Identification of Loss-of-function RyR2 Mutations Associated with Idiopathic Ventricular Fibrillation and Sudden Death

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Running Title: Loss of RyR2 Function and Ventricular Fibrillation

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Summary Statement:
Idiopathic ventricular arrhythmias and sudden cardiac death associated with loss of RyR2 function represents a new entity of ventricular arrhythmias distinct from catecholaminergic polymorphic ventricular tachycardia (CPVT). Different strategies would be required for the diagnosis and treatment of ventricular arrhythmias associated with enhanced or suppressed RyR2 function.
ABSTRACT

Mutations in cardiac ryanodine receptor (RyR2) are linked to catecholaminergic polymorphic ventricular tachycardia (CPVT). Most CPVT RyR2 mutations characterized are gain-of-function (GOF), indicating enhanced RyR2 function as a major cause of CPVT. Loss-of-function (LOF) RyR2 mutations have also been identified and are linked to a distinct entity of cardiac arrhythmia termed RyR2 Ca$^{2+}$ release deficiency syndrome (CRDS). Exercise stress testing (EST) is routinely used to diagnose CPVT, but it is ineffective for CRDS. There is currently no effective diagnostic tool for CRDS in humans. An alternative strategy to assess the risk for CRDS is to directly determine the functional impact of the associated RyR2 mutations. To this end, we have functionally screened 18 RyR2 mutations that are associated with idiopathic ventricular fibrillation (IVF) or sudden death. We found two additional RyR2 LOF mutations E4146K and G4935R. The E4146K mutation markedly suppressed caffeine activation of RyR2 and abolished store overload induced Ca$^{2+}$ release in HEK293 cells. E4146K also severely reduced cytosolic Ca$^{2+}$ activation and abolished luminal Ca$^{2+}$ activation of single RyR2 channels. The G4935R mutation completely abolished caffeine activation of and $[^3]$H]ryanodine binding to RyR2. Co-expression studies showed that the G4935R mutation exerted dominant negative impact on the RyR2 wildtype channel. Interestingly, the RyR2-G4935R mutant carrier had a negative EST, and the E4146K carrier had a family history of sudden death during sleep, which are different from phenotypes of typical CPVT. Thus, our data further support the link between RyR2 LOF and a new entity of cardiac arrhythmias distinct from CPVT.
INTRODUCTION

The cardiac ryanodine receptor (RyR2) is a sarcoplasmic reticulum (SR) Ca\(^{2+}\) release channel essential for excitation-contraction coupling in the heart [1, 2]. RyR2 is also a critical player in the pathogenesis of various cardiac arrhythmias and cardiomyopathies [2-5]. Naturally occurring mutations in RyR2 have frequently been associated with catecholaminergic polymorphic ventricular tachycardia (CPVT), but also with other phenotypes such as idiopathic ventricular fibrillation (IVF), atrial fibrillation (AF), and cardiomyopathies [6-19]. An unresolved important question is how mutations in the same \(RyR2\) gene can cause such a wide spectrum of cardiac disorders.

A major effort towards addressing this question has been to understand the functional impact of disease-causing RyR2 mutations. To date, many RyR2 mutations have been characterized at the molecular, cellular, or whole animal levels [3-5]. Interestingly, nearly all RyR2 mutations that have been characterized enhance the response of the RyR2 channel to stimuli (i.e. gain-of-function, GOF). For instance, single channel studies demonstrated that many CPVT-associated RyR2 mutations enhance the sensitivity of the RyR2 channel to luminal Ca\(^{2+}\) activation [20, 21]. Some CPVT RyR2 mutations have also been shown to increase the sensitivity of the channel to activation by luminal and/or cytosolic Ca\(^{2+}\) [20, 22-25]. At the cellular level, CPVT RyR2 mutations enhance spontaneous Ca\(^{2+}\) release in the form of Ca\(^{2+}\) sparks, Ca\(^{2+}\) oscillations, or Ca\(^{2+}\) waves under conditions of SR Ca\(^{2+}\) overload [20-22, 24, 26-30]. This store overload induced Ca\(^{2+}\) release (SOICR) can lead to delayed afterdepolarizations (DADs), triggered activities, and ventricular tachyarrhythmias [5, 31-33]. RyR2 mutations associated with dilated cardiomyopathy affect the termination of SR Ca\(^{2+}\) release by reducing the termination threshold, leading to prolonged SR Ca\(^{2+}\) release [34]. Furthermore, RyR2 mutations located in the central domain increase the sensitivity of the RyR2 channel to activation by cytosolic Ca\(^{2+}\) [35, 36]. Interestingly, some of these central domain RyR2 mutations are associated with atrial fibrillations [17, 19]. Recent three-dimensional structural studies revealed that a large number of disease-causing RyR2 mutations are clustered at domain interfaces [37-44]. These mutations are thought to weaken domain-domain interactions that are important for stabilizing the closed state of the channel, thus facilitating channel opening and spontaneous SR Ca\(^{2+}\) release [45]. Overall, these observations have led to a general belief that disease-linked RyR2 mutations cause GOF defects, leading to inappropriate activation of the channel and excessive SR Ca\(^{2+}\) release that can precipitate into cardiac arrhythmias, cardiomyopathies, or sudden death [5].

In addition to disease-associated RyR2 GOF mutations, we have recently shown that RyR2 loss-of-function (LOF) mutations are causative for a novel inherited cardiac arrhythmia syndrome that we have termed RyR2 Ca\(^{2+}\) release deficiency syndrome (CRDS) [46]. Importantly, the phenotypes of the RyR2-CRDS are distinct from those of CPVT. Unlike CPVT, RyR2-CRDS lacks the catecholamine-induced ventricular ectopy [46]. The arrhythmogenic mechanism of RyR2-CRDS is also different from that of CPVT. Animal studies suggest that VTs in RyR2-CRDS arises secondary to substantial electrophysiological remodeling that increases the susceptibility to ventricular arrhythmias via EAD-mediated re-entrant mechanism [46]. Because of these differences, the diagnosis, management, and treatment of RyR2-CRDS would be different from those of CPVT. However, although RyR2-CRDS is a life-threatening arrhythmogenic disorder distinct from CPVT, there are currently no diagnostic tests for the disorder in humans. The standard exercise-stress test for CPVT is unable to distinguish RyR2-CRDS from normal individuals.

