardium

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Online Supplement: Expanded Methods Section

1.1 Human myocardial tissue

Hearts from 31 males and 12 females were included with a mean age of 47.8 ± 2.2 years. The mean ejection fraction was $22.8 \pm 1.3\%$, cardiac index was 2.1 ± 0.1 L/min/m², and pulmonary capillary wedge pressure was 21.5 ± 11.4 mmHg. Premedication mostly consisted of angiotensin-converting enzyme inhibitors (55%) or angiotensin II type-1 blockers (27%), β -blockers (85%), cardiac glycosides (30%), and diuretics (91%).

After explantation, hearts were stored in a cooled cardioprotective solution (mmol/L): Na⁺ 152, K⁺ 3.6, Cl⁻ 135, HCO₃⁻ 25, Mg²⁺ 0.6, H₂PO₄⁻ 1.3, SO₄²⁻ 0.6, Ca²⁺ 2.5, glucose 11.2, 2,3-butanedione monoxime (BDM) 10, oxygenated with 95% O₂ and 5% CO₂.

All procedures involving humans were performed in compliance with the local ethical committee.

1.2 Large animal model

Four sheep were sedated with Telazol (6 mg/kg) and endotracheally intubated. Anesthesia was maintained with 1-2% isoflurane. The hearts were explanted via a left thoracotomy which was performed through the 4th intercostal space.

Animals received humane care in compliance with the Guide for Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee at Harvard Medical School.

1.3 Trabeculae preparation and experiments

Thin ventricular trabeculae (cross sectional dimensions: 0.38 ± 0.06 for KN-93 vs. 0.37 ± 0.06 mm² for KN-92, $P=0.9$) were prepared using stereoscopic microscopy in a dissection chamber containing BDM-solution.¹ For isometric force recordings, trabeculae were mounted in an organ chamber and connected to the force transducer.² Trabeculae were superfused with Krebs-Henseleit solution (mmol/L: NaCl₂ 116, KCL 5, NaH₂PO₄ 2, MgCl₂ 1.2, Na₂SO₄ 1.2, NaHCO₃ 20, CaCl₂ initially 0.25, glucose 10) that was oxygenated with 95% O₂ and 5% CO₂ (37°C) and stimulated at a basal stimulation frequency of 1 Hz (voltage 25% above threshold). Ca²⁺ (0.25 mmol/L) was added every 2 min until the final concentration of 2 mmol/L was reached. After an equilibration period of 30-60 min, the trabeculae were

gradually stretched until the maximum steady-state twitch force was achieved. When steady-state twitch force was achieved, stimulation was stopped and CaMKII-inhibition was performed by superfusing trabeculae for 20 min with KN-93 (2 $\mu\text{mol/L}$, Calbiochem). Control experiments were performed using the inactive analogue KN-92 (2 $\mu\text{mol/L}$, Calbiochem). Force-frequency relationships (FFR) were obtained at steady-state twitch force conditions using stimulation frequencies from 0.5 to 3Hz. Additional experiments were performed using the synthetic peptide CaMKII-inhibitor myristoylated AIP (autocamtide-2-related inhibitory peptide, 2.5 $\mu\text{mol/L}$). To measure SR Ca^{2+} -load, post-rest behaviour was assessed by using increasing rest intervals to 10, 30, and 120 s between beats at a basal stimulation frequency of 1 Hz.² 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.

1.4 Myocyte isolation

LV myocardium was rinsed, cut into small pieces and incubated at 36°C in a spinner flask filled with Joklik MEM solution containing 1.0 mg/ml collagenase (Worthington type 2, 250 U/mg), and 13% trypsin (25% trypsin).^{2,3} After 45 min, the supernatant was discarded and the remaining tissue was poured into the flask again. The remaining tissue was digested in a fresh JMEM solution containing only collagenase until myocytes were disaggregated using a Pasteur pipette. Solutions containing disaggregated cells were centrifuged (60 rpm, 3 min). In the following steps the same solution without trypsin was used and aliquots were incubated for 10-15 min. This procedure was repeated 4-5 times. Cells were stored for 1 h in KB medium containing (mmol/L): taurine 10, glutamic acid 70, KCl 25, KH_2PO_4 10, dextrose 22, EGTA 0.5 (pH 7.4, KOH, room temperature). Only elongated cells with cross striations and without granulation were selected for experiments.

