A Novel Ryanodine Receptor Mutation Linked to Sudden Death Increases Sensitivity to Cytosolic Calcium

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Rationale: Mutations in the cardiac type 2 ryanodine receptor (RyR2) have been linked to catecholaminergic polymorphic ventricular tachycardia (CPVT). CPVT-associated RyR2 mutations cause fatal ventricular arrhythmias in young individuals during β-adrenergic stimulation.

Objective: This study sought to determine the effects of a novel RyR2-G230C mutation and whether this mutation and RyR2-P2328S alter the sensitivity of the channel to luminal calcium (Ca^{2+}).

Methods and Results: Functional characterizations of recombinant human RyR2-G230C channels were performed under conditions mimicking stress. Human RyR2 mutant channels were generated by site-directed mutagenesis and heterologously expressed in HEK293 cells together with calstabin2. RyR2 channels were measured to examine the regulation of the channels by cytosolic versus luminal sarcoplasmic reticulum Ca^{2+}. A 50-year-old white man with repeated syncopal episodes after exercise had a cardiac arrest and harbored the mutation RyR2-G230C. cAMP-dependent protein kinase–phosphorylated RyR2-G230C channels exhibited a significantly higher open probability at diastolic Ca^{2+} concentrations, associated with a depletion of calstabin2. The luminal Ca^{2+} sensitivities of RyR2-G230C and RyR2-P2328S channels were WT-like.

Conclusions: The RyR2-G230C mutant exhibits similar biophysical defects compared with previously characterized CPVT mutations: decreased binding of the stabilizing subunit calstabin2 and a leftward shift in the Ca^{2+} dependence for activation under conditions that simulate exercise, consistent with a “leaky” channel. Both RyR2-G230C and RyR2-P2328S channels exhibit normal luminal Ca^{2+} activation. Thus, diastolic sarcoplasmic reticulum Ca^{2+} leak caused by reduced calstabin2 binding and a leftward shift in the Ca^{2+} dependence for activation by diastolic levels of cytosolic Ca^{2+} is a common mechanism underlying CPVT. (Circ Res. 2011;109:281-290.)

Key Words: CPVT ■ sudden cardiac death ■ ryanodine receptor ■ calstabin

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a rare inherited arrhythmogenic disease characterized by syncope or sudden death induced by emotional or physical stress. The mortality rate in untreated individuals ranges from 30% to 50% by the age of 40 years. Dominantly inherited forms of CPVT have been linked to mutations in the cardiac ryanodine receptor (RyR2).1,2 Currently, >70 RyR2 mutations have been identified, and RyR2 mutations may account for 50% of diagnosed cases of CPVT.2,3

We report a novel CPVT mutation in RyR2 in a 50-year-old white man with a history of repeated syncopal episodes after exercise, beginning at age 30 years. The proband had a cardiac arrest and was diagnosed with CPVT. RyR2 genetic analysis performed by direct sequencing of the patient’s DNA revealed a RyR2-G230C mutation. A brother died suddenly during exercise at age 16 years, and a son died suddenly of cardiac arrest after swimming at 12 years of age. A younger son was tested and does not carry the RyR2-G230C mutation.

Our group first elaborated a mechanism for CPVT by showing that CPVT and sudden cardiac death (SCD)-related RyR2 mutations decrease the binding of calstabin2 (FKBP12.6) to RyR2, which is further reduced by protein kinase A (PKA) phosphorylation during adrenergic stimulation.4–7 Moreover, we showed that CPVT-linked RyR2 mutations, including RyR2-S2246L, RyR2-R2474S, RyR2-R4497C, RyR2-P2328S, RyR2-Q4201R, and RyR2-V4653F,
shift the sensitivity for cytosolic calcium (Ca\(^{2+}\))-dependent activation of the channel to the left, resulting in channels that are inappropriately activated at low diastolic levels of cytosolic Ca\(^{2+}\), thereby causing a diastolic sarcoplasmic reticulum (SR) Ca\(^{2+}\) leak.\(^4\) Importantly, the leftward shift in the sensitivity for cytosolic Ca\(^{2+}\)-dependent activation of the CPVT mutant channels compared with WT channels was only observed after PKA phosphorylation of the channels (conditions that mimic the effect of exercise or stress on the channel). This is in agreement with the clinical phenotype of CPVT patients who have arrhythmias during exercise but not at rest.

Another group has proposed that increased sensitivity to luminal SR Ca\(^{2+}\) is the mechanism underlying leaky CPVT mutant channels. This group reported that CPVT, SCD, or arrhythmogenic right ventricular dysplasia–linked RyR2 mutations, including RyR2-E189D, RyR2-N4104K, RyR2-R4496C, and RyR2-N4895D, exhibited increased sensitivity to activation by luminal Ca\(^{2+}\), leading them to conclude that store overload–induced Ca\(^{2+}\) release (SOICR) is the cause of CPVT.\(^8\)–\(^10\) A third group, using the CPVT mutant model RyR2-R2474S, suggested that CPVT and SCD-linked mutations induce defective interdomain conformational changes that destabilize the closed state of the channel and enhance its sensitivity to Ca\(^{2+}\)\(^6\)–\(^11\),\(^12\) It has also been reported that RyR2-L433P and RyR2-N2386I mutant channels exhibited impaired sensitivity to Ca\(^{2+}\)-dependent channel inhibition.\(^13\) More recently, 3 additional studies at the cellular level reported that the CPVT RyR2-R4496C mutant is abnormally Ca\(^{2+}\) sensitive and “leaky” and pointed out the importance of SR Ca\(^{2+}\) load in the genesis of Ca\(^{2+}\) waves and arrhythmias.\(^14\)–\(^16\)

These disparate conclusions have impaired a clear understanding of the mechanism underlying CPVT and require further investigation to resolve the differences.

