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CaMKII-dependent myofilament Ca^{2+} desensitization contributes to the frequency-dependent acceleration of relaxation

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Abstract

Background—Previous studies suggest that CaMKII activity is required for frequency-dependent acceleration of relaxation (FDAR) in ventricular myocytes. We propose that the underlying mechanism involves CaMKII-dependent regulation of myofilament Ca^{2+} sensitivity.

Methods and Results—Cardiac function was measured in mice using murine echo machine. $[\text{Ca}^{2+}]_i$ and sarcomere length were measured by IonOptix Ca^{2+} image system. Increasing pacing rate from 0.5 to 4Hz in left ventricular myocytes induced frequency-dependent myofilament Ca^{2+} desensitization (FDMCD) and FDAR. Acute inhibition of PKA or PKC had no effect, whereas CaMKII inhibition abolished both FDMCD and FDAR. Co-immunoprecipitation of CaMKII and troponin I (TnI) has been detected and CaMKII inhibition significantly reduced serine residue phosphorylation of TnI. Finally, chronic inhibition of CaMKII in vivo reduced TnI phosphorylation and abolished both FDAR and FDMCD, leading to impaired diastolic function.

Conclusions—Our results suggest that CaMKII-dependent TnI phosphorylation is involved in FDMCD and the consequent FDAR and that CaMKII inhibition removes this mechanism and thus induces diastolic dysfunction.

Graphical abstract

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Conflict of interest statement

All authors confirm that there is no conflict of interest to disclose.

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Keywords

CaMKII; Myocytes; Contraction; Relaxation; Myofilament Ca^{2+} sensitivity

INTRODUCTION

Bowditch *et al.* first reported that increasing pacing frequency in ventricular myocytes produced a positive force-frequency relationship (FFR) and frequency-dependent acceleration of relaxation (FDAR). It is known that positive FFR is due, at least in part, to an increase in systolic Ca^{2+} amplitude [1], which is accompanied by a parallel increase in sarcoplasmic reticulum (SR) Ca^{2+} content [2]. However, FDAR presents when SR Ca^{2+} content increases, decreases or fails to change with frequency [3–5]. Some studies showed that blocking SR Ca^{2+} uptake abolishes FDAR, indicating that SR Ca^{2+} transport is important for FDAR [2,6].

In the heart, Ca^{2+} /calmodulin (Ca/CaM)-dependent protein kinase (CaMKII) is one of the major regulators for cardiac excitation-contraction coupling (ECC) by phosphorylating a number of Ca^{2+} -regulatory proteins, including phospholamban (PLB), ryanodine receptor (RyR), and the L-type Ca^{2+} channel (LTCC) [7]. Several studies showed that CaMKII activity is required for the FDAR [8,9], although different results have been reported by others [10]. The first proposed hypothesis was that repeated Ca^{2+} release activates CaMKII

and causes phosphorylation of PLB, enhancing SR Ca^{2+} uptake which induces FDAR. This is consistent with the finding that FDAR was diminished after inhibition of SR Ca^{2+} uptake or CaMKII. Indeed, increasing frequency enhances the rate of Ca^{2+} influx, leading to CaMKII activation [8]. Frequency-induced CaMKII-dependent PLB phosphorylation at Thr-17 is correlated with FDAR [11], and mutation of CaMKII-dependent phosphorylation sites in PLB blunts FDAR [12]. Interestingly, CaMKII inhibition by KN93 or AC3-I diminishes the frequency dependent relaxation in PLB ($-/-$) mice [9,13]. The time courses of both PLB and RyR phosphorylation by CaMKII and FDAR kinetic were clearly dissociated and the net increase in PLB and RyR phosphorylation was low [14]. CaMKII can also directly phosphorylate SERCA2a, which increases $V_{\max}$ for Ca^{2+} uptake. Indeed, in transgenic mice with SR-targeted CaMKII inhibition, the enhancement of SR Ca^{2+} uptake at higher frequency was found to be mediated by CaMKII-dependent increase of SERCA $V_{\max}$ [5]. However, it has been reported that SERCA2a was not directly phosphorylated by CaMKII and $V_{\max}$ of the SERCA2a was CaMKII independent [15]. Unfortunately, there is a great discrepancy in the literature and the molecular basis for FDAR regulation by CaMKII remains unclear.

Although SR Ca^{2+} handling plays a central role in FDAR, myofilament properties are also involved in FDAR. Crossbridge kinetics rather than the rate of Ca^{2+} decline may represent the rate-limiting step of relaxation [16]. For instance, the lack of frequency-dependent myofilament Ca^{2+} desensitization (FDMCD) impairs diastolic function [17]. Moreover FDMCD is involved in FDAR by increasing both cardiac Troponin I (TnI) and myosin light chain-2 phosphorylation [18]. It is also known that in the setting of β -adrenergic receptor (β -AR) stimulation, TnI phosphorylation plays an important role in relaxation [11,18]. TnI is the inhibitory element of troponin complex. TnI can be phosphorylated by protein kinase A (PKA), protein kinase C (PKC), cyclic GMP-dependent protein kinase, and p21-activated kinase at different sites, and the phosphorylation at specific serine or threonine can change the myofilament characteristics [19]. Up-to-date, only PKA and PKC are able to decrease the myofilament Ca^{2+} sensitivity by phosphorylating TnI at Ser22/23 (corresponding to 23/24 in mice) [20–24]. CaMKII can also change the myofilament properties by phosphorylation of myosin binding protein-C, titin and TnI [25,26]. However, the underlying mechanisms are unknown.

