Ryanodine Receptor Phosphorylation by Calcium/Calmodulin-Dependent Protein Kinase II Promotes Life-Threatening Ventricular Arrhythmias in Mice With Heart Failure

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Background—Approximately half of patients with heart failure die suddenly as a result of ventricular arrhythmias. Although abnormal Ca\(^{2+}\) release from the sarcoplasmic reticulum through ryanodine receptors (RyR2) has been linked to arrhythmogenesis, the molecular mechanisms triggering release of arrhythmogenic Ca\(^{2+}\) remain unknown. We tested the hypothesis that increased RyR2 phosphorylation by Ca\(^{2+}\)/calmodulin-dependent protein kinase II is both necessary and sufficient to promote lethal ventricular arrhythmias.

Methods and Results—Mice in which the S2814 Ca\(^{2+}\)/calmodulin-dependent protein kinase II site on RyR2 is constitutively activated (S2814D) develop pathological sarcoplasmic reticulum Ca\(^{2+}\) release events, resulting in reduced sarcoplasmic reticulum Ca\(^{2+}\) load on confocal microscopy. These Ca\(^{2+}\) release events are associated with increased RyR2 open probability in lipid bilayer preparations. At baseline, young S2814D mice have structurally and functionally normal hearts without arrhythmias; however, they develop sustained ventricular tachycardia and sudden cardiac death on catecholaminergic provocation by caffeine/epinephrine or programmed electric stimulation. Young S2814D mice have a significant predisposition to sudden arrhythmogenic death after transverse aortic constriction surgery. Finally, genetic ablation of the Ca\(^{2+}\)/calmodulin-dependent protein kinase II site on RyR2 (S2814A) protects mutant mice from pacing-induced arrhythmias versus wild-type mice after transverse aortic constriction surgery.

Conclusions—Our results suggest that Ca\(^{2+}\)/calmodulin-dependent protein kinase II phosphorylation of RyR2 Ca\(^{2+}\) release channels at S2814 plays an important role in arrhythmogenesis and sudden cardiac death in mice with heart failure. (Circulation. 2010;122:00-00.)

Key Words: arrhythmia • calcium • calcium-calmodulin-dependent protein kinase type 2 • heart failure • ryanodine receptor calcium release channel • sarcoplasmic reticulum

Congestive heart failure (HF) is a leading cause of mortality and morbidity worldwide. Approximately 50% of HF patients die of sudden cardiac death (SCD) attributed to ventricular arrhythmias (>300,000 in the United States annually).\(^1,2\) A large fraction of these arrhythmias are thought to be initiated by focal triggered mechanisms, such as spontaneous diastolic Ca\(^{2+}\) release from cardiac myocyte ryanodine receptors (RyR2) on the sarcoplasmic reticulum (SR), which activates an arrhythmogenic depolarizing inward Na\(^+\)/Ca\(^{2+}\) exchange (NCX) current.\(^3,4\) Indeed, in HF there is enhanced diastolic SR Ca\(^{2+}\) release and other changes in electrophysiological substrate that greatly enhance the propensity for triggered arrhythmias. Likewise, patients with inherited RyR2 point mutations exhibit catecholaminergic polymorphic ventricular tachycardia, a known cause of SCD with sensitivity to adrenergic conditions such as exercise or stress.\(^5,6\) HF is a chronic hyperadrenergic state, and a prominent theory suggested that β-adrenergic activation of protein kinase A (PKA) destabilized RyR2 through the loss of binding by FKBP12.6,7,8 contributing to SR Ca\(^{2+}\) leak and consequent systolic dysfunction and arrhythmogenesis. However, subsequent work showed that inhibition of Ca\(^{2+}\)/cal-

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modulin-dependent protein kinase II (CaMKII) rather than PKA was sufficient to reverse the arrhythmogenic SR Ca\(^{2+}\) leak in HF\(^9\) and that CaMKII mediates the β-adrenergic-induced increase in SR Ca\(^{2+}\) leak even in normal myocytes.\(^{10}\) Thus, in HF, CaMKII phosphorylation of RyR2 may be more important for arrhythmogenic events leading to SCD.

**Clinical Perspective on p 489**

CaMKII, which is upregulated and more active in HF,\(^4\) can phosphorylate and modulate numerous Ca\(^{2+}\) transport and ion channel proteins in cardiac myocytes, including RyR2 and voltage-gated Ca\(^{2+}\) and K\(^+\) channels, all of which could contribute to arrhythmogenesis.\(^{11}\) Moreover, transgenic overexpression of CaMKIV or CaMKIIα (the predominant myocyte isomorph) induces HF and cardiac arrhythmias,\(^{12–15}\) whereas inhibition and CaMKIIα knockout limit the progression of HF and arrhythmias.\(^{16,17}\) One weakness of currently available genetic models in which CaMKII activity is inhibited by gene deletion or transgenic expression of inhibitory peptides is that they do not permit selective evaluation of specific downstream phosphorylation targets affected by CaMKII upregulation or inhibition. Given the role of CaMKII phosphorylation of RyR2 in both HF and diastolic SR Ca\(^{2+}\) release, we sought to define the role of CaMKII phosphorylation of RyR2 specifically in cardiac arrhythmogenesis. Therefore, we generated and studied knockin mouse models in which the CaMKII phosphorylation site on RyR2 was either genetically inhibited or constitutively activated. Here we tested whether (1) the phosphomimetic S2814D mutant RyR2 alone increased the susceptibility to ventricular arrhythmias and (2) whether the nonphosphorylatable S2814A mutant prevented arrhythmias associated with cardiac hypertrophy and failure.

**Methods**

**Animals**

Generation of RyR2-S2814A knockin and AC3I transgenic mice was described previously.\(^{5,15}\) RyR2-S2814D knockin mice were created by an approach similar to that described for RyR2-S2814A knockin mice\(^5\) (see the online-only Data Supplement). All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and conforming to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health publication No. 85–23, revised 1996).

**Coimmunoprecipitation Assay**

RyR2 was immunoprecipitated from heart lysates with the use of an anti-RyR2 antibody (Thermo Scientific, Rockland, Ill) incubated with protein A-Sepharose beads (Rockland, Gilbertsville, Pa) at room temperature for 1 hour. For coimmunoprecipitation, antibody-attached beads were incubated with heart lysate aliquots containing 1000 μg total protein at 4°C overnight. After incubation, beads were washed with detergent-free coimmunoprecipitation buffer and resuspended in 2× lithium dodecyl sulfate buffer (Invitrogen, Carlsbad, Calif) containing β-mercaptoethanol. Samples were heated at 50°C for 15 minutes and were resolved on 4% to 20% Criterion sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (Bio-Rad) for detection of RyR2 and FKBP12.6.

**RyR2 Phosphorylation Assay**

RyR2 was immunoprecipitated, and beads were washed with phosphorylation-assay buffer containing 50 mmol/L Tris-Cl (pH 7.5), 10 mmol/L MgCl\(_2\), 2 mmol/L dithiothreitol, 0.1 mmol/L Na\(_3\)EDTA, 5 mmol/L NaF, 1 mmol/L Na\(_3\)VO\(_4\), 1× protease inhibitors, and 1× phosphatase inhibitors and resuspended in phosphorylation-assay buffer supplemented with 100 μmol/L cold ATP and 1.5 μCi [γ-\(^{32}\)P] ATP (Perkin Elmer, Waltham, Mass). The phosphorylation reaction was initiated by adding CaMKII (250 U per reaction), which was preactivated according to the manufacturer’s (New England Biolabs, Ipswich, Mass) instructions in the absence or presence of the CaMKII inhibitor KN-93 (10 μmol/L) (Calbiochem, San Diego, Calif). Reaction mixtures were incubated at 30°C for 20 minutes and stopped by adding 2× lithium dodecyl sulfate buffer (Invitrogen) containing β-mercaptoethanol. Samples were heated at 50°C for 10 minutes and resolved on 5% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels. The protein gels were dried and exposed to Kodak BioMax maximum resolution films.