We report a novel CPVT mutation, RyR2-G230C, and show that this mutation, like others we have reported,\(^4\)–\(^6\) results in increased depletion of calstabin2 from the RyR2 macromolecular complex and enhanced sensitivity to cytosolic Ca\(^{2+}\) (leftward shift in the Ca\(^{2+}\)-dependence for activation of the channel) and under conditions that simulate exercise-induced stress (β-adrenergic stimulation). Importantly, when examined at the single-channel level, both RyR2-G230C and RyR2-P2328S mutant channels exhibited normal luminal Ca\(^{2+}\) sensitivity compared with WT, indicating that their threshold for luminal Ca\(^{2+}\) activation is the same as WT channels. Thus, increased sensitivity to cytosolic Ca\(^{2+}\) can explain the leaky channel behavior of CPVT mutant RyR2 channels and indicate that altered sensitivity to luminal Ca\(^{2+}\) (SOICR) is not required to explain CPVT.

### Methods

Recombinant mutant channels were generated and expressed in HEK293 cells. HEK293 cell lines expressing RyR2 wt and mutants were generated with the use of QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). Single-channel measurements were performed to investigate the RyR2 channel properties in planar lipid bilayers. Measurements of Calstabin2 binding to immunoprecipitated RyR2 were performed under PKA phosphorylation. A detailed Methods section can be found in the Online Data Supplement available at http://circres.ahajournals.org.

### Results

#### Phenotype

Clinical evaluation including cardiac catheterization revealed no cardiac structural abnormalities. ECG at rest showed normal sinus rhythm including a normal QT interval. Treadmill stress test (Bruce protocol) induced polymorphic premature ventricular beats (Figure 1).

#### Molecular Characterization of CPVT-Linked RyR2 Mutation

Blood samples were obtained by venipuncture, and genomic DNA was extracted from peripheral blood lymphocytes. We performed genetic testing of the proband’s genomic DNA and confirmed it by dyeoxy sequencing method. We found on genetic testing that the proband has a missense mutation with a Guanine to Thymine substitution at the 688th base of the RyR2 gene nucleotide sequence (Ryr2–688G>T), which resulted in a glycine to cysteine substitution at the 230th position (G230C), close to the amino-terminus of the RyR2 protein (Online Figure I). This CPVT-linked RyR2 mutation has not been previously reported.

#### Pedigree of Index Family

The proband was a 50-year-old white man with a history of repeated syncopal episodes after exercise beginning at age 30 years. He had a cardiac arrest and was diagnosed with CPVT. His brother died suddenly during exercise at age 16 years, and his son died suddenly of cardiac arrest after swimming at 12 years of age. A younger son was tested and does not carry the RyR2-G230C mutation (Online Figure II).

#### Functional Characterization of CPVT-Linked Mutant RyR2-G230C Channels

To study the effects of the CPVT-associated RyR2-G230C mutations on the channel function, we characterized the biophysical properties of the single-channel activity (Figure 2). ER microsomes from HEK293 cells transiently coexpressing calstabin2 and either recombinant RyR2-WT or RyR2-G230C were fused into planar lipid bilayers. To test the functional properties of RyR2-G230C mutant channels under conditions simulating stress caused by increased activity of the sympathetic nervous system, we measured single-channel activity of the RyR2-WT and RyR2-G230C channels from microsomes that were subjected to PKA phosphorylation in vitro, using the planar lipid bilayer technique over a range of physiological Ca\(^{2+}\) concentrations, starting at 150

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nmol/L up to full activation of the channel (≈1 to 10 μmol/L Ca2⁺). At low cytosolic calcium concentration (ie, 150 nmol/L free Ca2⁺) that corresponds to the resting diastolic Ca2⁺ level under basal conditions (no PKA treatment), the activity of RyR2-WT and RyR2-G230C channels was similar, displaying a very low open probability (Po) (mean Po, 0.007±0.004 for RyR2-WT, n=5; versus mean Po, 0.009±0.004 for RyR2-G230C, n=5; P=NS; Figure 2A and 2C). As the cis (cytosolic), Ca2⁺ concentration was increased, and the Po of both channels increased to a similar degree (mean Po, 0.041/0.359/0.531 at 350/700/1000 nmol/L Ca2⁺ for RyR2-WT versus mean Po, 0.108/0.310/0.626 at 350/700/1000 nmol/L Ca2⁺ for RyR2-G230C; P=NS; Figure 2D).

After PKA treatment, channel activity was increased for both RyR2-WT and RyR2-G230C. However, the Po of RyR2-G230C mutant channels was significantly higher compared with RyR2-WT channels at 150 nmol/L cytosolic Ca2⁺ (mean Po, 0.068±0.020 for RyR2-WT, n=5, versus mean Po, 0.182±0.038 for RyR2-G230C, n=7; P<0.05; Figure 2B and 2C). The activity of RyR2-G230C channels was also higher, with 350 nmol/L cytosolic Ca2⁺ (mean Po, 0.174±0.064 for RyR2-WT, n=5, versus mean Po, 0.338±0.032 for RyR2-G230C, n=7; P<0.05; Figure 2D). It should be noted that the mean closed time was comparable (data not shown). Once the channel was activated with further cytosolic Ca2⁺ (>700 nmol/L, free Ca2⁺), no differences in Po were found between PKA-treated RyR2-G230C and WT channels (Figure 2D).

