Catecholaminergic Polymorphic Ventricular Tachycardia Is Caused by Mutation-Linked Defective Conformational Regulation of the Ryanodine Receptor

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Rationale: Catecholaminergic polymorphic ventricular tachycardia (CPVT) is caused by a single point mutation in a well-defined region of the cardiac type 2 ryanodine receptor (RyR2). However, the underlying mechanism by which a single mutation in such a large molecule produces drastic effects on channel function remains unresolved.

Objective: Using a knock-in (KI) mouse model with a human CPVT-associated RyR2 mutation (R2474S), we investigated the molecular mechanism by which CPVT is induced by a single point mutation within the RyR2.

Methods and Results: The R2474S/+ KI mice showed no apparent structural or histological abnormalities in the heart, but they showed clear indications of other abnormalities. Bidirectional or polymorphic ventricular tachycardia was induced after exercise on a treadmill. The interaction between the N-terminal (amino acids 1 to 600) and central (amino acids 2000 to 2500) domains of the RyR2 (an intrinsic mechanism to close Ca²⁺ channels) was weakened (domain unzipping). On protein kinase A–mediated phosphorylation of the RyR2, this domain unzipping further increased, resulting in a significant increase in the frequency of spontaneous Ca²⁺ transients. cAMP-induced aberrant Ca²⁺ release events (Ca²⁺ sparks/waves) occurred at much lower sarcoplasmic reticulum Ca²⁺ content as compared to the wild type. Addition of a domain-unzipping peptide, DPc10 (amino acids 2460 to 2495), to the wild type reproduced the aforementioned abnormalities that are characteristic of the R2474S/+ KI mice. Addition of DPc10 to the (cAMP-treated) KI cardiomyocytes produced no further effect.

Conclusions: A single point mutation within the RyR2 sensitizes the channel to agonists and reduces the threshold of luminal [Ca²⁺] for activation, primarily mediated by defective interdomain interaction within the RyR2. (Circ Res. 2010;106:1413-1424.)

Key Words: ryanodine receptor ■ calcium ■ ventricular tachycardia ■ sarcoplasmic reticulum

To date, more than 70 cardiac ryanodine receptor (RyR2) missense mutations have been identified that are linked with 2 inherited forms of sudden cardiac death: catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right ventricular cardiomyopathy type 2. These mutations cluster in 3 well-defined regions of the RyR2 that correspond to malignant hyperthermia or the central core disease mutable regions, designated as the N-terminal domain (amino acids 1 to 600), central domain (amino acids 2000 to 2500), and the C-terminal transmembrane channel domain of the skeletal muscle–type ryanodine receptor (RyR1). This suggests that the RyR2 shares a common domain-mediated channel regulation mechanism with RyR1. Mutations at different positions in each of these domains result in the nearly identical phenotype of channel dysfunctions such as hyperactivation of the Ca²⁺ channel and hypersensitization to agonists. To account for these phenomena, Ikemoto et al proposed the so-called “domain switch hypothesis” and stated that in the resting or nonactivated state, the N-terminal domain and the central domain make close contact at several subdomains (domain zipping). Then, on physiological or pharmacological stimulation, these critical interdomain contacts are weakened, resulting in the loss of conformational constraints (domain unzipping), thus lowering the energy barrier for Ca²⁺ channel opening. Consistent with this hypothesis, single particle analysis of the 3D structure of the RyR2 molecule revealed that the N-terminal and central domains (located in domains 5 and 6 of the so-called clamp

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region, respectively) are in a close apposition to each other.\(^4,5\)

Recent reports deal with 3 types of knock-in (KI) mice with human CPVT/arhythmogenic right ventricular cardiomyopathy–associated RyR2 mutations: R4496C,\(^6\) R176Q,\(^7\) and R2474S.\(^8\) Injection of caffeine plus epinephrine or exercise induces ventricular tachycardia (VT) in these mice, indicating that these point mutations can cause lethal arrhythmias. However, the underlying mechanism by which a single mutation causes lethal arrhythmia remains unresolved. We recently reported that in failing hearts, defective interdomain interaction within the RyR2 (aberrant unzipping of the N-terminal/central domain pair and channel activation in an otherwise resting state) causes diastolic Ca\(^{2+}\) leakage and contractile dysfunction.\(^9\) As shown in our previous report,\(^9\) pathological conditions (diastolic Ca\(^{2+}\) leakage and contractile dysfunction) are reproduced in the otherwise normal system by adding DPc10, a central domain peptide (Gly2460-Pro2495) of the RyR2 that interferes with the interaction between the N-terminal and central domains of the RyR2 and causes defective domain unzipping. George et al\(^{10}\) showed that functional coupling between the cytoplasmic and transmembrane domains of the RyR2 is mediated by the 3722 to 4104 residue region, called the I-domain and that sudden death (SCD)-linked mutations occurring in the 4610 residue region, called the I-domain (N4104K and R4496C) caused channel instability.

interaction hypothesis. In the present study, using the KI mouse model with a human CPVT-associated RyR2 mutation, R2474S, we investigated the molecular mechanism by which CPVT is induced by a single point mutation within the RyR2. The data presented here suggested that the introduced mutation, in fact, causes defective interdomain interaction in the RyR2, reduces the threshold of luminal Ca\(^{2+}\)-dependance for channel activation, sensitizes RyR2 to protein kinase (PK)A-dependent phosphorylation, and in turn leads to CPVT.

### Methods

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

### Animals

This study conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH (NIH Publication No. 85-23, revised 1996). The care of the animals and the protocols used were in accordance with guidelines established by the Animal Ethics Committee of Yamaguchi University School of Medicine.

### Statistics

Paired or unpaired \(t\) tests were used for statistical comparisons of data obtained during the 2 different situations, whereas ANOVA with a post hoc Scheffe’s test was used for statistical comparison of concentration-dependent data. All data are expressed as means±SE. A probability value of <0.05 was considered statistically significant.

### Results

#### No Appreciable Change in Structural or Functional Characteristics of R2474S/+ KI Mice During the Resting (Nonactivated) State

In the absence of activation, there was no appreciable difference in the structural or functional features of the hearts between wild-type (WT) and KI mice. Thus, the cross-sectional view showed the identical features (Figure 1A). Echocardiography revealed no functional difference between WT and KI mice (Figure 1B). There was no appreciable change in the KI mice regarding the expression or phosphorylation levels of any of the saroplasmic reticulum (SR) proteins examined (Online Figure III).

#### Exercise or Drug (Epinephrine and Caffeine) Administration Induced VT in R2474S/+ KI Mice

In the resting conscious condition, we frequently observed polymorphic ventricular premature contractions in KI mice in response to even very weak stimuli, like light or sound, but not in WT mice (data not shown). Injection of caffeine plus epinephrine (IP) or exercise on a treadmill induced bidirectional or polymorphic VT in KI mice, but not in WT mice (Figure 2A). The duration of VT in most KI mice was less than 30 seconds (Figure 2B).

#### Seizures Were Not Observed in R2474S/+ KI Mice

To determine whether seizures occurred in KI mice, as previously reported,\(^8\) we monitored the behavior of mice by video recording for 1 week and assessed their susceptibility to seizures by pharmacological induction (see expanded Methods in the Online Data Supplement). Thus far, we have not

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>CaMKII</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CPVT</td>
<td>catecholaminergic polymorphic ventricular tachycardia</td>
</tr>
<tr>
<td>DAD</td>
<td>delayed afterdepolarization</td>
</tr>
<tr>
<td>FDHM</td>
<td>full duration at half maximum</td>
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<tr>
<td>FWHM</td>
<td>full width at half maximum</td>
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<tr>
<td>KI</td>
<td>knock in</td>
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<tr>
<td>MCA</td>
<td>methylcoumarin acetamido</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
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<tr>
<td>SERCA</td>
<td>saroplasmic reticulum Ca(^{2+})-ATPase</td>
</tr>
<tr>
<td>SpF</td>
<td>Ca(^{2+}) spark frequency</td>
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<tr>
<td>SR</td>
<td>saroplasmic reticulum</td>
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<tr>
<td>VT</td>
<td>ventricular tachycardia</td>
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<td>WT</td>
<td>wild-type</td>
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observed spontaneous seizures in KI or WT mice. Furthermore, a pharmacological provocation test with 4-aminopyridine and caffeine showed no difference in the latency to the development of generalized tonic-clonic seizures (Online Figure IV).