**Western Blot Analyses**

Heart lysates were prepared from flash-frozen mouse hearts as described previously.\(^{18}\) Lysates were taken from mice at rest (Figure 1), after transverse aortic constriction (TAC) or after sham surgery (Figure 7 and Figure V in the online-only Data Supplement), immediately after pacing (Figure 5 and Figure IV in the online-only Data Supplement), and with/without exposure to pacing (Figure 7). For experimental details, please refer to the online-only Data Supplement.

**Histology**

A transverse section of the heart was fixed in 10% buffered formalin for 48 hours. After paraffin embedding and sectioning, 5-μm sections were stained with hematoxylin-eosin for cell morphology and Masson’s trichrome for interstitial fibrosis as described.\(^{18}\)

**Confocal Imaging**

Ca\(^{2+}\) sparks were recorded in saponin-permeabilized (50 μg/mL) or intact ventricular cardiomyocytes with the use of a Zeiss LSM510 confocal microscope. For experimental details, refer to the online-only Data Supplement.

**Single-Channel Recordings**

Single-channel recordings of wild-type (WT) or S2814D RyR2 were performed and analyzed under voltage-clamp conditions as described previously\(^{19}\) (see also the online-only Data Supplement).

**Transthoracic Echocardiography**

Mice were anesthetized with 1.5% isoflurane in 95% O\(_2\). Body temperature was maintained at 36°C to 37°C on a heated platform, and ECGs and temperature were continuously monitored. Cardiac function was assessed with the use of a VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Ontario, Canada) equipped with a high-frequency 30-MHz probe, as described.\(^{20,21}\) Data analysis was performed with the use of VisualSonics software (VisualSonics).

**ECG Telemetry**

Twenty-three animals (11 S2814D and 12 WT) were studied with ECG telemetry according to published methods\(^{22}\) (see the online-only Data Supplement).

**Programmed Electric Stimulation**

Atrial and ventricular intracardiac electrograms were recorded with the use of a 1.1F octapolar electrode catheter (EPR-800; Millar Instruments, Houston, Tex) inserted into the right ventricle via the right jugular vein, as described in the online-only Data Supplement.\(^{23}\)
Transverse Aortic Constriction
TAC was performed as described previously in detail (see also the online-only Data Supplement).21,24,25

Statistical Analysis
Continuous variables were expressed as mean±SEM; whenever the distribution was skewed, medians with the first and third quartiles were expressed. Continuous variables were evaluated with an unpaired Student t test or ANOVA. The Mann-Whitney test was used to compare continuous variables with a skewed distribution. Categorical data were expressed as percentages and were compared with the Fisher exact test. The Kaplan-Meier survival curve was evaluated by the log-rank test. 

P≤0.05 was considered statistically significant.

Results
We generated a knockin mouse model in which aspartic acid replaces serine at RyR2–2814 (S2814D) to mimic constitutive phosphorylation of RyR2 by CaMKII (Figure I in the online-only Data Supplement). CaMKII can phosphorylate RyR2 immunoprecipitated from WT hearts but not from S2814D mice (Figure 1A). Furthermore, CaMKII inhibition by KN-93 prevents RyR2 phosphorylation by CaMKII in WT hearts but has no effect in S2814D hearts, indicating that S2814 is the major CaMKII target site of RyR2 (Figure 1A).

FKBP12.6 binding to RyR2 can alter RyR2 function, but we found that the S2814D mutation did not alter FKBPP12.6 binding to RyR2 in cardiac homogenates (Figure 1B), as found in an earlier report.19 At baseline, cardiac structure and function are similar in young (3-month-old) WT and S2814D mice as determined by echocardiography (Table and Table I in the online-only Data Supplement). Transverse hematoxylin-eosin–stained sections from WT and S2814D hearts showed that there were no significant differences in right ventricular wall thickness (WT, 0.58±0.17 mm; S2814D, 0.47±0.14 mm; P=0.10), left ventricular (LV) posterior wall thickness (WT, 0.74±0.21 mm; S2814D, 0.74±0.22 mm; P=0.91), or LV anteroposterior diameter (WT, 3.51±1.02 mm; S2814D, 3.63±1.05 mm; P=0.41) (Figure 1C and 1D). Quantitative analysis of Masson’s trichrome stainings revealed no differences in the amount of interstitial fibrosis when WT (0.06±0.02% of total surface area) and S2814D mouse hearts (0.07±0.02%; P=0.61) (Figure 1E and 1F) were compared. Wheat germ agglutinin staining and quantification of myocardial cell size revealed no differences in myocyte surface area in WT (2311±166 μm²) and S2814D (2315±256 μm²) mouse hearts (P=0.99) (Figure 1G).

Figure 1. Baseline molecular and structural characteristics of S2814D mice. A, The S2814D mutation prevents CaMKII-mediated phosphorylation of RyR2. Equal amounts of RyR2 protein were immunoprecipitated from WT and S2814D hearts and phosphorylated with CaMKII in the presence or absence of KN93. Shown is a representative example of an experiment repeated twice. B, Coimmunoprecipitation showing that the binding of FKBPP12.6 to RyR2 was not altered in S2814D mice. IB indicates immunoblot; IP, immunoprecipitant. C, Representative histological sections of 3-month-old mouse hearts stained with hematoxylin and eosin (H&E). Bar=1 mm. D, Cardiac wall dimensions assessed from hematoxylin-eosin–stained histological sections. LV anteroposterior diameter (APD), LV posterior wall diameter (LVPWD), and right ventricular wall diameter (RVD) were similar among WT and S2814D hearts. E, Masson’s trichrome (MT) staining for fibrosis of histological sections of 3-month-old mouse hearts. Bar=30 μm. F, Percent fibrosis quantified from Masson trichrome stain. G, Representative wheat germ agglutinin (WGA) fluorescence stains of mouse cardiomyocytes. Bar=10 μm. H, Cardiomyocyte surface area (SA) calculated from wheat germ agglutinin stains. I, Western blot and phosphorylation assays showing baseline protein expression and phosphorylation of RyR2 at baseline. On Western blot, expressions of the cardiac RyR2, the L-type Ca²⁺ channel (Cav1.2), NCX, and CaMKII were not statistically different between WT and S2814D mouse hearts at rest. GAPDH was used as a loading control for Western blots. Number of animals (number of cells) is indicated in bar graphs.
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**Table.** Echocardiographic Parameters of WT and S2814D Mice at 3 and 12 Months of Age

<table>
<thead>
<tr>
<th></th>
<th>Age 3 mo WT (n=14)</th>
<th>S2814D (n=14)</th>
<th>Age 12 mo WT (n=14)</th>
<th>S2814D (n=14)</th>
<th>P</th>
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<tr>
<td>Heart rate, bpm</td>
<td>448.1±12.2</td>
<td>455.5±11.0</td>
<td>484.6±12.4</td>
<td>459.6±10.6</td>
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<td>Ejection fraction, %</td>
<td>59.4±0.7</td>
<td>59.1±0.7</td>
<td>56.9±0.7</td>
<td>52.4±1.8</td>
<td>0.87</td>
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<tr>
<td>LV fractional shortening, %</td>
<td>31.2±1.1</td>
<td>31.0±0.7</td>
<td>30.3±0.7</td>
<td>27.4±0.9</td>
<td>0.85</td>
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<td>End-systolic diameter, mm</td>
<td>2.68±0.10</td>
<td>2.76±0.07</td>
<td>2.85±0.06</td>
<td>3.13±0.09</td>
<td>0.53</td>
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<tr>
<td>End-diastolic diameter, mm</td>
<td>3.89±0.09</td>
<td>3.99±0.07</td>
<td>4.09±0.06</td>
<td>4.30±0.08</td>
<td>0.39</td>
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<td>IVSs, mm</td>
<td>0.90±0.03</td>
<td>0.89±0.03</td>
<td>0.94±0.02</td>
<td>0.95±0.03</td>
<td>0.82</td>
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<tr>
<td>IVSd, mm</td>
<td>0.67±0.03</td>
<td>0.67±0.03</td>
<td>0.72±0.03</td>
<td>0.77±0.03</td>
<td>0.93</td>
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<tr>
<td>LVPWs, mm</td>
<td>0.99±0.05</td>
<td>0.95±0.03</td>
<td>1.06±0.03</td>
<td>1.13±0.05</td>
<td>0.84</td>
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<tr>
<td>LVPWd, mm</td>
<td>0.68±0.05</td>
<td>0.66±0.03</td>
<td>0.73±0.03</td>
<td>0.84±0.04</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. IVSs/IVSd indicates intraventricular septal wall thickness in systole/diastole; LVPWs/LVPWd, LV posterior wall thickness in systole/diastole. Student’s t test was used to compare intragroup differences. P values between WT and S2814D mice at 3 and 12 months are shown.

