Sudden Death in Familial Polymorphic Ventricular Tachycardia Associated With Calcium Release Channel (Ryanodine Receptor) Leak

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Background—Familial polymorphic ventricular tachycardia (FPVT) is characterized by exercise-induced arrhythmias and sudden cardiac death due to missense mutations in the cardiac ryanodine receptor (RyR2), an intracellular Ca\(^{2+}\) release channel required for excitation-contraction coupling in the heart.

Methods and Results—Three RyR2 missense mutations, P2328S, Q4201R, and V4653F, which occur in Finnish families, result in similar mortality rates of \(\approx 33\%\) by age 35 years and a threshold heart rate of 130 bpm, above which exercise induces ventricular arrhythmias. Exercise activates the sympathetic nervous system, increasing cardiac performance as part of the fight-or-flight stress response. We simulated the effects of exercise on mutant RyR2 channels using protein kinase A (PKA) phosphorylation. All 3 RyR2 mutations exhibited decreased binding of calstabin2 (FKBP12.6), a subunit that stabilizes the closed state of the channel. After PKA phosphorylation, FPVT-mutant RyR2 channels showed a significant gain-of-function defect consistent with leaky Ca\(^{2+}\) release channels and a significant rightward shift in the half-maximal inhibitory Mg\(^{2+}\) concentration (IC\(_{50}\)). Treatment with the experimental drug JTV519 enhanced binding of calstabin2 to RyR2 and normalized channel function.

Conclusions—Sympathetic activation during exercise induces ventricular arrhythmias above a threshold heart rate in RyR2 mutation carriers. Simulating the downstream effects of the sympathetic activation by PKA phosphorylation of RyR2 channels containing these FPVT missense mutations produced a consistent gain-of-function defect. RyR2 function and calstabin2 depletion were rescued by JTV519, suggesting stabilization of the RyR2 channel complex may represent a molecular target for the treatment and prevention of exercise-induced arrhythmias and sudden death in these patients. (Circulation. 2004;109:3208-3214.)

Key Words: calcium ■ death, sudden ■ arrhythmia ■ sarcoplasmic reticulum ■ drugs

Sudden cardiac death (SCD) due to ventricular arrhythmias is a leading cause of mortality in patients with heart disease. The mechanisms that trigger fatal arrhythmias, however, are incompletely understood, and treatment and prevention remain largely ineffective. Familial polymorphic ventricular tachycardia (FPVT) is an autosomal-dominant disease associated with highly reproducible ventricular tachycardias during physical or emotional stress and SCD in the absence of structural heart disease.1 FPVT was initially linked to 3 missense mutations (P2328S, Q4201R, and V4653F) in the cardiac ryanodine receptor (RyR2) gene in Finnish families (Figure 1A).2 Distinct RyR2 missense mutations were reported in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT)3 and in patients with arrhythmogenic right ventricular dysplasia and exercise-induced arrhythmias.4

The RyR2 gene encodes the major intracellular Ca\(^{2+}\) release channel on the sarcoplasmic reticulum (SR) of cardiomyocytes. During excitation-contraction coupling, activation of a voltage-dependent L-type Ca\(^{2+}\) channel triggers a several-times-larger intracellular SR Ca\(^{2+}\) release via RyR2 that activates cardiac muscle contraction via Ca\(^{2+}\)-induced Ca\(^{2+}\) release.5 RyR2 is a tetrameric channel complex comprised of 4 RyR2 monomers, each associated with 1 calstabin2 protein.6,7 Calstabin2, also known as the FK506-binding protein (FKBP12.6), stabilizes the RyR2 channel in the

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been shown to decrease intracellular Ca\textsuperscript{2+} because of small carrier number. Average thresholds as indicated by dashed lines are not significantly different (P \textsuperscript{0.58}) from skeletal muscle containing missense mutations, increasing susceptibility to malignant hyperthermia. B, Kaplan-Meyer analysis of cumulative mortality rates for P2328S, Q4201R, and V4653F in RyR2 mutation carriers. At 35 years of age, mortality reaches 30% to 33% for all mutations. C, Threshold heart rates at which ventricular arrhythmias were initiated during exercise testing in P2328S or V4653F carrier. At age 40 years, heart rate at which frequent premature complexes (PVCs) and nonsustained episodes of ventricular tachycardias (NSVTs) (3 or more premature ventricular complexes) first appeared was determined. All mutation carriers received \beta-adrenergic blocker therapy after completion of diagnostic testing combined with implantation of automatic implantable cardioverter-defibrillators in cases of incomplete protection.