In light of the link between RyR2 LOF and RyR2-CRDS, another approach to assess the risk for RyR2-CRDS is to screen for RyR2 mutations and functionally characterize their impact. To date, there are 10 RyR2 LOF mutations (Q3774L, I3995V, D4112N, T4196I, K4594R/I2075T, D4646A, I4855M, A4860G, Q4879H, and S4938F) that have been reported [46]. There are certainly more RyR2 LOF mutations and identifying these LOF RyR2 mutations is critical to prevent sudden death. To this end,
In the present study, we determined the functional impact of a large number of known RyR2 mutations that are associated with IVF or sudden unexplained death. Our site-directed mutagenesis studies and functional screening led to the identification of 2 additional LOF RyR2 mutations (E4146K and G4935R) associated with IVF and sudden death. Thus, ventricular arrhythmias associated with RyR2 LOF represents a significant portion of RyR2-linked arrhythmogenic disorders that have yet to be explored.
Materials and Methods

Materials: Human embryonic kidney 293 (HEK293) cell line, plasmid pcDNA3, plasmid pcDNA5, Tris-HCl (pH 8.8), MgSO4, Triton X-100, bovine serum albumin (BSA), dATP, dCTP, dGTP and dTTP (Amersham), DNA polymerase (Stratagene), QIA quick PCR Purification Kit, Flp-In T-Rex Core Kit (Invitrogen), phosphate-buffered saline (PBS) (137 mM NaCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, and 2.7 mM KCl), hygromycin (Invitrogen), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, HEPES buffer (274 mM NaCl, 1.8 mM Na2HPO4 and 50 mM HEPES, pH 7.04), KRH (Krebs-Ringer-HEPES) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 6 mM glucose, 1.2 mM MgCl2 and 25 mM HEPES, pH 7.4), CaCl2, fluo 3, sulfinpyrazone, Fura-2 acetoxyethyl ester (Fura-2 AM), tetracycline, pluronic F-127, caffeine, EGTA, EDTA, Tris, CHAPS, soybean phosphatidylcholine, DTT, benzamidine, leupeptin, pepstatin A, aprotinin, PMSF, L-glutamine, penicillin, nonessential amino acids, Tween-20, skimmed-milk powder, anti-RyR antibody (34c), anti-mouse IgG (H&L) antibodies, enhanced chemiluminescence kit (Pierce), [3H]ryanodine (PerkinElmer), ryanodine (Abcam).

Methods

Construction of RyR2 mutations

All RyR2 point mutations were generated by using the overlap extension PCR method as described previously [47, 48]. The PCRs were carried out in a 100 µl reaction buffer containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 2.0 mM MgSO4, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin, 50 ng of each DNA primer, 200 µM each of dATP, dCTP, dGTP and dTTP (Amersham), 1 unit of pfu DNA polymerase (Stratagene) and 100 ng of template cDNA. All of the PCR products were purified using the QIA quick PCR Purification Kit. cDNA fragments containing the desired mutations were removed from the PCR products and used to replace the corresponding wild-type (WT) fragments in the full-length mouse RyR2 cDNA in the expression plasmid pcDNA5 [49]. The mutations and sequence of the PCR products were confirmed by DNA sequencing.

Generation of stable, inducible HEK293 cell lines expressing RyR2 WT or mutants

Stable, inducible HEK293 cell lines expressing RyR2 WT or the E4146K mutant were generated using the Flp-In T-Rex Core Kit from Invitrogen. Briefly, Flp-In T-Rex-293 cells were co-transfected with the inducible expression vector pcDNA5/FRT/TO containing the mutant cDNAs and the pOG44 vector encoding the Flp recombinase in 1: 5 ratios using the Ca2+ phosphate precipitation method. The transfected cells were washed with phosphate buffered saline (PBS: 137 mM NaCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl) 24 h after transfection followed by a change into fresh media for 24 h. The cells were then washed again with PBS, harvested, and plated onto new dishes. After the cells had attached (~4 h), the growth medium was replaced with a selection medium containing 200 µg/ml hygromycin (Invitrogen). The selection medium was changed every 3-4 days until the desired number of cells was grown. The hygromycin-resistant cells were pooled, aliquoted, and stored at ~80 °C. These positive cells are believed to be isogenic, because the integration of RyR2 cDNA is mediated by the Flp recombinase at a single FRT site.

Caffeine-induced Ca2+ release measurements

Free cytosolic Ca2+ concentration in transfected HEK293 cells was measured using the fluorescence Ca2+ indicator dye fluo-3-AM as described previously [49]. HEK293 cells grown on 100-mm tissue culture dishes for 18-20 h after subculture were transfected with 12-16 µg of WT or mutant RyR2 cDNA. Cells grown for 18-20 hr after transfection were washed four times with PBS and incubated in KRM buffer without MgCl2 and CaCl2 (KRH buffer: 125 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 6
mM glucose, 1.2 mM MgCl₂, 2 mM CaCl₂, and 25 mM HEPES, pH 7.4) at room temperature for 40 min, and at 37 °C for 40 min. After being detached from culture dishes, cells were collected by centrifugation at 1,000 rpm for 3 min. in a Beckman TH-4 rotor. Cell pellets were washed twice with KRH buffer and loaded with 10 µM fluo 3 in Dulbecco’s Modified Eagle Medium at room temperature for 60 min., followed by washing with KRH buffer three times and resuspended in 150 µl KRH buffer plus 0.1 mg/ml BSA and 250 µM sulfinpyrazone. The fluo 3 loaded cells were added to 2 ml (final volume) KRH buffer in a cuvette. Fluorescence intensity of fluo 3 at 530 nm was measured before and after repeated additions or single additions of various concentrations of caffeine in an SLM-Aminco series 2 luminescence spectrometer with 480 nm excitation at 25 °C (SLM Instruments, Urbana, IL). The peak level of each caffeine-induced Ca²⁺ release was determined and normalized to the highest level (100%) of caffeine-induced Ca²⁺ release for each experiment. The normalized data were fitted with the Hill equation.