1.5 Intracellular Ca^{2+} -imaging

Measurement of Ca^{2+} -sparks

Isolated myocytes were placed on laminin-coated recording chambers and incubated for 20 min at room temperature with a 10 $\mu\text{mol/L}$ Fluo-3AM (Molecular Probes) loading buffer which also contained either CaMKII inhibitor KN-93 (1 $\mu\text{mol/L}$) or its inactive analogue KN-92 (1 $\mu\text{mol/L}$).³ Similar experiments were performed using AIP (1 $\mu\text{mol/L}$). Experimental solution contained (mmol/L): 136 NaCl, 4 KCl, 0.33 NaH_2PO_4 , 4 NaHCO_3 , 2 CaCl_2 , 1.6 MgCl_2 , 10 HEPES, 10 glucose (pH 7.4, NaOH,

room temperature) as well as either KN-93 vs. KN-92 or AIP vs. control. Cells were continuously superfused during experiments. To wash out the loading buffer and remove any extracellular dye, as well as to allow enough time for complete de-esterification of Fluo-3AM, cells were superfused with experimental solution for 5 min before experiments were started. Ca^{2+} spark measurements were performed with a laser scanning confocal microscope (LSM 5 Pascal, Zeiss) using a 40x oil-immersion objective. Fluo-3 was excited by an argon ion laser (488 nm) and emitted fluorescence was collected through a 505 nm long-pass emission filter. Fluorescence images were recorded in the line-scan mode with 512 pixels per line (width of each scanline 38.4 μm) and a pixel time of 0.64 μs . Ca^{2+} -spark frequency (CaSpF) was measured at resting conditions and normalized to cell volume and scan rate ($\text{pL}^{-1}\cdot\text{s}^{-1}$). Spark amplitude was estimated by dividing peak fluorescence by baseline fluorescence (F/F_0) after subtraction of the background fluorescence. Spark frequency (CaSpF) was measured after loading the SR with Ca^{2+} by repetitive field stimulation (10 pulses at 1 Hz) and normalized to cell volume and scan rate ($\text{pL}^{-1}\cdot\text{s}^{-1}$). Spark size (CaSpS) was calculated as product of amplitude (F/F_0), duration and width. From this, we inferred the average leak per cell by multiplication of CaSpS with the mean spark frequency of the respective cell.

Determination of SR Ca^{2+} -content

The chambers containing dye loaded myocytes were mounted on the stage of an inverted microscope (Nikon Eclipse TE2000-U) and superfused. Fluo-3 was excited at 480 ± 15 nm and fluorescence was measured at 535 ± 20 nm. After a washout period in order to eliminate the external dye, myocytes were field-stimulated at 1 Hz. SR Ca^{2+} -content was assessed by caffeine-induced Ca^{2+} -transients. During steady-state conditions at 1 Hz, stimulation was stopped for 1 s and caffeine solution (10 mmol/L) was applied directly to one myocyte leading to immediate and complete SR Ca^{2+} -release. Ca^{2+} -transient amplitudes (F/F_0) were calculated by dividing fluorescence F by the baseline fluorescence F_0 after subtraction of the background fluorescence (IonWizard, IonOptix Corp). The amplitudes of the caffeine-induced Ca^{2+} -transients were used as a measure for SR Ca^{2+} -content.⁴

1.6 Specification of antibodies

Ventricular myocardium was homogenized in buffer containing (mmol/L): 20 Tris-HCl, pH 7.4, 200 NaCl, 20 NaF, 1 Na_3VO_4 , 1 DTT, 1% Triton X-100 and complete

protease inhibitor cocktail (Roche Diagnostics). Protein concentration was determined by BCA assay (Pierce Biotechnology). Denatured tissue (30 min, 37°C for PLB and 5 min, 95°C for CaMKII, RyR2 and LTCC β_{2a} subunit in 2% β -mercaptoethanol) were subjected to Western blotting (4-15% gradient and 10% SDS-polyacrylamide gels) using anti-CaMKII δ (1:15000, a gift from D.M. Bers), anti-CaMKII γ (1:2000, Santa Cruz), anti-phospho-CaMKII Thr-286 (1:1500, Affinity Bio Reagents), anti-PLB (1:5000, Millipore), anti-phospho-PLB Thr-17 (1:5000, Badrilla), anti-LTCC CP β_{2a} subunit (1:200, Santa Cruz) anti-RyR2 and anti-phospho-RyR2 2809 (1:1000), anti-phospho-RyR2 2815 (1:1000, gifts from A.R. Marks), and anti-GAPDH (1:20000, Biotrend Chemikalien GmbH) antibodies. Chemiluminescent detection was done with ImmobilonTM Western (Millipore). Phosphorylation values were normalized to protein expression.

References

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