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Calmodulin kinase II inhibition prevents arrhythmias in RyR2^{R4496C}^{+/-} mice with catecholaminergic polymorphic ventricular tachycardia

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ABSTRACT

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmogenic disease characterized by life-threatening arrhythmias elicited by adrenergic activation. CPVT is caused by mutations in the cardiac ryanodine receptor gene (RyR2). In vitro studies demonstrated that RyR2 mutations respond to sympathetic activation with an abnormal diastolic Ca²⁺ leak from the sarcoplasmic reticulum; however the pathways that mediate the response to adrenergic stimulation have not been defined. In our RyR2^{R4496C}^{+/-} knock-in mouse model of CPVT we tested the hypothesis that inhibition of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) counteracts the effects of adrenergic stimulation resulting in an antiarrhythmic activity. CaMKII inhibition with KN-93 completely prevented catecholamine-induced sustained ventricular tachyarrhythmia in RyR2^{R4496C}^{+/-} mice, while the inactive congener KN-92 had no effect. In ventricular myocytes isolated from the hearts of RyR2^{R4496C}^{+/-} mice, CaMKII inhibition with an autocamtide-2 related inhibitory peptide or with KN-93 blunted triggered activity and transient inward currents induced by isoproterenol. Isoproterenol also enhanced the activity of the sarcoplasmic reticulum Ca²⁺-ATPase (SERCA), increased spontaneous Ca²⁺ release and spark frequency. CaMKII inhibition blunted each of these parameters without having an effect on the SR Ca²⁺ content. Our data therefore indicate that CaMKII inhibition is an effective intervention to prevent arrhythmogenesis (both in vivo and in vitro) in the RyR2^{R4496C}^{+/-} knock-in mouse model of CPVT. Mechanistically, CaMKII inhibition acts on several elements of the EC coupling cascade, including an attenuation of SR Ca²⁺ leak and blunting catecholamine-mediated SERCA activation. CaMKII inhibition may therefore represent a novel therapeutic target for patients with CPVT.

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1. Introduction

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited disease characterized by adrenergically mediated polymorphic or bidirectional ventricular tachycardia (VT) leading to syncope and sudden cardiac death [1]. We previously demonstrated that autosomal dominant CPVT is caused by mutations in the cardiac ryanodine receptor (RyR2) [2]. Effective therapy is lacking; beta-adrenergic blockers are the mainstay therapy for CPVT patients, but approximately 30% of patients still experience cardiac arrhythmias and eventually require an implantable cardioverter defibrillator [3].

The causal association between stress and arrhythmic symptoms in patients with CPVT highlights the importance of the adrenergic signaling pathway [4]. The β-adrenergic receptor signals through an axis that involves G-proteins, adenylyl cyclase, cAMP and protein kinase A (PKA) to phosphorylate essential target proteins involved in EC coupling. These include RyR2 and phospholamban, which synergistically act to modulate Ca²⁺ flux and sarcoplasmic reticulum (SR) function. β-Adrenergic receptor signaling may also be associated with Ca²⁺ overload, spontaneous Ca²⁺ leak, delayed afterdepolarizations (DADs) and triggered activity [5]. These phenomena are especially pronounced in the RyR2^{R4496C} mutant [6].

Despite the well-described involvement of PKA in β-adrenergic signaling, there is emerging evidence for an additional role of the Ca²⁺/calmodulin-dependent serine–threonine protein kinase II (CaMKII) [7,8]. The relative importance and functional consequences of CaMKII signaling on Ca²⁺ handling are still evolving [9] and under debate [10]. Interestingly, however, CaMKII appears to phosphorylate the same targets as PKA, including RyR2 and phospholamban [9]. Of note,

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several studies have implicated CaMKII signaling in arrhythmogenic events. For example, it has been suggested that the SR Ca^{2+} leak induced by β -adrenergic stimulation is mediated principally by CaMKII [11]. Another study suggested that the abnormal SR Ca^{2+} leakiness in a rabbit model of non-ischemic, arrhythmogenic heart failure was mitigated by CaMKII inhibition but was unaltered by PKA inhibition [12]. These studies highlight the possibility that CaMKII might similarly be mechanistically involved in arrhythmogenesis of CPVT. In the present study, therefore, we assessed the effects of CaMKII inhibition on life-threatening arrhythmias in a $\text{RyR2}^{\text{R4496C}/+/-}$ CPVT knock-in mouse model previously developed in our laboratory. We found that CaMKII inhibition is an effective intervention to prevent arrhythmogenesis in the $\text{RyR2}^{\text{R4496C}}$ knock-in mouse model, both in vivo and in vitro. CaMKII inhibition therefore represents a novel therapeutic target for patients with CPVT.

2. Materials and methods

An expanded Materials and Methods section can be found in the online Supplementary material.

2.1. In vivo drug testing

ECG radiotelemetry monitors were implanted as previously described [13]. Mice were treated with the CaMKII inhibitory agent KN-93 (30 $\mu\text{mol/kg}$ IP bolus) or the inactive congener KN-92 (30 $\mu\text{mol/kg}$ IP bolus). 10 min later, drug stress with co-injection of epinephrine and caffeine was performed as previously described [13]. All experiments were performed in accordance with the regulations of our Institutional Animal Care and Use Committees.

2.2. Isolation of adult mice ventricular myocytes

Ventricular myocytes were isolated using an established enzymatic digestion protocol [14].

2.3. Electrophysiological recordings in isolated ventricular myocytes

Transmembrane potentials and currents were recorded in whole-cell mode. Only quiescent, Ca^{2+} -tolerant, rod-shaped cells with clear cross striations were used for electrophysiological recordings. For action potential recording, myocytes were electrically stimulated by intracellular current injection through patch electrodes using depolarizing pulses with a duration of 3 ms and amplitude of 1.5 to 2.5 nA. The transient inward current (I_{ti}) was elicited by repeated trains of 100-ms voltage-clamp steps from -80 to 50 mV, voltage steps of 3 s were followed by repolarizing steps to potentials between -100 and 20 mV. I_{ti} amplitude was measured as difference between the peak of the transient current and the mean of current just before and after the transient current. The CaMKII inhibitor peptide (AIP) was loaded into myocytes in a Ca^{2+} free Tyrode's solution [11].