**Single cell cytosolic Ca²⁺ imaging of HEK293 cells**

Cytosolic Ca²⁺ levels in stable, inducible HEK293 cells expressing RyR2 WT or the E4146K mutant channels were monitored using single-cell Ca²⁺ imaging and the fluorescent Ca²⁺ indicator dye Fura-2 acetoxymethyl ester (Fura-2 AM) as described previously [20, 21]. Briefly, cells grown on glass coverslips for 18-22 hours after induction by 1 µg/ml tetracycline were loaded with 5 µM Fura-2/AM in KRH (Kreb's-Ringer-HEPES) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 6 mM glucose, 1.2 mM MgCl₂ and 25 mM HEPES, pH 7.4) plus 0.02% pluronic F-127 and 0.1 mg/ml BSA for 20 min at room temperature (23 °C). The coverslips were then mounted in a perfusion chamber (Warner Instruments, Hamden, CT, U.S.A.) on an inverted microscope (Nikon TE2000-S). The cells were continuously perfused with KRH buffer containing increasing extracellular Ca²⁺ concentrations (0, 0.1, 0.2, 0.3, 0.5, 1.0, and 2.0 mM). Caffeine (10 mM) was applied at the end of each experiment to confirm the expression of active RyR2 channels. Time-lapse images (0.25 frame/s) were captured and analyzed with the Compix Simple PCI 6 software (Compix Inc., Sewickley, PA, USA). Fluorescence intensities were measured from regions of interest centered on individual cells. Only cells that responded to caffeine were analyzed. The filters used for Fura-2 imaging are exciters: 340 ± 26 nm and 387 ± 11 nm, and emitter: 510 ± 84 nm with a dichroic mirror (410 nm).

**Single channel recordings in planar lipid bilayers**

Recombinant RyR2 WT and mutant channels were purified from cell lysate prepared from HEK293 cells transfected with RyR2 WT or the E4146K mutant cDNAs by sucrose density gradient centrifugation as described previously [50]. Bilayers were formed across a 250 µm hole in a Delrin partition separating two chambers. The trans chamber (800 µl) was connected to the head stage input of an Axopatch 200A amplifier (Axon Instruments, Austin, TX). The cis chamber (1.2 ml) was held at virtual ground. A symmetrical solution containing 250 mM KCl and 25 mM HEPES (pH 7.4) was used for all recordings, unless indicated otherwise. A 4-µl aliquot (≈ 1 µg of protein) of the sucrose density gradient-purified recombinant RyR2 WT or E4146K mutant channels was added to the cis chamber. Spontaneous channel activity was always tested for sensitivity to EGTA and Ca²⁺. The chamber to which the addition of EGTA inhibited the activity of the incorporated channel presumably corresponds to the cytosolic side of the Ca²⁺ release channel. The direction of single channel currents was always measured from the luminal to the cytosolic side of the channel, unless mentioned otherwise. Recordings were filtered at 2,500 Hz. Data analyses were carried out using the pclamp 8.1 software package (Axon Instruments). Note that in our single channel studies, we used a wide range of Ca²⁺ concentrations from 45 nM [50, 51].
to 40 mM. It is challenging to prepare the exact Ca\(^{2+}\) concentrations in the nanomolar and submicromolar range confirmed with a Ca\(^{2+}\) electrode. Hence, we estimated the free Ca\(^{2+}\) concentrations using the computer program of Fabiato and Fabiato [51]. To minimize potential impact of variable estimations of free Ca\(^{2+}\) concentrations, we always use the same Ca\(^{2+}\) stock solutions to test the Ca\(^{2+}\) response of the WT and mutant channels under the same conditions.

**Western blotting**

HEK293 cells transfected with RyR2 WT or the RyR2-E4146K mutant cDNAs were washed with PBS plus 2.5 mM EDTA and harvested in the same solution by centrifugation at 2,000 rpm for 10 min in a Beckman TH-4 rotor. The cells were then washed with PBS without EDTA and centrifuged again at 2,000 rpm for 10 min. The PBS washed cells were solubilized in a lysis buffer containing 25 mM Tris, 50 mM HEPES (pH 7.4), 137 mM NaCl, 1% CHAPS, 0.5% soybean phosphatidylcholine, 2.5 mM DTT, and a protease inhibitor mix (1 mM benzamidine, 2 µg/ml leupeptin, 2 µg/ml pepstatin A, 2 µg/ml aprotinin, 0.5 mM PMSF). This mixture was incubated on ice for 1 hr. Cell lysate was obtained by centrifuging twice at 16,000 x g in a microcentrifuge at 4°C for 30 min to remove unsolubilized materials. The RyR2 WT and mutant proteins were subjected to 6% SDS-PAGE [52] and transferred to nitrocellulose membranes at 45 V for 18–20 h at 4°C in the presence of 0.01% SDS [53]. The nitrocellulose membranes containing the transferred proteins were blocked for 30 min with PBS containing 0.5% Tween-20 and 5% skimmed-milk powder. The blocked membrane was incubated with the anti-RyR antibody (34c) (1:1000) and then incubated with the secondary anti-mouse IgG (H&L) antibodies conjugated to horseradish peroxidase (1:20000). After washing for 5 min, three times, the bound antibodies were detected using an enhanced chemiluminescence kit from Pierce.

**\(^{3}\text{H}\)**Ryanodine Binding Assay

Equilibrium \(^{3}\text{H}\)ryanodine binding to cell lysates was performed as described previously [49, 54] with some modifications. Cell lysates were incubated with 5 nM \(^{3}\text{H}\)ryanodine at 37°C for 2h in 300 µl of a binding solution containing 500 mM KCl, 25 mM Tris, 50 mM Hepes (pH 7.4). Free [Ca\(^{2+}\)] (0.1 nM – 100 µM) was adjusted by EGTA and CaCl\(_2\) solutions using the computer program of Fabiato and Fabiato [51]. At the completion of incubation, samples were diluted with 5 ml of ice-cold washing buffer containing 25 mM Tris (pH 8.0) and 250 mM KCl, and filtered through Whatman GF/B filters presoaked with 1% polyethyleneimine. Filters were washed immediately with 2 x 5 ml of the same buffer. The amount of \(^{3}\text{H}\)ryanodine retained in filters was determined by liquid scintillation counting. Specifically bound \(^{3}\text{H}\)ryanodine was calculated by subtracting nonspecific binding that was determined in the presence of 50 µM unlabeled ryanodine. All binding assays were performed in duplicate. \(^{3}\text{H}\)ryanodine binding data were fitted with the Hill equation using the Prism 8 (GraphPad Software, San Diego, California USA).

**Statistical Analysis**

All values shown are mean ± SEM unless indicated otherwise. To test for differences between groups, we used Student's t test (2-tailed) or one-way ANOVA with post hoc tests. A P value <0.05 was considered to be statistically significant.