In addition to an increased sensitivity to low [Ca2⁺]cyt, amplitude histograms indicated an increased prevalence of subconductance states in the PKA-phosphorylated RyR2-G230C channels (Figure 2B) as previously observed for RyR channels that were PKA-phosphorylated and depleted of calstabin.5,17,18 Taken together, these results indicate that under stress conditions (mimicked by PKA phosphorylation) the CPVT-associated RyR2 mutation RyR2-G230C results in increased sensitivity to cytosolic Ca2⁺ causing “leaky” channels. These results are consistent with the observed functional effect of previously characterized CPVT and SCD-related RyR2 mutations4–6 and with the clinical observations that cardiac arrhythmias in CPVT patients occur during physical or emotional stress.

Biochemical Characterization of RyR2-G230C Mutation

Previous studies have shown that the depletion of calstabin2 from the RyR2 channel complex destabilizes the closed state of RyR2.4,5 This destabilization leads to enhanced diastolic Ca2⁺ sensitivity manifested as an increased RyR2 Po in the presence of low activating [Ca2⁺]=150 nmol/L.5,17,19 In support of this model, our group has demonstrated that recombinant CPVT and SCD-related RyR2 mutants, expressed in HEK293 cells, exhibit a reduced affinity for calstabin2 compared with WT channels.4,5
To determine whether the increased Po and gating changes observed for the PKA-phosphorylated RyR2-G230C mutant channels were associated with depletion of the stabilizing subunit calstabin2 protein, RyR2 macromolecular complexes were immunoprecipitated from HEK293 cell membranes. Whereas untreated channels revealed only a basal level of PKA phosphorylation, PKA-treated RyR2-WT and RyR2-G230C channels exhibited a comparable high level of PKA phosphorylation (Figure 3A and 3B) and significantly lower amounts of calstabin2 bound to the PKA-phosphorylated RyR2-G230C mutant channels compared with the RyR2-WT treated channels (Figure 3A and 3C). The significantly lower amount of calstabin2 bound to the PKA-phosphorylated RyR2-G230C mutant channels suggests that the CPVT-associated mutation decreases the binding affinity of calstabin2 to the mutant RyR2, as previously reported for other CPVT-linked RyR2 mutations.4,5

**CPVT-Linked RyR2-G230C and RyR2-P2328S Mutations Do Not Affect Luminal Ca^{2+} Sensitivity**

To study the effects of RyR2-G230C mutation on the luminal Ca^{2+} sensitivity, we performed single-channel measurements.
using Ba\(^{2+}\) as the charge carrier with added Ca\(^{2+}\) to the luminal (trans) side at low cytosolic (cis) Ca\(^{2+}\) in absence of allosteric modulators (ie, no PKA treatment, no Mg-ATP nor caffeine). Addition of 1 mmol/L Ca\(^{2+}\) to the luminal side did not significantly increase the single Po of recombinant RyR2-G230C and WT channels (mean Po, 0.016±0.011 at 0 mmol/L luminal [Ca\(^{2+}\)], n=8, versus mean Po, 0.032±0.015 at 1 mmol/L luminal [Ca\(^{2+}\)] for RyR2-WT, n=5, and mean Po, 0.013±0.006 at 0 mmol/L luminal [Ca\(^{2+}\)], n=10, versus mean Po, 0.032±0.015 at 1 mmol/L luminal [Ca\(^{2+}\)] for RyR2-G230C, n=8; P=NS; Figure 4A and 4C). Addition of 10 mmol/L luminal Ca\(^{2+}\) did not increase Po significantly in either recombinant channel groups (mean Po, 0.047±0.008 for RyR2-WT, n=5, versus mean Po, 0.033±0.012 for RyR2-G230C, n=7; P=NS; Figure 4B and 4C). Addition of 10 mmol/L luminal Ca\(^{2+}\) induced comparable significant increases in Po in both WT and CPVT channels when compared with the Po obtained at 0 mmol/L luminal Ca\(^{2+}\) (mean Po, 0.016±0.011 at 0 mmol/L luminal [Ca\(^{2+}\)], n=8, versus mean Po, 0.100±0.024 at 10 mmol/L luminal [Ca\(^{2+}\)], n=5, for RyR2-WT, P<0.01; and mean Po, 0.013±0.006 at 0 mmol/L luminal [Ca\(^{2+}\)], n=10, versus mean Po, 0.084±0.034, n=5, for RyR2-G230C, P<0.05; Figure 4B and 4C).

However, even at 10 mmol/L luminal [Ca\(^{2+}\)], no difference in sensitivity to activation by luminal Ca\(^{2+}\) was observed between WT and RyR2-G230C recombinant channels (mean Po, 0.100±0.024 for RyR2-WT, n=5, versus mean Po, 0.084±0.034 for RyR2-G230C, n=5; P=NS; Figure 4C).

To reduce the baseline effect of divalent cations on the luminal side of the channel and to avoid any contaminating Ca\(^{2+}\) in the Ba\(^{2+}\) solution, we also tested the effect of luminal Ca\(^{2+}\) in absence of other divalent ions. We performed single-channel measurements using Cs\(^{+}\) as the primary current charge carrier with added Ca\(^{2+}\) to the luminal (trans) side in presence of EGTA. In agreement with the above Ba\(^{2+}\) experiments, Po obtained with 0, 1, 5, and 10 mmol/L free Ca\(^{2+}\) to the luminal side was comparable between RyR2-G230C and WT (mean Po, 0.230/0.336/0.330/0.498 at 0/1/5/10 mmol/L Ca\(^{2+}\); n=5 for each, for RyR2-WT versus mean Po, 0.262/0.288/0.400/0.317 at 0/1/5/10 mmol/L Ca\(^{2+}\), n=4 for each, for RyR2-G230C; P=NS; Online Figure III).