In the present study, we hypothesized that CaMKII-dependent TnI phosphorylation contributes to the FDAR and that CaMKII inhibition impairs the diastolic function by elimination of FDMCD.

METHODS

Use of Vertebrate Animals

All experiments using live C57BL/6 male mice were performed in accordance with the animal protocol specifically for this study approved by Emory Institutional Animal Care and Use Committee (EMORY IACUC). 32 mice at 6 weeks of age were divided into 3 groups and injected intraperitoneally (IP) with saline solution (NaCl 9%), KN92 (10 μ mol/Kg) or KN93 (10 μ mol/Kg) for consecutive 7 days. Lungs and hearts were removed to obtain weights. Heart weight included both atria and ventricles.

Echocardiography

Cardiac function was measured in mice using a high resolution murine echo machine (Visualsonics RMV707B with a 30 MHz transducer). A short-axis two-dimensional image-guided M-mode view of the LV was acquired at a sound speed of 1540 m/s and stored digitally. The following parameters were measured digitally from the M-mode tracings, including LV dimensions at diastole and systole (LVDd, LVDs) and heart rate (HR). Based on these measurements, left ventricular volume systole was calculated as $[(7.0/(2.4 + LVDs))^3 - (7.0/(2.4 + LVDd))^3] \times LVDd/3$; left ventricular volume diastole was calculated as $[(7.0/(2.4 + LVDd))^3 - (7.0/(2.4 + LVDd))^3] \times LVDd/3$; ejection fraction (EF) was calculated as $[(LV \text{ Vold} - LV \text{ VolDs})/LV \text{ Vold}] \times 100\%$. All measurements were made as the average from 5 adjacent cardiac cycles. *In vivo* diastolic function was investigated in anesthetized mice with inhalation of a mixture of 1.5% isoflurane and 100% oxygen at a flow rate of 1.0 L/min. Peak velocity of early (E) and late (A) diastolic mitral inflow and peak velocity of early (Em) and late (Am) diastolic mitral valve are measured from apical 4-chamber view using B-mode images and pulsed-wave Doppler images and tissue-Doppler image, respectively.

Isolation of adult mouse left ventricular myocytes

Mouse LV myocytes were isolated enzymatically by a protocol described previously [27,28]. In brief, after retrograde perfusion with Krebs-Ringer solution at 2ml/min (5 minutes), the heart was then perfused by a fresh solution containing 0.8mg/ml collagenase (Worthington type II) for about 15 minutes. The apex was then removed and the LV wall was cut and put into a culture dish filled with “KB” solution. LV wall were minced into small pieces in “KB” solution, triturated, and studied within 4–6 h. All steps were performed at 37°C in solutions gassed with 95% O₂ + 5% CO₂. Only calcium-tolerant, quiescent and rod-shaped cells showing clear cross striations were used for internal Ca²⁺ ([Ca²⁺]_i) and sarcomere shortening recording.

Intracellular [Ca²⁺]_i and sarcomere shortening measurements

[Ca²⁺]_i and sarcomere shortening was recorded using methods described previously [29]. Myocytes were loaded with 2μmol/L fura-2/AM for 40 minutes and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (IonOptix). After loading, cells were washed and resuspended in Tyrodes solution and then placed in the cell chamber, stimulated at 0.5Hz until steady state was achieved. Then stimulation frequency was set to 2Hz and 4Hz. After recordings in basal condition, cells were then perfused for 5–10 minutes with either CaMKII inhibitor KN93 (1μmol/L; Enzo Life Science), PKA inhibitor H89 (1 μmol/L; LC Laboratories), PKC inhibitor chelerythrine (CHE; 1μmol/L; LC Laboratories), or Isoproterenol (ISO; 1μmol/L; Sigma). The background fluorescence for each myocyte was determined by moving the myocyte out of the view and recording the fluorescence from the solution alone. Cell shortening was measured in myocytes that were loaded with fura-2/AM. This concentration of fura-2/AM did not affect contractility (compared with unloaded cells, data not shown). The myofilament Ca²⁺ sensitivity was characterized using sarcomere length and internal [Ca²⁺]_i kinetic difference. Sarcomere length and [Ca²⁺]_i were plotted point to point over the whole twitch cycle to perform sarcomere length-[Ca²⁺]_i hysteresis analysis. Ca²⁺ transient is the

difference between the peak Ca^{2+} during twitch and diastolic Ca^{2+} . The fractional shortening is the diastolic sarcomere length (i.e. baseline) percentage of peak sarcomere length, i.e. $(\text{baseline}-\text{peak})/\text{baseline} \times 100$.