**Human Studies**

Human studies were approved by IRCCS Fondazione Maugeri ethical IRB and in agreement with the Declaration of Helsinki. Patients were referred to the Molecular Cardiology Clinics of the Maugeri...
Foundation for evaluation of family history of SCD and underwent clinical evaluation and genetic testing. All patients or their guardians provided written informed consent.
RESULTS
Identification of loss-of-function RyR2 mutations associated with sudden death
Among hundreds of disease-associated RyR2 mutations, there are only a handful of known loss-of-function (LOF) RyR2 mutations characterized to date [8, 46, 55-58]. The impact of most of the disease-associated RyR2 mutations has not been functionally characterized. It has become imperative to identify patients with RyR2 LOF mutations, as their phenotypes are effectively concealed prior to their onset of ventricular arrhythmias or sudden death. To this end, we performed functional screening of a large number of RyR2 mutations associated with idiopathic ventricular fibrillation (IVF) and/or sudden unexplained death (SUD) (Supplementary Information). These include RyR2 mutations: I217V[59], R414C[60], P466A[61], E1127G (new), G2145R[62], F2331S[60], G2337V[63], A2387T[61], Y2392C[13], R2401L[60], A3442E (new), I3476T (new), R3570W[62], N4097S[59], E4146K [59], I4848V[61], G4935R [64], and R4959Q[65]. Of these mutations, E1127G, A3442E, and I3476T are novel. Most of these mutations are located in one of the four disease hotspots in RyR2 (Fig. 1). All these mutation carriers were labelled as IVF patients or were associated with SUD. It is unclear whether these mutations are associated with the typical CPVT that is caused by GOF RyR2 mutations or with the newly identified RyR2-CRDS that is caused by LOF RyR2 mutations [46].

To address this question, we assessed the impact of each of the 18 mutations on RyR2 function by measuring caffeine induced Ca²⁺ release in HEK293 cells. Figure 2 shows intracellular Ca²⁺ release induced by sequential additions of increasing concentrations of caffeine in HEK293 cells transfected with the RyR2 wild type (WT), the E4146K or G4935R mutants using the fluorescent Ca²⁺ indicator dye fluo-3 AM. HEK293 cells expressing RyR2 WT responded to caffeine with an activation threshold of ~0.05 mM. The level of Ca²⁺ release increased progressively with each consecutive addition of caffeine from 0.05 mM up to 1.0 mM, and then decreased with further additions of caffeine (2.5 and 5 mM). This decrease is likely due to the depletion of the intracellular Ca²⁺ stores by the prior additions of caffeine (0.025 to 1.0 mM) (Fig. 2A, D). On the other hand, the E4146K mutation markedly suppressed the caffeine response with an activation threshold of ~ 1 mM, while the G4935R mutation completely abolished caffeine response (Fig. 2B, C, D). Immunoblotting analysis showed that the expression of the E4146K mutant in HEK293 cells was markedly reduced, whereas the expression of the G4935R mutant was unchanged compared to that of the RyR2 WT (Fig. 2E, F, Supplementary Figs. 1, 2). Other RyR2 mutants, including I217V, R414C, P466A, E1127G, G2145R, F2331S, G2337V, A2387T, Y2392C, R2401L, A3442E, I3476T, R3570W, N4097S, I4848V, and R4959Q, exhibited a caffeine response similar to that of the RyR2 WT (Figs. 3 and 4). Note that the R414C mutation was previously reported to have no significant impact on the Ca²⁺ dependent activation of [³H]ryanodine binding to RyR2 [24]. Therefore, these functional screening identified 2 additional RyR2 mutations (E4146K and G4935R) that substantially suppress or abolish RyR2 function. However, it is important to emphasize that RyR2 mutations that displayed a caffeine response similar to that of RyR2 WT may alter other aspects of channel function. Thus, the pathogenicity of RyR2 mutations with a WT-like caffeine response is unclear and has yet to be determined.

The RyR2 E4146K mutation abolishes SOICR
We have previously shown that CPVT-linked RyR2 mutations enhance the propensity for SOICR [20, 21]. To assess the effect of RyR2 mutation E4146K on SOICR, HEK293 cells expressing RyR2 WT or the E4146K mutation were perfused with elevating extracellular Ca²⁺ (0 - 2.0 mM) to induce spontaneous Ca²⁺ oscillations as described previously [20, 21]. The resultant SOICR was then monitored by using a fluorescence Ca²⁺ indicator, fura-2 AM, and single cell Ca²⁺ imaging. As shown in Fig. 5, elevating extracellular Ca²⁺ induced SOICR in HEK293 cells expressing RyR2 WT (Fig. 5A, C), whereas, no SOICR was detected in HEK293 cells expressing the RyR2-E4146K mutant (Fig. 5B, C),
Despite the increased ER store Ca\(^{2+}\) content compared with that in WT cells (Fig. 5D). Thus, opposite to the effect of CPVT-linked RyR2 GOF mutations, the RyR2 E4146K LOF mutation abolishes SOICR in HEK293 cells.

**The E4146K mutation diminishes luminal Ca\(^{2+}\) activation of single RyR2 channels**

The impact of the LOF RyR2-E4146K mutation on channel function was further characterized at the single channel level. We first assessed whether the E4146K mutation affects luminal Ca\(^{2+}\) activation of RyR2. To this end, we incorporated single RyR2 WT or the E4146K mutant channels into planar lipid bilayers and determined their single channel properties (open probability, Po, mean open time, To, and mean closed time Tc) in the presence of 45nM cytosolic Ca\(^{2+}\) and a wide range of luminal Ca\(^{2+}\) concentrations (45 nM to 40 mM). As shown in Fig. 6, luminal Ca\(^{2+}\) up to 40 mM did not activate single E4146K mutant channels, whereas, single RyR2 WT channels were activated by luminal Ca\(^{2+}\) under the same conditions (Fig. 6A, B). Thus, the E4146K mutation diminishes luminal Ca\(^{2+}\) activation of single RyR2 channels.

**The E4146K mutation suppresses cytosolic Ca\(^{2+}\) activation of RyR2**

We next assessed whether the E4146K mutation alters the cytosolic Ca\(^{2+}\) activation of RyR2. We performed single channel analysis of the RyR2 WT and the E4146K mutant in the presence of various cytosolic Ca\(^{2+}\) concentrations (45 nM – 100 µM) and in the near absence of luminal Ca\(^{2+}\) (~45 nM). As shown in Fig. 7, single RyR2 WT channels were activated by cytosolic Ca\(^{2+}\) (~100 nM), whereas, the E4146K mutation substantially reduced the cytosolic Ca\(^{2+}\) activation of RyR2 (Fig. 7A, B). Taken together, these single channel analyses indicate that RyR2 mutation E4146K suppresses both the cytosolic and luminal Ca\(^{2+}\) activation of RyR2.