Thus, our results indicate that the sensitivity of RyR2-G230C mutant channel to activation by luminal Ca\(^{2+}\) is the same as that of WT channels, demonstrating that there is no requirement to invoke the SOICR hypothesis to explain the mechanism of leak observed for the CPVT-linked RyR2-G230C channels.

RyR2-P2328S is a CPVT-linked missense mutation that is located in the central region of the RyR2 gene near the FKBP12.6 binding site.\(^{20}\) We first observed that recombinant human PKA–phosphorylated RyR2-P2328S mutant exhibits decreased binding of calstabin2 and has a significant gain-of-function defect consistent with a “leaky” channel at low cytosolic Ca\(^{2+}\) and with a rightward shift in the half-maximal inhibition Mg\(^{2+}\) concentration (IC\(_{50}\)).\(^{7}\) The murine model of RyR2-P2328S mutation was then characterized by Goddard et al.,\(^{21}\) who observed altered cellular Ca\(^{2+}\) handling linked to CPVT-linked arrhythmias with a more pronounced effect in the homozygote than the heterozygote.

To determine whether the RyR2-P2328S mutation exhibits a reduced threshold for luminal Ca\(^{2+}\), we measured the effects of increasing luminal [Ca\(^{2+}\)] in the presence of 3 mmol/L Mg-ATP and 0.6 mmol/L MgCl\(_2\) at 150 mmol/L [Ca\(^{2+}\)]\(_{cyt}\) (Figure 5A and 5B).

Data were averaged and Po increased as luminal Ca\(^{2+}\) increased, with no significant difference between RyR2-P2328S and WT recombinant channels (eg, mean Po, 0.062±0.037 at 0.2 mmol/L luminal [Ca\(^{2+}\)], n=5, versus mean Po, 0.110±0.070 at 1 mmol/L luminal [Ca\(^{2+}\)] for RyR2-WT, n=4, and mean Po, 0.026±0.022 at 0.2 mmol/L luminal [Ca\(^{2+}\)], n=4, versus mean Po, 0.042±0.037 at 1 mmol/L luminal [Ca\(^{2+}\)] for RyR2-P2328S, n=4; P=NS; Figure 5A and 5C).

Increased [Ca\(^{2+}\)] on the luminal side of the channel slightly increased single-channel Po in both groups, with no signifi-
Differences between WT and RyR2-P2328S channels (eg, mean Po, 0.160 ± 0.060 at 5 mmol/L luminal [Ca2+]i, n=4, versus mean Po, 0.209 ± 0.086 at 10 mmol/L luminal [Ca2+]i) for RyR2-WT, n=4, and mean Po, 0.161 ± 0.109 at 5 mmol/L luminal [Ca2+]i, n=4, versus mean Po, 0.070 ± 0.036 at 10 mmol/L luminal [Ca2+]i for RyR2-P2328S, n=4; P=NS; Figure 5B and 5C). Thus, like the hRyR2-G230C channels, the CPVT-linked recombinant hRyR2-P2328S channels exhibit the same sensitivity to luminal [Ca2+]i as WT RyR2 channels.

Discussion

In the present study, we report a novel CPVT-linked RyR2 mutation that results in the substitution of cysteine for glycine at position 230, RyR2-G230C. The mutation is located in the disease-associated “hot spot” N-terminal domain of cardiac RyR2.20,22–24 We coexpressed recombinant hRyR2 channels and the stabilizing subunit calstabin2. Our experiments indicate that the functional consequences of the RyR2-G230C mutation are a “leaky” RyR2 channel at diastolic Ca2+ (low cytosolic [Ca2+]c) associated with reduced binding of calstabin2 to the RyR2 channel that occurs only under stress conditions. Moreover, because previous CPVT/arrhythmogenic right ventricular dysplasia RyR2 mutants were proposed to have increased luminal Ca2+ sensitivity,8–10 we compared the effects of luminal (trans) Ca2+ on RyR2-G30C mutant channel properties and did not find any differences in the CPVT channels compared with WT channels. Additionally, we also compared the luminal Ca2+ dependence of another CPVT mutant, RyR2-P2328S, known to be hypersensitive to cytosolic Ca2+.7,21 Like the RyR2-G230C channels, the RyR2-P2328S mutant channels do not display any abnormalities in responsiveness to luminal [Ca2+]i. These results show that the threshold for luminal Ca2+ activation is the same in WT and 2 CPVT mutant channels, and therefore the SOICR mechanism is not a generalized mechanism for CPVT.

Overall, our results reinforce the molecular mechanism by which, under the β-adrenergic stimulation, CPVT-associated RyR2 mutations lead to a pathologically increased cytosolic Ca2+ sensitivity associated with a depletion of calstabin2. Furthermore, our results indicate that the RyR2-G230C mu-
tant channels exhibit increased cytosolic Ca\(^{2+}\) sensitivity that is not dependent on luminal Ca\(^{2+}\) and thus do not support the concept of store overload–induced Ca\(^{2+}\) release as a generalized mechanism for CPVT.\(^8\)–\(^10\),\(^25\) In the present report, we found a single-base substitution in the \(\text{RyR2}\) gene in a 50-year-old man. The proband had repeated syncope episodes after exercise beginning at age 30 years, had a cardiac arrest, and was diagnosed with CPVT. This single-base substitution in \(\text{Ryr2}\) resulted in a novel \(\text{RyR2-G230C}\) mutation close to the amino-terminus with similar biophysical defects as other mutations in the middle and carboxy-terminus of the \(\text{RyR2}\) channel and is associated with decreased binding of the stabilizing subunit calstabin2.