2.4. Cardiac myocyte Ca^{2+} transient

Isolated ventricular myocytes were incubated with 2 μM Fura-2 acetoxymethyl ester for 6 min at room temperature and washed twice with Tyrode's solution containing 250 μM probenecid. All experiments were conducted at 35°C . In some experiments, cells were loaded with Fura-4F (K_d : 0.77 μM). Intracellular Ca^{2+} transients were simultaneously recorded using a dual-beam excitation fluorescence photometry setup. Ca^{2+} level is proportional to the fluorescence ratio at 360 nm and 380 nm excitation. Since Fura-2/AM compartmentalizes into intracellular organelles, calculating intracellular Ca^{2+} concentrations from Fura-2 fluorescence ratios can be problematic in intact cells. Thus, Ca^{2+} level measurements are reported as fluorescence ratios (F_{ratio}) [15]. SR Ca^{2+} load was measured as the amplitude

of the 10 mmol/L caffeine-induced Ca^{2+} transient. At higher frequencies where Ca^{2+} transient decline was incomplete, the time constant was calculated from the last transient after cessation of electrical stimulation, therefore allowing complete Ca^{2+} transient decline and accurate calculation of Tau.

2.5. Calcium spark measurement

Ca^{2+} sparks were recorded in intact myocytes loaded with fluo-4 AM on a laser scanning confocal microscope (SP5, Leica Microsystems CMS GmbH, Mannheim, Germany) using Tyrode's solution. Myocytes were loaded with 5 $\mu\text{mol/L}$ fluo-4 AM for 20 min at room temperature. All experiments were conducted at 23°C . Line-scan mode was used (400 Hz) with a Leica $\times 60$ oil-immersion objective (n.a. 1.3). Spontaneous Ca^{2+} sparks were obtained in quiescent cells after 1 Hz stimulation (for 1 min) to reach a steady state and to ensure equal SR Ca^{2+} load. The dye was excited at 488 nm and emission was collected at >510 nm. Image analyses were performed with spark master in Image J software [16].

2.6. Western blot

For Western blot assays, we use monoclonal anti-RyR2, polyclonal anti-phospho-RyR2 (Ser-2814), monoclonal anti-PLB and polyclonal anti-Thr17-phosphorylated PLB antibodies.

2.7. Data analysis

Data were expressed as mean $\pm$ SEM. Statistical analysis was performed using the SPSS statistical package (version 17). The Students t-test was used to determine statistical significance between 2 groups. To determine statistical significance with multiple groups, we used a 1-way ANOVA followed by a Tukey test for multiple comparisons. Chi-square and Fisher's exact tests were used as appropriate for non-parametric data. Comparisons yielding a p value ≤ 0.05 were considered statistically significant.

3. Results

3.1. CaMKII inhibition prevents ventricular tachycardia in $\text{RyR2}^{\text{R4496C}/+/-}$ mice

Neither WT nor $\text{RyR2}^{\text{R4496C}/+/-}$ mice exhibited spontaneous arrhythmias as evidenced from continuous ECG telemetric recordings. In agreement with our previous data [13], combined IP administration of epinephrine (2 mg/kg) and caffeine (120 mg/kg) induced sustained VT or bidirectional VT in 50% of the $\text{RyR2}^{\text{R4496C}/+/-}$ mice (6/12). In contrast, none of the WT mice developed arrhythmias (0/12; $p < 0.05$; Fig. 1A). In a separate set of experiments, the CaMKII inhibitor KN-93 (30 $\mu\text{mol/kg}$ IP; $n = 12$) was administered for 10 min and had no effect on heart rate (761 ± 56 versus 773 ± 43 bpm before drug stress; $p = 0.89$). When pretreated with KN-93, epinephrine/caffeine failed to induce arrhythmias in any of the $\text{RyR2}^{\text{R4496C}/+/-}$ mice (0/12; $p < 0.05$ versus no drug). In contrast, when pretreated with the inactive congener KN-92 (30 $\mu\text{mol/kg}$ IP), $\text{RyR2}^{\text{R4496C}/+/-}$ mice still developed sustained VT following the epinephrine/caffeine challenge (6/12; $p = 1$ versus no drug). In a small set of experiments, we examined the steady state effects of KN-93 (30 $\mu\text{mol/kg/d}$ for 3 days in osmotic pump, $n = 3$) on the arrhythmogenesis in $\text{RyR2}^{\text{R4496C}/+/-}$ mice. None of these mice developed sustained VT upon exposure to epinephrine/caffeine, whereas 2 of the 3 time-matched control animals (saline infusion) developed bidirectional VT. Our in vivo data therefore demonstrate that in vivo CaMKII inhibition is protective in this mouse model of CPVT.