**The LOF G4935R mutation exerts dominant negative impact on RyR2 WT function**

The G4935R mutant does not form a caffeine-sensitive Ca\(^{2+}\) release channel in HEK293 cells when expressed alone. Besides abolishing its own channel function, the G4935R mutation may affect the function of the RyR2 WT channel when co-expressed with the WT. To test this possibility, we co-transfected HEK293 cells with RyR2 WT and the G4935R mutant plasmid cDNAs (in 1:1 ratio) and assessed the caffeine induced Ca\(^{2+}\) release in these co-transfected cells. As shown in Fig. 8, co-expression of the G4935R mutant significantly suppressed the caffeine response of RyR2 WT (Fig. 8A, B, C). We also assessed the impact of the G4935R mutation on \(^{3}\text{H}\)ryanodine binding to RyR2. Similarly, we found that the G4935R mutation completely abolished Ca\(^{2+}\) dependent activation of \(^{3}\text{H}\)ryanodine binding to RyR2 when it was expressed alone (Fig. 8D). Furthermore, co-expression of the G4935R mutant with RyR2 WT substantially inhibited Ca\(^{2+}\) dependent activation of \(^{3}\text{H}\)ryanodine binding to RyR2 (Fig. 8D). Thus, the LOF G4935R mutation exerts a dominant negative effect on the function of the RyR2 WT channel.
DISCUSSION

It is generally believed that RyR2-associated ventricular arrhythmias (VAs) and sudden cardiac death (SCD) result from gain-of-function (GOF) defects of the RyR2 channel [2-5]. However, we and others have recently shown that a number of RyR2 mutations associated with idiopathic ventricular fibrillation (IVF) and SCD with negative exercise-stress testing suppress the function of RyR2 [8, 46, 55-58]. Since only a fraction of known disease-associated RyR2 mutations have been functionally characterized, it is possible that additional LOF RyR2 mutations may exist. To test this possibility, we assessed, in the present study, the functional impact of 18 RyR2 mutations that were labelled as IVF or associated with sudden death. We found 2 additional LOF RyR2 mutations (E4146K and G4935R). A hallmark of CPVT patients with GOF RyR2 mutations is reproducible exercise-induced bidirectional and/or polymorphic VAs [2-5]. Given their opposite impact on the RyR2 channel, LOF RyR2 mutations would expect to be associated with different clinical phenotypes in patients. Indeed, exercise stress testing showed no evidence of arrhythmias in the patient carrying the LOF G4935R mutation associated with SCD [64]. The LOF RyR2-E4146K mutation was discovered by postmortem genetic testing in a 14 years old male with a family history of sudden death who died during sleep [59]. Thus, consistent with those reported recently [46], LOF RyR2 mutations are associated with VAs that are distinct from the typical CPVT.

The existence of both GOF and LOF RyR2 mutations has profound implication for the understanding of RyR2-associated cardiac arrhythmias. The GOF RyR2 mutations are believed to enhance the propensity for spontaneous Ca²⁺ waves under conditions of SR Ca²⁺ overload such as during physical and emotional stress [5]. These spontaneous Ca²⁺ waves are well-known cause of delayed afterdepolarizations (DADs), which, in turn, can lead to triggered activity, triggered arrhythmia, and SCD [5, 31-33]. However, the exact mechanism by which LOF RyR2 mutations causes sudden death has yet to be defined. We have recently shown that the RyR2 LOF mutation D4646A resulted in substantial electrophysiological and structural remodeling and enhanced the propensity for Ca²⁺ alternans, early-afterdepolarizations (EADs), and re-entrant activities [46]. Therefore, there are at least two distinct mechanisms by which RyR2 mutations could lead to VAs: a DAD-based mechanism associated with enhanced RyR2 function and an EAD-mediated re-entrant mechanism associated with suppressed RyR2 function.

Given these fundamental differences in their functional impact, the diagnosis and treatment strategies for ventricular arrhythmias associated with GOF or LOF RyR2 mutations would have to be different. Exercise stress testing is widely used for the diagnosis of typical CPVT, which is associated with GOF RyR2 mutations. However, such a test would be ineffective in identifying patients carrying LOF RyR2 mutations because of their lack of response to exercise stress test [46]. In this regard, it is of interest to know that the LOF RyR2 G4935R mutation identified in an 8 years old girl who suffered from sudden unexplained death was initially given a clinical diagnosis of epilepsy [64]. Hence, a specific diagnostic test for individuals with RyR2 LOF mutations is pressingly needed. We have recently established a programed electrical stimulation sequence that encompasses a long burst, a long pause, and a short-coupled extra-stimulus (LBLPS). We showed that this LBLPS stimulation sequence triggers VAs in RyR2 LOF mutant mice, but not in RyR2 GOF mutant mice or WT control mice. However, the specificity and sensitivity of the LBLPS protocol in humans remain to be determined. From a clinical standpoint, there are currently no studies directly comparing the response to therapies of GOF vs LOF RyR2 mutant carriers. Drugs that suppress RyR2 activity would be appropriate for treating patients with enhanced RyR2 function. However, these drugs would be ineffective for patients with suppressed RyR2 function as they would exacerbate the LOF defects. For an effective treatment, one would need to suppress the GOF of RyR2, whereas reverse the LOF of RyR2. It is, therefore, necessary and important to characterize the functional impact of all disease-associated RyR2 mutations, so that
one could distinguish patients with enhanced RyR2 function from those with suppressed RyR2 function for a proper diagnosis and treatment of RyR2-associated cardiac arrhythmias.

It is of interest to note that although mutations G4935R, S4938F and R4959Q are all located in the CTD, their impact on RyR2 channel activation is very different. R4959Q does not significantly alter caffeine activation of RyR2, while S4938F markedly suppresses caffeine activation, and G4935R completely abolishes caffeine activation. Thus, it would be challenging to predict whether a mutation will impact channel function solely based on the domain it is located in.