1G and 1H). On Western blots, protein expression from WT and S2814D heart lysates with the use of antibodies against cardiac RyR2, L-type Ca\(^{2+}\) channel, NCX, and CaMKII was not statistically different (L-type Ca\(^{2+}\) channel, P=0.66; NCX, P=0.17; CaMKII, P=0.61) (Figure 1I).

At the myocyte level, HF and CaMKII overexpression (and activation) enhance SR Ca\(^{2+}\) leak (manifested as increased Ca\(^{2+}\) sparks or waves mediated by RyR2) and can thus serve as the molecular trigger for arrhythmia. To determine how SR Ca\(^{2+}\) leak is altered in young S2814D and S2814A mice, we used confocal microscopy to image local SR Ca\(^{2+}\) release events, or Ca\(^{2+}\) sparks, in permeabilized isolated cardiomyocytes (Figure 2A). At baseline, Ca\(^{2+}\) spark frequency was significantly increased in S2814D (9.8±0.5/s per 100 μm) versus WT mice (6.4±0.3/s per 100 μm; P<0.001; Figure 2A and 2B). Activation of endogenous CaMKII significantly increased Ca\(^{2+}\) spark frequency in WT myocytes (9.9±0.5/s per 100 μm) but had no additional effect in S2814D cells (which were already at this higher level: 10.2±0.6/s per 100 μm). In S2814A myocytes, Ca\(^{2+}\) spark frequency was comparable to WT at baseline both before (5.1±0.1/s per 100 μm) and after activation of CaMKII (6.1±0.2/s per 100 μm). The small rise with CaMKII was entirely attributable to enhanced SR Ca\(^{2+}\) content (Figure 2C and 2D) and did not significantly alter Ca\(^{2+}\) spark amplitude, full duration at half-maximum, full width at half-maximum, or maximum Ca\(^{2+}\) release (Figure II in the online-only Data Supplement). Inclusion of the specific CaMKII inhibitory peptide AIP (1 μmol/L) prevented CaMKII-dependent activation of Ca\(^{2+}\) spark frequency in WT cells but did not alter Ca\(^{2+}\) spark frequency in S2814D myocytes (Figure 2B). Combined, these results show that constitutive RyR2-S2814 pseudophosphorylation mimics maximal CaMKII activation of WT RyR2 in ventricular myocytes and that S2814D is the only functionally important CaMKII site with respect to these measurements.

We also measured Ca\(^{2+}\) transients, Ca\(^{2+}\) sparks, and SR Ca\(^{2+}\) load in intact ventricular myocytes. Twitch Ca\(^{2+}\) transient amplitude during electric field stimulation (1 Hz)
was similar in WT, S2814D, and S2814A cardiomyocytes (P=0.43; Figure 3A and 3B). However, the time constant of twitch [Ca\(^{2+}\)] decline was significantly lower in S2814D (403.8±18 ms; P<0.05) compared with WT (274.8±13 ms) and S2814A (238±18 ms). Moreover, SR Ca\(^{2+}\) load (assessed by rapid application of 10 mmol/L caffeine) was 50% lower in S2814D compared with WT or S2814A myocytes (P=0.02; Figure 3A and 3C). This is presumably due to the slightly reduced SERCA2a function and higher diastolic SR Ca\(^{2+}\) leak in S2814D myocytes (see above) and also evidenced by significantly higher Ca\(^{2+}\) spark frequency in intact S2814D myocytes versus WT or S2814A myocytes (Figure IIIA and IIIB in the online-only Data Supplement). However, other parameters such as Ca\(^{2+}\) spark amplitude, full duration at half-maximum, full width at half-maximum, and rate of rise were unchanged among the groups (Figure IIC through IIIF in the online-only Data Supplement Figure). Note that CaMKIIδc transgenic mouse myocytes and rabbit HF myocytes also exhibit enhanced leak and reduced SR Ca\(^{2+}\) load.9,12 There was no statistical difference in NCX function, measured as the time constant of [Ca\(^{2+}\)] decline during a caffeine-induced Ca\(^{2+}\) transient, among the 3 mouse groups: WT (τ=1.9±0.2 seconds), S2814D (τ=2.2±0.6 seconds), and S2814A (τ=1.7±0.2 seconds) (P=0.54). However, the S2814D mice exhibited enhanced fractional SR Ca\(^{2+}\) release (ratio of twitch/caffeine-induced Ca\(^{2+}\) transient) compared with WT or S2814A mice (P<0.001; Figure 3D). Thus, S2814D mice maintain normal Ca\(^{2+}\) transients (and cardiac function) with a smaller SR Ca\(^{2+}\) load but a larger fractional release at each contraction. This is consistent with prior work suggesting that CaMKII-dependent RyR2 phosphorylation sensitizes RyR2 to a given Ca\(^{2+}\) current trigger at a given SR Ca\(^{2+}\) load.27 Thus, RyR2 phosphorylation at S2814 activates both diastolic and systolic RyR2 Ca\(^{2+}\) release.

To directly assess RyR2 single-channel opening, we extracted microsomes containing RyR2 from young WT and S2814D mouse hearts and reconstituted them in lipid bilayers. RyR2-S2814D channels exhibited much higher open probability (54.3% [interquartile range, 25.8% to 86.5%]) compared with RyR2 from WT mice (1.0% [interquartile range, 0.9% to 2.5%]; P<0.001; Figure 3E and 3F). It is likely, however, that the relative increase in open probability of S2814D mutant channels will be more modest in vivo because the frequency of Ca\(^{2+}\) sparks in S2814D myocytes was only increased 2-fold. The single-channel and whole-cell experiments demonstrate that pseudophosphorylation of RyR2 at the S2814 CaMKII site increases the open probability of RyR2, resulting in diastolic Ca\(^{2+}\) leak.

We then used young (3- to 4-month-old) S2814D knockin mice to evaluate the effects of CaMKII-mediated RyR2 phosphorylation on arrhythmogenesis in structurally normal hearts. ECG telemeters were implanted in both WT and S2814D mice to allow recording of ambulatory ECG waveforms. S2814D mice had normal heart rhythm at rest, with unaltered electrophysiological parameters such as heart rate, depolarization intervals (PQ, QRS), and repolarization intervals (QTc) (Table II in the online-only Data Supplement). Moreover, the ventricular effective refractory period was also unaffected in S2814D mice (Table III in the online-only Data Supplement). However, when challenged with the β-adrenergic agonist isoproterenol (0.5 mg/kg IP), S2814D mice exhibited a significantly higher increase in premature ventricular complexes versus WT (10.0 [interquartile range, 6.0 to 14.0] versus 3.8 [interquartile range, 1.0 to 4.8];
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P = 0.002), which is indicative of ventricular ectopic arrhythmic activity (Figure 4A and 4B).