**Ryanoine Receptor Expression and Purification**

Chameleon site-directed mutagenesis (Stratagene) was used to generate missense mutations of the hRyR2 gene in the pBS-SK\textsuperscript{+} vector. HEK293 cells grown in MEM supplemented with 10% (vol/vol) FBS (Invitrogen), penicillin (100 U/mL), streptomycin (100 \mu g/mL), and L-glutamine (2 mmol/L) were cotransfected with 20 \mu g of wild-type or different FPVT-mutant cDNA and 2.5 \mu g of calstabin2 cDNA by Ca\textsuperscript{2+} phosphate precipitation for the expression of homotetrameric channels (RyR2-WT, RyR2-P2328S, RyR2-Q4201R, and RyR2-V4653F). In a separate experiment, RyR2-WT and RyR2-P2328S subunits were coexpressed at a 1:1 ratio (10 \mu g cDNA each) with 2.5 \mu g of calstabin2 cDNA (RyR2-WT×P2328S for heterotetrameric channels)\textsuperscript{12,14}.

**Phosphorylation of Ryanoine Receptors and Single-Channel Recordings**

Microsomes containing recombinant RyR2 isolated from transfected HEK293 cells were in vitro phosphorylated by the PKA catalytic subunit as described\textsuperscript{9}. PKA phosphorylation of RyR2 aimed at maximal phosphorylation of all subunits, because FPVT patients develop syncope and SCD only during intense stress and because we found similar degrees of RyR2 phosphorylation during maximal exercise in vivo\textsuperscript{12}. RyR2 single-channel recordings were performed under voltage-clamp conditions in lipid bilayers at variable concentration of cis (cytoplasmic) Ca\textsuperscript{2+} or Mg\textsuperscript{2+} as described previously\textsuperscript{12,14}. The single-channel currents were filtered at 1 kHz with an 8-pole Bessel filter (Warner Instruments) and digitized at 4 kHz. Data are expressed as mean±SEM. Statistical analysis was performed with unpaired Student’s t test, and P<0.05 was considered significant. Please refer to the online Data Supplement for details.

**Results**

**Genotype-Phenotype Correlation in RyR2 Mutation Carriers**

The P2328S missense mutation was found in 17 family members. Exercise bicycle testing induced ventricular arrhythmias in 10 of 12 tested P2328S mutation carriers (Data Supplement Table). In the second family, 3 subjects were positive for the Q4201R mutation, and 1 obligate carrier had died suddenly at age 27 years. Exercise-induced ventricular arrhythmias occurred in 2 Q4201R carriers. The V4653F mutation occurred in 9 family members, and 8 carriers displayed ventricular arrhythmias during exercise. A DNA sample from 1 family member, who had died suddenly at the
age of 50 years in the absence of any diagnostic findings during autopsy, demonstrated a positive V4653F carrier status, and 5 members had a history of SCD. On average, 91% of the RyR2 mutation carriers showed exercise-induced ventricular arrhythmias, which is similar to previously reported results from 8 unrelated carriers with distinct RyR2 missense mutations.3 All FPVT mutation carriers had a normal QTc interval at rest and during exercise, and epsilon waves or negative T waves in leads V1 through V3 were excluded. Kaplan-Meyer analysis revealed similar mortality rates of 30% to 33% by the age of 35 years for the P2328S, Q4201R, and V4653F mutation carriers (Figure 1B). The threshold heart rates at which ventricular arrhythmias occurred during exercise testing were not significantly different between P2328S (129±17 bpm) and V4653F (133±20 bpm) mutation carriers (P=0.58; Figure 1C; 2 Q4201R mutation carriers not included). None of the mutation carriers had structural cardiac abnormalities or heart failure, as evidenced by echocardiography or ventriculography excluding ARVD2.2,4

The characteristic pattern of arrhythmia induction in RyR2 mutation carriers suggests that 3 unrelated RyR2 mutations result in a highly reproducible phenotype of exercise-induced ventricular arrhythmias in the absence of structural heart disease.