Co-immunoprecipitation experiments

Myocytes were treated with either CaMKII inhibitor KN93 ($1\mu\text{mol/L}$; Enzo Life Science), PKA inhibitor H89 ($1\mu\text{mol/L}$; LC Laboratories), or PKC inhibitor Chelerythrine (CHE; $1\mu\text{mol/L}$; LC Laboratories) for 30 minutes in Dulbecco's modified Eagle's medium (DMEM)/M199 (3:1) containing 10% FBS with 5% CO_2 at 37°C . Myocytes were then centrifuged at 8000 rpm for 3 minutes. Supernatant was removed and pellet was lysed with $200\mu\text{L}$ lysis buffer (mmol/L) EDTA 2, DTT 1, (v/v) Triton X-100 1%, NaCl 150, NaF 20, Na_2VO_3 1, Tris-HCl 20, PMSF 1 and phosphatase Inhibitor Cocktail) on ice. To compare TnI phosphorylation at serine residue, we used the method described previously (Huke and Bers, 2007). Briefly, myocytes were perfused for 5–10 minutes with KN93 and stimulated at 0.5Hz until steady state was reached, followed by 4Hz pacing for 5 minutes. Myocytes were then removed to lysis buffer and immediately frozen in liquid N_2 . The myocytes lysates were transferred to a fresh microcentrifuge tubes containing $50\mu\text{L}$ Protein G-Sepharose resin to preclear the myocytes lysates and then incubated for 2h at 4°C with constant rotation. Resin was then removed by centrifugation at 9000 rpm for 5 minutes. The procedure was repeated using conjugated Protein-G with anti-TnI or anti-CaMKII antibodies, and the mixture was incubated at 4°C for 12–16 h. After incubation, resin was collected by centrifugation and washed thoroughly with wash buffer 1 (in mmol/L : EDTA 0.1, (v/v) Triton-X 100 0.1%, NaCl 150, Tris-HCl 20, pH adjusted to 7.5 with NaOH) and wash buffer 2 (mmol/L) EDTA 0.1, Tris-HCl 20, pH adjusted to 7.5 with NaOH). Proteins were eluted with $2\times$ SDS-PAGE sample loading buffer containing 2% (v/v) 2-mercaptoethanol and heated up to and 95°C for 10 min. Total lysates and immunoprecipitates were subjected to SDS-PAGE and immunoblotting.

Western blot analysis

Primary antibodies used in this study include mouse monoclonal anti-Phospho-CaMKII Thr 287 antibody (corresponding to Thr286 in human; MA1-047; Thermo), rabbit polyclonal anti-CaMKII M-176 antibody (sc-9035; Santa Cruz Biotechnology), rabbit polyclonal anti-Phospho-TnI Ser23/24 (corresponding to Ser 22/23 in human; 4004, cell signaling technology), mouse monoclonal anti-TnI (sc-52266; Santa Cruz Biotechnology), mouse monoclonal anti-Phospho-Serine, clone 4A4 (05-1000 $\times$; Milipore) and a mouse monoclonal anti-GAPDH (sc-66163; Santa Cruz Biotechnology). Secondary antibodies used for Western blot were goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP (all from Santa Cruz Biotechnology).

Statistical analysis

Statistical analysis was performed using SigmaStat for Windows (Jandel Scientific). Paired and unpaired t -tests and 1-way ANOVA analysis were used for comparisons and $p < 0.05$ was regarded as significant. Mann-Whitney rank sum tests were performed if tests for normality or equal variance failed.

RESULTS

CaMKII regulates FDMCD

Myofilament Ca^{2+} desensitization is required for FDAR [18]. CaMKII, PKA and PKC are the major regulators for EC coupling in myocytes. To understand the role these kinases in FDMCD, we first investigated their involvement in FFR and FDAR. Fig 1A–D shows typical example of Ca^{2+} transient and fractional shortening (FS) at 0.5 and 4Hz in control and after acute CaMKII inhibition (KN93) in isolated ventricular myocytes, respectively. Increasing frequency from 0.5 to 4Hz significantly enhanced Ca^{2+} transient and fractional shortening (FS) while KN93 significantly decreased Ca^{2+} transient and FS. However, both PKA inhibitor H89 and PKC inhibitor Chelerythrine (CHE) did not affect Ca^{2+} transient and FS amplitude (data not shown). Increasing frequency from 0.5 to 4Hz also slowed the time constant (Tau) of the decline phase for both Ca^{2+} transient (from 162 ± 13 to 60 ± 5 ms, $n=35$) and sarcomere relaxation (from 36 ± 2 to 18 ± 1 ms, $n=35$), respectively ($p < 0.05$, for the changes of both values, Fig 1). H89 and CHE had no effect on myocytes relaxation (Fig 1E–F; dark gray dash line and light gray line). However, KN93 significantly slowed Tau of Ca^{2+} decline and sarcomere relaxation at 2 and 4Hz, respectively ($p < 0.05$; Fig 1). These data suggest that CaMKII plays a major role in the regulation of FDAR in ventricular myocytes.

Furthermore, we observed that increasing frequency from 0.5 to 4Hz shifts the hysteresis loop to the right (Fig 2A), indicating FDMCD. PKA (H89) and PKC (CHE) inhibition had no effect on the Ca^{2+} desensitization (Fig 2C–D). In contrast, the loop shifting was blunted by CaMKII inhibition (Fig 2B), suggesting diminishment of FDMCD. To quantify myofilament Ca^{2+} sensitivity, we measured internal Ca^{2+} at 50% of twitch activation (systole; table 1) and internal Ca^{2+} at 50% of twitch relaxation (diastole, table 1). FDMCD was calculated as 4/0.5Hz ratio at systole phase and diastolic phase. Increasing pacing frequency from 0.5 to 4Hz leads to an increase in $[\text{Ca}^{2+}]_i$ at 50% of twitch activation and relaxation to $14.52 \pm 2.61\%$ and $11.92 \pm 1.62\%$, respectively. CaMKII inhibition decreased $[\text{Ca}^{2+}]_i$ at 50% of twitch activation and relaxation to $3.41 \pm 1.72\%$ and $1.63 \pm 1.61\%$, respectively ($p < 0.05$, Fig 2E). These results suggest the involvement of CaMKII in FDMCD.