Relaxation Phase of Cell Shortening and Ca$^{2+}$/H$^{+}$ Transient Was Prolonged in the Isoproterenol-Activated R2474S/+ KI Mice

There was no statistically significant difference in the contour or kinetic parameters of Ca$^{2+}$/H$^{+}$ transient and cell shortening at baseline between the WT and KI mice (Figure 3A and Online Table I). In response to isoproterenol, however, the time from the peak to 80% decline in cell shortening or Ca$^{2+}$/H$^{+}$ transient was prolonged in KI cardiomyocytes, suggesting a delay in the inactivation of Ca$^{2+}$ release and/or spontaneous Ca$^{2+}$ release events. Moreover, the SR Ca$^{2+}$ content, determined by caffeine application, was significantly lower in KI cardiomyocytes than in WT cardiomyocytes, both before and after the addition of isoproterenol (Figure 3B). SR Ca$^{2+}$-ATPase (SERCA)2 activity could contribute to the slowed decay kinetics of the Ca$^{2+}$/H$^{+}$ transient during heart failure. Thus, we next measured the SERCA2-mediated Ca$^{2+}$ uptake by monitoring the time-dependent change in the intra-SR [Ca$^{2+}$] (Online Figure V). As shown, there was no appreciable change in the time course of SR Ca$^{2+}$ uptake.

RyR2 Ca$^{2+}$ Channels of R2474S/+ KI Cardiomyocytes Were Hypersensitive to Channel Activation by Isoproterenol, PKA Phosphorylation, and Luminal Calcium

In KI cardiomyocytes, Ca$^{2+}$ spark frequency (SpF) was higher than that in WT cardiomyocytes, both before and after the addition of isoproterenol (Figure 4A; for 3D images, see Online Figure VI, A). In the presence of a higher concentration of isoproterenol (100 nmol/L), the frequency of spontaneous Ca$^{2+}$ waves was much higher in KI cardiomyocytes than in WT cardiomyocytes and the duration of local Ca$^{2+}$ release provoked by the transmission of the Ca$^{2+}$ waves was markedly prolonged (Online Figure VII). In both KI and WT cardiomyocytes, isoproterenol (10 nmol/L) increased the peak amplitude, full width at half maximum (FWHM), and full duration at half maximum (FDHM) (Online Table II). In KI cardiomyocytes, however, FDHM increased approximately twice as much as in WT cardiomyocytes (Online Table II). In response to isoproterenol (10 nmol/L), the level of PKA-dependent phosphorylation at Ser2808 of the RyR2 showed a rather modest increase that was slightly less than half of maximum phosphorylation (see Online Figure II). However, in this case, the extent of the increase was nearly the same in the KI and WT cardiomyocytes (Figure 4B). These results suggest that the sensitivity of the channel to both SR content (luminal [Ca$^{2+}$]) and PKA phosphorylation is markedly increased in KI channels.
For further analysis of the increased sensitivity of KI channels to PKA phosphorylation and the SR Ca\(^{2+}\) content, we measured the PKA phosphorylation level at Ser2808 of RyR2 after addition of cAMP under the same conditions as the Ca\(^{2+}\) spark assay ([Ca\(^{2+}\)]\(_{30}\) nmol/L, buffered by 0.5 mmol/L EGTA). For this purpose, we added the Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMK)II inhibitor KN-93 (1 μmol/L) to inhibit the effect of intrinsic CaMKII on the phosphorylation of the RyR2. We then measured both SpF and SR Ca\(^{2+}\) content in the absence and in the presence of 1 μmol/L cAMP in the saponin-permeabilized cardiomyocytes. There was no significant difference in the PKA

![Figure 2](image)

Figure 2. Effect of epinephrine plus caffeine or exercise on ventricular arrhythmia. A, Representative ECG recordings in WT and R2474S/+, KI mice. In all KI mice examined, bidirectional or polymorphic ventricular tachycardia (VT) was induced by epinephrine (2 mg/kg of body weight [BW] IP) and caffeine (120 mg/kg of body weight IP) (n=10) (top) or exercise with treadmill (n=6) (bottom). In WT mice, neither epinephrine plus caffeine (n=8) nor exercise (n=6) produced VT. B, Durations of VT and numbers of VT episodes observed during the 5 minutes period in WT and KI mice. VT was induced by only epinephrine (2 mg/kg of body weight IP) without caffeine. N indicates the number of mice examined.

![Figure 3](image)

Figure 3. Cell shortening and intracellular Ca\(^{2+}\) transient in intact cardiomyocytes. A, Representative recordings of cell length and fura-2 fluorescence signal, at a pacing rate of 2 Hz, with isoproterenol (10 nmol/L) activation (ISO) and without it (baseline). B, Representative recording of fura-2 fluorescence signal after addition of caffeine (top), which is a measure of the SR Ca\(^{2+}\) content, and the summarized data of SR Ca\(^{2+}\) content (bottom). Caffeine-induced Ca\(^{2+}\) transient was measured by first applying a stimulation train at 2 Hz and then by rapidly switching the superfusing solution to a solution containing 20 mmol/L caffeine for 5 to 6 seconds. N indicates the number of cells from 3 to 9 hearts.
phosphorylation level at Ser2808 between the KI and WT cardiomyocytes, both in the absence and the presence of cAMP (Figure 4C). Because there is marked variation in specificity among antibodies generated against Ser2808, we compared the phosphorylation level at Ser2808 of RyR2 by using another different antibody against Ser2808 (Badrilla, Leeds, UK). There was no significant difference in the PKA phosphorylation level at Ser2808 between KI and WT cardiomyocytes (Online Figure VIII).

To test the postulated possibility that destabilization of the RyR2 caused by the dissociation of FK506 binding protein 12.6 (FKBP12.6) from the RyR2 is a common pathogenic mechanism underlying heart failure and lethal arrhythmia, we determined the RyR2-bound FKBP12.6 by using a pull-down assay. As shown in Figure 4D, between the WT and KI mice, there was no significant difference in the RyR2-bound FKBP12.6 in the absence or the presence of cAMP (1 μmol/L).

Figure 5A shows the representative traces of Ca²⁺ sparks in the presence of various concentrations of cAMP (0.1 to 1 μmol/L) (for 3D images of Ca²⁺ sparks, see Online Figure VI, B). The dependence of SpF on the SR Ca²⁺ content is plotted in Figure 5B. To obtain the point at lower SR Ca²⁺ content, thapsigargin was added to the cardiomyocytes. Then, both Ca²⁺ sparks and SR Ca²⁺ content were measured 3, 5, and 10 minutes after the addition of thapsigargin. SpF was higher in KI than in WT cardiomyocytes, although there was a considerable reduction in the SR Ca²⁺ content in KI cardiomyocytes. As a result, there was a considerable left-shift of the SpF versus SR Ca²⁺ content plot in the case of KI cardiomyocytes (Figure 5B). The effect of cAMP on Ca²⁺ spark characteristics are summarized in Online Table III.

Compared to the WT cardiomyocytes, both the peak amplitude and FWHM decreased whereas FDHM increased in KI cardiomyocytes, suggesting a delay in the inactivation of the RyR2. These results suggest that the threshold of luminal [Ca²⁺] for channel opening decreased considerably owing to the single R2474S CPVT mutation of the RyR2. To assess the maximum capacity of Ca²⁺ loading of SR, we added tetracaine (1 mmol/L) to inhibit Ca²⁺ release and then evaluated SR Ca²⁺ content by caffeine application. After treatment with the Ca²⁺ release blocker tetracaine (arrows, Online Figure IX), the SR Ca²⁺ content increased and reached nearly the same level (≅4 F/F₀) in both WT and KI cardiomyocytes. This suggests that the reduced SR Ca²⁺ content in the KI cardiomyocytes was not attributable to reduced capacity of
Figure 5. Decreased threshold of SR Ca\(^{2+}\) content to induce spontaneous Ca\(^{2+}\) sparks in saponin-permeabilized cardiomyocytes. A, Representative line-scan images of cardiomyocytes after addition of cAMP (0.1 to 1 \(\mu\)mol/L), at 30 nmol/L [Ca\(^{2+}\)] buffered by 0.5 mmol/L EGTA. B, Relationship between Ca\(^{2+}\) spark frequency (SpF) and SR Ca\(^{2+}\) content. SR Ca\(^{2+}\) content was measured by addition of 10 mmol/L caffeine. To obtain the point at lower SR Ca\(^{2+}\) content, thapsigargin was added to the cardiomyocytes. Then, both Ca\(^{2+}\) sparks and SR Ca\(^{2+}\) content were measured 3, 5, and 10 minutes after the addition of thapsigargin [WT (n=6 to 12 hearts): (--) thapsigargin: 107 to 441 cells for SpF and 10 to 84 cells for SR Ca\(^{2+}\) content; (+) thapsigargin: 4 cells for SpF and 4 cells for SR Ca\(^{2+}\) content, R2474S/ KI (n=6 to 9 hearts): (--) thapsigargin: 91 to 361 cells for SpF and 14 to 46 cells for SR Ca\(^{2+}\) content; (+) thapsigargin: 4 cells for SpF and 4 cells for SR Ca\(^{2+}\) content]. C, Effect of PKA phosphorylation on Ca\(^{2+}\) spark frequency at comparable SR [Ca\(^{2+}\)] content in WT and R2474S/ KI saponin-permeabilized cardiomyocytes. For PKA phosphorylation of the RyR2, both cAMP (1 \(\mu\)mol/L) and okadaic acid (1 \(\mu\)mol/L) were added to the cardiomyocytes at 30 nmol/L [Ca\(^{2+}\)] buffered by 0.5 mmol/L EGTA.
Ca\(^{2+}\) loading but to increased activation of the Ca\(^{2+}\) release flux relative to the influx.