Cardiac arrhythmia susceptibility genes that have been identified predominantly encode ion channels.\(^26\),\(^27\) Our work strongly suggests that the single point mutation \(\text{RyR2-G230C}\) is linked to the cardiac pathological phenotype observed in the proband. A critical feature of CPVT is that triggering of an arrhythmogenic episode occurs in the absence of structural alterations to the heart and requires the presence of both a causal mutation and stressful conditions that induce β-adrenergic stimulation. Our results support the model by which CPVT-associated mutations in the \(\text{RyR2}\), under stress conditions (ie, mimicked by the PKA phosphorylation of \(\text{RyR2}\) in vitro), induce a significant increase in the cytosolic Ca\(^{2+}\) sensitivity and highlight the link with the calstabin2 binding from the \(\text{RyR2}\) macromolecular complex.

Several studies have confirmed that CPVT and SCD-related \(\text{RyR2}\) mutations lead to a cytosolic Ca\(^{2+}\) hypersensitivity, providing support for the Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism rather than the SOICR hypothesis.\(^4\)–\(^6\),\(^14\) In line with this view, Fernandez-Velasco et al\(^14\) have observed an increase in Ca\(^{2+}\) spark frequency in \(\text{RyR2-4496C}\) permeabilized cardiomyocytes, dependent on the diastolic intracellular Ca\(^{2+}\) concentration (ie, nmol/L range of [Ca\(^{2+}\)]) as well as an increase in cytosolic Ca\(^{2+}\) sensitivity in crude SR membrane of \(\text{RyR2-4496C}\) using \(^{[3]H}\) ryanodine binding experiments.

![Figure 5. Effects of luminal Ca\(^{2+}\) on recombinant human \(\text{RyR2-P2328S}\) mutant. A and B. Representative single-channel current traces of recombinant \(\text{RyR2-WT}\) and \(\text{RyR2-P2328S}\) channels measured at increasing luminal Ca\(^{2+}\) concentrations as indicated on the left (0 and 1 mmol/L [mM] luminal [Ca\(^{2+}\)], first and second rows in A, and 5 and 10 mmol/L luminal [Ca\(^{2+}\)], first and second rows in B) with Ba\(^{2+}\) as the charge carrier at 0 mV. The cytosolic (cis) side contained 3 mmol/L Mg-ATP, 0.6 mmol/L MgCl\(_2\), and 150 nmol/L free [Ca\(^{2+}\)]. Channel openings are shown as upward deflections; the closed (c-) state of the channel is indicated by horizontal bars in the beginning of each tracing and the Po, To (average open time), and Tc (average closed time) of the illustrative trace, calculated over 2 minutes of continuous recording, are shown above each trace. C, Luminal Ca\(^{2+}\) dependence of single-channel Po in \(\text{RyR2-WT}\) (white bars) and \(\text{RyR2-P2328S}\) (black bars) channels. All Po measurements were made from 4 to 8 different channels at different luminal [Ca\(^{2+}\)] concentrations (0, 0.02, 0.2, 0.6, 1, 2, 5, and 10 mmol/L).]
It is technically difficult to distinguish the SOICR mechanism from the Ca$^{2+}$-induced Ca$^{2+}$ release mechanism because luminal Ca$^{2+}$ may flow through the channel and modulate the cytosolic Ca$^{2+}$ binding sites of RyR2.28,29 Indeed, previous studies have shown that luminal Ca$^{2+}$ regulates the cardiac RyR2 through direct feedback by binding to cytosolic Ca$^{2+}$ activation and inactivation sites.30 Therefore, any experiments performed using high concentrations of luminal Ca$^{2+}$, which passes through the channel, will be subject to the limitation that this luminal Ca$^{2+}$ can activate cytosolic sites after it passes through the channel. Therefore, if CPVT channels are more sensitive to cytosolic [Ca$^{2+}$] than WT channels, luminal Ca$^{2+}$ passing through the channel could result in increased Po in CPVT channels not because of a reduced threshold for activation by luminal Ca$^{2+}$ (as proposed by the SOICR hypothesis) but rather because of increased sensitivity to cytosolic Ca$^{2+}$, as we have shown previously.4–7 To circumvent this limitation, we used Ba$^{2+}$ as the charge carrier in the luminal (trans) bilayer chamber and tightly controlled the free cytosolic (cis) [Ca$^{2+}$] with EGTA in a range from $\approx 150$ nmol/L to 10 $\mu$mol/L at 0 mV. Although Ba$^{2+}$ can pass through the RyR2 pore, Ba$^{2+}$ ions cannot activate RyR2 channels and therefore cannot explain any increases of the channel Po. To determine whether the RyR2-G230C mutation increases the sensitivity to activation by luminal Ca$^{2+}$ and thus increases the open probability of the channel, we examined the effects of Ca$^{2+}$ added to luminal (trans) chamber, in the presence of low cytosolic Ca$^{2+}$ (ie, 150 nmol/L free Ca$^{2+}$) and in the absence of any agonist (eg, no ATP, no caffeine, and no PKA phosphorylation), because the SOICR mechanism has been previously reported for some CPVT mutants but only in absence of PKA phosphorylation of the channel. Adding Ca$^{2+}$ on the luminal side of the channel induced a slight increase of Po, with no significant difference between RyR2-G230C and WT channels. Furthermore, we performed experiments on RyR2-P2328S channels, testing increasing concentrations of luminal Ca$^{2+}$ in the presence of the physiological channel modulators Mg-ATP and MgCl$_2$ and did not find any differences in Po compared with WT channels. Our data show that SOICR is not a generalized mechanism for CPVT; however, we cannot exclude that regulation of RyR2, by luminal SR proteins such as calsequestrin, can modulate the Ca$^{2+}$ sensitivity of the channel.28,29 Thus, our results and those of others4–6,14 indicate that the Ca$^{2+}$-induced Ca$^{2+}$ release mechanism can explain CPVT and that there is no need to invoke SOICR as an alternative mechanism, as recently explained.28