Another interesting observation is that the presence of caffeine (10 mM), but not the absence of caffeine, induced Ca\(^{2+}\) oscillations in RyR2-E4146K mutant expressing HEK293 cells. On the other hand, high concentrations of caffeine induced a single peak of Ca\(^{2+}\) release in RyR2-WT expressing HEK293 cells. The exact mechanism underlying this difference is not completely understood. We have shown previously that caffeine induces Ca\(^{2+}\) release/Ca\(^{2+}\) oscillations in RyR2-expressing HEK293 cells by reducing the threshold for store-overload induced Ca\(^{2+}\) release (SOICR) [66]. The observation that SOICR in RyR2-E4146K expressing HEK293 cells occurs only in the presence, but not in the absence of high concentrations of caffeine is consistent with the notion that the threshold for SOICR in RyR2-E4146K expressing HEK293 cells is markedly increased. We hypothesize that this markedly elevated SOICR threshold prevents store overload induced spontaneous Ca\(^{2+}\) oscillations in the absence of caffeine. Whereas, in the presence of 10 mM caffeine, the SOICR threshold in RyR2-E4146K expressing HEK293 cells would be reduced to a level at which spontaneous Ca\(^{2+}\) oscillations can now occur. On the other hand, the normal SOICR threshold in RyR2-WT expressing HEK293 cells would be dramatically reduced by 10 mM caffeine, resulting in the depletion of ER Ca\(^{2+}\) stores, which may explain the lack of Ca\(^{2+}\) oscillations in RyR2-WT expressing HEK293 cells in the presence of 10 mM caffeine [66].

In summary, the present study reveals novel LOF RyR2 mutations associated with IVF and SCD with negative exercise stress test. IVF and SCD associated with loss of RyR2 function represents a new entity of ventricular arrhythmias distinct from CPVT. Different strategies and protocols will be required for the diagnosis and treatment of ventricular arrhythmias associated with enhanced or suppressed RyR2 function.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

Data and materials availability: All data are available in the main text or the supplementary information.

AUTHOR CONTRIBUTIONS

XZ, RW, PPJ, CN, SGP, SRWC designed the experiments; XZ, WG, JW, YT, YL, JZZ, VHT, LZ, RW, CN, SGP performed the experiments and collected the data; XZ, WG, JW, YT, YL, JZZ, VHT, LZ, RW, CN, SGP, SRWC performed the analysis; and XZ, RW, PPJ, SGP, SRWC wrote the paper.
THE ABBREVIATIONS USED
SCD, sudden cardiac death
aSCD, aborted sudden cardiac death
CPVT, catecholaminergic polymorphic ventricular tachycardia
IVF, idiopathic ventricular fibrillation
EPS, electrophysiological study
EST, Exercise stress testing
ICD, implantable cardioverter defibrillator
ECG, electrocardiogram
DAD, delayed afterdepolarization
SR, sarcoplasmic reticulum
RyR2, cardiac ryanodine receptor
GOF, gain-of-function
LOF, loss-of-function
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FIGURES AND LEGENDS:

Figure 1

(A) A schematic diagram of the linear sequence of RyR2. Major structural domains of RyR2 are depicted as solid blue boxes. The orange boxes indicate four disease-associated mutation clusters (mutation hotspots) in RyR2. IVF- and SCD-associated RyR2 mutations identified were listed underneath their corresponding domains.

(B) Locations of RyR2 mutations in the three-dimensional
structure of RyR2 (PDB: 6JI0). The three-dimensional locations of RyR2 mutations, F2331S, G2337V, A3442E, I3476T, and R3570W have not been resolved and thus were not shown in the three-dimensional structure. The bindings sites for Ca^{2+}, caffeine, and ATP are also shown.
Figure 2. Effects of RyR2 E4146K and G4935R mutations associated with sudden death on caffeine-induced Ca\(^{2+}\) release and their expression in HEK293 cells.

HEK293 cells were transfected with RyR2 WT (A), E4146K (B), and G4935R (C). Fluorescence intensity of the fluo-3-loaded transfected cells was monitored continuously before and after each caffeine addition. The numbers (under the traces) indicate caffeine concentrations. Traces shown are from representative experiments. (D) Cumulative caffeine concentration–Ca\(^{2+}\) release relationships in HEK293 cells transfected with RyR2 WT, E4146K, and G4935R. Data shown are mean ± SEM (n=3). (E, F) HEK293 cells were transfected with RyR2 WT, E4146K, or G4935R. Cell lysates were prepared
from these transfected cells and used for immunoblotting analysis. The same amount of cell lysate was used for immunoblotting using the anti-RyR2 antibody. Data shown are mean ± SEM (n=4).
Figure 3. Effect of RyR2 mutations on caffeine-induced Ca^{2+} release in HEK293 cells.

HEK293 cells were transfected with RyR2 WT (A) or mutants (B-Q). Fluorescence intensity of the fluo-3-loaded transfected cells was monitored continuously before and after each caffeine addition. The numbers (under the traces) indicate caffeine concentrations. Traces shown are from representative experiments (n=3).
Figure 4. Impact of RyR2 mutations on caffeine-induced Ca\textsuperscript{2+} release in HEK293 cells.

HEK293 cells were transfected with RyR2 WT or mutants. Fluorescence intensity of the fluo-3-loaded transfected cells was monitored continuously before and after each caffeine addition. (A-C) Cumulative caffeine concentration–Ca\textsuperscript{2+} release relationships in HEK293 cells transfected with RyR2 WT or
mutants. Note that the caffeine responses of these mutants are similar to that of RyR2 WT. Data shown are mean ± SEM (n=3).
Figure 5.

The RyR2-E4146K mutation abolishes store-overload induced Ca\(^{2+}\) release (SOICR) in HEK293 cells

Stable, inducible HEK293 cells expressing RyR2 WT or E4146K were loaded with 5 μM Fura-2 AM in KRH buffer. The cells were then perfused continuously with KRH buffer containing increasing levels of extracellular Ca\(^{2+}\) (0 - 2 mM) to induce SOICR. Fura-2 ratios of representative RyR2 WT (A) and E4146K (B) cells were recorded using single cell Ca\(^{2+}\) imaging. (C) The percentages of RyR2 WT (691 cells) and E4146K (466) cells that display Ca\(^{2+}\) oscillations at various extracellular Ca\(^{2+}\) concentrations. Note that no SOICR was detected in HEK293 cells expressing the E4146K mutant. (D) ER store Ca\(^{2+}\) content in RyR2 WT or E4146K mutant expressing HEK293 cells estimated by measuring the amplitude of caffeine (10 mM) induced Ca\(^{2+}\) release. Data shown are mean ± SEM (n = 3-5).
Figure 6