PKA phosphorylation modulates not only RyR2 function but also SR Ca\(^{2+}\) load dependence of channel activation. Thus, to clarify the direct effect of PKA phosphorylation on RyR2 function, we measured the SpF and SR Ca\(^{2+}\) content in the presence of 0.3 \(\mu\)mol/L thapsigargin (inhibitor of SERCA2a activity) (Figure 5C). At comparable SR Ca\(^{2+}\) content, PKA phosphorylation increased SpF in KI cardiomyocytes, but not in WT cardiomyocytes. This suggests that PKA-dependent phosphorylation sensitized diastolic SR Ca\(^{2+}\) release, but only in CPVT, and not WT RyR2 channels.

**Domain Unzipping Peptide DPc10 Mimics the Phenotype of KI Channels in WT Cardiomyocytes**

As shown previously, DPc10 (a peptide corresponding to the 2460 to 2495 region of the central domain) mimics the channel disorder in the CPVT mutant (R2474S) by interfering with the channel-stabilizing interdomain interactions between the N-terminal and central domains (ie, domain unzipping). To assess the effect of domain unzipping on the dependence of SpF on the SR Ca\(^{2+}\) content, we added DPc10 to the WT saponin-permeabilized cardiomyocytes. Successful incorporation of DPc10 was confirmed by the intracellular fluorescence signal of Alexa fluor 488–labeled DPc10 (Online Figure X). DPc10 (50 \(\mu\)mol/L, applied externally) induced Ca\(^{2+}\) sparks in WT cardiomyocytes (Figure 5D, top). Importantly, addition of DPc10 caused a left-shift of the SpF/SR Ca\(^{2+}\) content relationship in WT cardiomyocytes (Figure 5D, bottom), resulting in the nearly identical SpF/SR Ca\(^{2+}\) profile as that of KI cardiomyocytes (Figure 5B). Similar to cAMP-treated KI cardiomyocytes, addition of DPc10 to WT cardiomyocytes prolonged FDHM (21.32 ± 0.31 ms [n = 27 cells]; 23.82 ± 0.54 ms [n = 48 cells]; P < 0.01), but decreased the peak (1.74 ± 0.03 to 1.69 ± 0.01 arbitrary units; P = 0.068) and FWHM (2.05 ± 0.03 to 2.00 ± 0.02 \(\mu\)m; P < 0.01). Addition of DPc10 to the (cAMP-treated) KI cardiomyocytes produced no further effect (Figure 5E). To assess whether suppression of domain unzipping reversed the left-shift of the SpF/SR Ca\(^{2+}\) content relationship in KI cardiomyocytes, we added dantrolene, which has corrected aberrant domain unzipping and prevented the development of heart failure.14 Interestingly, dantrolene shifted back the SpF/SR Ca\(^{2+}\) content relationship to the right, almost toward the point corresponding to that of WT cardiomyocytes (Figure 5E). Dantrolene also prevented the DPc10-induced left-shift of the SpF/SR Ca\(^{2+}\) content relationship in WT cardiomyocytes (Figure 5D). Collectively, these findings indicate that the defective interdomain interaction within the RyR2 caused by either point mutation (R2474S) or domain unzipping peptide (DPc10) reduced the threshold of luminal [Ca\(^{2+}\)] for channel activation, leading to the phenotype of CPVT (hyper-activation of CPVT).
the RyR2 channel). This suggests that as in the case of malignant hyperthermia, correction of the defective interdomain interaction by dantrolene may provide an effective method to treat CPVT.

Luminal $[\text{Ca}^{2+}]$ of SR in Saponin-Permeabilized WT and KI Cardiomyocytes

To confirm the above notion that the level of SR $\text{Ca}^{2+}$ load was decreased in KI cardiomyocytes, we monitored luminal $[\text{Ca}^{2+}]$ in both cardiomyocytes using fluo-5N as a luminal $\text{Ca}^{2+}$ probe. The average level of free diastolic SR $\text{Ca}^{2+}$ decreased in KI cardiomyocytes, and on caffeine-induced discharge of luminal $\text{Ca}^{2+}$, the luminal $\text{Ca}^{2+}$ reached the same basal level in both WT and KI cardiomyocytes (Figure 6).

Spectroscopic Evidence That the Interdomain Interaction Is Defective in R2474S/+ KI Cardiomyocytes

To investigate whether the interdomain interaction was defective in KI mice, we used the fluorescence quench technique that permits spectroscopic monitoring of the state of the interdomain interaction (zipped or unzipped). The methylcoumarin acetamido (MCA) probe that has been attached to the critical domain would be inaccessible to a bulky fluorescence quencher (QSY-BSA conjugate) in a zipped configuration of the interacting domains, whereas it would become accessible to the quencher on unzipping. As in our previous study with DPc10, site-specific intense fluorescence labeling of the RyR2 band was achieved using DPc10 as a carrier (Figure 7A, left lane), but there was no MCA labeling when DPc10-mut was used as a carrier (middle lane). As shown in the “cold-chase” experiment (right lane), excess unlabeled DPc10 (10 mmol/L) prevented DPc10-mediated MCA labeling. DPc10 increased the slope of the Stern–Volmer plot ($K_Q$), a measure of the extent of unzipping between the N-terminal (amino acids 1 to 600) and central (amino acids 2000 to 2500) domains in WT SR (Figure 7B, left). Addition of cAMP (1 μmol/L) had no effect (no unzipping) in WT SR (Figure 7B, left). In contrast, KI SR showed a high $K_Q$ value (Figure 7B, right). Addition of cAMP (1 μmol/L) further increased the $K_Q$, which was comparable with the value of

![Figure 7. Spectroscopic monitoring of domain–domain interaction between the N-terminal (amino acids 1 to 600) and central (amino acids 2000 to 2500) domains in the RyR2.](http://circres.ahajournals.org/).
WT SR with added DPc10 (Figure 7B, right). Addition of DPc10 on the top of cAMP (1 µmol/L) produced no further increase in $K_Q$. Interestingly, dantrolene (1 µmol/L) partially reversed the $K_Q$ that had been increased by cAMP (Figure 7B, right). Dantrolene also prevented the DPc10-induced unzipping in WT SR (Figure 7B, left). These $K_Q$ values are summarized in Figure 7C.

**Arrhythmogenic Characteristics of the Membrane Potential in R2474S/+ KI Cardiomyocytes**

Because altered membrane potential events represent an important landmark in arrhythmogenesis, we measured $m_R$ (00–2500) a higher the WT cardiomyocytes organized Ca"_t eased SpF only in KI cardiomyocytes. ICR, membrane potentials of WT and KI cardiomyocytes by recording the di-8-ANEPSS fluorescence while pacing in the presence of 30 nmol/L isoproterenol (Online Figure XI). WT cardiomyocytes showed no spontaneous after potential (Figure 8A) and no spontaneous Ca$^{2+}$ transient (Figure 8B). However, KI cardiomyocytes showed spontaneous Ca$^{2+}$ transients and spontaneous after potentials in response to isoproterenol (30 nmol/L) when we increased the pacing rate from 1 to 5 Hz (Figure 8A and 8B). Interestingly, the spontaneous after potentials and Ca$^{2+}$ transients disappeared in the presence of dantrolene (1 µmol/L), which corrected the defective inter-domain interaction between the N-terminal and central domains (Figure 8A and 8B).

**Discussion**

Many point mutations have been found in RyR2 in patients with CPVT. Several pieces of biochemical evidence suggest that these mutations cause defective channel gating, leading to diastolic Ca$^{2+}$ leakage. More direct evidence that a single point mutation in the RyR2 is the primary cause of channel dysfunctions in CPVT patients have been obtained by recent studies with a KI mouse model. A single point mutation (R4496C) introduced in the RyR2 was found to cause bidirectional or polymorphic VT in the KI mice. In isolated cardiomyocytes from the R4496C KI mice, both delayed afterdepolarization (DAD) and triggered activity were induced on stimulation with isoproterenol. A more recent study using the same model showed that a dramatic increase in the Ca$^{2+}$ sensitivity of the RyR2 channel resulted in the increased frequency of Ca$^{2+}$ sparks and Ca$^{2+}$ waves, which was further amplified by either isoproterenol or high pacing rates. Further studies confirmed mutation-linked dysfunc-
tion of RyR2, namely spontaneous Ca$^{2+}$ release events and DAD. These studies show a close inter-relationship between the single point mutation, increased Ca$^{2+}$ sensitivity of the channel gating, and the resulting lethal arrhythmia. However, the underlying mechanism by which a single mutation in such a large molecule causes drastic effects on cardiac function has remained unclear.