CaMKII-dependent phosphorylation of TnI

TnI phosphorylation plays an important role in the regulation of myofilament Ca^{2+} sensitivity [19]. Our results demonstrated that CaMKII regulates myofilament sensitivity to Ca^{2+} . Thus, we hypothesize that CaMKII may be involved in TnI phosphorylation. To test this, we performed TnI immunoprecipitation in lysates of ventricular myocytes (Fig 3A). CaMKII proteins were then detected from the resulting immunoprecipitates using anti-CaMKII antibody. A clear band at molecular weight ≈ 50 kDa representing CaMKII proteins was exclusively detected in TnI immunoprecipitates, indicating co-immunoprecipitation (co-IP) of TnI and CaMKII proteins (Fig 3A). We confirmed this result by performing the reverse co-IP (Fig 3B). These data demonstrated that CaMKII and TnI can form a stable molecular complex in ventricular myocytes.

We then tested TnI phosphorylation at Ser 23/24, which is known to reduced myofilament Ca^{2+} sensitivity [20,22–24]. We found that both PKA and PKC inhibition significantly decreased the TnI phosphorylation at Ser23/24 (Fig 3F). However, KN93 treatment has no effect on Ser 23/24 phosphorylation (Fig 3C). Note that all of the inhibitors did not affect the endogenous level of TnI expression (Fig 3F). These results showed that TnI-Ser23/24 is phosphorylated by PKA and PKC, but not by CaMKII.

Furthermore, we performed TnI immunoprecipitation in lysates of ventricular myocytes and used anti-phospho-serine antibody to detect the phosphorylation level of serine residues in TnI. A clear band at molecular weight $\approx 25\text{kDa}$ was observed, representing TnI phosphorylation at serine residues (Fig 3D). Consistent with our previous result, Western blot showed that PKA and PKC inhibition significantly decreased TnI phosphorylation at serine residues (a reduction of $43.9 \pm 15.5\%$ and $52.8 \pm 17.3\%$, respectively, $p < 0.05$; Fig 3F). We also observed similar reduction of TnI phosphorylation at serine residues by CaMKII inhibition ($32.4 \pm 6.7\%$, $p < 0.05$; Fig 3F), indicating that CaMKII can also phosphorylate TnI at serine residues. Finally, we show that increasing pacing from 0.5 to 4Hz increased TnI phosphorylation at serine residues, consisting with the notion that TnI phosphorylation is involved in FDAR (Fig 3E). CaMKII inhibition blunted the frequency-dependent increasing of TnI phosphorylation at serine residues. These data suggest that CaMKII-dependent phosphorylation of TnI contributes to FDAR.

Role of CaMKII-dependent FDMCD in the setting of β -AR stimulation

It is known that myofilament Ca^{2+} sensitivity contributes to the β -adrenergic-dependent acceleration of relaxation [30]. We used β -adrenergic agonist isoproterenol (ISO) to study the role of CaMKII-dependent myofilament Ca^{2+} desensitization in myocyte relaxation during β -AR stimulation. We found that ISO significantly accelerated the time constant (Tau) of Ca^{2+} decline and sarcomere relaxation (Fig 4A & B, black line), indicating accelerated FDAR. KN93 blunted FDAR and significantly slowed the time constants of both Ca^{2+} decline and sarcomere relaxation at each frequency (Fig 4A, B, dark gray line). PKA inhibition although slowed the Tau of Ca^{2+} decline and sarcomere relaxation (Fig 4A, B; gray line), but the FDAR still presents (Fig 4A & B). These data suggest that the CaMKII-dependent FDAR is not mainly mediated by SR Ca^{2+} handling. Instead, CaMKII-dependent TnI phosphorylation and the consequent change of myofilament sensitivity to Ca^{2+} play an important role. Indeed, we observed that increasing frequency from 0.5 to 4Hz shifted the hysteresis loop to the right in the presence of ISO or H89+ISO where the FDAR is observed but not in KN93+ISO where the FDAR was inhibited (Fig 4C–E). KN93 significantly decreased the FDMCD for both myofilament contraction and relaxation (Fig 4F). To evaluate ISO-dependent myofilament Ca^{2+} desensitization (IDMCD), we compared the $[\text{Ca}^{2+}]_i$ at 50% of twitch activation (systole) and 50% of twitch relaxation (diastole) at 0.5 and 4Hz before and after β -AR stimulation, respectively. We found that only PKA inhibition significantly reduced the IDMCD for both activation and relaxation (Fig 4G, H). These data suggest that PKA is important for IDMCD while CaMKII plays a major role in FDMCD.