**RyR2 Missense Mutations Cause a Gain-of-Function Defect**

To determine the functional effects of the RyR2 mutations, we coexpressed homotetrameric (RyR2-WT, RyR2-P2328S, RyR2-Q4201R, and RyR2-V4653F) and heterotetrameric (RyR2-WT×P2328S) channels (Supplementary Figure, A). All FPVT-mutant channels were phosphorylated to the same degree as RyR2-WT by PKA, as evidenced by immunoblotting using the RyR2-Ser2809 phospho-epitope antibody. Specificity of PKA phosphorylation and dissociation of calstabin2 was shown in the presence or absence of the PKA inhibitor PKI14-22 (Supplementary Figure, B and C).12

FPVT-mutant RyR2 channels showed significantly decreased binding affinity of 35S-labeled calstabin2 (Figure 2). The dissociation constants (Kd) of homotetrameric channels (RyR2-P2328S, 223±23 nmol/L; RyR2-Q4201R, 242±38 nmol/L; and RyR2-V4653F, 191±29 nmol/L) and heterotetrameric channels (RyR2-WT×P2328S, 153±21 nmol/L) were significantly increased compared with control (P<0.001 versus RyR2-WT 107±19 nmol/L) at similar Bmax values (53.3 to 55.1 pmol/mg per nmol). The significant increase in Kd as determined from Scatchard analysis (n=3) indicates decreased calstabin2 binding affinities of FPVT mutant RyR2.

Unphosphorylated RyR2-WT Ca2+ release channels exhibited low Popen (0.2±0.1%) at 150 nmol/L cytosolic Ca2+ in the presence of 1 nmol/L Mg2+ as expected, because under diastolic conditions, RyR2 channels have to be tightly closed to allow for relaxation of the heart muscle. Accordingly, the low Popen of homotetrameric FPVT-mutant channels (RyR2-P2328S, RyR2-Q4201R, and RyR2-V4653F) was not significantly different from RyR2-WT (Figures 3A and 3B). Because arrhythmias in FPVT patients are characteristically triggered by exercise, we simulated the effects of sympathetic activation on RyR2 by in vitro PKA phosphorylation of the channels, as confirmed by a phosphospecific antibody against RyR2-Ser2809 (Supplementary Figure, B). PKA phosphorylation significantly increased the Popen of all FPVT-mutant RyR2 channels, consistent with a gain-of-function defect (Figures 3C and 3D). Accordingly, the distribution of opening events was shifted to higher current amplitudes, as shown in respective histograms. Mutagenesis studies of the PKA phosphorylation site RyR2-Ser2809 in the full-length channel have excluded additional PKA phosphorylation sites.11 The unchanged activity of nonphosphorylated FPVT mutant channels and the gain-of-function defect consistently observed in all phosphorylated FPVT mutant channels support the concept that PKA phosphorylation of Ser2809 is a necessary event to activate the functional defect.

FPVT occurs as an autosomal-dominant trait, where both mutant and WT subunits are thought to be present in heterotetrameric RyR2 complexes.2 To investigate the phenotype of heterotetrameric FPVT-mutant RyR2 channels, we coexpressed RyR2-WT and RyR2-P2328S mutant subunits at a 1:1 ratio and characterized the resulting heterotetrameric channel (RyR2-WT×P2328S). In the presence of the PKA inhibitor PKI14-22, RyR2-WT×P2328S resembled the low Popen seen in the homotetrameric channels (Figures 3A and 3B). Typical recordings of PKA phosphorylated heterotetrameric RyR2-WT×P2328S channels resemble the gain-of-function defects seen in homotetrameric RyR2-P2328S channels (Figures 3C and 3D).

**FPVT-Mutant RyR2 Are Resistant to Mg2+ Inhibition**

Phosphorylated heterotetrameric mutant RyR2 channels showed resistance to inhibition by millimolar Mg2+ (Figure 4A). Whereas RyR2-WT channel activity was significantly inhibited (P=0.01%) at Mg2+ concentrations ≥2.0 mmol/L, the Popen of mutant RyR2-P2328S channels remained significantly increased. Inhibition of average Popen 1.0% required 10 mmol/L Mg2+ in homotetrameric FPVT-mutant RyR2 channels (Figure 4B). Similarly, heterotetrameric RyR2-WT×P2328S channels showed a significant resistance to inhibition by Mg2+ (Figure 4B). Thus, after PKA phosphorylation, heterotetrameric and homotetrameric FPVT-mutant
RyR2 channels displayed a significant gain-of-function abnormality that was not inhibited within the physiological range of Mg²⁺ concentrations. The resistance of FPVT-mutant RyR2 to Mg²⁺ inhibition involved a significant rightward shift of the half-maximal inhibitory Mg²⁺ concentration (IC₅₀ in mmol/L: RyR2-P2328S, 2.89±0.08; RyR2-WT, 2.49±0.03; RyR2-Q4201R, 2.72±0.12; RyR2-V4653F, 3.53±0.12; P<0.01 versus RyR2-WT 1.53±0.03). These data suggest that the FPVT-associated RyR2 mutations render these channels partially resistant to inhibition by an important endogenous channel modulator, Mg²⁺.