Given that hearts of S2814D mice were structurally and electrically normal at rest but exhibited ventricular ectopy upon β-adrenergic stimulation, we considered that the model phenotype might resemble catecholaminergic polymorphic ventricular tachycardia. To further test S2814D mice for predisposition to ventricular arrhythmias under more stringent catecholaminergic conditions,5,28 we injected caffeine and epinephrine (120 mg/kg and 2 mg/kg IP, respectively). There was a significantly increased incidence of sustained ventricular tachycardia in S2814D mice (71%) compared with WT mice (13%; P = 0.04) (Figure 4C and 4D). This is consistent with the presence of a proarrhythmogenic substrate caused by CaMKII pseudophosphorylation of RyR2. One S2814D mouse exhibited persistent ventricular tachycardia after caffeine and epinephrine that deteriorated into bradycardia and then asystole (Figure 4E). Thus, CaMKII-mediated RyR2 phosphorylation promotes in vivo ventricular arrhythmias and increases the risk of SCD.

To further differentiate the role of CaMKII activation of RyR2 from the effects of PKA activation, we sought to examine the role of elevated heart rate on arrhythmogenesis by performing in vivo intracardiac electrophysiology studies. Programmed electric stimulation with the use of ventricular burst pacing was performed to compare cardiac susceptibility with ventricular ectopic activity in WT and S2814D mice. Burst pacing evoked sustained ventricular tachycardia in 53% of S2814D mice compared with 6% of WT mice (P = 0.006; Figure 5A and 5B). Consistent with previous studies,19 rapid pacing induced CaMKII but not PKA phosphorylation of RyR2, whereas the S2814D mutation inhibited CaMKII phosphorylation of RyR2 underpaced and unpaced conditions (Figure 5 and Figure IVA and IVB in the online-only Data Supplement). In contrast, pacing increased phospholamban phosphorylation at the CaMKII site T17 (Figure 5E and 5F) but not the PKA site S16 (Figure IVC and IVD in the online-only Data Supplement). In contrast, pacing increased phospholamban phosphorylation at the CaMKII site T17 (Figure 5E and 5F) but not the PKA site S16 (Figure IVC and IVD in the online-only Data Supplement).

Finally, treatment with the β-adrenergic receptor blocker propranolol (3 mg/kg) did not significantly alter the incidence of ventricular arrhythmia induction (S2814D, 50%; WT, 0%) (Figure 5B). Because sudden changes in heart rate may induce a reflex sympathetic response and change in blood pressure, we also performed control experiments in which the arterial blood pressure was continuously monitored while the right ventricle was paced from 500 to 800 bpm at 100-bpm intervals. The blood pressure at a pacing rate of 800 bpm was significantly higher (3.1 ±0.1%; P = 0.88) than at 500 bpm, thus excluding the possibility that a reflex sympathetic response due to blood pressure changes is evoked in mice receiving electrophysiology studies.

To test whether CaMKII targets other than RyR2 may contribute to the observed arrhythmogenesis, we crossed S2814D mice with AC3I transgenic mice, in which the CaMKII-inhibitory peptide AC3I reduces CaMKII activity in the heart.17 S2814D:AC3I double-mutant mice experienced a slight decline in arrhythmia incidence (33% versus 53% in S2814D), suggesting that the effects of CaMKII on other targets, such as L-type Ca2+ current29 or phospholamban, might promote SR Ca2+ loading and arrhythmogenesis. However, when these experiments were repeated after injec-
tion of the β-adrenergic receptor agonist isoproterenol (0.5 mg/kg IP), the incidence of sustained ventricular tachycardia was not reduced in S2814D-AC3I mice (66%) (Figure 5B). These results suggest that PKA activation and phosphorylation of Ca^{2+} handling proteins (eg, phospholamban, L-type Ca^{2+} channel, and RyR2), even in the absence of CaMKII activation, can also enhance SR Ca^{2+} loading and promote arrhythmias in S2814D mice.

Previously, other groups have demonstrated that transgenic overexpression of CaMKII-δ induces HF in mice, and therefore we sought to define the specific role of CaMKII phosphorylation of RyR2 in progression to HF. As mentioned above, S2814D mice had no significant echocardiographic differences compared with WT littermates at 3 months of age (Table). However, at 12 months of age, S2814D mice demonstrated a significant increase in LV posterior wall diameter and end-diastolic diameter and a small but significant decrease in ejection fraction (52.4±1.8%) compared with WT mice (56.9±0.7%; P=0.005) (Figure 6A through 6C and Table; P<0.05).

Additionally, we performed TAC in young (3- to 4-month-old) S2814D and WT mice to evaluate the effects of constitutive CaMKII phosphorylation of RyR2 on the development of HF and arrhythmias. At 4 weeks after TAC, CaMKII phosphorylation of RyR2 was not significantly elevated in WT mice (Figure VA and VB in the online-only Data Supplement). Moreover, phosphorylation of S2808 on RyR2 was not altered in WT and S2814D mice after TAC (Figure VC and VD in the online-only Data Supplement). However, survival was significantly lower for S2814D mice (40%) compared with WT mice (91%; P=0.02) 3 weeks after TAC surgery (Figure 6D). To determine whether the difference in survival was caused by arrhythmias, we repeated TAC studies in 5 WT and 5 S2814D mice in which a telemetric ECG transmitter was implanted 1 week before TAC. These studies revealed that the 2 S2814D mice in this group that died after TAC experienced episodes of ventricular arrhythmias immediately preceding death, whereas none of the WT mice died within 3 weeks after TAC (Figure 6E). These results implicate CaMKII phosphorylation of RyR2 as an important factor contributing to arrhythmogenesis and sudden death in HF.

Next, we assessed whether prevention of CaMKII-mediated phosphorylation of RyR2 at S2814 could ameliorate ventricular arrhythmogenesis in mice with HF. To test this hypothesis, we used knockin mice in which S2814 of RyR2 is replaced by alanine (S2814A) to genetically inhibit CaMKII phosphorylation of RyR2. Surgical TAC was performed in young (3- to 4-month-old) WT and S2814A mice to induce
HF. After echocardiography at 8 weeks after TAC, WT and S2814A mice were matched such that, on average, both groups exhibited equal levels of cardiac dysfunction (Table IV in the online-only Data Supplement). Similar to patients with HF,30 WT mice subjected to TAC developed an increased propensity toward ventricular arrhythmias. Programmed electric stimulation revealed that 75% (6 of 8) of WT mice developed nonsustained ventricular tachycardia after overdrive pacing at 8 weeks after TAC (Figure 7A and 7B). In contrast, only 14% (1 of 7) of S2814A mice developed nonsustained ventricular tachycardia (P=0.04). Western blots with the use of a phosphoepitope-specific

**Figure 6.** CaMKII phosphorylation of RyR2 causes cardiac dilation, loss of contractility, and early death from arrhythmias. Echocardiographic measurements of LV posterior wall diameter (LVPWD) (A), end-diastolic diameter (EDD) (B), and ejection fraction (EF) (C) in S2814D and WT mice from 3 to 12 months are shown. S2814D mice have significantly increased diastolic dimensions and reduction in ejection fraction vs WT mice (n=14, both groups). D, Kaplan-Meier survival curve in S2814D (n=10) and WT (n=11) mice 3 weeks after TAC. E, Representative ECG tracing of an arrhythmogenic death in a S2814D mouse that died after TAC surgery. Sudden death after ventricular tachycardia (VT) was observed only in S2814D mice and in none of the WT mice after TAC. *P<0.05 vs WT. Student t test was used to compare echocardiographic data. A log-rank test was used to evaluate survival analysis.