The RyR2-G230C mutation is located in the N-terminal portion of the channel. The N-terminus of RyR2 is one of the three major regions that harbor disease-causing mutations.20,22,23,31 Several biochemical and functional studies have indicated that disease-associated RyR mutations are located along the domain interface between the N-terminal and central regions of RyRs and weaken interdomain interactions (the domain unzipping hypothesis).11,12,32 A recent crystal structure provided an insight into the hotspot domain I of RyR and revealed the exact locations of more than 50 disease-associated mutations.24 Interestingly, these results indicated that Glycine 230 of hRyR2 is located in a critical subunit interface area that contains more than 19 disease-associated mutations in RyR1 and RyR2. Therefore, it is reasonable to propose that a single point mutation such as G230C may destabilize the contact and/or the folding of individual RyR2 domains, destabilizing the closed state of the channel. G230C mutation may also induce a decrease of the RyR2 protein level and/or the associated calstabin2. Our results show that the protein levels of immunoprecipitated recombinant RyR2-G230C and WT as well as the associated calstabin2 are similar. Because we use HEK293 cells heterologously expressing RyR2 and calstabin2, we cannot make any statement about RyR2 or calstabin2 protein levels in vivo. However, we have previously reported that knock-in mice expressing RyR2 harboring human CPVT mutations have normal levels of RyR2 and calstabin2 proteins.4 Our results also indicate that the RyR2-G230C mutation alters the channel activity only after PKA phosphorylation and leads to greater depletion of calstabin2 from the channel complex associated with a higher sensitivity to low cytosolic [Ca$^{2+}$] compared with WT. Thus, using immunodetection of Western-blotted proteins, we do not observe any difference in the associated calstabin2 level between RyR2-G230C and WT when the channels are not PKA-phosphorylated. Previous results from our group indicate that using $^{35}$S-labeled Calstabin2, CPVT-associated mutants RyR2-S2226L, RyR2-R2474S, and RyR2-R4497C have decreased basal affinity for Calstabin2.5,7 We also previously reported that a transgenic mouse overexpressing calstabin2 can prevent leaky RyR2 and heart failure progression.33 Interestingly, the PKA phosphorylation level of pS2809 is similar between RyR2-G230C and WT. Thus, the consequences of G230C mutation on RyR2 might be dependent on allosteric or charge effects of the PKA phosphorylation that promote channel openings. These observations are in agreement with previous published results showing that CPVT-associated RyR2 mutations, located at different regions of the amino acid sequence (eg, RyR2-R2474S and RyR2-V4653F), exacerbate the effect of PKA phosphorylation, as evidenced by an increased depletion of calstabin2 from the RyR2 complex.5,7

Although our results rule out the possibility that G230C mutation may affect the closed state of the channel (ie, mean closed time was comparable), more experiments will be needed to investigate the Ca$^{2+}$-dependent channel inhibition of RyR2-G230C channels, because some CPVT-related RyR2 mutants exhibit sensitivity to Ca$^{2+}$-dependent channel inhibition.13 Moreover, RyR has highly reactive cysteines able to form disulfide bonds.24 Therefore, the cysteine introduced by the CPVT mutation may interact with other cysteines in the channel, disturbing folding or inducing conformational changes to the channel. Further experiments under reducing conditions are needed to determine whether the substitution of cysteine for glycine induces conformational changes that destabilize the channel and increase sensitivity to cytosolic Ca$^{2+}$. Our recent results from a murine model indicate that catecholamine treatment can result in oxidization of RyR2 in addition to causing PKA phosphorylation. The combination of oxidation and PKA phosphorylation can activate the channel and pro-
mote diastolic SR Ca\(^{2+}\) leak.\(^{35}\) The addition of an extra cysteine in RyR2-G230C could increase the effect of channel oxidation during stress. However, other CPVT mutations that do not involve addition of a cysteine, such as RyR2-P2328S, result in similar effects on channel properties.

In conclusion, we have demonstrated a novel, functionally significant CPVT mutation in RyR2. The consequences of this mutation are an increased sensitivity to cytosolic Ca\(^{2+}\) under stress, associated with a depletion of calstabin2 from the RyR2 macromolecular complex, when the PKA phosphorylation level at serine 2809 is similar to the level in WT. These observations reinforce the model of “leaky” RyR2 channel through an altered calstabin2 binding as a mechanism by which CPVT-related RyR2 mutations cause arrhythmias.

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Disclosures
A.R.M. is a consultant for ARMGØ Pharma, Inc, a biotech company targeting RyR2 treatment for prevention of CPVT.