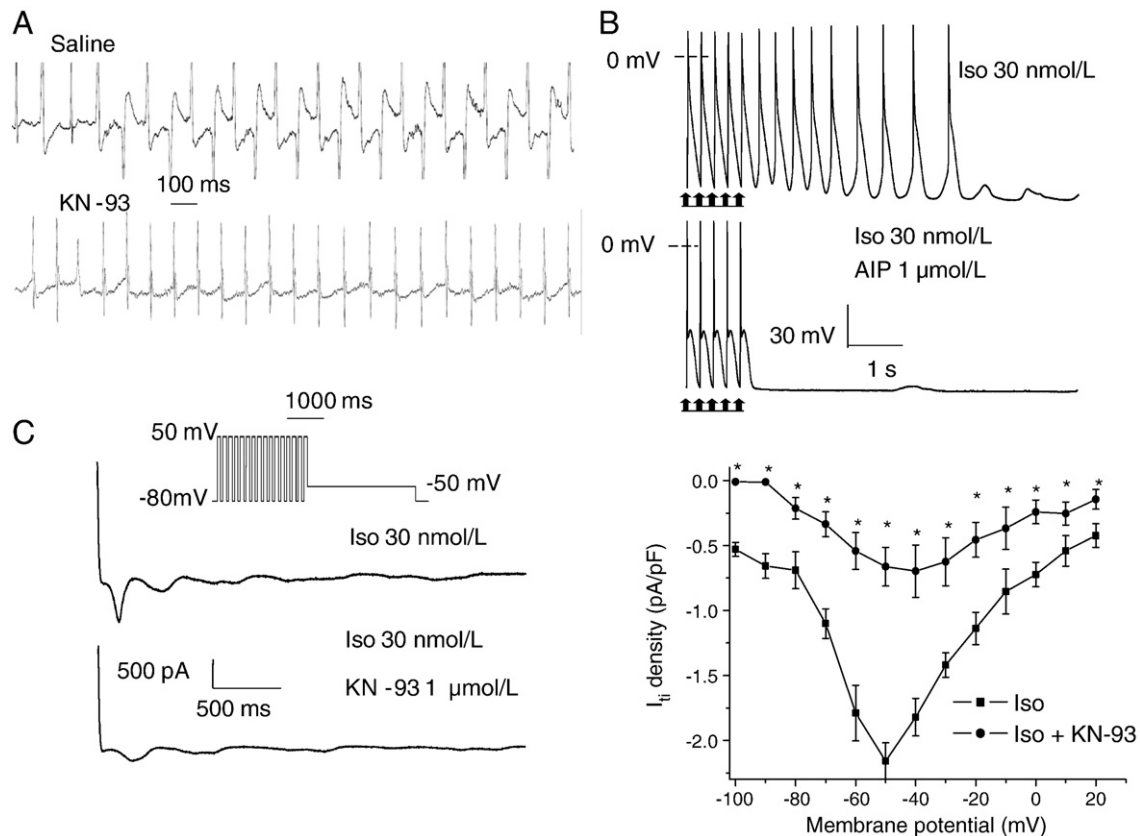


Fig. 1. (A) ECG recordings in RyR2^{R4496C}/+ mice. Upper trace: bidirectional VT induced in a saline treated mouse after epinephrine and caffeine administration, only isolated premature ventricular beats were observed in a KN-93 treated mouse after epinephrine and caffeine administration (lower trace). (B) Iso induced sustained TA in a RyR2^{R4496C}/+ myocyte (upper trace), which was prevented by AIP (lower trace). (C) Transient inward current (I_{ti}) in RyR2^{R4496C}/+ myocytes was elicited by trains of voltage-clamp steps (fourteen 100-ms steps from -80 to 50 mV, left panel) and plotted as a function of the test potential (right panel). KN-93 (1 μ mol/L) significantly reduced the amplitude of I_{ti} at all test potentials ($n = 5$, * $p < 0.001$).

3.2. CaMKII inhibition prevents triggered activity in RyR2^{R4496C}/+ myocytes

We next investigated the effects of CaMKII inhibition on arrhythmogenic mechanisms using isolated RyR2^{R4496C}/+ ventricular myocytes. As demonstrated previously [14], in the presence of isoproterenol (Iso; 30 nmol/L) and when paced at 5 Hz, the majority (61%; 20/33; $p < 0.05$) of RyR2^{R4496C}/+ myocytes developed triggered activity (TA). In contrast, none of the WT myocytes (0/12) developed TA under these conditions. When RyR2^{R4496C}/+ myocytes were pretreated with 1 μ mol/L CaMKII inhibitory peptide for 60 min (AIP; 0/15 Fig. 1B) or with 1 μ mol/L KN-93 (0/15), no TA was observed upon cessation of electrical stimulation. However similar to the control group, 67% (10/15) of RyR2^{R4496C}/+ myocytes pretreated with the inactive stereoisomer KN-92 (1 μ mol/L) developed TA. The DADs and TA reflect membrane depolarizations that are induced by the transient inward current (I_{ti}) [17]. We therefore investigated effects of CaMKII inhibition on the I_{ti} density. Iso (30 nmol/L) significantly increased the amplitude of I_{ti} . After perfusion with 1 μ M KN-93, the amplitude of I_{ti} was significantly decreased in the RyR2^{R4496C}/+ myocytes at all voltages tested (Fig. 1C; for example, at -50 mV; I_{ti} decreased from -2.16 ± 0.14 to -0.66 ± 0.15 pA/pF within 5 min, $n = 5$; $p < 0.01$). These data demonstrate that triggered activity caused by DADs (and the underlying I_{ti}) is abolished by CaMKII inhibition.

3.3. CaMKII inhibition diminishes spontaneous Ca^{2+} release from SR

We used Fura-2 loaded myocytes to investigate Ca^{2+} handling during field stimulation (1–5 Hz). As expected, the incidence of

spontaneous Ca^{2+} release (SCR) events was higher at elevated pacing rates (Figs. 2A and B). AIP (1 μ mol/L) had a tendency to decrease the incidence of SCR in both WT and RyR2^{R4496C}/+ myocytes (similar data were obtained with 1 μ mol/L KN-93, Supplemental Fig. 1). However, this decrease was statistically significant only at 5 Hz in the RyR2^{R4496C}/+ group (from 32.6% to 10.3%, $p < 0.05$). In the presence of Iso (30 nmol/L for 5 min) the incidence of SCR was significantly elevated in RyR2^{R4496C}/+ myocytes compared to WT myocytes (Figs. 2A and B). This Iso-induced increase was prevented by CaMKII inhibition with AIP (1 μ mol/L; Fig. 2C) or KN-93 (1 μ mol/L; Supplemental Fig. 1).

3.4. Effects of CaMKII inhibition on the Ca^{2+} transient

We next investigated the consequences of CaMKII inhibition on cytosolic Ca^{2+} transient amplitudes and kinetics. Under our experimental conditions the Ca^{2+} transient amplitude was not modified by rate (1–5 Hz) with a trend towards an increased Ca^{2+} transient at higher stimulation rates (Fig. 3). In the presence of AIP (1 μ mol/L) this trend did not occur and the Ca^{2+} transient amplitudes were decreased at higher stimulation rates. This decrease was also observed in the presence of Iso (30 nmol/L) and it occurred both in the WT and RyR2^{R4496C}/+ myocytes. AIP (1 μ mol/L) partially prevented the effects of Iso on Ca^{2+} transient amplitude at the faster pacing rates, consistent with the notion that CaMKII is predominantly activated at fast pacing rates.

In mouse myocytes, the function of SERCA is reflected in the time course of the Ca^{2+} transient decay [18]. An involvement of CaMKII in SERCA function is demonstrated by the finding that Iso accelerated the Ca^{2+} transient decay at all stimulation frequencies and this effect was