**Figure 7.** Genetic blockade of CaMKII phosphorylation of RyR2 reduces risk for pacing-induced ventricular ectopy. A, Representative ECG tracing of pacing-induced nonsustained ventricular tachycardia (NSVT) in WT mice 8 weeks after TAC (top), whereas the S2814A TAC mice typically showed sinus rhythm (bottom). Arrows indicate two last paced beats and final extrastimulus. B, Bar graph showing incidence of pacing-induced nonsustained ventricular tachycardia in WT and S2814A mice at 8 weeks after TAC. C, Representative Western blots showing total RyR2 and CaMKII-phosphorylated RyR2 at S2814 in WT and S2814A mice at 8 weeks after TAC. D, Bar graphs showing averaged ratio between phosphorylated RyR2-pS2814 and total RyR2. E, Representative Western blots showing total RyR2 and PKA-phosphorylated RyR2 at S2808 in WT and S2814A mice at 8 weeks after TAC. F, Bar graphs showing averaged ratio between phosphorylated RyR2-pS2808 and total RyR2. N is shown in bar graphs. Fisher exact test was used to compare nonsustained ventricular tachycardia incidence; Student t test was used to compare Western blot data. *P<0.05 vs WT surgically matched control.
antibody revealed increased CaMKII phosphorylation of RyR2 in WT mice after TAC, whereas the S2814 phosphorylation site could not be phosphorylated in S2814A mice, as expected (Figure 7C and 7D). In contrast, phosphorylation of the PKA site S2808 was not altered in WT and S2814A mice compared with sham-operated controls (Figure 7E and 7F). These results suggest that CaMKII phosphorylation of S2814 on RyR2 is an essential signaling event that promotes ventricular arrhythmias in TAC-induced HF. Taken together, our data in S2814D and S2814A mice demonstrate that CaMKII phosphorylation of RyR2 at this site is critical for the development of cardiac arrhythmia.

**Discussion**

Previous work has demonstrated that increased CaMKII activity in failing hearts may contribute to abnormal Ca\(^{2+}\) handling, contractile failure, and arrhythmogenesis.\(^9,13\) The novel knockin mice, which express only the CaMKII phosphomimetic S2814D-mutant RyR2 or only nonphosphorylatable S2814A at this RyR2 site, have allowed unique tests of the importance of CaMKII phosphorylation of RyR2 at the cardiomyocyte and whole-animal level. First, both biochemical and functional data (Figure 1A and 1B) indicate that RyR2 phosphorylation at S2814 is the principal and possibly only site mediating CaMKII phosphorylation of RyR2 in mouse myocytes under physiological conditions. There may be other CaMKII target sites on RyR2,\(^31\) but they are obvious neither in our CaMKII-dependent \(^32\)P incorporation into S2814D mutant RyR2 nor at the functional level, as evidenced by Ca\(^{2+}\) release events. Additionally, CaMKII activation in WT myocytes did not produce a stronger increase in the Ca\(^{2+}\) spark frequency than the S2814D myocytes exhibit at baseline. In addition, in the S2814A myocytes, the very small CaMKII-induced increase in Ca\(^{2+}\) spark frequency was explained by an enhanced SR Ca\(^{2+}\) load (presumably secondary to SR Ca\(^{2+}\)-ATPase stimulation via CaMKII-dependent phospholamban phosphorylation). Note that a primary enhancement of RyR2 activity would decrease rather than increase SR Ca\(^{2+}\) load.

A second major conclusion is that constitutive activation of the RyR2 CaMKII site increases the risk for ventricular arrhythmias in vivo, even in the absence of structural heart disease in young mice. Quantitative analysis of cardiac dimensions, fibrosis, and cardiac myocyte size revealed that these dimensions, fibrosis, and cardiac myocyte size revealed that structural remodeling is unlikely to be a significant cause of arrhythmogenesis in young S2814D mice. The elevated arrhythmia risk is also independent of altered binding of FKBP12.6 and can occur in the absence of increased PKA activity on elevated heart rate. Interestingly, even though S2814D mice exhibit an increased incidence of Ca\(^{2+}\) sparks, ectopic activity was not observed under resting conditions. At baseline, the myocytes may be effectively compensated by the combination of reduced SR Ca\(^{2+}\) load and increased fractional SR Ca\(^{2+}\) release during excitation-contraction coupling, such that the diastolic SR Ca\(^{2+}\) leak is insufficient to produce triggered arrhythmias unless SR Ca\(^{2+}\) load is driven toward WT levels (for example, after phosphorylation of phospholamban).

Our findings have potentially important clinical implications with respect to arrhythmias in HF because CaMKII expression, activity, and phosphorylation of RyR2 at S2814, with resultant diastolic SR Ca\(^{2+}\) leak, are all enhanced in patients and animals with HF.\(^9,16\) The level of S2814 phosphorylation is increased by \(\sim 50\%\) to 100\% in failing hearts compared with nonfailing control hearts.\(^9,16,32,33\) Most of our studies were performed in homozygous S2814D mice to determine the physiological consequences of RyR2 phosphorylation. Whereas it is unlikely that S2814 on RyR2 will be maximally phosphorylated for prolonged amounts of time in patients or animals with HF, this model enabled us to elucidate the specific effects of RyR2 phosphorylation by CaMKII. Preliminary studies in heterozygous S2814D mice revealed arrhythmia incidences similar to those of homozygous S2814D mice (2 of 3 developed pacing-induced arrhythmia after isoproterenol administration), suggesting that our data may also be relevant as a model of RyR2 hyperphosphorylation seen in failing hearts.

Indeed, our findings suggest that CaMKII-dependent RyR2 phosphorylation may be a critical mediator of the high incidence of arrhythmias in human HF. Moreover, our data suggest that among the several CaMKII targets that have the potential to be proarrhythmic (Na\(^+\), Ca\(^{2+}\), and K\(^+\) channels), the RyR2 effects may predominate. The activating effect of CaMKII on RyR2 resembles the gain-of-function phenotype seen in inherited RyR2 mutations that are associated with catecholaminergic polymorphic ventricular tachycardia. On the other hand, our data show that ventricular arrhythmias can be triggered in S2814D mice in the absence of increased PKA activation and PKA phosphorylation of RyR2 and phospholamban. Conversely, \(\beta\)-adrenergic stimulation increased the likelihood of arrhythmogenesis, possibly because of enhanced Ca\(^{2+}\) entry with increased SR Ca\(^{2+}\) loading via SERCA2a/phospholamban. On the basis of prior studies,\(^34\) enhanced SR Ca\(^{2+}\) release via RyR2 is likely sufficient to trigger Ca\(^{2+}\) waves and depolarizing transient inward current via NCX, although this remains to be confirmed in S2814D mice in future studies.

A third conclusion is that when RyR2 S2814 cannot be phosphorylated (S2814A), arrhythmias associated with the development of HF are prevented. This extends the aforementioned conclusion in an important way: In addition to being proarrhythogenic in normal and failing hearts, CaMKII-dependent phosphorylation of RyR2-S2814 may be an essential component for the triggered arrhythmias in HF, at least in the context of pressure overload–induced HF, as examined here. This may extend to recent observations that CaMKIIΔ knockout mice exhibit reduced transition to HF (and reduced RyR2-S2814 phosphorylation) during similar pressure overload–induced HF.\(^16\) The dramatic increase in diastolic SR Ca\(^{2+}\) leak (and Ca\(^{2+}\) spark frequency) in CaMKIIΔ transgenic mice reduces SR Ca\(^{2+}\) load severely enough to depress systolic dysfunction, despite enhanced fractional SR Ca\(^{2+}\) release.\(^13\) Thus, CaMKII-dependent phosphorylation of RyR2-S2814 may be critical in both arrhythmogenesis and systolic dysfunction in HF.

Prior reports suggested that PKA-dependent hyperphosphorylation of RyR2 at S2808 and consequent FKBP12.6
dissociation are causative in the enhanced SR Ca\(^{2+}\) leak and arrhythmias in HF.\(^{35,36}\) Although key aspects of these observations remain highly controversial.\(^{37}\) Our results here do not resolve this controversy, although, in our TAC model, RyR2 phosphorylation at the CaMKII site and not the PKA site was increased. Moreover, it is clear that CaMKII-dependent phosphorylation of RyR2 (or pseudophosphorylation) at S2814, which occurs in HF, can strongly activate RyR2 in myocytes and increase the propensity for arrhythmias and may even accelerate the transition from hypertrophy to HF.\(^{21}\)

It has been shown that inhibition of calmodulin binding to RyR2 by selective mutations in the binding site leads to a severe phenotype characterized by early-onset cardiomyopathy and postnatal death between days 9 and 16.\(^{38}\) Because calmodulin is thought to inhibit RyR2, disrupting the calmodulin binding site on RyR2 may lead to increased Ca\(^{2+}\) release. However, the functional effects of disrupting calmodulin binding appear to be more severe compared with increased Ca\(^{2+}\) release by constitutive CaMKII phosphorylation (RyR2-ADA mice are lethal before weaning; RyR2-S2814D mice appear to have a normal lifespan). Thus, SR Ca\(^{2+}\) leak and ultimately death from ventricular arrhythmias but not severe enough to cause early-onset cardiomyopathy due to activation of Ca\(^{2+}\)-dependent hypertrophic signaling pathways.