JTV519 Rescues FPVT-Mutant Channel Function by Rebinding of Calstabin2

Recently, the 1,4-benzothiazepine derivative JTV519 was shown to inhibit progression of canine heart failure, possibly by increasing the binding of calstabin2 to RyR2.13,15 Therefore, we examined whether JTV519 affects the activity of mutant RyR2-P2328S channels (Figure 5, A and B). PKA-phosphorylated RyR2-P2328S channels showed a significant leftward shift to half-maximal activation by increasing cys (cytosolic) Ca²⁺ concentrations (EC₅₀ shift in nmol/L from 389.8±44.5 to 204.3±21.7; P<0.001 versus unphosphorylated RyR2-P2328S, each n=11), which was significantly more pronounced than in phosphorylated RyR2-WT (EC₅₀=308.6±36.2 nmol/L; P<0.005 vs RyR2-P2328S+PKA, 228.3±21.7 nmol/L; P<0.05 vs RyR2-P2328S+PKA).
to PKA-phosphorylated RyR2-WT treated with JTV519 (Figure 5, A and C; EC$_{50}$, 399.8±11.9 nmol/L; n=10). Treatment of RyR2-P2328S with 1.0 μmol/L JTV519 resulted in rebinding of calstabin2 to unphosphorylated levels in the channel complex, confirming the effects of our single-channel experiments (Figure 5C). These results indicate that the experimental drug JTV519 may rescue the gain-of-function defect in RyR2-P2328S channels via increased binding of the stabilizing calstabin2 subunit to the channel complex.

**Discussion**

RyR2 missense mutations have been identified in some familial forms of exercise-induced ventricular tachycardias and sudden death. All mutations resulted in high mortality rates in early adulthood, and no phenotypic differences were observed in the appearance of ventricular arhythmias. Arrhythmogenic activity correlated closely with the degree of exercise increasing from premature ventricular contractions to nonsustained ventricular tachycardias. These data suggest that structural changes and sympathetic activation of FPVT-mutant channels combine to trigger arrhythmias in RyR2 mutation carriers.

Recombinant RyR2 channels containing the missense mutations found in mutation carriers (RyR2-P2328S, RyR2-Q4201R, and RyR2-V4653F) showed a significant gain-of-function defect. The ~10-times increased P$_o$ of FPVT-mutant RyR2 channels occurred specifically during PKA phosphorylation, which is a downstream effector of sympathetic activation. When PKA phosphorylation was specifically inhibited with the peptide PKI5-24, FPVT-mutant RyR2 channels were not different from RyR2-WT. Observed differences in channel activity were tested in at least 3 different preparations from parallel PKA reactions to exclude experimental variability, and equal phosphorylation levels were confirmed in immunoblots (Supplementary Figure, B). In accordance with our results in homotetrameric FPVT-mutant RyR2, we found similar functional and structural defects related to PKA phosphorylation in heterotetrameric RyR2-WT×P2328S channels. These findings exclude the possibility that the RyR2-WT subunit in the tetrameric channel may rescue the functional phenotype. Although we used a constant molar ratio for mutant and wild-type subunit expression, we were not able to determine the exact subunit composition on an individual channel basis. However, the fact that the degree of the gain-of-function defect increased from heterotetrameric RyR2-WT×P2328S to homotetrameric RyR2-P2328S channels is consistent with a heterotetrameric subunit composition in the former. Unmasking the gain-of-function defect by PKA phosphorylation closely reflects the disease phenotype where ventricular arrhythmias are triggered only above heart rates of 130 bpm and SCD or arrhythmias do not occur in the absence of sympathetic activation.