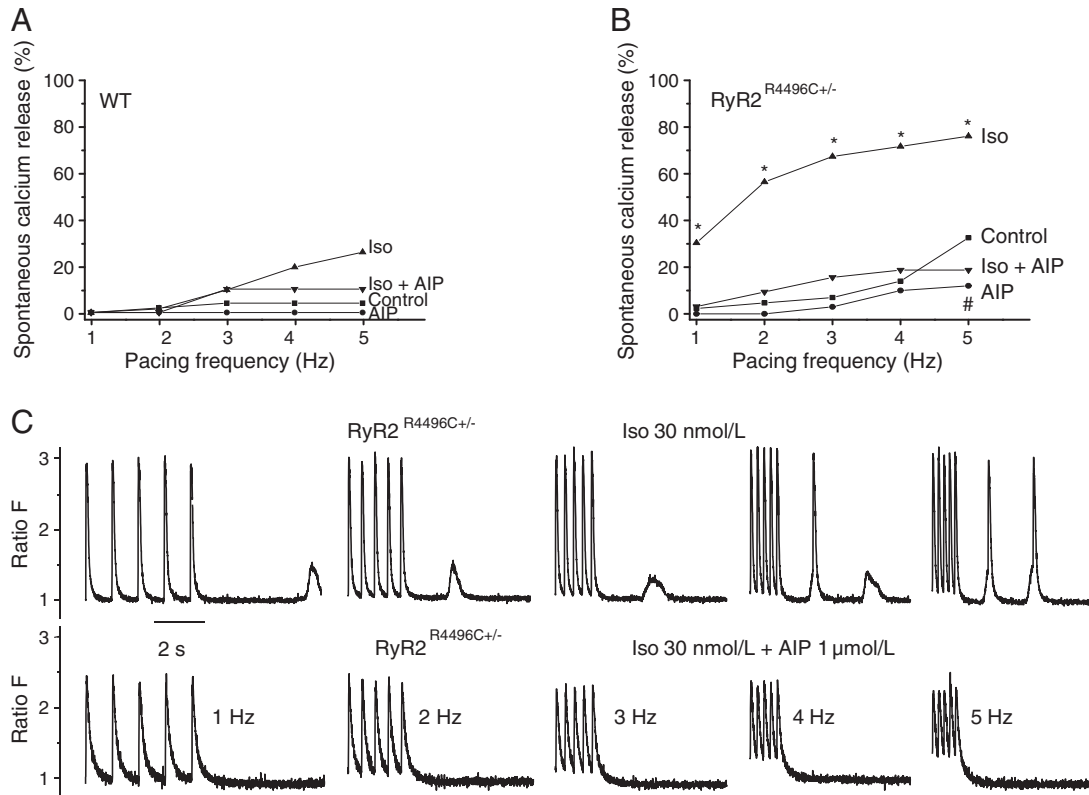


Fig. 2. (A and B) Relative occurrence of spontaneous Ca^{2+} releases upon cessation of stimulation at 1, 2, 3, 4 and 5 Hz stimulation with or without CaMKII inhibition with AIP in WT and $\text{RyR2}^{\text{R4496C+/-}}$ myocytes, $n = 32\text{--}46$ in each group. Higher pacing frequency increased the occurrence of spontaneous Ca^{2+} release. AIP prevented Ca^{2+} release events induced by pacing and isoproterenol. * $p < 0.001$ versus $\text{RyR2}^{\text{R4496C+/-}}$ myocytes in the absence of Iso at the same stimulation frequency. # $p < 0.05$ versus $\text{RyR2}^{\text{R4496C+/-}}$ myocytes in the absence of Iso. (C) Spontaneous Ca^{2+} releases elicited upon cessation of electrical field stimulation (1 to 5 Hz) in a $\text{RyR2}^{\text{R4496C+/-}}$ myocyte incubated with Iso (30 nmol/L). AIP prevented the occurrence of spontaneous Ca^{2+} releases even at the fastest pacing rate of 5 Hz.

largely reversed by pretreatment with AIP (Figs. 4A and B) or KN-93 (1 μmol/L; Supplemental Fig. 2). We observed a frequency-dependent acceleration of relaxation (FDAR) of Ca^{2+} transients at 5 Hz on component to 1 Hz (Fig. 4C). Interestingly, 1 μmol/L AIP also significantly diminished FDAR.

3.5. Effects of CaMKII inhibition on

3.5.1. SR Ca^{2+} content

We assessed the SR Ca^{2+} content as the increase in cytosolic Ca^{2+} with rapid caffeine application (10 mmol/L) following pacing at 1, 3 or 5 Hz. The SR Ca^{2+} content had a tendency to increase at higher pacing

frequencies (Fig. 5). There was no significant difference in SR Ca^{2+} content between WT and $\text{RyR2}^{\text{R4496C+/-}}$ myocytes.

Pretreatment with AIP had minor effects on SR Ca^{2+} content; an 8% reduction was observed only in the 5 Hz group (from 1.7 ± 0.06 to 1.6 ± 0.07 in WT myocytes, from 1.8 ± 0.10 to 1.6 ± 0.11 $\text{RyR2}^{\text{R4496C+/-}}$ myocytes). Iso (30 nmol/L) significantly increased the SR Ca^{2+} content at all pacing frequencies; both in the WT and $\text{RyR2}^{\text{R4496C+/-}}$ groups. Although there was a trend for a larger increase of SR Ca^{2+} content at higher stimulation frequencies (e.g. respectively 12% and 4% higher at 5 Hz relative to 1 Hz in WT and $\text{RyR2}^{\text{R4496C+/-}}$ myocytes), these differences were not statistically significant. Similarly, pretreatment with AIP had no significant effects on the SR Ca^{2+} content (a decrease of 4% and 2% was respectively noted in WT and $\text{RyR2}^{\text{R4496C+/-}}$ myocytes).

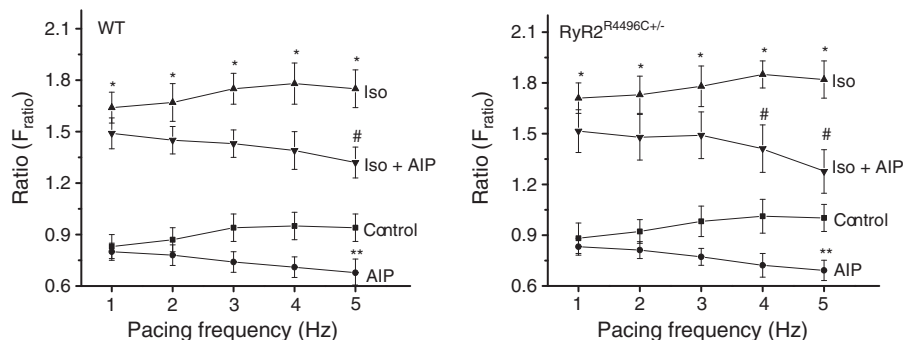


Fig. 3. Frequency dependence of Ca^{2+} transient amplitude in WT and $\text{RyR2}^{\text{R4496C+/-}}$ myocytes, $n = 18\text{--}32$ in each group. * $p < 0.001$ versus control cells at the same frequency; ** $p < 0.05$ versus control cells at the same frequency; # $p < 0.05$ versus Iso treated cells at the same frequency.