References

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**Novelty and Significance**

**What Is Known?**

- Individuals with catecholaminergic polymorphic ventricular tachycardia (CPVT)-linked mutations in the cardiac ryanodine receptor (RyR2) have exercise-induced arrhythmias, normal ECGs, and no arrhythmias at rest.
- Fatal ventricular arrhythmias are caused by intracellular calcium leak through mutant CPVT-linked RyR2 channels on the sarcoplasmic reticulum.
- CPVT mutations in the central and carboxy terminal domains of RyR2 cause decreased binding to the channel-stabilizing subunit calstabin2, rendering the RyR2 channels leaky, and the calcium leak is exacerbated by exercise, leading to fatal arrhythmias.

**What New Information Does This Article Contribute?**

- A new clinically significant mutation RyR2-G230C that leads to CPVT is reported.
- The novel RyR2-G230C mutation near the amino terminus induces calcium leak by decreasing the binding of calstabin2 to the RyR2 macromolecular complex, resulting in increased sensitivity of the channel to activation by diastolic calcium levels during stress.
- CPVT-linked mutations RyR2-G230C and RyR2-P2328S do not affect the threshold for luminal calcium activation and do not support an alternative mechanism called store overload–induced calcium release.

In this article, we report the identification and characterization of a new CPVT-linked RyR2-G230C mutation. To investigate the functional properties of the RyR2-G230C mutation, we generated and expressed recombinant human RyR2 mutant channels and tested the molecular mechanisms for CPVT. We show that RyR2-G230C mutant channels exhibit a calcium leak during stress because of decreased binding to calstabin2, which results in increased sensitivity of the mutant channels to activation by intracellular calcium levels. The results also indicate that RyR2-G230C, as well as a previously reported RyR2-P2328S CPVT-linked mutant channel, exhibits normal thresholds for activation by luminal SR calcium. These results show that there is no need to invoke the store overload--induced calcium release mechanism. The present study shows that a CPVT-linked mutation located near the amino terminus of RyR2 shares a common mechanism for triggering arrhythmias by rendering the channel leaky as the result of decreased calstabin2 binding and increased sensitivity to activation by low levels of cytosolic calcium.
A Novel Ryanodine Receptor Mutation Linked to Sudden Death Increases Sensitivity to Cytosolic Calcium

Albano C. Meli, Marwan M. Refaat, Miroslav Dura, Steven Reiken, Anetta Wronska, Julianne Wojciak, Joan Carroll, Melvin M. Scheinman and Andrew R. Marks

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Materials and Methods

Clinical evaluation
We obtained a detailed medical history with particular emphasis on syncope, cardiac arrest or symptoms during exertion or emotional stress; medical records, including cardiac evaluation, and a blood sample for genomic DNA extraction for each participant. Treadmill stress tests were performed using a Bruce protocol. The diagnosis of CPVT was based on the criteria which, in short, required stress-induced reproducible ventricular arrhythmias in patients with a normal resting ECG and no detectable structural heart abnormalities. Family screening was performed and blood samples were obtained from those who agreed to a genetic evaluation and provided written consent. The diagnosis of CPVT was made either on a clinical basis or after identification of a mutation in the RyR2 gene.

Genetic Studies
Genomic DNA was extracted from whole blood using standard methods. The RyR2 coding regions were amplified using polymerase chain reaction (PCR) and analyzed by denaturing high-performance liquid chromatography.

Ryanodine receptor mutagenesis and expression
hRyR2-G230C and hRyR2-P2328S recombinant RyR2 were generated using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). For the novel hRyR2-G230C, an N-terminal fragment of hRyR2 in pBS vector was mutated using primers: 5’-gcccaaggtatctcatt(tgt)gggtgtcctcag-3’ (codon G230C in parentheses) and its complementary reverse equivalent. A NotI-BstEII fragment of the mutant construct was subsequently subcloned into recombinant WT human RyR2 in the pCMV5 vector, resulting in a pCMV5/hRyR2-G230C construct, which was confirmed by sequencing. The hRyR2-P2328S mutant channel was generated as previously described.

ER vesicle preparation
ER vesicles from HEK293 cells co-expressing calstabin2 and RyR2-WT, RyR2-G230C or RyR2-P2328S mutants were prepared by homogenizing cell pellets on ice using a Teflon-glass dounser (50 times) with 2 volumes of: 20 mmol/L (mM) Tris-maleate (pH 7.4), 1 mM EDTA, 1 mM DTT and protease inhibitors (Roche). Homogenate was then centrifuged at 4,000 g for 15 min at 4°C and the following supernatant was centrifuged at 40,000 g for 30 min at 4°C. The final pellet, containing the ER fractions, was resuspended and aliquoted in 250 mM sucrose, 10 mM MOPS (pH 7.4), 1 mM EDTA, 1 mM DTT and protease inhibitors (Roche). Samples were frozen in liquid nitrogen and stored at -80°C. For PKA-phosphorylated channel experiments, ~200 ug of microsomes were in vitro phosphorylated with 40 units of PKA catalytic subunit (Sigma-Aldrich) for 30 min at 30°C in presence of the following buffer: 50 mM Tris/PIPERs (pH 7.0), 8 mM MgCl2, 1 mM Mg-ATP, and 1 mM EGTA. The samples were then centrifuged for 10 min at 100,000 g in a Beckman centrifuge using a 70Ti rotor. The resulting pellets were washed 4 times with wash buffer (300 mM sucrose, 10 mM imidazole, pH 7.4). Aliquots were frozen in liquid nitrogen and stored at -80°C.