Single channel activities of the E4146K mutant (A) were recorded in a symmetrical recording solution containing 250 mM KCl and 25 mM HEPES (pH 7.4). The Ca²⁺ concentration on both the cytoplasmic and the luminal face of the channel was adjusted to ~45 nM (panel a). The luminal Ca²⁺ concentration was then increased to various levels by an addition of aliquots of CaCl₂ solution (panels b-d). Recording potentials were -20mV. Openings were downward and baselines indicated (short bars). Open probability (Po), mean open time (To), and mean closed time (Tc) are shown. (B) The relationships between Po and luminal Ca²⁺ concentrations (pCa) of single E4146K (open squares) mutant channels are shown. Data points shown are mean ± SEM from 7 E4146K single channels. The Po-luminal Ca²⁺ relationship (dashed line) of single RyR2 WT channels was taken from a previous study [46] where E4146K was part of the study.
Figure 7

Figure 7. Impact of the E4146K mutation on cytosolic Ca\(^{2+}\) activation of single RyR2 channels

Single channel activities of the E4146K mutant (A) were recorded in a symmetrical recording solution containing 250 mM KCl and 25 mM HEPES (pH 7.4) and in the presence of ~45 nM luminal Ca\(^{2+}\) and various concentrations of cytosolic Ca\(^{2+}\) (panels a-c). (B) The relationship between Po and cytosolic Ca\(^{2+}\) concentrations (pCa) of single E4146K (open squares) mutant channels are shown. Data points shown are mean ± SEM from 14 E4146K single channels. The Po-cytosolic Ca\(^{2+}\) relationship (dashed line) of single RyR2 WT channels was taken from a previous study [46] where E4146K was part of the study.
Figure 8. The RyR2 LOF G4935R mutation has dominant negative effect on RyR2 WT

HEK293 cells were transfected with RyR2 WT (A) or co-transfected with RyR2 WT and G4935R (B). Fluorescence intensity of the fluo-3-loaded transfected cells was monitored continuously before and after each caffeine addition. (C) Cumulative caffeine concentration–Ca$^{2+}$ release relationships in HEK293 cells transfected with RyR2 WT or co-transfected with RyR2 WT/G4935R. Data shown are mean ± SEM (n=3). (D) $[^{3}H]$ryanodine binding to cell lysates prepared from HEK293 cells transfected with RyR2 WT or the G4935R mutant or co-transfected with RyR2 WT/G4935R was carried out at various Ca$^{2+}$ concentrations (0.1 nM to 100 µM). Data shown are mean ± SEM (n = 5 separate experiments for WT, 3 for RyR2 WT/G4935R, and 4 for G4935R).
Supplementary Fig. 1 Effect of IVF-associated RyR2 mutations on channel expression
HEK293 cells were transfected with RyR2 WT, E4146K, or G4935R. Cell lysates were prepared from these transfected cells and used for immunoblotting analysis. The same amount of cell lysate was used for immunoblotting using the anti-RyR2 antibody.
Supplementary Figure 2

A Uncropped gels

RyR2

250kDa —

WT E4146K

75kDa —

50kDa —

37kDa —

GAPDH

25kDa —

20kDa —

WT E4146K

B

1.5

1.0

0.5

0

WT

E4146K

Supplementary Fig. 2 Effect of E4146K mutation on channel expression in HEK293 cells

Cell lysates were prepared from stable, inducible HEK293 cells expressing RyR2 WT or the E4146K mutant and used for immunoblotting analysis (A, B). The same amount of cell lysate was used for immunoblotting using the anti-RyR2 antibody. Uncropped gels for anti-RyR2 and anti-GAPDH immunoblots are shown (A). Data shown are mean ± SEM (n=4) (B).
Supplementary Information

Of the 18 RyR2 mutations tested in the present study, three are novel and the rest have been reported previously. A detailed description of the clinical evaluation for each of the three novel RyR2 mutations (E1127G, A3442E, and I3476T) was provided below. A brief summary of the major phenotypes for each of the previously reported RyR2 mutations was also included. Please refer to the published literature for more detailed description of the previously reported RyR2 mutations [6, 11, 13, 59-65].

I217V
The RyR2-I217V mutation was reported by Tester et al. [59] through molecular autopsy in a 21-year-old male who died suddenly and had syncope and a positive family history of SCD, but the SUD event was nonspecific [59].

R414C
The RyR2-R414C mutation was reported by Creighton et al. [60] through molecular autopsy in a 16-year-old female who died suddenly while swimming competitively. The patient had a history of attention deficit hyperactivity disorder and syncope. Evaluations with electroencephalogram, computed tomography scan, and 24-hour Holter monitoring were negative. Histological analyses showed unremarkable myocardium, coronary arteries and conduction system. Exercise stress test was not available [60].

P446A
The RyR2-P446A mutation was reported by Tester et al. [61] in a 9-year-old boy who had ventricular fibrillation when he was 11-month-old and a family history of sudden infant death syndrome (SIDS) and SCD. Exercise stress test was not available. The patient had syncope during breath holding and aborted cardiac arrest [61].

E1127G
The RyR2-E1127G mutation has not been reported previously. The patient is a young, completely asymptomatic female who experienced an aborted sudden cardiac death (April 2011) at 25 years of age. At the moment of the event the patient was resting waiting for the start of a theatre act where she was supposed to sing. She experienced a sudden loss of consciousness and ventricular fibrillation was observed on the cardiac monitor by paramedics who were on place; sinus rhythm was promptly restored with DC shock. The subsequent clinical and instrumental investigations showed normal findings (ECG, echocardiography, coronary angiography and heart MRI). Exercise stress test and ECG Holter monitoring did not show any arrhythmias. Therefore, on May 2012 the patients underwent ICD implantation and was referred to our center for genetic investigations. Genetic screening of KCNQ1, KCNH2, SCN5A, Kir2.1, KCNE1 and KCNE2 were negative while the screening of RyR2 led to the identification of the E1127G mutation. Collection of family history showed a case of sudden death in a young male who died suddenly at rest at the age of 13. Unfortunately, no DNA was available for cascade genetic screening in the family. The E1127G mutation was inherited from the asymptomatic father who presented with a normal ECG (but refused additional clinical investigations). The patient was treated with beta-blockers and remained asymptomatic thereafter with no arrhythmias detected by the device.

G2145R
The RyR2-G2145R mutation was reported by Marjamaa et al. [62] in the index patient who was 41 years old at the time of death. The patient had a syncopal event at exercise two months before his death. A medico-legal autopsy and histological and toxicological analyses revealed a structurally normal heart and provided no apparent explanation for the sudden death. The index patient’s daughter is also RyR2 G2145R mutation positive, but showed no structural or electrical abnormalities on clinical evaluation at the age of 23 years old [62].