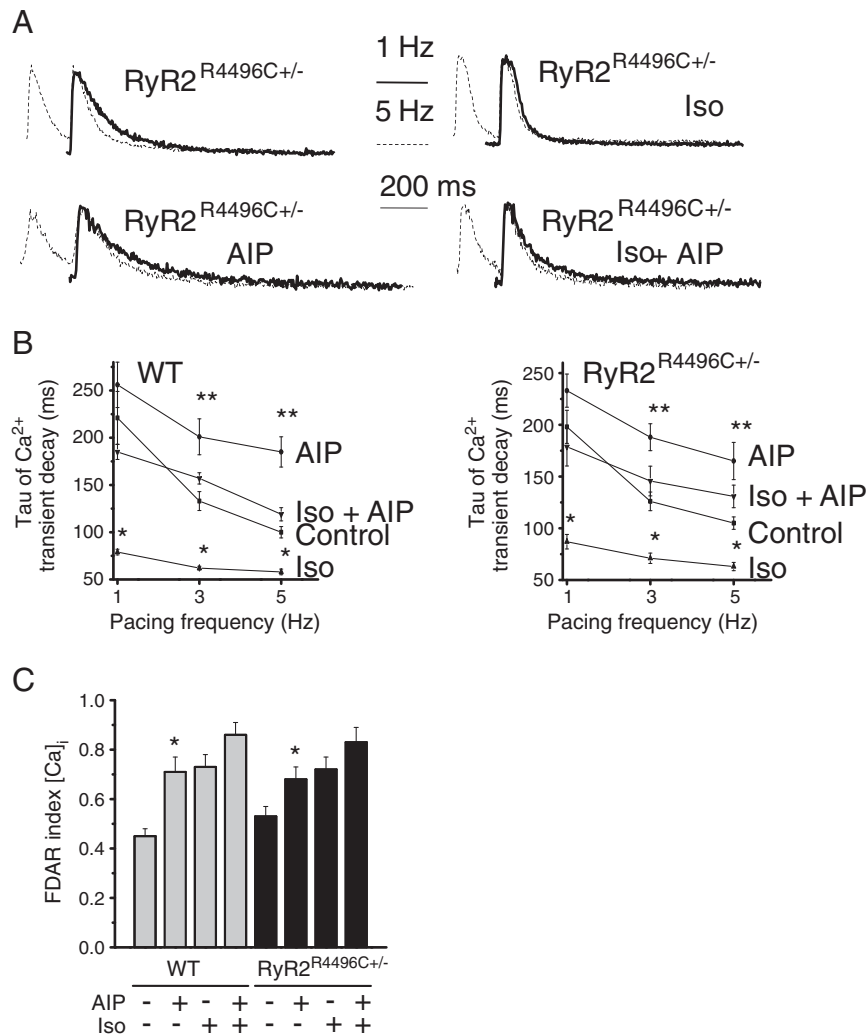


Fig. 4. (A) Normalized Ca^{2+} transients at 1 and 5 Hz in $\text{RyR2}^{\text{R4496C+/-}}$ myocytes. (B) Averaged single exponential time constant of Ca^{2+} transient decay in WT and $\text{RyR2}^{\text{R4496C+/-}}$ myocytes, $n = 18\text{--}41$ in each group. * $p < 0.001$ versus control cells and Iso + AIP treated cells at the same frequency; ** $p < 0.05$ versus control cells at the same frequency. (C) FDAR index in WT and $\text{RyR2}^{\text{R4496C+/-}}$ myocytes.

Experiments were also performed using a lower affinity Ca^{2+} indicator dye, Fura-4F, to ensure that dye saturation was not an issue and similar data were obtained (not shown).

3.5.2. SR fractional release

The fractional SR Ca^{2+} release was calculated by dividing the amplitude of the Ca^{2+} transient induced by electrical stimulation by the caffeine releasable SR Ca^{2+} . As expected, Iso increased the fractional SR Ca^{2+} release (Fig. 5) [19]. This effect was attenuated by AIP (particularly at the higher stimulation frequencies). Similar data were obtained with KN-93 (Supplemental Fig. 3).

3.5.3. NCX function

In the presence of caffeine, SR Ca^{2+} uptake by SERCA is inhibited and Ca^{2+} efflux occurs predominantly through the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Consequently, we measured the exponential time constant of Ca^{2+} decay during the 10 s caffeine-induced Ca^{2+} transients. There were no significant differences in this parameter between WT and $\text{RyR2}^{\text{R4496C+/-}}$ myocytes. Furthermore, this parameter was not affected either by AIP, KN-93 or Iso (Supplemental Table 1), suggesting that the $\text{Na}^+/\text{Ca}^{2+}$ exchange activity was largely unaffected by CaMKII activity under our experimental conditions.

3.6. Effects of PKA inhibition on Ca^{2+} handling in $\text{RyR2}^{\text{R4496C+/-}}$ myocytes

PKA is a major signaling pathway of beta-adrenergic stimulation. We therefore also accessed the effects of PKA inhibition on Ca^{2+} handling in $\text{RyR2}^{\text{R4496C+/-}}$ myocytes using the PKA inhibitor H-89. We found that 5 $\mu\text{mol/L}$ H-89 prevented the Iso-induced SCR in $\text{RyR2}^{\text{R4496C+/-}}$ myocytes and significantly blunted the Iso-induced enhancement of Ca^{2+} transient amplitudes, Ca^{2+} transient decay and SR Ca^{2+} content (Fig. 6).

3.7. Effects of CaMKII inhibition on Ca^{2+} sparks in $\text{RyR2}^{\text{R4496C+/-}}$ myocytes

Ca^{2+} sparks are the elementary events of local SR Ca^{2+} release and the occurrence of Ca^{2+} sparks under resting conditions represents spontaneous Ca^{2+} leak from SR [20]. After 1 min pacing at 1 Hz to achieve constant SR Ca^{2+} load, pacing was stopped and Ca^{2+} sparks were measured for 5 s. The frequency of Ca^{2+} spark occurrence was significantly increased in $\text{RyR2}^{\text{R4496C+/-}}$ myocytes compared to WT myocytes (0.82 ± 0.29 versus 0.43 ± 0.16 $100 \mu\text{m}^{-1} \text{s}^{-1}$, $P < 0.01$), suggestive of an increased SR Ca^{2+} leak. The spark frequency was unaffected by AIP in the absence of Iso. In the presence of Iso (30 nmol/L), the spark frequency was significantly elevated in both genotypes.