In conclusion, our data suggest that increased CaMKII phosphorylation of RyR2 Ca\(^{2+}\) release channels at S2814 promotes the development of ventricular arrhythmias in mice even in the absence of structural heart disease. Our findings may have clinical implications with respect to arrhythmias in patients with congestive HF because the activity of CaMKII is chronically enhanced in failing hearts. We have also demonstrated that inhibition of CaMKII phosphorylation of RyR2 can prevent ventricular arrhythmias in mice with HF. Thus, the effects of CaMKII on RyR2 may be a critical factor in the generation of arrhythmias in patients with HF, although this remains to be studied in humans. Our findings may lead to the development of more specific therapies that could modify the level of CaMKII phosphorylation of RyR2, thus reducing diastolic Ca\(^{2+}\) leak and ultimately death from arrhythmias. Such novel antiarrhythmic agents, once tested in randomized trials, may ultimately provide new avenues for treating ventricular arrhythmias and preventing sudden cardiac death in patients with HF.

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Disclosures

Dr Anderson is a named inventor on several patents that claim to treat arrhythmias by CaMKII inhibition.

References

Despite recent therapeutic advances including β-adrenergic blockers and implantable cardioverter-defibrillators, ventricular arrhythmias remain a prominent cause of death in patients with heart failure. Diastolic Ca\(^{2+}\) leak from the sarcoplasmic reticulum is believed to contribute to arrhythmia initiation in failing hearts, although the underlying mechanisms remain poorly understood. The expression and activity of the enzyme Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) are implicated in arrhythmogenesis. Although increased CaMKII activity has been implicated in arrhythmogenesis, the specific CaMKII targets contributing to arrhythmia susceptibility have remained elusive. Our data revealed that mice in which the CaMKII phosphorylation site on the cardiac ryanodine receptor is constitutively activated exhibit an increased likelihood of ventricular arrhythmias. Moreover, constitutive CaMKII phosphorylation of ryanodine receptor causes an increase in arrhythmogenic sudden cardiac deaths after induction of experimental heart failure. Conversely, mice with genetic ablation of the CaMKII site on ryanodine receptor shows protection from intracellular calcium leak and arrhythmias. Protection from cardiac arrhythmia through ryanodine receptor-stabilizing protein calstabin2. Science. 2004;304:292–296.

CLINICAL PERSPECTIVE

van Oort et al  CaMKII Phosphorylation of RyR2 Causes Arrhythmias

"leak" leads to an increased susceptibility to ventricular tachycardia in mice. Proc Natl Acad Sci U S A. 2006;103:7906–7910.

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Ryanodine Receptor Phosphorylation by Calcium/Calmodulin-Dependent Protein Kinase II Promotes Life-Threatening Ventricular Arrhythmias in Mice With Heart Failure

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Animals. Generation of RyR2-S2814A knock-in and AC3I transgenic mice was described previously \(^1\)\(^2\). RyR2-S2814D knock-in mice were created using a similar approach described for RyR2-S2814A knock-in mice \(^1\). Briefly, a genomic clone containing exons 56 and 57 of the mouse Ryr2 gene was cloned into a pDTA4B vector using homologous recombination. The S2814D mutation was introduced along with a silent Clal restriction site into exon 56 of Ryr2 by site-directed mutagenesis. The final targeting vector was obtained by cloning a lox P-flanked Neo cassette into intron 56 of Ryr2. After linearization with Pmel, the targeting vector was electroporated into AB2.2 129Sv/J ES cells. Successfully targeted ES cell clones were identified by Southern blot and injected into blastocysts to generate chimeric mice. Germline transmission was verified by PCR analysis and positive offspring was crossed with Meox2-Cre mice to allow genetic excision of the Neo cassette. Heterozygous RyR2-S2814D mice were mated to obtain RyR2-S2814D homozygous knock-in mice and WT littermates. The RyR2-S2814A and RyR2-S2814D knock-in mice, and AC3-I transgenic mice, have all been backcrossed into C57Bl/6 mice for 7-10 generations. Homozygous RyR2-S2814A and RyR2-S2814D knock-in mice were littermates of the WT mice. Numbers of mice studied includes: WT (n = 89), S2814D (n = 66), S2814D x AC3I (n = 24), and S2814A (n = 16). All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine conforming the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Co-Immunoprecipitation Assay. RyR2 was immunoprecipitated from heart lysates using an anti-RyR2 antibody (Thermo Scientific, Rockland, IL) incubated with Protein A-Sepharose beads
(Rockland, Gilbertsville, PA) at room temperature for 1 h. For co-immunoprecipitation, antibody-attached beads were incubated with heart lysate aliquots containing 1000 μg total protein at 4°C overnight. The 1 mL reaction consisted of Co-IP buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% CHAPS, 20 mM NaF, 1 mM Na₃VO₄, 1x protease inhibitors and 1x phosphatase inhibitors (Roche, Basel, Switzerland) and 0.3% Triton x-100. Post-incubation, beads were washed with detergent-free Co-IP buffer, and resuspended in 2x LDS buffer (Invitrogen, Carlsbad, CA) containing β-mercaptoethanol. Samples were heated at 50°C for 15 min, and were resolved on 4-20% Criterion SDS-PAGE gels (Bio-Rad) for detection of RyR2 and FKBP12.6. Western blotting on resolved gels was performed as described below using monoclonal anti-RyR2 antibody and polyclonal anti-FKBP12.6 antibody (Thermo Fisher).

**Western blot analyses.** Heart lysates were prepared from flash-frozen mouse hearts as described previously ³. Lysates were taken from mice at rest (Fig. 1), post-TAC or post-sham surgery (Fig. 7 and S5), immediately after pacing (Fig. 5 and S4), and with/without exposure to pacing (Fig. 7). Heart lysate aliquots were size-fractionated on 6% (for RyR2) or 12% (for Cav1.2, NCX1, SERCA2a, CaMKII, and GAPDH) or 15% (for PLN) SDS-polyacrylamide gels. For PLN-monomer blots, heart lysates were heated at 70 °C for 10 min in 1x sample loading buffer containing 5% β-mercaptoethanol before loading on gels. The resolved gels were electro-transferred on PVDF membranes. The membranes were probed with anti-pSer2808-RyR2 (1:1,000), anti-pSer2814-RyR2 (1:500) (both custom-made for our lab ¹ ³), anti-pSer16-PLN (1:5,000), anti-pThr17-PLN (1:2,500) (latter two from Badrilla Ltd., Leeds, United Kingdom), anti-CaMKIIδ (1:200), anti-Cav1.2 (1:200; Alomone Labs, Jerusalem), anti-NCX1 (1:500; Swant, Bellinzona, Switzerland), anti-SERCA2a (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal antibody, anti-RyR2 (1:5,000), anti-PLN (1:1000) (both Thermo Fisher Scientific (Pierce), Rockford, IL), or anti-GAPDH (1:5,000; Millipore, Temecula, CA) monoclonal antibody
at 4°C either overnight or at room temperature for 4 h. They were developed using Alexa-Fluor680-conjugated anti-mouse (Invitrogen Molecular Probes, Carlsbad, CA) and/or IR800Dye-conjugated anti-rabbit fluorescent secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA), and scanned on an Odyssey infrared scanner (Li-Cor, Lincoln, NE). Integrated densities of protein bands were measured using ImageJ Data Acquisition Software (National Institute of Health, Bethesda, MD). Protein-signal densities were normalized to the corresponding GAPDH-signal densities, while phosphorylation-signal densities were normalized to the corresponding total protein-signal densities, and used for plotting data. Student T-tests were used to compare data in two sample groups.