Single channel data acquisition and analysis
SR vesicles were prepared from murine hearts as previously described. Planar lipid bilayers were formed by painting a mixture of phosphatidylethanolamine and phosphatidylcholine (3:1 ratio; Avanti Polar Lipids, Alabaster, AL) across a 200-µm aperture in
polysulfonate cup (Warner Instruments) separating 2 chambers. The final concentration of lipids was 40 mg/ml dissolved in decane. Membrane thinning was assayed by applying a triangular wave test pulse. Typical capacitance values were 50 – 150 pF. The trans chamber (1.0 ml), representing the intra-SR (luminal) compartment, was connected to the head stage input of a bilayer voltage clamp amplifier. The cis chamber (1.0 ml), representing the cytoplasmic compartment, was held at virtual ground. In experiments using Ba\(^{2+}\) as the primary charge current carrier, the chamber solutions were as follows: 1 mmol/L (mM) EGTA, 250/125 mM Hepes/Tris, 50 mM KCl, 0.64 mM CaCl\(_2\), pH 7.35 as cis solution and 53 mM Ba(OH)\(_2\), 50 mM KCl, 250 mM Hepes, pH 7.35 as trans solution. Single RyR2 channels were reconstituted by spontaneously fusing crude ER vesicles into the planar lipid bilayer and after incorporation the RyR2 channel activity was measured at 0 mV. In experiments using Cs\(^{+}\) as the primary charge current carrier, crude ER vesicles were added to the cis chamber, which contained 700 mM CsCl, 10 mM HEPES, pH 7.35, 1 mM EGTA and 8 \(\mu\)M free Ca\(^{2+}\), while the trans chamber contained 250 mM CsCl, 10 mM HEPES, pH 7.35 and 1 mM EGTA. After fusion of vesicles, the cis chamber was rapidly perfused with 10 ml (10x volume wash) of a solution containing 250 mM CsCl, 10 mM HEPES, pH 7.35, 1 mM EGTA and ~150 nM free Ca\(^{2+}\). Single channel activity was measured at +30 mV with respect to the cis (ground) side. The concentration of free Ca\(^{2+}\) in the cis and trans chambers was calculated with WinMaxC program (version 2.50; www.stanford.edu/~cpatton/maxc.html). Single channel currents were recorded using a Bilayer Clamp BC-525D amplifier (Warner Instruments). In experiments using Ba\(^{2+}\), single channel currents were filtered with a low-pass Bessel filter eight pole (Warner Instruments) at 1 kHz and then sampled at 4 kHz, whereas in experiments using Cs\(^{+}\), they were filtered at 2 kHz and sampled at 10 kHz. Data acquisition was performed using Digidata 1322A and Axoscope 10 software (Axon Instruments). Po was determined over 2 min of continuous recording using the method of 50% threshold analysis \(^4\). The recordings were analyzed by using Clampfit 10.1 (Molecular Devices) and Sigma Plot software (ver. 10.0, Systat Software) and Prism (ver.5.0, GraphPad). The data in Ca\(^{2+}\)-dependence experiments were fitted with the sigmoidal three-parameters equation using Sigma Plot software.

### Immunoprecipitation and immunoblot analyses

RyR2 channels were immunoprecipitated using an anti-RyR antibody as described \(^5\). RyR2 was immunoprecipitated from samples by incubating 75 \(\mu\)g of ER vesicles with anti-RyR antibody (2 \(\mu\)l 5029 Ab) in 0.5 ml of a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na\(_3\)VO\(_4\), 0.5% Triton-X100, and protease inhibitors) for 2 hrs at 4°C. The samples were incubated with protein A sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) at 4°C for 1 hr, after which, the beads were washed five times with 1.0 ml RIPA. Samples were heated to 95°C and size fractionated by PAGE (6% for RyR, 15% for calstabin2). Proteins were transferred to nitrocellulose membranes and immunoblots were developed using the following antibodies: anti-calstabin (1:1,000) \(^5\), anti-RyR (5029; 1:3,000) \(^5\), anti-phospho-RyR2-pSer\(^{2808}\) (1:5,000) \(^6\). Levels of RyR2 bound proteins were normalized to the total RyR2 immunoprecipitated (arbitrary units). All immunoblots were developed using the Odyssey system (LI-COR, Inc., Lincoln, NE) with IR labeled anti-mouse and anti-Rabbit IgG (1:10,000 dilution) secondary antibodies.
References


Online Figure I

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![Mutation Diagram]
Online Figure III

RM Pedigree

Diagram of a family pedigree with symbols indicating unaffected, affected, and deceased individuals.
Online Figure III

![Bar chart showing open probability vs. luminal calcium concentration (mM) for WT-RyR2 and GC-RyR2.](chart.png)
Legend

**Online Figure I. Sequence electropherogram.** A point mutation resulted in a G to T substitution at the 688th base of the RyR2 gene. The mutation resulted in an amino acid substitution of a cysteine (C) for glycine (G) at position 230.

**Online Figure II. Pedigree of the index family carrying the CPVT G230C-RyR2 mutation.** Square = male; circle = female; solid = affected; open = unaffected; slash through the symbol = deceased. The arrow indicates the proband III-3.

**Online Figure III. Effects of luminal Ca\textsuperscript{2+} on recombinant human RyR2-G230C mutant channels using Cs\textsuperscript{+} as the primary current charge carrier.** Bar graph summarizing Po at 0, 1, 5 and 10 mmol/L (mM) free luminal [Ca\textsuperscript{2+}] in RyR2-WT (white bars, n = 5 for each) and RyR2-G230C (black bars, n = 4 for each) channels.