**Defective Interdomain Interaction Is the Source Mechanism of Mutation-Linked Channel Disorder**

The most important new aspect of the present study is the finding that introduction of the R2474S CPVT mutation into the central domain of RyR2 induced a defective interaction between the central domain and the N-terminal domain, as predicted from the “domain switch hypothesis,” and this caused channel dysfunction similar to that of CPVT patients in KI mice. The 3 lines of evidence are consistent with this. First, DPC10 (amino acids 2460 to 2495), which contains the mutable R2474 residue and is known to interfere with normal interdomain interaction between the N-terminal and central domains, reproduced the abnormal cellular Ca$^{2+}$ events seen in the R2474S/+ KI mice (eg, increased frequency of Ca$^{2+}$ sparks) in an otherwise normal system (ie, in cardiomyocytes isolated from WT mice). However, the addition of DPC10 to the (cAMP-treated) R2474S KI cardiomyocytes produced no further effect, suggesting that the defective interdomain interaction (aberrant domain unzipping) had already taken place in the KI cardiomyocytes. Second, The R2474S mutation, introduced into the central domain of RyR2 of KI mice, did produce defective interdomain interaction between the N-terminal and central domain (aberrant domain unzipping), as evidenced by the accessibility of the fluorescent probe MCA attached to the N-terminal domain to a high molecular weight fluorescence quencher QSY7-BSA was considerably higher in the KI RyR2 than the WT RyR2. Finally, dantrolene, which corrects aberrant domain unzipping, did suppress aberrant phenomena characteristic of CPVT KI mice, such as reduced threshold of luminal Ca$^{2+}$ for channel activation, spontaneous Ca$^{2+}$ sparks, and DAD.

**PKA-Dependent Phosphorylation of the RyR2 at Ser2808 Facilitates Domain Unzipping Only in the CPVT Mutant Ryanodine Receptor**

Interesting new finding in the present study is that the threshold of luminal [Ca$^{2+}$] for activation of Ca$^{2+}$ sparks was much lower in R2474S/+ KI mice than in WT mice. In other words, the sensitivity of the RyR2 channel to activation by luminal [Ca$^{2+}$] was increased in R2474S/+ KI mice. More importantly, we could reproduce this sensitized channel gating to luminal [Ca$^{2+}$] that is characteristic of the R2474S/+ KI mice, in WT cardiomyocytes by adding DPC10 (Figure 5D). This provides further support for the notion that the aberrant channel gating in R2474S/+ KI mice is produced by defective interdomain interaction between the N-terminal and central domains.

This study also showed that the level of PKA-dependent phosphorylation of the RyR2 at Ser2808 was virtually indistinguishable between KI and WT RyR2s (Figure 4B and 4C), yet PKA phosphorylation produced a much larger effect in increasing the frequency of Ca$^{2+}$ sparks (SpF) in the KI cardiomyocytes than the WT myocytes (Figures 4A and 5A). This suggests that the CPVT mutation also sensitizes the channel to PKA phosphorylation-dependent activation. In the 3D image of the RyR2, Ser2808, the site of PKA phosphorylation, has been localized in the vicinity of the boundary between the N-terminal (amino acids 1 to 600) and the central domains (amino acids 2000 to 2500). Earlier cryoelectron microscopy single particle study of RyR1 also showed that domain 5 (including the N-terminal domain) and domain 6 (including the central domain) at the clamp region are indeed in close apposition to each other in the resting state, whereas these domains become separated in the activated (channel-open) state (eg, in the presence of cAMP and activating Ca$^{2+}$). Thus, it is tempting to suggest that PKA phosphorylation at Ser2808 accelerates domain unzipping in the KI channel, where domain unzipping has already progressed because of a weakened interdomain interaction caused by the CPVT mutation.

**A New Molecular Mechanism for CPVT**

Although recent studies using KI mouse models have demonstrated that CPVT is caused by mutation-linked dysregulation of intracellular Ca$^{2+}$ events and membrane potential events (DAD and triggered activity), it is still unclear how a single mutation changes the conformational state of the RyR2, leading to leaky channel. We propose a new molecular mechanism underlying CPVT (Online Figure XII). In the normal channel, domain–domain interaction between the N-terminal (amino acids 1 to 600) and central (amino acids 2000 to 2500) domains is maintained in a zipped state, and thus, the channel is stabilized, preventing Ca$^{2+}$ leakage and DAD at a physiological range of SR Ca$^{2+}$ contents. In the mutant channel, the stabilized interdomain interaction is disrupted, causing aberrant domain unzipping; domain unzipping is further aggravated by the PKA phosphorylation of Ser2808, located at the boundary between the 2 domains at the clamp region. In turn, the threshold of luminal [Ca$^{2+}$] for channel activation is decreased (cf, elsewhere). Together, this results in SR Ca$^{2+}$ leakage, DAD, and lethal arrhythmia. It should be noted, however, that DAD-triggered arrhythmia can also be induced by intracellular Ca$^{2+}$ overload, for example, the toxic arrhythmogenic effects of the cardiac glycosides.
which may account for the destabilized channel gating in heart failure. Thus, either a CPVT-type mutation or FKBP dissociation in heart failure commonly induces domain unzipping as an independent trigger, resulting in aberrant Ca\(^{2+}\) release in diseased hearts.

The reduction in SR Ca\(^{2+}\) content in heart failure may be partly attributable to increased Na\(^{+}\)/Ca\(^{2+}\) exchanger function and the increased SR Ca\(^{2+}\) leakage (in addition to reduced SERCA function), causing contractile dysfunction as well as arrhythmia. We previously demonstrated that dantrolene corrects defective interdomain interactions within the RyR2 in failing hearts, inhibits spontaneous Ca\(^{2+}\) leakage, and in turn improves cardiomyocyte function in failing hearts.\(^{14}\) In this study, we showed that dantrolene was equally effective in the CPVT-type mutated RyR2 as in failing hearts. This indicates that channel dysfunction in CPVT and heart failure are caused by a common mechanism, that is, defective interdomain interaction within the RyR2.

According to a recent report by Lehnhart et al.,\(^{8}\) R2474S KI mice, which harbors the same mutation as those used in the present study, exhibited spontaneous generalized tonic-clonic seizures. In our KI mice, however, we did not observe spontaneous tonic-clonic seizures. We have no explanation for this difference. It may be ascribable to differences in the background of the mouse model. In generating the RyR2 KI mice, we used ES cells derived from C57BL/6J mouse (no difference in the background between ES cell line mice and KI mice), but Lehnhart et al. used those from 129 mice, followed by back-crossing with C57BL/6J mice.

In this study, we evaluated the phosphorylation status only at Ser2808. However, because \(\beta\)-adrenergic stimulation has been reported to activate CaMKII,\(^{11,23}\) a further investigation is clearly needed to assess the role of other phosphorylation sites (eg, Ser2814 and Ser2030) on CPVT-type channel disorder.

In conclusion, a single point mutation within the RyR2 sensitizes the RyR2 channel to activation by luminal [Ca\(^{2+}\)] (ie, a decreased threshold of luminal [Ca\(^{2+}\)] for channel activation), and in turn induces spontaneous Ca\(^{2+}\) sparks and DAD, leading to CPVT. More importantly, this aberrant channel opening is primarily mediated through defective interdomain interaction between the N-terminal (amino acids 1 to 600) and central (amino acids 2000 to 2500) domains.

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Disclosures

None.

References

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Novelty and Significance

What Is Known?

- Catecholaminergic polymorphic ventricular tachycardia (CPVT) is caused by single point mutation in cardiac, type 2, ryanodine receptor (RyR2).
- Aberrant Ca\(^{2+}\) release occurs in CPVT-type mutant RyR2.

What New Information Does This Article Contribute?

- Defective interdomain interaction (namely, domain unzipping) within the RyR2 is a source mechanism of catecholamine-induced aberrant Ca\(^{2+}\) release in CPVT.
- Correction of the defective interdomain interaction could be a new strategy against CPVT.