**Confocal imaging.** Ca\(^{2+}\) sparks were recorded in saponin-permeabilized (50 µg/ml) or intact ventricular cardiomyocytes. Permeabilized cells were placed in an internal solution (in mM): EGTA 0.5; HEPES 10; K-aspartate 120; MgATP 5; free MgCl\(_2\) 1; reduced glutathione 10; free [Ca\(^{2+}\)] 50nM; creatine phosphokinase 5U/ml; phosphocreatine 10; dextran (MW: 40,000) 4%; fluo-4 K-salt 0.025; pH 7.2). PKA activity was prevented by using PKA inhibitory peptide PKI (15 µmol/L). Endogenous CaMKII activation was obtained by elevated [Ca\(^{2+}\)] (500 nM), exogenous calmodulin (1.2 µM) and okadaic acid (2 µM) as previously described. After incubation with endogenous CaMKII activation solution for 1 minute, myocytes were superfused with original internal solution containing 10 µM okadaic acid to wash off phosphorylating solution and preserve phosphorylation while Ca\(^{2+}\) sparks were recorded again. CaMKII activity was inhibited by AIP administration (1 µM). Intact ventricular myocytes were loaded with Fluo-3 AM (5 µM, Molecular Probes) and Ca\(^{2+}\) transients were recorded as previously described. Ca\(^{2+}\) transients were obtained by field stimulation at 1 Hz in normal Tyrode’s solution (in mM): 140 NaCl, 4 KCl, 1.1 MgCl\(_2\), 10 HEPES, 10 glucose, 1.8 CaCl\(_2\); pH7.4 with NaOH. SR Ca\(^{2+}\) load was evaluated by Ca\(^{2+}\) transient upon rapid caffeine application (10 mM). Experiments were performed on confocal microscopy (BioRad, Radiance 2100, 40x objective) using line scan mode with argon
laser ($\lambda_{\text{ex}}$ 488 nm, $\lambda_{\text{em}}$ >505 nm). Image analysis used ImageJ software and homemade routines in IDL (interactive data language).

**Single channel recordings.** The *trans* chamber (1.0 ml of 250 mM HEPES and 53 mM Ca(OH)$_2$, 50 mM KCl, pH 7.35) representing the intra-SR compartment was connected to the head stage input of a bilayer voltage-clamp amplifier. The *cis* chamber (1.0 ml of 250 mM HEPES, 125 mM Tris, 50 mM KCl, 1.0 mM EGTA, and 0.5 mM CaCl$_2$, pH 7.35) representing the cytoplasmic compartment was held at virtual ground. Free $[Ca^{2+}]$ was calculated by CHELATOR software. At the conclusion of each experiment, ryanodine (5 µmol/L) or ruthenium red (20 µmol/L) was applied to confirm RyR2 channel identity.

**ECG telemetry.** Transmitters (Data Sciences International, St. Paul, MN) were implanted in the abdominal cavity with subcutaneous electrodes in lead II configuration. Telemetry was recorded > 48 h after surgery in ambulatory, unanesthetized mice for 24 hours total for baseline measurements. Interventional analysis was performed at baseline and also after intraperitoneal (i.p.) injection of 100 µg isoproterenol (Sigma Aldrich, St. Louis). Additionally, on a separate day, ECG’s were recorded before and after caffeine and epinephrine (120 mg/kg and 2 mg/kg, respectively, Sigma Aldrich) injection i.p. Data collection, which started 1 hour before the intervention and continued for 2-3 hours after injection, was performed using Dataquest software. Off-line data analyses was performed by using ECG Auto analysis software, version 4.1 (Data Sciences International).

**Programmed electrical stimulation.** Mice were anesthetized using 1.5 % isofluorane in 95% O$_2$. ECG channels were amplified (0.1 mV/cm) and filtered between 0.05 and 400 Hz). A computer-based data acquisition system (Emka Technologies) was used to record a 6-lead body surface ECG, and up to 4 intracardiac bipolar electrograms. Bipolar right atrial pacing and
right ventricular pacing was performed using 2-ms current pulses delivered by an external stimulator (STG-3008, MultiChannel Systems, Reutlingen, Germany). Standard clinical electrophysiologic pacing protocols were used to determine all basic electrophysiologic parameters. Ventricular effective refractory period was determined at three drive cycle lengths. Overdrive pacing and single, double, and triple extrastimuli were delivered to determine inducibility of VT, which were tested twice. After baseline measurements were completed, and VT inducibility was tested via pacing, isoproterenol (0.5 mg/kg, Sigma Aldrich, St. Louis) or propranolol (3 mg/kg, Sigma Aldrich, St. Louis) were administered, and pacing protocols were repeated to assess the effects on conduction and refractoriness.

**Transverse aortic constriction.** Mice were anesthetized using a mixture of 2% isoflurane and 95% O₂. TAC was created by subjecting the aorta to a defined, 27-gauge constriction using a 6-0 suture between the first and second truncus of the aortic arch. A computer-based Doppler signal processor (Indus Instruments, Houston, TX) was used to measure Doppler velocities in the right and left carotid arteries. Right/left carotid peak velocity ratios were similar in S2814A and WT littermates, or S2814D and WT littermates, respectively.
SUPPLEMENTAL TABLES

Table S1.

ANOVA Evaluation of Echocardiographic Parameters

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<td>3.1</td>
<td>0.04</td>
</tr>
<tr>
<td>LVPWd(mm)</td>
<td></td>
<td>3</td>
<td>0.09</td>
<td>4.6</td>
<td>0.006</td>
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</tbody>
</table>

HR = heart rate; bpm = beats per minute; EF = ejection fraction; FS = left ventricular fractional shortening; ESD = end-systolic diameter; EDD = end-diastolic diameter; IVSs/ IVSd = intraventricular septal wall thickness in systole/diastole; LVPWs/ LVPWd = left ventricular posterior wall thickness in systole/diastole. 2-way ANOVA was used to compare groups. Degrees of freedom (df), mean square, the F statistic, and p values are displayed in the ANOVA statistics heading.
Table S2.

Electrophysiological intervals recorded using ECG telemetry in WT, S2814D and S2814D:AC3I mice.

<table>
<thead>
<tr>
<th></th>
<th>WT (n = 10)</th>
<th>S2814D (n = 13)</th>
<th>S2814D:AC3I (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR (ms)</td>
<td>91.6 ± 2.1</td>
<td>97.5 ± 4.4</td>
<td>99.5 ± 5.0</td>
</tr>
<tr>
<td>PQ (ms)</td>
<td>27.5 ± 0.9</td>
<td>28.4 ± 1.1</td>
<td>32.7 ± 1.7 *</td>
</tr>
<tr>
<td>QRS (ms)</td>
<td>8.9 ± 0.5</td>
<td>9.2 ± 0.4</td>
<td>10.1 ± 0.2 *</td>
</tr>
<tr>
<td>QT (ms)</td>
<td>35.1 ± 1.4</td>
<td>32.5 ± 1.5</td>
<td>38.3 ± 1.0 *</td>
</tr>
<tr>
<td>QTc (ms)</td>
<td>60.0 ± 1.4</td>
<td>55.5 ± 1.9</td>
<td>60.7 ± 2.0</td>
</tr>
<tr>
<td>SCL (ms)</td>
<td>92.3 ± 2.3</td>
<td>97.2 ± 4.6</td>
<td>98.6 ± 5.4</td>
</tr>
<tr>
<td>AV (ms)</td>
<td>33.9 ± 0.9</td>
<td>35.0 ± 0.9</td>
<td>38.7 ± 2.0</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. RR, time interval between two consecutive RR waves; PQ, interval from the beginning of the P wave to the peak of the Q wave; QRS, duration of the interval between beginning of Q wave to peak of S wave; QT, interval from beginning of Q wave to the end of the T wave; QTc, QT interval corrected for heart rate; SCL, sinus cycle length time; AV, interval from the beginning of the P wave to the beginning of the QRS complex. *P<0.05 versus WT.
Table S3.
Electrophysiological intervals in WT and S2814D mice.