CaMKII-dependent FDMCD is required to maintain diastolic function

We have investigated the effect of chronic CaMKII inhibition on diastolic function in ventricular myocytes by *in vivo* daily injection with KN93 for 1 week. A $50.0 \pm 1.8\%$ reduction in CaMKII autophosphorylation was detected in the KN93 injected mice compared to the KN93 inactive analogue KN92 injected mice (Fig 5F, H). We found that FDAR was abolished in the LV myocytes isolated from the mice treated with KN93 (Fig 5A, B). Compared to the KN92 injected mice, chronic CaMKII inhibition significantly decreased FDMCD for both activation and relaxation (Fig 5C, D). The inhibition of FDMCD in KN93 treated mice was associated with a significant reduction of TnI phosphorylation at serine residues (Fig 5G, H). Note that the TnI phosphorylation at Ser23/24 (the known PKA and PKC sites) is not affected by KN93 treatment (Fig 5E) and KN92 did not affect CaMKII activity, CaMKII dependent TnI phosphorylation, FDAR and FDMCD compared to saline injected mice (Fig 5). These results demonstrated that both acute and chronic CaMKII inhibitions of TnI phosphorylation blunt FDAR and FDMCD in ventricular myocyte, which is associated with CaMKII-dependent phosphorylation of TnI at sites distinct from the Ser23/24.

To assess the effect of CaMKII-dependent myofilament Ca^{2+} sensitivity on diastolic filling, we performed echocardiography in mice injected with KN93. Chronic CaMKII inhibition did not change cardiac morphology and systolic function (table 2). To evaluate the diastolic function, we measured the peak velocity of early diastolic mitral inflow (E) and the peak velocity of early diastolic mitral valve (Em) (Fig 6A–D) and used E/Em ratio as an index of diastolic function. After 1 week KN93 injection, we observed a dramatic increase in E/Em ratio (from 24.6 ± 1.3 to 35.1 ± 1.7), indicating deterioration of diastolic function (Fig 6E). Consistent with the impairment of ventricular relaxation, the left atrium (LA) was enlarged (the size of LA was increased from $2.3 \pm 0.1 \text{ mm}^2$ in control to $2.81 \pm 0.1 \text{ mm}^2$ in KN93 mice $p < 0.01$; Fig 6F). Note that KN92 injection had no effect on cardiac morphology and function compared to saline injected mice (table 2). These results implicate CaMKII in maintaining diastolic function.

DISCUSSION

The present study demonstrated that (i) CaMKII-dependent myofilament Ca^{2+} sensitivity plays a major role in regulating FDAR; (ii) PKA is important in β -adrenergic-dependent myofilament sensitivity to Ca^{2+} ; (iii) CaMKII binds and phosphorylates cardiac TnI at sites distinct from the PKA and PKC phosphorylation sites; and CaMKII is required to maintain the diastolic function. Our results also showed that CaMKII and PKA act in concert to regulate myofilament Ca^{2+} sensitivity in response to β -adrenergic stimulation.

Several studies showed that CaMKII increases SERCA V_{max} for Ca^{2+} uptake [5] and that the beat-to-beat CaMKII activation phosphorylates PLB which increases SERCA function and SR Ca^{2+} uptake, allowing a faster relaxation at fast heart rate. However, FDAR is still observed in the PLB (–/–) mice and sensitive to CaMKII inhibitors [9,13]. In addition, the time course of PLB phosphorylation by CaMKII and the FDAR kinetics were found to be dissociated [14]. Although SERCA V_{max} is reduced at fast pacing in mice with SR-targeted CaMKII inhibition, FDAR still presents [5]. Taken together, it is clear that the change in

SR- Ca^{2+} uptake is not the only mechanism underlying FDAR regulation. Previously, myofilament sensitivity to Ca^{2+} has been suggested to be sensitized [1], unchanged [10], or desensitized at higher frequencies [26]. Using analytic method of dynamic force- $[\text{Ca}^{2+}]_i$ phase-plane loops approach, Tong *et al.* [26] and Varian *et al.* [18] showed that FDAR involves myofilament Ca^{2+} sensitivity. Although the conclusion of these studies is similar to us, force and calcium are only in equilibrium during certain period of diastole but not the entire phase of force decline, which could explain the discrepancy result addressing FDAR regulation. Indeed, crossbridge kinetics rather than the rate of Ca^{2+} decline may represent the rate-limiting step of relaxation, especially at higher rate where the diastolic Ca^{2+} is high [16]. In line with these studies, CaMKII inhibition abolished FDCMD and induced myofilament Ca^{2+} sensitization in myocyte (Fig 2 B). For the first time, our data showed that the CaMKII-dependent myofilament Ca^{2+} sensitivity plays a major role in FDAR regulation, especially at higher frequency.