CPVT is known to be caused by single point mutation taking place in well defined regions of the RyR2. A single point mutation introduced in the RyR2 has been found to cause bidirectional or polymorphic VT in the knock-in mice, showing a close inter-relationship among the single point mutation and the resultant lethal arrhythmia. However, the underlying mechanism by which a single mutation in such a large molecule causes drastic effects on channel function has remained unresolved. Here, we report that introduction of R2474S CPVT mutation into the central domain of mouse RyR2 interferes with a normal tight interaction between the central domain (amino acids 2000 to 2500) and the N-terminal domain (amino acids 1 to 600), which reduces the threshold of luminal [Ca\(^{2+}\)] for channel activation, sensitizes to the protein kinase A–dependent phosphorylation, and in turn leads to CPVT. Correction of the defective interdomain interaction by dantrolene stops the aberrant Ca\(^{2+}\) release and spontaneous after potential characteristic of CPVT. These results provide a new pathogenic mechanism of CPVT and a novel therapeutic strategy against CPVT.
Catecholaminergic Polymorphic Ventricular Tachycardia Is Caused by Mutation-Linked Defective Conformational Regulation of the Ryanodine Receptor
Hitoshi Uchinoumi, Masafumi Yano, Takeshi Suetomi, Makoto Ono, Xiaojuan Xu, Hiroki Tateishi, Tetsuro Oda, Shinichi Okuda, Masahiro Doi, Shigeki Kobayashi, Takeshi Yamamoto, Yasuhiro Ikeda, Tomoko Ohkusa, Noriaki Ikemoto and Masunori Matsuzaki

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Hitoshi Uchinoumi, et al. Catecholaminergic polymorphic ventricular tachycardia is caused by mutation-linked defective conformational regulation of the ryanodine receptor.

EXPANDED MATERIALS AND METHODS

Generation of knock-in mice with the RyR2 R2474S mutation (Online Figure I).

We isolated the template clone containing exon 47-54 from C57BL/6J mouse genomic library (BAC clone No.RPCI-23-138016). The 5’-arm 2.0Kb MfeI-SexAI fragment from exon 47 to 48 was amplified by PCR using the template. To introduce a point mutation R2474S, 58bp of 5’-reagion and 170bp of 3’-reagion of the exon 49 were connected at their NcoI sites. After insertion of the mutant fragment into the SmaI site of the pBluescript II KS+ (pBSK, Stratagene) vector, LoxP-Neo was introduced at the 3’-end of the vector. This vector was inserted into the 5’-arm fragment to construct 5’-arm 2.0Kb / R2474S / LoxP-Neo / pBSK. The exons 50-54 3’-arm 6.9Kb fragment isolated from the template plasmid was inserted into the 3’-end of the 5’-arm 2.0Kb / R2474S / LoxP-Neo / pBSK, and then the diphtheria toxin A (DT-A) fragment for negative selection was introduced at the 5’-end the vector.

The targeting vector, which was constructed as above, was linearized with ClaI and transfected into the CMT (CMTI-2 EmbryoMax ; Millipore co.) embryonic stem (ES) cells by electroporation. Cells were plated in 100 mm dishes and were incubated for 24 h. Positive and negative selections were performed using Geneticin and diphtheria toxin A (DT-A) fragment, respectively. About 100 clones were selected and analyzed by PCR using the neo P1 (5’ - GCCCTTCTTGAGCAGTTCTTCTCTGTA-3’) and exon primer PCR3 (5’ - ATTCCTGCCTGTATGCTG- 3’) and then confirmed by Southern blot analysis. The selected ES cells were injected into the blastocysts of BALB/c mice and chimeric animals were obtained. The chimeric mice gave offspring with germ-line transmission, and mice heterozygous for the targeted RyR2 +/RyR2R2474S-neo were established. Sperms from the heterozygous mice were externally fertilized with eggs from C57BL/6 female mice. The neo cassette was removed by injection of the Cre-expression circular plasmid (pCAGGS-Cre) 1 into the oocytes. The genotypes of the F1 generations were determined by PCR and Southern blot analysis of genomic tail DNA. The positive clone was injected into the blastocysts of BALB/C (JCL) mice and chimeric animals were obtained. Chimeric male mice were bred to C57BL/6J female mice to establish a hybrid line. The chimeric mice gave offspring with germ-line transmission, and mice heterozygous for the targeted RyR2 R2474S/+ were established. The genotypes from the F1 and F2 generations were determined by PCR on DNA from tail biopsy
specimens.

**Histology.**

Hearts from R2474S/+ KI and WT mice, aged 16-22 weeks, were fixed with 10% formalin. A complete, full-circumferential section, at the level of the two left ventricular papillary muscles, was selected for morphometric analysis. Hematoxylin-Eosin and Trichromic Masson stains were performed for each section of the ventricle.

**Echocardiography.**

Cardiac function was analyzed by a HDI-5000 ultrasound machine (Philips, Netherlands) equipped with a 15-MHz probe. R2474S/+ KI and WT mice were initially anesthetized with 4-5% isoflurane (mixed with oxygen) and maintained with 1-2% isoflurane during echocardiography.

**ECG telemetry.**

ECG was monitored in a conscious state for R2474S/+ KI and WT mice by using ECG telemetry. Briefly, transmitters (Data Sciences International, St. Paul, MN) were implanted in the back space with s.c. electrodes in a lead II configuration. Telemetry was recorded 48 hours after surgery in a conscious state at baseline and after the injection of only epinephrine (2 mg/kg of body weight i.p.) for measurement of the number of VT episodes for 5 minutes. A subset of telemetered KI (n=10) and WT mice (n=8) were injected with epinephrine (2 mg/kg of body weight i.p.) and caffeine (120 mg/kg of body weight i.p.) and monitored for 30 minutes. Another set of KI (n=6) and WT mice (n=6) were exercised with treadmill (Panlab, Barcelona, Spain).

**Seizure experiments.**

To assess whether a spontaneous seizure occurs in R2474S/+ KI mice, we videorecorded the behavior of mice for 1 week, as previously described. Then, three observers blinded to the experimental protocol analyzed the videorecordings focusing on the occurrence of seizure. In a different set of experiments, we assessed the susceptibility of generalized tonic-clonic seizures by an administration of 2.5 mg/kg 4-aminopyridine, followed 25 minutes later by caffeine (250 mg/kg) either at one time addition or at an incremental addition of dose (by 50mg /5min) up to 250 mg/Kg. In either case, all mice showed suddenly generalized tonic-clonic seizure, followed by sudden death in 5 minutes. During the seizure, ECG shows bradyarrythmia, but not tachyarrythmia like ventricular tachycardia or fibrillation. Mice were directly observed and videorecorded for later analysis during a 60-minute observation period.
Preparation of SR vesicles.

We prepared SR vesicles from mice LV, with the modifications described previously.\textsuperscript{3} Left ventricles ($n=10$) were homogenized in a solution containing 30 mmol/L Tris-malate, 0.3 mol/L sucrose, protease inhibitor cocktail (complete, EDTA-free, Roche), at pH 7.0. The homogenate was centrifuged at 4,000 g for 15 minutes and the resultant supernatant was filtered through four layers of cheesecloth before centrifugation at 10,000 g for 15 minutes. The supernatant was again filtered through cheesecloth and centrifuged at 60,000 g for 30 minutes. The pellet was resuspended in a solution containing 0.1 mol/L KCl, 20 mmol/L Tris-malate, 0.3 mol/L sucrose, protease inhibitor cocktail (complete, EDTA-free), at pH 7.0 to give a final concentration of about 10-20 mg protein/mL. This fraction was rapidly frozen in liquid nitrogen and stored at -80°C.

Peptides used and peptide synthesis.

We used the 2 domain peptides, DPc10 and DPc10-mut, as described previously;\textsuperscript{4} DPc10: DPc2460-2495
\begin{verbatim}
2460GFCPDHKAAMVLFLDRVYGIIEVQDFLLHLLEVGLP2495
\end{verbatim}
DPc10-mut: DPc2460-2495-mut R2474S
\begin{verbatim}
2460GFCPDHKAAMVLFLD5VYGIIEVQDFLLHLLEVGLP2495.
\end{verbatim}
Peptides were synthesized on an Applied Biosystems model 431A synthesizer employing Fmoc (N-(9-fluorenyl)methoxycarbonyl) as the alpha-amino protecting group. The peptides were cleaved and de-protected with 95% trifluoroacetic acid and purified by reversed-phase high-pressure liquid chromatography.

Site-directed fluorescent labeling of the RyR2.

Specific fluorescent labeling of the RyR2 in SR vesicles was performed using the cleavable hetero-bifunctional cross-linking reagent sulfosuccinimidyl 3-((2-(7-azido-4-methylcoumarin-3-acetamido) ethyl) dithio)propionate (SAED) from PIERCE, with DPc10 as a site-specific carrier, as described previously.\textsuperscript{4,5} First, peptide-SAED conjugate was formed by incubating 0.5 mmol/L peptide with 0.5 mmol/L SAED in a 20 mmol/L HEPES (pH 7.5) solution for 60 minutes at 22 °C in the dark. The reaction was quenched by 20 mmol/L lysine. Free SAED was removed using ion exchange column (GE HiTrap Q XL). The peptide-SAED conjugate (5 µmol/L in a final concentration) was mixed with 2 mg/mL SR protein in the sample solution containing a 1 mmol/L EGTA/calcium buffer (1.0 µmol/L free Ca\textsuperscript{2+}) in the dark and photolyzed with UV light in a Pyrex tube at 4 °C for 2 minutes. Beta-mercaptoethanol was added (100 mmol/L in a final concentration) to cleave the disulfide
bond of SAED. After incubation on ice for 1 hour, the mixture was centrifuged at 100,000g for 15 minutes, and the sedimented vesicles were resuspended in the sample solution to a final protein concentration of 10 mg/mL.