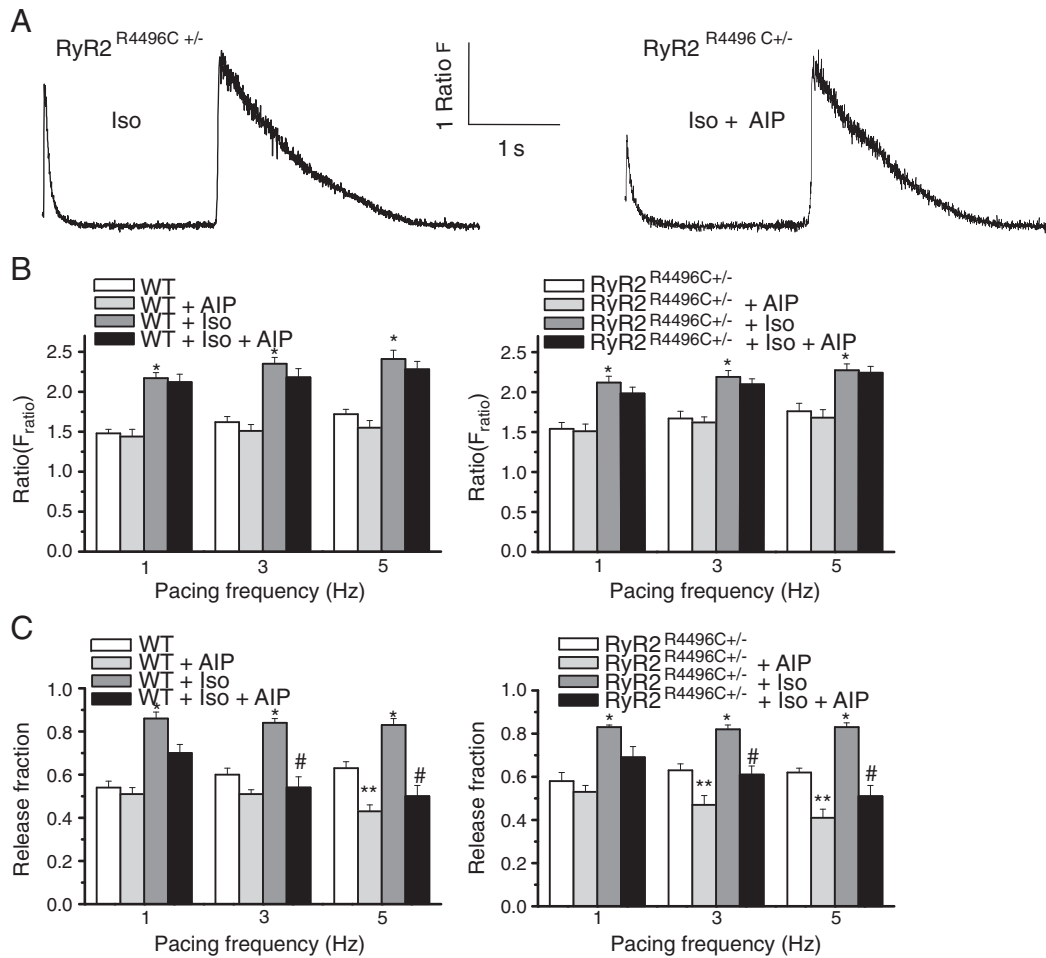


Fig. 5. (A) Original recording of caffeine (10 mmol/L) induced Ca^{2+} transient in WT and $\text{RyR2}^{\text{R4496C+/-}}$ myocytes. (B) Averaged SR Ca^{2+} content in WT and $\text{RyR2}^{\text{R4496C+/-}}$ myocytes ($n=18$ –25 in each group) at 1, 3, and 5 Hz pacing. * $p<0.01$ versus control cells at the same frequency. (C) Averaged release fraction in WT and $\text{RyR2}^{\text{R4496C+/-}}$ myocytes at 1, 3, and 5 Hz pacing. * $p<0.01$ and ** $p<0.05$ versus control cells at the same frequency; # $p<0.05$ versus Iso treated cells at the same frequency.

This increase was attenuated by AIP (Fig. 7), KN-93 (Supplemental Fig. 4) and 5 $\mu\text{mol/L}$ H-89.

3.8. Effect of CaMKII inhibition on RyR2 and PLB phosphorylation

To assess the phosphorylation state of RyR2 and PLB in the $\text{RyR2}^{\text{R4496C+/-}}$ myocytes, we performed Western blotting with phospho-specific antibodies against RyR2 Ser2814 and PLB Thr17 (Fig. 7). Experiments were performed with or without Iso (30 nmol/L) stimulation. We also assessed the effect of KN-93 (1 $\mu\text{mol/L}$). Iso significantly increased the phosphorylation of RyR2 Ser2814 and PLB Thr17. KN-93 blunted the Iso-induced increase in both of these phosphorylation sites.

4. Discussion

The evidence that CPVT is triggered by an increase of the adrenergic tone and reflexes such as exercise or emotional stress provides a clinical rationale for the use of beta-adrenergic blockers in CPVT patients [3,4]. In the initial clinical observations, beta-adrenergic blockers appeared to be able to prevent the occurrence of cardiac events in CPVT patients [4]; however, incomplete protection from sudden cardiac death in CPVT patients and in CPVT transgenic mouse has been subsequently reported [3,13]. These findings prompted us to test several compounds acting on targets that are critical for calcium homeostasis in our

$\text{RyR2}^{\text{R4496C+/-}}$ CPVT mouse model [21,22]. We found that CaMKII inhibition is a powerful approach to prevent arrhythmogenesis in $\text{RyR2}^{\text{R4496C}}$ knock-in mice in vivo. Then we decided to investigate the underlying mechanisms for these strong antiarrhythmic effects.

4.1. Cellular mechanisms for spontaneous Ca^{2+} release in CPVT

Although there is some debate regarding the underlying cellular and molecular mechanisms, there are several lines of evidence to demonstrate that mutant RyR2 channels increase the sensitivity to luminal and/or cytosolic Ca^{2+} and decrease the threshold for Ca^{2+} release under basal conditions [23]. Phosphorylation of the mutant RyR2 channel by adrenergic stimulation aggravates this defective behavior and leads to a further increase of RyR2 open probability and Ca^{2+} release events [23,24]. It has been proposed that increased RyR2 open probability (or Ca^{2+} leak) by itself is insufficient to produce sustained arrhythmogenic diastolic SR Ca^{2+} release [25]. These arrhythmogenic events will cease if the SR Ca^{2+} declines below this threshold value. Maintaining high SR Ca^{2+} content during catecholamine stimulation (which is primarily determined by SERCA function) can therefore be regarded as a major determinant for the spontaneous Ca^{2+} leak and arrhythmogenesis. Theoretically, preventing spontaneous Ca^{2+} release during CPVT can therefore be achieved by either enhancing the Ca^{2+} release threshold of the mutant RyR2 channel and/or avoiding SR Ca^{2+} overload [25].