It has been reported that CaMKII regulates myofilament properties by phosphorylation of myosin binding protein-C, titin and TnI [25,26]. In mouse, phosphorylation of myosin regulatory light chain is not an essential determinant of FDAR [31]. CaMKII-dependent phosphorylation of titin has been found to reduce passive force [25]. In the line of this study, we found that CaMKII inhibition blunted the positive FFR (Fig 1B and D). In addition to the finding that TnI phosphorylation is involved in FDMCD [18], studies from Tong *et al.* [26] and Cazorla *et al.* [32] observed CaMKII-dependent TnI phosphorylation using back phosphorylation assays and phosphoprotein staining, respectively. Using co-immunoprecipitation and Western blot analysis, we found that CaMKII and TnI can form a molecular complex (Fig 3) and that CaMKII inhibition decreased the total phosphorylation of serine residues in TnI at 0.5 and 4Hz (Fig 3). On the other hand, CaMKII inhibition have no effect on TnI phosphorylation at Ser23/24 (Fig 3), suggesting that CaMKII phosphorylates TnI at distinct serine residues other than the PKA and PKC sites Ser23/24. The PKA and PKC dependent phosphorylation of Ser23/24 (corresponding to Ser 22/23 in human) is the only known pathway to reduce the myofilament Ca^{2+} sensitivity by changing TnI phosphorylation. However, FDAR is still detected in mice with mutated Ser 23/24 [33], and our data clearly demonstrated that neither PKA nor PKC is involved in FDAR (Fig 1). Instead, CaMKII-dependent TnI phosphorylation directly contributes to the FDAR. Further studies are needed to identify the CaMKII-dependent TnI phosphorylation sites. While CaMKII-dependent TnI phosphorylation is critical in regulating FDAR, we cannot exclude the involvement of CaMKII-dependent phosphorylation of myosin binding protein-C and titin herein.

Several studies report that upon β -AR stimulation, TnI phosphorylation plays an important role in myocyte relaxation [30]. Stimulation of β -AR by catecholamine induces positive inotropic and chronotropic effects. β -AR stimulation activates CaMKII in parallel with or as downstream of PKA signaling cascade [34]. Our results showed that only CaMKII inhibition blunted both FDAR and FDMCD. In contrast, PKA inhibition abolished IDCMD without inhibition of FDAR and FDMCD, while CaMKII had no effect on IDCMD (Fig 4). We conclude that PKA plays an important role in IDCMD while CaMKII contributes to FDMCD. Although ISO accelerated FDAR, the FDAR itself is not PKA-dependent. We

hypothesized that β -AR stimulation activates both PKA and CaMKII in which PKA contributes to IDCMD and thereby accelerates relaxation while CaMKII contributes to FDAR *via* FDMCD.

CaMKII inhibition protects against structural heart disease and improves contractility in human failing myocardium [35]. However, we recently reported that although CaMKII inhibition improves cellular contraction, the *in vivo* cardiac reserve to β -adrenergic regulation is reduced and the diastolic function is deteriorated [3]. It has been also shown that SR-targeted inhibition of CaMKII accelerates cardiac remodeling [36]. Recently, Hamdani et al. report that CaMKII-dependent phosphorylation of titin contributes to diastolic function in HF [25]. In line with these findings, our data here show that inhibition of CaMKII-dependent myofilament Ca^{2+} sensitivity impairs myocyte relaxation, leading to *in vivo* diastolic dysfunction (Fig 6). Indeed, Varian *et al.* reported that the lack of FDMCD impaired diastolic function [17]. Furthermore, Belin *et al.* observed a depressed myofilament function secondary to dysfunction of the regulatory troponin complex and the increased phosphorylation of TnI in 2 distinct rat models of ventricular failure [37]. It has been reported that more than 50% of HF patients have preserved left ventricular EF, so called diastolic HF [38,39]. Our data support the notion that failure of FDMCD leads to inadequate filling of the left ventricle and thus reducing of the overall cardiac output, which challenges the recent idea that CaMKII inhibition is a beneficial treatment strategy in patients with structural heart disease by pointing out a caution of diastolic dysfunction.

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Highlights

Frequency-dependent acceleration of relaxation (FDAR) is an intrinsic mechanism in ventricular myocytes allowing a faster ventricular relaxation and preservation of diastolic filling at fast heart rates. Previous studies suggest that CaMKII activity is required for FDAR but the underlying molecular targets remain elusive. Several studies showed that CaMKII increases SERCA $V_{\max}$ for Ca^{2+} uptake and that the beat-to-beat CaMKII activation phosphorylates PLB which increases SERCA function and SR Ca^{2+} uptake, allowing a faster relaxation at fast heart rate. However, FDAR is still observed in the PLB $(-/-)$ mice and sensitive to CaMKII inhibitors. In addition, the time course of PLB phosphorylation by CaMKII and the FDAR kinetics were found to be dissociating. Although SERCA $V_{\max}$ is reduced at fast pacing in mice with SR-targeted CaMKII inhibition, FDAR still presents. Taken together, it is clear that SR- Ca^{2+} uptake is not the only mechanism underlying FDAR regulation.

Excessive activation of Ca^{2+} /Calmodulin-dependent kinase II (CaMKII) is linked to the development of cardiomyopathy and dysfunction of cardiac excitation-contraction coupling. Thus, CaMKII has been proposed to be a viable therapeutic target. However, we recently reported that CaMKII inhibition significantly deteriorates diastolic function.

In the current study, we found that CaMKII binds and phosphorylates cardiac troponin I at sites distinct from the PKA and PKC sites Ser 23/24 and therefore regulates myofilament sensitivity to Ca^{2+} . This finding can explain why CaMKII inhibition, but not PKA or PKC inhibition can blunt FDAR and frequency-dependent myofilament Ca^{2+} desensitization (FDMCD). This mechanism also significantly contributes to the CaMKII inhibition-induced diastolic dysfunction.