**Fluorescence quenching of the MCA fluorescence attached to the DPc10 binding site.**

The zipped and unzipped states of the RyR2 were evaluated as described previously.4,5 The principle of the fluorescence quenching assay of domain unzipping is that a large-size quencher QSY®_7 -BSA is inaccessible to the attached MCA in the zipped state, whereas it becomes accessible to the MCA site in the unzipped state. To form the quencher, QSY®_7 carboxylic acid, was conjugated with BSA by incubating 5 mmol/L QSY®_7 carboxylic acid with 0.5 mmol/L BSA in 20 mmol/L HEPES (pH 7.5) for 60 minutes at 22°C in the dark. Unreacted QSY®_7 carboxylic acid was removed by means of Sephadex G50 gel filtration. Fluorescence quenching by QSY®_7-BSA conjugate (a large-size quencher) was performed by measuring steady-state fluorescence of labeled MCA (excitation at 368 nm, emission at 455 nm) in the presence or absence of chemicals. The data were analyzed using the Stern-Volmer equation.

\[
\frac{F_0}{F} = 1 + K_Q [Q]
\]

where \( F \) and \( F_0 \) are fluorescence intensities in the presence and in the absence of added quencher, respectively; \( K_Q \), quenching constant, which is the measure of the accessibility of the protein-bound probe to the quencher; \([Q]\), the quencher concentration. The principle of the fluorescence quenching assay of domain unzipping is that a large-size quencher QSY®_7 -BSA is not accessible to the attached MCA in the zipped state, whereas it becomes accessible to the MCA in the unzipped state.

**Isolation of cardiac cardiomyocytes.**

The enzymatic isolation of mice cardiomyocytes was performed as described previously.6 In brief, R2474S/+ KI and WT mice (2 to 3 months) were anesthetized with pentobarbital sodium (70 mg/kg of body weight i.p.), intubated and ventilated with ambient air. An incision in the chest was made, and the heart was quickly removed and retrogradely perfused with a collagenase-free buffer via the aorta under constant flow. The LV myocardium was minced with scissors in a fresh collagenase-containing buffer and the rod-shaped adult mice cardiomyocytes were prepared by retrograde perfusion of the hearts with 95%O₂/5%CO₂-bubbled Minimal Essential Medium (Sigma, St Louis, MO, USA) supplemented with 50 µmol/L [Ca²⁺], 0.5 mg/mL collagenase B, 0.5 mg/mL, collagenase D, and 0.02 mg/mL protease type XIV. The Ca²⁺ concentration was then gradually increased to a final concentration of 1 mmol/L by changing the incubation medium (50 µmol/L, 100 µmol/L, 300 µmol/L, 600 µmol/L and then 1 mmol/L).
The isolated mice cardiomyocytes were transferred to laminin-coated glass culture dishes, and incubated for 12 hours at 37°C in a 5%CO₂/95%O₂ atmosphere. To assess SR Ca^{2+} content, caffeine (10 mmol/L) was rapidly perfused. In case of intact cells, caffeine was perfused after field-stimulation at 2 Hz. Experiments were carried out at room temperature.

**Cell shortening and Ca^{2+} transient measurement.**

Measurements of myocyte cell shortening and intracellular Ca^{2+} were performed using fura-2 AM, as described previously. Cells were stimulated by a field electric stimulator (IonOptix) at a stimulation frequency of 2 Hz. After stimulation of the cells, the cell shortening and peak Ca^{2+} transient gradually increased and reached a steady state within 2 minutes. At the steady state (2 minutes after initiation of pacing), the intracellular calcium concentration was monitored by a dual-excitation spectrofluorometer as the ratio of the fluorescence emission intensities (at 505 nm) of fura-2 AM elicited by excitation at 340 and 380 nm.

**Analysis of local Ca^{2+} release events with laser scanning confocal microscopy.**

The Ca^{2+} sparks were measured as previously described using a laser scanning confocal microscope system (LSM-510, Carl Zeiss) equipped with an argon ion laser coupled to an inverted microscope (Axiovert 100, Carl Zeiss) with a Zeiss x40 oil-immersion Plan-Neofluor objective (numerical aperture, 1.3; excitation at 488 nm; emission > 505 nm). Briefly, intact cardiomyocytes were loaded with fluo-4 AM (20 µmol/L; Molecular Probes) for 30 minutes at room temperature. Line-scan mode was used, where a single cardiomyocyte was scanned repeatedly (520.8 Hz) along a line parallel to the longitudinal axis, avoiding nuclei. To monitor Ca^{2+} sparks, cardiomyocytes were stimulated until the Ca^{2+} transient reached a steady state, then stimulation was stopped, and Ca^{2+} sparks recorded during the subsequent ~10 s rest.

The Ca^{2+} events under a fixed intracellular [Ca^{2+}] condition were also measured in saponin-permeabilized cardiomyocytes, as described previously. Ventricular myocytes were superfused with relaxing solution containing EGTA 0.1 mmol/L, ATP 5 mmol/L, HEPES 10 mmol/L, potassium aspartate 150 mmol/L, MgCl₂ 0.25 mmol/L, and reduce-glutathione 10 mmol/L, at 23°C. The sarcolemma was permeabilized with saponin (50 µg/mL) for 30s. After permeabilization, myocytes were placed in a solution containing EGTA 0.5 mmol/L, HEPES 10 mmol/L, K-aspartate 120 mmol/L, ATP 5 mmol/L, free MgCl₂ 1 mmol/L, reduced glutathione 10 mmol/L, free [Ca^{2+}] 30 nmol/L (calculated using MaxChelator (http://www.stanford.edu/~cpatton/webmaxcS.htm)), creatine phosphokinase 5 U/ml, phosphocreatine 10 mmol/L, dextran (Mr: 40,000) 4%; Rhod-2 0.02 mmol/L, pH 7.2. Rhod-2 were excited by 543 nm laser lines, and fluorescence was acquired at wavelengths of >560 nm. Ca^{2+} spark images were obtained from permeabilized ventricular myocytes before and after the
addition of cAMP (0.1-1 µmol/L) in the presence of the CaMKII inhibitor KN-93 (1 µmol/L) and okadaic acid (1 µmol/L) to prevent dephosphorylation by endogenous phosphatases. 

Data were analyzed with SparkMaster, an automated analysis program which allows rapid and reliable spark analysis. The analysis includes general image parameters (number of detected sparks, spark frequency) as well as individual spark parameters (Amplitude, FWHM: full width at half maximum, FDHM: full duration at half maximum).

Measurement of intra-SR [Ca^{2+}].

According to the methods by Belevych et al, myocytes were first loaded with 5 µmol/L fluo-5N AM for 3-6 hours at 37°C, and then the fluo-5N-loaded cells were permeabilized with saponin (50 µg/mL) for 30s, in the relaxing solution containing EGTA 0.1 mmol/L, ATP 5 mmol/L, HEPES 10 mmol/L, potassium aspartate 150 mmol/L, MgCl2 0.25 mmol/L, and reduce-glutathione 10 mmol/L, at 23°C. After permeabilization, the internal solution was replaced with that for measurement of cytosolic [Ca^{2+}] containing EGTA 0.5 mmol/L, HEPES 10 mmol/L, K-aspartate 120 mmol/L, ATP 5 mmol/L, free MgCl2 1 mmol/L, reduced glutathione 10 mmol/L, free [Ca^{2+}] 30 nmol/L, creatine phosphokinase 5 U/ml, phosphocreatine 10 mmol/L, dextran (Mr: 40,000) 4%; Rhod-2 0.02 mmol/L, pH 7.2 (for simultaneous recoding of cytosolic [Ca^{2+}] as well). Fluo-5N was excited with a 488 nm laser line and emission acquired at 500-530 nm. For quantitative studies, the temporal dynamics in fluorescence was expressed as \( \frac{\Delta F_{CAFF}}{\Delta F_{MAX}} = \frac{(F - F_{CAFF})}{(F_{MAX} - F_{CAFF})} \), where F represents fluorescence at a time t, \( F_{CAFF} \) represents the fluorescence level of the cells after the application of 20 mmol/L caffeine, and \( F_{MAX} \) represents fluo-5N fluorescence in the presence of 10 mmol/L [Ca^{2+}]. In permeabilized myocytes \( F_{MAX} \) was determined by application of 10 mmol/L [Ca^{2+}] in the presence of 10 mmol/L BDM and 1 µmol/L ionomycin.