We have recently linked decreased calstabin2 binding affinities to PKA phosphorylation closely reflects the disease mechanism in patients with CPVT. Now we find significantly decreased calstabin2 binding affinities and a gain-of-function defect in distinct FPVT-mutant RyR2 channels. The gain-of-function defect during maximal PKA phos-
phorylation of FPVT-mutant RyR2 channels paralleled the effects of PKA phosphorylation on RyR2 channel function in calstabin2−/− mice, which was sufficient to induce arrhythmias and sudden death.12 We propose that the common final pathway of all RyR2 missense mutations may be reduced calstabin2 levels in the RyR2 channel complex during PKA phosphorylation and increased Ca2+ leak, which in turn would activate inwardly depolarizing membrane currents, delayed afterdepolarizations, and triggered arrhythmias.12,16,17

We observed a significantly increased resistance to inhibition by Mg2+ in all PKA-phosphorylated FPVT-mutant RyR2 channels. In cardiac muscle, free intracellular Mg2+ concentrations of approximately 1 mmol/L have been reported.18,19 Millimolar Mg2+ concentrations stabilize the closed state of RyR2-WT channels under diastolic conditions20,21 and inhibit rapid Ca2+ release from SR vesicles.22 During exercise, sympathetic stimulation decreases Mg2+ in heart muscle cells by up to 20%, which may additionally increase the propensity of arrhythmias in RyR2 mutation carriers.23 Decreased plasma levels of Mg2+ were shown to increase the propensity for ventricular arrhythmias and sudden cardiac death,24–26 whereas interventions that increase Mg2+ plasma levels were shown to decrease the incidence of fatal arrhythmias in heart failure, ischemic heart disease, and other conditions with an increased propensity for SCD.27–29 Therefore, significantly decreased RyR2 sensitivity to inhibition by Mg2+ may represent an additional mechanism that contributes to SCD in RyR2 mutation carriers.

Previously, we and others have shown that PKA hyperphosphorylation of RyR2 channels in failing human hearts significantly increases RyR2 activity by depletion of calstabin2 from the channel complex, resulting in increased Ca2+ leak.9,30 In performing experiments to examine the affects of PKA phosphorylation of RyR2 or the FPVT mutations on the binding of calstabin2 to the channel, we are careful to maintain physiological ratios of calstabin2 to RyR2, as addressed in a recent article.15 Overexpression of calstabin2 outside the physiological range may counteract the shift in Kd induced by PKA phosphorylation and explain different findings reported by other groups.31,32 Treatment with β-adrenergic receptor blockers reverses PKA hyperphosphorylation and calstabin2 depletion in heart failure, and the beneficial effects of β-blocker treatment in patients with exercise-induced arrhythmias may be related to prevention of RyR2-mediated SR Ca2+ leak.33

Recently it was reported that the 1,4-benzothiazepine derivative JTV519 inhibits FK506-induced intracellular Ca2+ leak in the heart and may normalize leaky RyR2 in failing hearts.13,15 Treatment of phosphorylated RyR2-P2328S channels with 1.0 μmol/L JTV519 completely normalized the gain-of-function defect, and the significant leftward shift of Ca2+ sensitivity was rescued (Figure 5). In contrast to Mg2+ (data not shown), JTV519 treatment resulted in significantly increased calstabin2 levels in RyR2-P2328S channels. These studies demonstrate a molecular mechanism whereby JTV519 may prevent diastolic Ca2+ leak through the FPVT-mutant RyR2 channels. Thus, the present studies not only provide a basis for treating exercise-induced cardiac arrhythmias that cause SCD but also indicate that JTV519 could be beneficial in the treatment of heart failure, which is associated with aberrant SR Ca2+ leak via calstabin2-depleted RyR2 channels.9,33 Furthermore, these studies confirm results with a high-affinity calstabin2-D37S mutant that normalized the channel function of constitutively PKA-phosphorylated RyR2 channels.12

In summary, genotype-phenotype studies in RyR2 mutation carriers showed high mortality rates and a reproducible threshold heart rate above which ventricular arrhythmias occur. The electrophysiological phenotype of FPVT mutation carriers is characterized by exercise-induced polymorphic ventricular arrhythmias above a heart rate threshold of approximately 130 bpm and incomplete suppression of arrhythmias and sudden death by β-blockers. Three structurally unrelated RyR2 missense mutations exhibited a significant gain-of-function defect at the single-channel level with a resistance to inhibition by Mg2+. This defect was specific to conditions that simulate adrenergic activation, in agreement with the clinical phenotype. The experimental drug JTV519 normalized FPVT-mutant RyR2 channel function by rebinding of calstabin2 to the channel complex. Therefore, stabilization of the closed state of mutant RyR2 channels by increased calstabin2 binding may represent a novel pharmacological principle to prevent arrhythmias and sudden death in this population and may have broader implications, ranging from genetic forms of arrhythmias to complex diseases associated with a high incidence of SCD, such as heart failure.

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