The data have partially presented in AHA scientific session meeting. This paper has not been published previously or under consideration for publication elsewhere.

Figure 1. Effects of CaMKII, PKA and PKC inhibition in ventricular myocytes

A–B: Typical traces of Ca^{2+} transient recorded at 0.5 (black line) and 4 Hz (gray line) in control and in the presence of CaMKII inhibitor KN93, respectively. **C–D:** Representative fractional shortening at 0.5 (black line) and 4 Hz (gray line) in control and in the presence of KN93, respectively. **E–F:** Mean value of time constant τ for Ca^{2+} decline and sarcomere length relaxation recorded at 0.5, 2 and 4 Hz in normal Tyrode's solution (black, n=35) and in the presence of KN93 (gray, n=19), PKA inhibitor H89 (dark gray dash line, n=13) and

PKC inhibitor CHE (light gray, n=9), respectively. *: $p<0.05$ compared with control; #: $p<0.05$ compared with 0.5 Hz.

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Figure 2. Effect of CaMKII, PKA and PKC inhibition on myofilament Ca^{2+} sensitivity
A–D: Representative traces of sarcomere length vs. corresponding $[\text{Ca}^{2+}]_i$ at 0.5 (dash line), 2 (gray) and 4Hz (black) and in the presence of KN93, H89 and CHE, respectively. **E:** Summary of frequency-dependent myofilament Ca^{2+} desensitization (FDMCD) evaluated by the comparison between $[\text{Ca}^{2+}]_i$ at 50% of twitch activation (systole) and $[\text{Ca}^{2+}]_i$ at 50% of twitch relaxation (diastole) at 0.5 and 4Hz. *: $p < 0.05$ compared to Control.

Figure 3. CaMKII-dependent TnI phosphorylation

A, B: Co-purifications of TnI and CaMKII were detected in the lysates of ventricular myocytes. TnI was immunoprecipitated, and then blots were detected with anti-CaMKII and anti-TnI antibodies. Each immunoblotting represents 3 independent experiments. **C:** TnI phosphorylation at Ser 23/24 was evaluated using a specific anti-body that detects endogenous levels of TnI that is phosphorylated at Ser 23/24. **D–E:** Phosphorylation levels of serine residues in the immunoprecipitated TnI proteins using anti-phospho-serine antibody (P-Ser). **F:** Summary of changes of TnI phosphorylated at Ser 23/24 TnI (n=3), the

total TnI (n=3), and the TnI phosphorylated at serine residues (n=4) by KN93 at 0.5 and 4HZ, H89 and CHE (n=3). * $p<0.05$, ** $p<0.01$ compared to Control.

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Figure 4. Effects of β -AR stimulation on Ca^{2+} transient and sarcomere shortening in ventricular myocytes

A, B: Tau of Ca^{2+} decline and sarcomere relaxation recorded in ventricular myocytes at 0.5, 2 and 4 Hz in the condition of control (dash line, $n=9$), ISO (black, $n=9$), KN93 + ISO (dark gray, $n=11$) and H89 + ISO (light gray, $n=8$), respectively. **C–E:** Typical example of sarcomere length vs. corresponding $[\text{Ca}^{2+}]_i$ at 0.5 (dash line), 2 (gray) and 4 Hz (black) in the presence of ISO, KN93+ISO and H89+ISO. **F:** Summary of FDMCD after CaMKII (KN93) and PKA (H89) inhibition in response to β -AR stimulation (ISO). **G, H:** Summary of ISO-

dependent myofilament Ca^{2+} desensitization (IDMCD) evaluated by the comparison of $[\text{Ca}^{2+}]_i$ at 50% of twitch activation (G) and $[\text{Ca}^{2+}]_i$ at 50% of twitch relaxation (H) before and after β -adrenergic stimulation. $^{\#}p<0.05$ compared to 0.5Hz; $^*p<0.05$ compared to ISO.

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Figure 5. FDAR and FDMCD in KN93 treated mice

A, B: Tau of both Ca^{2+} decline and sarcomere relaxation recorded at 0.5, 2 and 4 Hz from saline treated (dash line; n=18), KN92 (black, n=10) and KN93 treated (KN93; gray; n=12) mice. **C:** Representative sarcomere length vs. corresponding $[\text{Ca}^{2+}]_i$ at 0.5 (gray), 2 (dark gray) and 4 Hz (black) in KN92 (dash line) and KN93 (solid line) mice. **D:** Summary of FDMCD in saline, KN92 and KN93 treated mice. **E:** TnI phosphorylation level at Ser 23/24. **F:** CaMKII autophosphorylation at Thr 287 detected using anti-phospho Thr 287 CaMKII antibody. **G:** Phosphorylation levels of serine residues in the immunoprecipitated TnI

proteins using antiphospho- serine antibody (P-Ser). **H:** Summary of the changes in the autophosphorylated CaMKII, total CaMKII, TnI phosphorylation at Ser 23/24, phospho-serine of TnI and the total TnI (n=3). * $p<0.05$ compared to KN92 mice. # $p<0.05$ compared to 0.5Hz.