Measurement of membrane potential in cardiomyocytes.

Isolated cardiomyocytes were attached to a laminin-coated glass culture dish. To measure the membrane potential of cardiomyocytes, cells were stained with 1 µmol/L di-8-ANEPPS (Molecular Probes) in standard Tyrode’s solution at room temperature for 4 minutes in the dark, with slight modifications of the previously described method. The membrane potential was measured by a laser scanning confocal microscope (LSM-510, Carl Zeiss) equipped with an argon ion laser coupled to an inverted microscope (Axiovert 100, Carl Zeiss) with a Zeiss x40 oil-immersion Plan-Neofluor objective (excitation at 488 nm; emission: 505-545 nm and greater than 650 nm). The membrane potential was recorded with the ratiometry of values of these fluorescence emissions to distinguish membrane potential from motion artifact of cardiomyocyte and exclude the effect of the fluorescence breaching. Cardiomyocytes were
stimulated at a rate of 1 or 5 Hz for 30 sec, and then abruptly stopped the field stimulation to observe the following membrane potential fluctuation.

**Immunoblot analysis.**

We performed immunoblot analyses for RyR2, phosphorylated RyR2 at Ser2808 (P-Ser2808-RyR2), SR Ca$^{2+}$-ATPase (SERCA2a), phospholamban (PLB), phosphorylated PLB at serine 16 (P-Ser16-PLB), phosphorylated PLB at threonine 17 (P-Thr17-PLB), calsequestrin (CASQ), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Specific antibodies against RyR2 (Sigma), SERCA2a (Affinity Bioregents), PLB (Upstate biotech), P-Thr17-PLB (Badrilla), CASQ (Affinity Bioregents), GAPDH (Chemicon). Specific antibody to P-Ser2808-RyR2 (epitope 2803RTRRISQTSQV2813) was an order-made from Sigma. PKA-dependent phosphorylation of Ser2808-RyR2 at various phosphorylation states$^{12}$ was shown in Online Figure II.

**Evaluation of FKBP12.6 association with the RyR2.**

FKBP12.6 bound to the mouse RyR2 was detected by immunoprecipitation and immunoblot analysis. Briefly, left ventricles were homogenized in a solution containing 30 mmol/L Tris-malate, 0.3 mol/L sucrose, protease inhibitor cocktail (complete, EDTA-free, Roche), at pH 7.0. The homogenate was centrifuged at 4,000 g for 15 minutes and the resultant supernatant was filtered through four layers of cheesecloth before centrifugation at 100,000 g for 30 minutes. The pellet was resuspended in RIPA buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L Na$_3$VO$_4$, 1 mmol/L NaF, 0.25% TritonX-100, and protease inhibitors) for immunoprecipitation, in the presence of anti-RyR2 antibody (MA3-925, Affinity BioReagents Inc) for 4 hours at 4°C. After incubating the samples with protein G-Sepharose beads (Amersham; GE Healthcare) at 4°C for 2 hour, proteins were separated in a 4% (for RyR2) or 15% (for FKBP12.6) SDS-PAGE gel, transferred to PVDF membranes, and immunoblotted using the following antibodies: anti-FKBP12.6 (1:1,000) (AF4174, R&D systems), anti-RyR (1:1,000).

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Online Figure legends

**Online Figure I.**
Schematic representation of the genomic structure of the mouse RyR2; the targeting vector used to generate the knock-in RyR2 R2474S mouse strain and the recombining genomic structure of RyR2 R2474S.

**Online Figure II.**
Protein kinase A (PKA)-dependent phosphorylation of Ser2808-RyR2. Four different conditions were evaluated in crude homogenates prepared from WT (n=3) and R2474S/+ KI (n=3) hearts. A, representative Western blotting. (1) minimal phosphorylation (with protein phosphatase 1 (PP1): 20 U/mL; Sigma, and protein kinase A inhibitor (PKI): 150 µmol/L; Calbiochem), (2) control condition (no modulators), (3) phosphorylation (with 1 µmol/L cAMP + 1 µmol/L okadaic acid (OA), (4) maximal phosphorylation (with 10µmol/L cAMP + 10 µmol/L OA + 2 mmol/L NaF). B, The summarized data of the phosphorylated(P)-RyR2 at Ser2808. The ratio of P-Ser2808-RyR2/total RyR2 is normalized as % of the control condition.

**Online Figure III.**
(Top) Western blots of various Ca$^{2+}$ regulatory proteins: RyR2, cardiac ryanodine receptor; P-Ser2808-RyR2, phosphorylated RyR2 at Ser2808; SERCA2α, SR Ca$^{2+}$-ATPase; PLB, phospholamban; P-Ser16-PLB, phosphorylated PLB at serine 16; P-Thr17-PLB, phosphorylated PLB at threonine 17; CASQ, calsequestrin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (Bottom) Summarized data of Western blots. Open bar: WT; closed bar: KI. Data represent the mean ± SE of 4 hearts.

**Online Figure IV.**
Average latency to development of generalized tonic-clonic seizures. Mice were injected with 2.5 mg/kg 4-aminopyridine, followed 25 minutes later by caffeine (250 mg/kg ) either at one time addition (A) or at an incremental addition (by 50mg /5min) up to 250 mg/Kg (B). In either case, there was no significant difference in the latency to development of generalized tonic-clonic seizures. N means the number of mice.

**Online Figure V.**
SERCA2-mediated Ca$^{2+}$ uptake. A, Time course of SR Ca$^{2+}$ uptake in WT and R2474S/+ KI saponin-permeabilized cardiomyocytes, in which fluo-5N was pre-loaded. The SR [Ca$^{2+}$] was first depleted with 10 mmol/L caffeine in Ca$^{2+}$- free solution, and SR Ca$^{2+}$ uptake was initiated
by addition of 100 nmol/L Ca\(^{2+}\) in the presence of 10 µmol/L ruthenium red, 1 µmol/L okadaic acid (OA), and 1 µmol/L cAMP. \(\textbf{B}\), Average time constants derived by exponential fitting of SR Ca\(^{2+}\) uptake curve. \(N\): the number of cells from 3-5 hearts.

**Online Figure VI.**
Representative 3D images of Ca\(^{2+}\) sparks. The Ca\(^{2+}\) sparks were shown 3-dimensionally in intact or saponin-permeabilized cardiomyocytes. \(\textbf{A}\) Isoproterenol (ISO:10 nmol/L) or \(\textbf{B}\) cAMP (1 µmol/L) was added, respectively.

**Online Figure VII.**
Spontaneous Ca\(^{2+}\) waves. Top: Recordings of line-scan images of fluo-4 AM fluorescence with or without isoproterenol (ISO:100 nmol/L) in intact WT and R2474S/+ KI cardiomyocytes. Bottom: Summarized data of the frequency of Ca\(^{2+}\) wave. \(N\): the number of cells from 3-5 hearts.

**Online Figure VIII.**
Comparison of P-Ser2808-RyR2 by immunoblotting using antibody against Ser2808 (Badrilla, UK: ref. 11) in KI and WT cardiomyocytes. ISO-induced phosphorylation in intact cardiomyocytes. In the presence of isoproterenol (10 nmol/L), KI and WT cardiomyocytes were incubated in the lysis buffer, and centrifuged. Then, the supernatant fraction containing crude homogenate was used for phosphorylation assay. \(N\): the number of cell lysate from 4 hearts.

**Online Figure IX.**
Effect of tetracaine on SR Ca\(^{2+}\) load. Top: Representative line-scan images of cardiomyocytes after addition of tetracaine (1 mmol/L), at 30 nmol/L [Ca\(^{2+}\)] buffered by 0.5 mmol/L EGTA. Bottom: Relationship between SpF and SR Ca\(^{2+}\) content. Arrow indicates the shift of the data point by the addition of tetracaine. \(N\)= 19-35 cells from 3-5 hearts.

**Online Figure X.**
DPc10 introduction into saponin-permeabilized cardiomyocytes. Note that Alexa Fluor 488-labeled DPc10 (showed as green) is clearly seen in saponin-permeabilized cardiomyocytes. Alexa Fluor 633-labeled wheat germ agglutinin (WGA) binds to cell membrane (shown as red).

**Online Figure XI.**
Representative recording of di-8-ANEPPS fluorescence in intact cardiomyocytes. Arrows
indicate the line scan site on the plasma membrane. The fluorescence ratio (505-545/650-) reveals membrane potential.