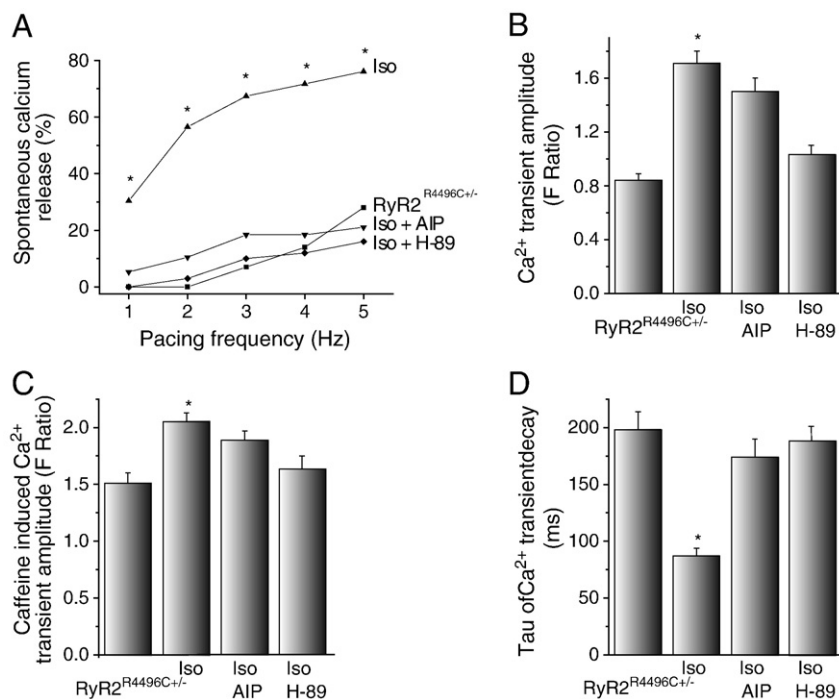


Fig. 6. (A) Relative occurrence of spontaneous Ca^{2+} releases upon cessation of stimulation at 1, 2, 3, 4 and 5 Hz stimulation with CaMKII inhibition with AIP and PKA inhibition with H-89 in $\text{RyR2}^{\text{R4496C}/-}$ myocytes. * $p < 0.01$ versus $\text{RyR2}^{\text{R4496C}/-}$ myocytes in the absence of Iso, the cells treated by AIP (1 $\mu\text{mol/L}$) and the cells treated by H-89 (5 $\mu\text{mol/L}$) at the same stimulation frequency. (B) Averaged Ca^{2+} transient amplitude at 1 Hz pacing. * $p < 0.01$ versus control cells and the cells treated by H-89 (5 $\mu\text{mol/L}$). (C) Averaged caffeine-induced Ca^{2+} transient amplitude at 1 Hz pacing. * $p < 0.01$ versus control cells and the cells treated by H-89 (5 $\mu\text{mol/L}$). (D) Averaged Ca^{2+} transient decay at 1 Hz pacing. * $p < 0.01$ versus control cells, the cells treated by AIP (1 $\mu\text{mol/L}$) and the cells treated by H-89 (5 $\mu\text{mol/L}$).

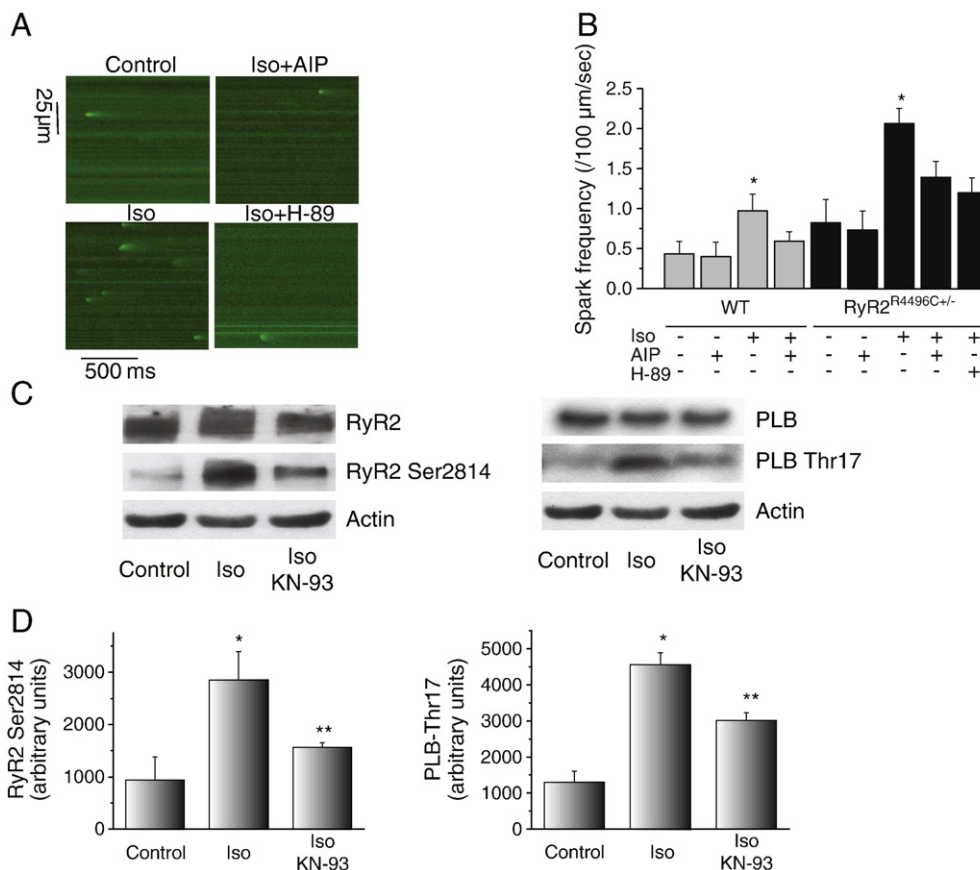


Fig. 7. (A) Representative confocal line-scan images of Ca^{2+} sparks in $\text{RyR2}^{\text{R4496C}/-}$ myocytes. (B) Averaged Ca^{2+} spark frequency (n = 10 for each group as indicated). * $p < 0.05$, versus control cells, Iso + AIP treated cells and Iso + H-89 treated cells (5 $\mu\text{mol/L}$). (C) Representative Western blots, analysis of protein expression and phosphorylation in $\text{RyR2}^{\text{R4496C}/-}$ mice. Effect of CaMKII inhibition with KN-93 on RyR2 and PLB phosphorylation in isolated hearts stimulated with Iso as compared to control cells. Actin was used to verify the amount of loaded samples. (D) Data pooled from 5 experiments for RyR2 S2814 and PLB Thr17 respectively, * $p < 0.001$, versus control cells and ** $p < 0.01$, versus Iso treated cells.

4.2. CaMKII inhibition enhances the threshold for spontaneous Ca^{2+} release

It is not entirely clear from the literature how the R4496C mutation affects RyR2 function or how this mutation predisposes the heart to catecholamine-dependent arrhythmias. In vitro studies have suggested that the basal activity of the RyR2 channel is elevated [26] and that the threshold for SR-overload-induced Ca^{2+} release is reduced [27]. Cardiac myocytes isolated from RyR2^{R4496C/+/-} knock-in mice exhibit delayed afterdepolarizations and abnormal Ca^{2+} release that has been attributed to an increased Ca^{2+} sensitivity of RyR2^{R4496C}, which induces diastolic Ca^{2+} release upon catecholamine stimulation and lowers the threshold for triggered activity. We demonstrate in this study that the mutant RyR2^{R4496C/+/-} myocytes have a significantly elevated Ca^{2+} spark frequency in response to β -adrenergic stimulation and that spark frequency is attenuated by interventions that inhibit CaMKII activity.