F2331S
The RyR2-F2331S mutation was reported by Creighton et al. [60] through molecular autopsy in an 8-year-old male who became unresponsive while climbing a rock wall. The individual had prior seizure episodes. Previous clinical evaluation did not show any evidence of long QT syndrome. Histological analyses at autopsy showed unremarkable myocardium, coronary arteries and conduction system [60].

G2337V
The RyR2-G2337V mutation was reported by Haugaa et al. [63] through molecular genetic screening for CPVT and long QT syndrome in a family with eight members who died suddenly. Post-mortem genetic testing in three of them revealed heterozygous RyR2 G2337V mutation. All patients had normal resting ECG and none of the subjects experienced sustained ventricular tachycardias or required external cardioversion [63].

A2387T
The RyR2-A2387T mutation was reported by Tester et al. [61] in an 18-year-old female who experienced aborted cardiac arrest (ACA) and had a positive family history of SCD. Exercise stress testing was not available [61].

Y2392C
The RyR2-Y2392C mutation was reported by Bauce et al.[13] through screening families with effort-induced polymorphic ventricular arrhythmias, syncope, and sudden death. The 12-lead ECG showed normal sinus rhythm and atrioventricular conduction and postmortem examination showed structurally normal heart [13].

R2401L
The RyR2-R2401L mutation was reported by Creighton et al.[60] through molecular autopsy in a 12-year-old male who died from a syncopal/arrhythmic episode while running. The patient had a history of exercise-induced ventricular tachycardia and sinus node dysfunction. A previous catheterization study showed normal hemodynamics. Histological analyses showed unremarkable myocardium, coronary arteries and conduction system [60].

A3442E
The RyR2-A3442E mutation has not been reported previously. The proband is a previously asymptomatic female patient who presented with syncopal episode with detection of ventricular fibrillation treated with DC Shock and external cardiac massage. The event was triggered by acute emotion. At the moment of the event (April 2002) the patient was 16 years old. Since then, she performed several tests including MRI and electrophysiological study with flecainide with negative results. Ventricular extrasystoles (couplets and bidirectional triplets) during exercise testing were observed. After about a month, new pre-syncopal event after an intense emotion
occurred. During the visit in August 2002 at our center, the presence of exercise-induced PVCs was confirmed. On this base, even if there was no clear evidence of repetitive life-threatening arrhythmias we decided to start with beta-blocker therapy (nadolol 40 mg/day approx 1 mg/kg). Another exercise stress test on therapy showed no arrhythmias. However, in agreement with the family an ICD was implanted (October 2002). The device was replaced in March 2010 (including ICD lead). During the visit (November 2013) the patient was regularly taking 40 mg/day of nadolol, no arrhythmias were detected at device interrogation. Exercise stress test showed ventricular bigeminy inducible with FC >120 bpm (reached at high workload after 10 minutes of exercise at Bruce IV), echocardiogram was normal; Holter monitoring showed isolated PVCs and one couplet during HR increases. Genetic screening resulted positive for the presence of a RyR2 A3442E mutation. Both parents were negative (sporadic case).

I3476T
The RyR2-I3476T mutation has not been reported previously. The proband is a female subject born on March 1989 who was referred for cardiologic evaluation for recurrent syncopal spells starting at the age of 2 years usually triggered by acute emotional stress. In 1995 while the patient was rushed to the ER due to a new episode of loss of consciousness a polymorphic VT was recorded (in the available medical record the arrhythmias was referred as “torsade de pointes-like”). This evidence in combination with a QTc at the upper limit immediately after restoration of sinus rhythm led the local referring cardiologist to suspect the diagnosis of LQTS and beta-blocker therapy was undertaken. The patient was referred to our center for clinical and genetic evaluation. The initial evaluation of the available ECGs showed normal QTc. We performed cardiac echo (normal), additional ECGs (unremarkable) and 12-leads Holter monitoring (normal QT no arrhythmias). However, polymorphic ventricular couplets and triplets were reproducibly induced at the exercise stress test. For these reasons we carried out molecular analysis of cardiac Ryanodine receptor that led to the identification of the I3476T mutation. The analysis of the major LQTS genes was negative. Cascade genetic testing in the family led to the identification of the same genetic defect in the proband’s father (asymptomatic, with normal cardiac findings including exercise stress test).

R3570W
The RyR2-R3570W mutation was reported by Marjamaa et al. [62] independently in two victims of SCD from Eastern Finland. The index patient in one family died at the age of 17 years old during a volleyball game. An autopsy and toxicological examination revealed a moderately enlarged and dilated heart. The index patient in another family was a 55-year-old male died suddenly while carrying a water bucket. Postmortem investigations uncovered a moderately enlarged and dilated heart. In these two families, 20 individuals out of 64 family members screened were carriers of the RyR2 R3570W mutation. None of the relatives was reported to have syncopal events or palpitations [62].

N4097S
The RyR2-N4097S mutation was discovered by molecular autopsy in an 18-year-old male who died suddenly and had a positive family history of SCD, but the SUD event was nonspecific [59].

E4146K

34
The RyR2-E4146K heterozygous mutation was reported by Tester et al. [59] through molecular autopsy in an 14-year-old male who died suddenly during sleep and had a positive family history of SCD [59].

**I4848V**
The RyR2-I4848V mutation was reported by Tester et al. [61] in two females (14 and 35 years old) who had near-drowning experiences and a positive family history of SCD. One patient also had aborted cardiac arrest during exertion [61].

**G4935R**
The RyR2-G4935R heterozygous mutation was reported by Johnson et al. [64] in a female patient presented with episodes of abrupt loss of consciousness while running at the age of 4 years old. The patient had a prenatal history of bradycardia in utero with no identified etiology. There was family history of seizures, but no history of sudden unexplained death. Neurologic examination was normal. Multiple ECGs were performed, all of which were normal. Exercise stress testing also showed no evidence of arrhythmia. At 8 years of age while running, the patient abruptly fell to the ground and had a prolonged generalized tonic-clonic seizure and died from cardiopulmonary arrest [64].

**R4959Q**
The RyR2-R4959R mutation was reported by Laitinen et al. [65] in a 60-year-old female proband during screening for RyR2 mutations in CPVT families. The proband had experienced syncope at the age of 40 years old. Resting ECG was normal, and cardiac imaging studies did not demonstrate any evidence of cardiac structural abnormalities [65]. The same mutation was also reported by Tester et al. [61] in a 31-year-old female and a 12-year-old female who presented with seizure and syncope during exertion and had a family history of sudden cardiac death. Exercise stress testing showed PVCs, ventricular bigeminy, and couplets.