**Online Figure XII.**
Proposal of a new molecular mechanism of CPVT. In the normal channel, domain-domain interaction between the N-terminal (aa 1-600) and central (aa 2000-2500) domains is maintained in a zipped state, then the closed state of the channel is stabilized. In the mutant channel, however, evident domain unzipping, which is facilitated by the protein kinase A (PKA)-phosphorylation of Ser2808 located at the boundary between these regulatory domains, occurs in the mutant channel. This induces sequential abnormal events: reduction of the threshold SR Ca\textsuperscript{2+} content for channel activation (or increased sensitivity to the luminal Ca\textsuperscript{2+}), Ca\textsuperscript{2+} leak, and delayed afterdepolarization (DAD) (spontaneous after potential induced by an inward Na\textsuperscript{+} current via Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange (NCX)). Dantrolene, which corrects defective domain unzipping to a normal zipped state, stops these abnormal events.
Online Figure I. Schematic representation of the genomic structure of the mouse RyR2: the targeting vector used to generate the knock-in RyR2 R2474S mouse strain and the recombining genomic structure of RyR2 R2474S.
Online Figure II. Protein kinase A (PKA)-dependent phosphorylation of Ser2808-RyR2. Four different conditions were evaluated in crude homogenates prepared from WT (n=3) and R2474S/+ KI (n=3) hearts. A, representative Western blotting. (1) minimal phosphorylation (with protein phosphatase 1 (PP1): 20 U/mL; Sigma, and protein kinase A inhibitor (PKI): 150 µmol/L; Calbiochem), (2) control condition (no modulators), (3) phosphorylation (with 1 µmol/L cAMP + 1 µmol/L okadaic acid (OA), (4) maximal phosphorylation (with 10µmol/L cAMP + 10 µmol/L OA + 2 mmol/L NaF). B, The summarized data of the phosphorylated(P)-RyR2 at Ser2808. The ratio of P-Ser2808-RyR2/total RyR2 is normalized as % of the control condition.
Online Figure III. (Top) Western blots of various Ca\textsuperscript{2+} regulatory proteins: RyR2, cardiac ryanodine receptor; P-Ser2808-RyR2, phosphorylated RyR2 at Ser2808; SERCA2a, SR Ca\textsuperscript{2+}-ATPase; PLB, phospholamban; P-Ser16-PLB, phosphorylated PLB at serine 16; P-Thr17-PLB, phosphorylated PLB at threonine 17; CASQ, calsequestrin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (Bottom) Summarized data of Western blots. Open bar: WT; closed bar: KI. Data represent the mean±SE of 4 hearts.
Online Figure IV. Average latency to development of generalized tonic-clonic seizures. Mice were injected with 2.5 mg/kg 4-aminopyridine, followed 25 minutes later by caffeine (250 mg/kg) either at one time addition (A) or at an incremental addition (by 50 mg/5 min) up to 250 mg/Kg (B). In either case, there was no significant difference in the latency to development of generalized tonic-clonic seizures. N means the number of mice.
Online Figure V. SERCA2-mediated Ca\(^{2+}\) uptake.\(^{10}\) A, Time course of SR Ca\(^{2+}\) uptake in WT and R2474S/+ KI saponin-permeabilized cardiomyocytes, in which fluo-5N was pre-loaded. The SR [Ca\(^{2+}\)] was first depleted with 10 mmol/L caffeine in Ca\(^{2+}\)-free solution, and SR Ca\(^{2+}\) uptake was initiated by addition of 100 nmol/L Ca\(^{2+}\) in the presence of 10 µmol/L ruthenium red, 1µmol/L okadaic acid (OA), and 1 µmol/L cAMP. B, Average time constants derived by exponential fitting of SR Ca\(^{2+}\) uptake curve. N: the number of cells from 3-5 hearts.
Online Figure VI. Representative 3D images of Ca\textsuperscript{2+} sparks. The Ca\textsuperscript{2+} sparks were shown 3-dimensionally in intact or saponin-permeabilized cardiomyocytes. (A) Isoproterenol (ISO:10 nmol/L) or (B) cAMP (1 µmol/L) was added, respectively.
Online Figure VII. Spontaneous Ca^{2+} waves. Top: Recordings of line-scan images of fluo-4 AM fluorescence with or without isoproterenol (ISO:100 nmol/L) in intact WT and R2474S/+ KI cardiomyocytes. Bottom: Summarized data of the frequency of Ca^{2+} wave. N: the number of cells from 3-5 hearts.
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**Online Table I.**

Effect of isoproterenol (ISO) on cell shortening and Ca\(^{2+}\) transient in WT and R2474S/+ KI cardiomyocytes

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>ISO (10 nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n=47)</td>
<td>KI (n=56)</td>
</tr>
<tr>
<td><strong>Cell shortening</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell shortening (% decrease from baseline)</td>
<td>2.5 ± 0.3</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>Time from peak to 80% decline (×10(^{-2})sec)</td>
<td>33.4 ± 1.0</td>
<td>33.5 ± 0.7</td>
</tr>
<tr>
<td><strong>Ca(^{2+}) transient</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak of Ca(^{2+}) transient (% increase from baseline)</td>
<td>30.9 ± 2.4</td>
<td>35.7 ± 1.9</td>
</tr>
<tr>
<td>Time from peak to 80% decline (×10(^{-2})sec)</td>
<td>29.9 ± 0.6</td>
<td>30.8 ± 0.4</td>
</tr>
</tbody>
</table>

N: the number of cells from 3 hearts. *P<0.01 vs WT ISO 10nmol/L; †P<0.01 vs WT Baseline; †P<0.01 vs KI Baseline.
### Online Table II.

Effect of isoproterenol on Ca\(^{2+}\) spark characteristics in intact cardiomyocytes

<table>
<thead>
<tr>
<th>ISO (10nmol/L)</th>
<th>Peak (F/F(_0))</th>
<th>FWHM (µm)</th>
<th>FDHM (ms)</th>
<th>No. of Sparks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(-)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT (n=8)</td>
<td>1.27±0.01</td>
<td>1.63±0.03</td>
<td>16.93±0.72</td>
<td>555</td>
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<tr>
<td>KI (n=6)</td>
<td>1.25±0.00</td>
<td>1.80±0.03</td>
<td>37.71±1.35*</td>
<td>1081</td>
</tr>
<tr>
<td><strong>(+)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT (n=8)</td>
<td>1.44±0.02*</td>
<td>2.01±0.04*</td>
<td>23.17±0.80*</td>
<td>1250</td>
</tr>
<tr>
<td>KI (n=6)</td>
<td>1.41±0.01#</td>
<td>2.00±0.04#</td>
<td>52.73±1.81# †</td>
<td>1321</td>
</tr>
</tbody>
</table>

N: the number of hearts. FWHM: full width at half maximum, FDHM: full duration at half maximum.

*\(p<0.01\) vs. WT ISO (-), †\(p<0.01\) vs. WT ISO (+).
## Online Table III.

Effect of cAMP on Ca\(^{2+}\) spark characteristics in saponin-permeabilized cardiomyocytes

<table>
<thead>
<tr>
<th>cAMP (µmol/L)</th>
<th>Peak (F/F(_0))</th>
<th>FWHM (µm)</th>
<th>FDHM (ms)</th>
<th>No. of Sparks</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WT (n=12)</td>
<td>1.75 ± 0.00</td>
<td>2.00 ± 0.01</td>
<td>23.68 ± 0.08</td>
<td>15405</td>
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<tr>
<td>KI (n=9)</td>
<td>1.49 ± 0.00*</td>
<td>1.71 ± 0.00*</td>
<td>26.65 ± 0.22*</td>
<td>14903</td>
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<tr>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WT (n=6)</td>
<td>2.13 ± 0.01</td>
<td>2.07 ± 0.01</td>
<td>25.13 ± 0.13</td>
<td>5915</td>
</tr>
<tr>
<td>KI (n=6)</td>
<td>1.62 ± 0.00*</td>
<td>1.83 ± 0.01*</td>
<td>26.64 ± 0.35*</td>
<td>8559</td>
</tr>
<tr>
<td>0.3</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>WT (n=6)</td>
<td>2.21 ± 0.01</td>
<td>2.18 ± 0.01</td>
<td>28.17 ± 0.23</td>
<td>5399</td>
</tr>
<tr>
<td>KI (n=6)</td>
<td>1.69 ± 0.00*</td>
<td>1.94 ± 0.01*</td>
<td>31.27 ± 0.36*</td>
<td>11770</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>WT (n=6)</td>
<td>2.21 ± 0.01</td>
<td>2.20 ± 0.01</td>
<td>34.51 ± 0.47</td>
<td>6766</td>
</tr>
<tr>
<td>KI (n=6)</td>
<td>1.77 ± 0.01*</td>
<td>2.01 ± 0.01*</td>
<td>36.25 ± 0.61#</td>
<td>5667</td>
</tr>
</tbody>
</table>

N: the number of hearts. FWHM: full width at half maximum, FDHM: full duration at half maximum.

*\(p<0.05\) vs. WT, \(^{*}p<0.01\) vs. WT.