We demonstrate an important role of the CaMKII signaling pathway in the regulation of mutant RyR2 channel physiology. These data complement earlier studies of Bers and colleagues [28], who dissected the relative roles of PKA and CaMKII phosphorylation on wild type RyR2. They reported that CaMKII directly phosphorylates normal RyR2, leading to an increased Ca^{2+} spark frequency without altering the SR calcium load. They also demonstrated that PKA inhibition with 1 μM H-89 did not prevent the Iso-induced SCR in the failing myocytes, suggesting a key role for non-PKA mediated phosphorylation [11,29]. Our data demonstrate that the mutant RyR2 channel behaves in a similar manner: we found an increased Ca^{2+} spark induced by β -adrenergic activation that is attenuated by CaMKII inhibition – this occurred without changes in the SR Ca^{2+} content. We found that PKA inhibition with 5 μM /L H-89 also significantly prevents SCR under these conditions in RyR2^{R4496C/+/-} myocytes and significantly diminished Ca^{2+} spark frequency. These data are consistent with a recent report by Niggli and colleagues [30], who demonstrated that both KN-93 and H-89 (5 μM /L) suppressed the Iso-induced increase in Ca^{2+} spark frequency in guinea pig myocytes. Interestingly in agreement with Niggli's and Bers' studies [30], we found that PKA inhibition, but not CaMKII inhibition, reverses the Iso-induced increase in SR Ca^{2+} content despite comparable increases in Ca^{2+} spark frequency, which suggests that phosphorylation by CaMKII, rather than PKA, regulates RyR2 induced Ca^{2+} leak.

4.3. CaMKII inhibition attenuates SERCA function

Ca^{2+} sequestration into the SR by SERCA is strongly inhibited by phospholamban. Consistent with published data [31,32], we found that β -adrenergic stimulation induced phosphorylation of phospholamban at Thr-17. It was reported that CaMKII-mediated phospholamban phosphorylation is responsible for the more rapid SR Ca^{2+} uptake. In this study, we found phosphorylation of Thr-17 to be reduced by CaMKII inhibition, which might be a reason for the inhibition of SERCA function by CaMKII inhibition (a slower Ca^{2+} transient decay). These data are in agreement with data from AIP transgenic mouse [33]. Since CaMKII inhibition reduces SERCA function (and thereby mitigates the probability for the Ca^{2+} release trigger to be reached), it is an important mechanistic target for the antiarrhythmic potential of CaMKII inhibition.

Interestingly, our data demonstrate that rapid pacing is associated with an accelerated Ca^{2+} transient decay, indicative of facilitated SERCA Ca^{2+} reuptake (also named FDAR). This occurred even in the absence of catecholamine stimulation. The mechanism of this phenomenon has not been fully resolved but may be mediated by CaMKII phosphorylation of phospholamban at Thr-17 [34–36], which manifests an accelerated SERCA-mediated SR Ca^{2+} reuptake. This is consistent with our data demonstrating that the Ca^{2+} transient decay is particularly sensitive to CaMKII inhibition at faster pacing rates. This

finding may be clinically relevant because in the majority of patients ventricular extrasystoles and/or VT are elicited when the heart rate exceeds 110–130 beats per minute [1]. Since CaMKII inhibition significantly decreases SCR in RyR2^{R4496C/+/-} myocytes even in the absence of catecholamine stimulation, the possibility is raised that CaMKII activity can facilitate SCR independent of β -adrenergic stimulation in the setting of mutant RyR2.

In addition to the effects of CaMKII inhibition on the RyR2 and SERCA, it has been reported that CaMKII contributes to the facilitation of the $\text{I}_{\text{Ca,L}}$ and mode 2 gating [37], thus enhancing the Ca^{2+} influx and myocytes Ca^{2+} loading. This concept is in agreement with studies in which $\text{I}_{\text{Ca,L}}$ blockers have been shown to prevent CPVT in human and CPVT mouse models [38,39]. Although modification of $\text{I}_{\text{Ca,L}}$ alone has very little effect on SR Ca^{2+} content [40], computer modeling studies suggest a major role for the L-type Ca^{2+} current in the regulation of CaMKII signaling [41]. It should be noted, however, that we previously failed to observe beneficial effect therapeutic concentration of verapamil on CPVT [21]. This is in line with our clinical experience and published data, which demonstrated that verapamil is not an effective antiarrhythmic agent in patients with CPVT [42]. The precise role of $\text{I}_{\text{Ca,L}}$ in the arrhythmogenesis of CPVT clearly needs further clarification.

4.4. CaMKII inhibition as a promising therapeutic strategy for CPVT

The existing treatment for CPVT is not satisfactory since 30% of patients treated with β -blockers still experience cardiac arrhythmias [3]. It is therefore necessary to examine the efficacy of novel antiarrhythmic agents in transgenic mouse models in order to develop effective treatment regimens for CPVT patients. There have been several recent attempts in this regard [42–44], such as to examine S107 in the RyR2^{R2474S/+/-} mouse or the effects of Mg^{2+} and flecainide in Casq2 transgenic mice. The underlying mechanisms for these drugs in combating CPVT relied on the blockade of RyR2 opening. However, these drugs proved to be ineffective in preventing SR Ca^{2+} uptake induced by adrenergic stimulation. Our study demonstrates that CaMKII inhibition not only prevents RyR2 opening but also limits SR Ca^{2+} uptake, suggesting that CaMKII represent a reasonable therapeutic target for an abnormal Ca^{2+} leak. Our finding is not without precedent. For example, Ai et al. [12] observed that CaMKII inhibition blocks SR Ca^{2+} leak in cardiac myocytes with heart failure and increases SR Ca^{2+} content. More recently Terentyev et al. [45] reported that miR-1 overexpression enhances the abnormal Ca^{2+} leak and reduces SR Ca^{2+} content and CaMKII inhibition can completely reverse the effects of miR-1, even in the presence of adrenergic stimulation. In the present study, we found CaMKII inhibition to produce a dramatic therapeutic effect in RyR2^{R4496C/+/-} myocytes and also inhibit SCR in WT myocytes. Since acute CaMKII inhibition is beneficial in treating abnormal SR Ca^{2+} leak in different settings, CaMKII may be a common pathway for abnormal Ca^{2+} leak from SR.

In summary, we demonstrate that CaMKII inhibition is a promising approach to prevent adrenergically-induced arrhythmogenesis in the RyR2^{R4496C/+/-} CPVT mouse model. CaMKII inhibition exerts its antiarrhythmic activity by synergistically targeting multiple Ca^{2+} homeostasis pathways including 1) reduction of SERCA function and importantly, by 2) attenuation of diastolic SR Ca^{2+} leakage through mutant RyR2 channels. Although CaMKII is a downstream effector of adrenergic stimulation, CaMKII inhibition might be a novel target for the antiarrhythmic treatment of CPVT patients.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at [doi:10.1016/j.jmcc.2010.10.001](https://doi.org/10.1016/j.jmcc.2010.10.001).

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