<table>
<thead>
<tr>
<th></th>
<th>WT (n = 8)</th>
<th>S2814D (n = 9)</th>
<th>S2814D:AC3I (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AERP (ms)</td>
<td>31.1 ± 3.3</td>
<td>30.7 ± 0.7</td>
<td>32.5 ± 1.7</td>
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<tr>
<td>VERP (ms)</td>
<td>26.2 ± 1.5</td>
<td>24.1 ± 2.3</td>
<td>24.6 ± 0.7</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. AERP, atrial effective refractory period; VERP, ventricular effective refractory period. *P<0.05 versus WT.
Table S4.

Echocardiographic parameters of WT and S2814A mice 8 weeks post-TAC surgery

<table>
<thead>
<tr>
<th></th>
<th>8 Weeks Post-TAC</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>WT (n = 14)</td>
<td>S2814A (n = 13)</td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>475.1 ± 22.0</td>
<td>474.3 ± 13.71</td>
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<tr>
<td>EF (%)</td>
<td>36.6 ± 6.1</td>
<td>34.2 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>FS (%)</td>
<td>17.8 ± 3.2</td>
<td>16.6 ± 2.3</td>
<td></td>
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<tr>
<td>ESD (mm)</td>
<td>3.69 ± 0.37</td>
<td>3.76 ± 0.3</td>
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</tr>
<tr>
<td>EDD (mm)</td>
<td>4.45 ± 0.29</td>
<td>4.45 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>0.87 ± 0.02</td>
<td>0.84 ± 0.02</td>
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<tr>
<td>IVSd (mm)</td>
<td>0.78 ± 0.01</td>
<td>0.75 ± 0.02</td>
<td></td>
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<tr>
<td>LVPWs</td>
<td>1.06 ± 0.05</td>
<td>1.05 ± 0.05</td>
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<tr>
<td>LVPWd (mm)</td>
<td>0.83 ± 0.03</td>
<td>0.89 ± 0.03</td>
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</table>

Data are expressed as mean ± SEM. HR = heart rate; bpm = beats per minute; EF = ejection fraction; FS = left ventricular fractional shortening; ESD = end-systolic diameter; EDD = end-diastolic diameter; IVSs/IVSd = intraventricular septal wall thickness in systole/diastole; LVPWs/LVPWd = left ventricular posterior wall thickness in systole/diastole. *P < 0.05 versus WT.
SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Generation of RyR2-S2814D knockin mice. (A-E) Schematic overview of gene targeting strategy. A genomic clone harboring exon 56 and 57 of Ryr2 was isolated from a BAC (A) and subcloned using homologous recombination (B). The nucleotide sequence coding for the S to D mutation was introduced along with a silent Clal site and a lox P-flanked Neo-cassette (C) to obtain the final targeting vector. This targeting vector was electroporated into ES cells for genomic recombination (D). Positive ES cells were injected into blastocysts to generate chimeric mice. Positive offspring was crossed with Meox2-Cre mice to establish excision of the Neo cassette (E). (F) Successful recombination in ES cells was verified by Southern blot after digesting DNA with EcoRV. (G) Heterozygous mice were mated to obtain homozygous RyR2-S2814D mice and WT littermates as determined by PCR on tail digests and subsequent Clal digestion.

Figure S2. Calcium spark characteristics for permeabilized cardiomyocytes. (A) Calcium spark amplitude in permeabilized myocytes in the absence (-) or presence (+) of CaMKII. There were no differences between the genotypes. (B) Calcium spark full duration at half-maximum (FDHM) was not different among WT, S2814D, and S2814A myocytes, with or without activation of endogenous CaMKII. (C) Full width at half-maximum (FWHM) was not different among WT, S2814D, and S2814A permeabilized myocytes in the presence or absence of CaMKII. (D) Maximum release of SR Ca$^{2+}$ was not different in WT, S2814D, and S2814A permeabilized myocytes in the presence or absence of CaMKII. N is shown in bar graphs.

Figure S3. Increased calcium spark frequency in intact cardiomyocytes from S2814D mice. (A) Ca$^{2+}$ spark frequency (CaSpF) in WT, S2814D, and S2814A intact cardiomyocytes, showing that myocytes from S2814D mice exhibited a significantly increased CaSpF. (B) Ratio
of CaSpF to SR Ca$^{2+}$ load. S2814D intact myocytes had a significantly higher ratio compared to WT myocytes. (C) Mean Ca$^{2+}$ spark amplitude in WT, S2814D, and S2814A intact myocytes were not different. (D) Ca$^{2+}$ spark full duration at half-maximum (FDHM) was not different in WT, S2814D, and S2814A intact myocytes. (E) Full width at half-maximum (FWHM) was not different among WT, S2814D, and S2814A intact myocytes. (F) Ca$^{2+}$ transient time to peak measured in seconds. There were no differences among WT, S2814D, and S2814A intact myocytes. Values are expressed as mean ± SEM. N is shown in bar graphs. *$P<0.05$, **$P<0.01$.

Figure S4. Intracardiac pacing does not alter phosphorylation of PKA targets. (A) Representative Western blot of the RyR2 PKA site (S2808) in WT and S2814D mice before and after intracardiac pacing. (B) Bar graph showing that pacing did not change PKA phosphorylation of RyR2. (C) Representative Western blot of the phospholamban (PLN) PKA site (S16). (D) Bar graph showing that pacing did not change PKA phosphorylation of PLN. N is shown in bar graphs.

Figure S5. TAC surgery does not increase RyR2 phosphorylation at 4 weeks. (A) Representative Western blots showing total RyR2 and CaMKII-phosphorylated RyR2 at 2814 in WT and S2814D mice at 4 weeks after transverse aortic constriction (TAC). (B) Bar graphs showing averaged ratio between phosphorylated RyR2-pS2814 and total RyR2. At four weeks post-TAC, WT mice showed a trend towards increased phosphorylation only; CaMKII phosphorylation of RyR2 was prevented in S2814D mice. (C) Representative Western blots showing total RyR2 and PKA-phosphorylated RyR2 at S2808 in WT and S2814D mice at 4 weeks after TAC. (D) Bar graphs showing averaged ratio between phosphorylated RyR2-pS2808 and total RyR2. There were no significant differences between the groups at the PKA site. N is shown in bar graphs. *$P<0.05$ versus surgically matched controls.
Figure S1

A

B

C

D

E

F

G

WT SD

-10 kb

-4 kb

S2814D

WT HET HOM

WT S2814D
Figure S2

A

B

C

D

CaMKII - + - + - +

Amplitude (F/Fo)

WT

S2814D

S2814A

CaMKII - + - + - +

FDHM (ms)

CaMKII - + - + - +

FWHM (mm)

CaMKII - + - + - +

Max Release (DF/DT)

CaMKII - + - + - +

13
Figure S3

A

CaSpF (n/s/100 nM)

B

CaSpF/ SR Ca Load

C

Spark Amplitude (F/F0)

D

FDHM (ms)

E

FWHM (mm)

F

Time to Peak (s)

15 21 15

15 21 15

15 21 15

15 21 15

15 21 15

15 21 15
Figure S4

A

B

C

D

RyR2

565 kDa

pS2808

565 kDa

Paced - + - +

WT S2814D

6 8 4 4

Paced - + - +

0.8

1.2

1.4

pS2808/RyR2 (A.U.)

0

11 kDa

pS16

11 kDa

Paced - + - +

WT S2814D

4 4 4 4

Paced - + - +

0.8

1.2

1.4

pS16/PLN (A.U.)
Figure S5

A

<table>
<thead>
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B

![Image](image9.png)

C

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D

![Image](image18.png)
Supplemental References