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Figure 6. CaMKII inhibition impairs diastolic function

Echocardiography performed before (day 0) and after (day 7) KN92 or KN93 injection. **A, B:** Typical pulsed-wave Doppler image for measuring the peak velocity of early (E) and late (A) diastolic mitral inflow. **C, D:** Representative tissue-Doppler image for measuring the peak velocity of early (Em) and late (Am) diastolic mitral valve. **E, F:** Summarized data of E/Em ratio measurements and left atrium (LA) area in saline (n=11), KN92 (n=10) and KN93 (n=11) injected mice. ** $p < 0.01$, *** $p < 0.001$ compared to the KN92 mice.

Table 1

Internal Ca^{2+} (F340/380) levels at 50% of maximal activation and relaxation

Frequency	Treatment	n	0.5 Hz		2 Hz		4 Hz	
			Systole	Diastole	Systole	Diastole	Systole	Diastole
	Control	35	1.53 ± 0.06	1.26 ± 0.04	1.66 ± 0.07	1.33 ± 0.05	1.79 ± 0.06*	1.43 ± 0.06*
	KN93	19	1.48 ± 0.08	1.22 ± 0.06	1.50 ± 0.07 [†]	1.23 ± 0.05	1.53 ± 0.08 ^{††}	1.24 ± 0.05 [†]
	H89	13	1.54 ± 0.05	1.27 ± 0.03	1.66 ± 0.07	1.34 ± 0.06	1.78 ± 0.09*	1.40 ± 0.08*
	Che	9	1.64 ± 0.07	1.37 ± 0.05	1.72 ± 0.05	1.56 ± 0.07	1.85 ± 0.05*	1.60 ± 0.09 ^{**}
	Iso	9	1.61 ± 0.09	1.37 ± 0.03	1.75 ± 0.12	1.45 ± 0.03	1.99 ± 0.05 [†]	1.54 ± 0.05*
	KN93+Iso	11	1.61 ± 0.05	1.34 ± 0.03	1.60 ± 0.04 [§]	1.35 ± 0.03	1.63 ± 0.05 ^{§§}	1.36 ± 0.03 [§]
	H89+Iso	8	1.55 ± 0.03	1.28 ± 0.02	1.68 ± 0.05	1.34 ± 0.03	1.80 ± 0.1*	1.43 ± 0.05*
	Sal. injection	18	1.51 ± 0.05	1.26 ± 0.06	1.65 ± 0.07	1.33 ± 0.07	1.80 ± 0.07 ^{**}	1.42 ± 0.06*
	KN93 injection	12	1.50 ± 0.05	1.22 ± 0.03	1.48 ± 0.03 [‡]	1.22 ± 0.02	1.52 ± 0.03 ^{‡‡}	1.23 ± 0.03 [‡]

Che: Chelerythrine; ISO: isoproterenol; Sal: Saline solution; Thaps: thapsigargin;

* : $p < 0.05$;

** : $p < 0.01$ compared to 0.5 Hz;

[†] : $p < 0.05$,

^{††} : $p < 0.001$ compared to control;

[§] : $p < 0.05$,

^{§§} : $p < 0.001$ compared to ISO;

[‡] : $p < 0.01$,

^{‡‡} : $p < 0.001$: compared to Sal. injection.

Table 2

Morphometric and functional parameters for mice after 1 week of saline or KN93 injection

Treatment	Saline solution (n=11)		KN93 (n=11)	
	0	7	0	7
Time (days)				
BW (g)	21.61 ± 0.19	22.05 ± 0.27	22.00 ± 0.34	22.63 ± 0.41
HW/BW	-	6.39 ± 0.30	-	6.51 ± 0.28
LW/BW	-	6.55 ± 0.12	-	6.37 ± 0.31
HR conscious (bpm)	754 ± 16	767 ± 12	755 ± 08	758 ± 13
HR unconscious (bpm)	413 ± 10	410 ± 12	395 ± 05	406 ± 09
LVDV (μl)	24.89 ± 1.36	24.85 ± 1.02	24.61 ± 2.08	27.40 ± 3.45
LVSF (μl)	2.83 ± 0.28	2.69 ± 0.33	2.46 ± 0.37	3.71 ± 3.24
mass (mg)	81.51 ± 3.92	90.94 ± 4.47	84.53 ± 5.7	92.38 ± 5.00
LA area (mm ²)	2.30 ± 2.16	2.31 ± 0.08	2.28 ± 0.09	2.81 ± 0.13**
EF (%)	86.39 ± 1.17	85.00 ± 1.15	88.25 ± 1.54	88.39 ± 2.11
FS (%)	54.95 ± 1.70	53.21 ± 1.38	54.87 ± 3.99	57.04 ± 3.68
E/Em	25.81 ± 2.01	24.60 ± 1.34	25.03 ± 1.76	35.10 ± 1.7***
E/A	1.53 ± 0.04	1.53 ± 0.09	1.5 ± 0.09	1.9 ± 0.05*

A: late diastolic mitral inflow; BW: body weight; bpm: beat per minute; E: peak velocity of early diastolic mitral inflow; EF: ejection fraction; Em: peak velocity of early diastolic mitral valve; FS: fractional shortening; HR: heart rate; HW: heart weight; LA: left atrium; Left ventricle interior dimension LV: left ventricle; LVDV: left ventricular diastolic volume; LVSF: left ventricular systolic volume; LW: lung weight;

**
: $p < 0.01$;

: $p < 0.001$ compared to saline solution.