Published in final edited form as:
Circ Res. 2013 January 18; 112(2): 298–308. doi:10.1161/CIRCRESAHA.112.274803.

Heterogeneity of Ryanodine Receptor Dysfunction in a Mouse Model Of Catecholaminergic Polymorphic Ventricular Tachycardia

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Abstract

Rationale—Most cardiac ryanodine receptor (RyR2) mutations associated with Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) are postulated to cause one distinctive form of Ca^{2+} release dysfunction. Considering the spread distribution of CPVT mutations, we hypothesized that dysfunctional heterogeneity was also feasible.

Objective—To determine the molecular and cellular mechanism(s) by which a novel RyR2-V2475F mutation associated with CPVT in humans triggers Ca^{2+}-dependent arrhythmias in whole hearts and intact mice.

Methods and Results—Recombinant channels harboring CPVT-linked RyR2 mutations were functionally characterized using [^{3}H]ryanodine binding and single channel recordings. Homologous recombination was used to generate a knock-in mouse bearing the RyR2-V2475F mutation. Ventricular myocytes from mice heterozygous for the mutation (RyR2-V2475F$^{+/-}$) and their wild-type (WT) littermates were Ca^{2+}-imaged by confocal microscopy under conditions that mimic stress. The propensity of WT and RyR2-V2475F$^{+/-}$ mice to develop arrhythmias was tested at the whole heart level and in intact animals. Recombinant RyR2-V2475F channels displayed a) increased cytosolic Ca^{2+} activation, b) abnormal PKA phosphorylation, and c) increased activation by luminal Ca^{2+}. The RyR2-V2475F mutation appears embryonic lethal in homozygous mice, but heterozygous mice have no alterations at baseline. Spontaneous Ca^{2+} release (SCR) events were

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DISCLOSURES
None.

Subject codes:
[5] Arrhythmias
[130] Animal models of human disease
[132] Arrhythmias-basic studies

more frequent and had shorter latency in isoproterenol-stimulated cardiomyocytes from RyR2-V2475F$^{+/-}$ hearts, but their threshold was unchanged with respect to WT. Adrenergically-triggered tachyarrhythmias were more frequent in RyR2-V2475F$^{+/-}$ mice.

Conclusions—The mutation RyR2-V2475F is phenotypically strong among other CPVT mutations and produces heterogeneous mechanisms of RyR2 dysfunction. In living mice, this mutation appears too severe to be harbored in all RyR2 channels, but remains undetected under basal conditions if expressed at relatively low levels. β-adrenergic stimulation breaks the delicate Ca^{2+} equilibrium of RyR2-V2475F$^{+/-}$ hearts and triggers life-threatening arrhythmias.

Keywords

CPVT; inherited arrhythmias; ryanodine receptor

INTRODUCTION

Type 2 ryanodine receptors (RyR2s) are the calcium release channels of sarcoplasmic reticulum (SR) that provide the majority of calcium ions (Ca^{2+}) necessary to induce contraction of cardiac cells.[1] In their intracellular environment, RyRs are regulated by a variety of cytosolic and luminal factors so that their output signal (Ca^{2+}) induces finely-graded cell contraction without igniting cellular processes that may lead to aberrant electrical activity (ventricular arrhythmias).[2] The importance of RyR2 dysfunction has been recently highlighted with the demonstration that point mutations in *RYR2*, the gene encoding RyR2 channels, are associated with Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT), an arrhythmogenic syndrome characterized by the development of adrenergically-mediated ventricular tachycardia in individuals with an apparently normal heart.[3] Equivalent mutations engineered in the murine *RyR2* gene also result in the development of phenotypes that recapitulate the major clinical manifestations of CPVT. Hence, CPVT mice represent a *bona fide* experimental model where the role of deranged Ca^{2+} homeostasis in the triggering of arrhythmias (Ca^{2+}-dependent arrhythmogenesis) may be integrally assessed. However, the molecular mechanisms that link a mutation in the RyR2 protein and the development of tachyarrhythmia remain incompletely understood.

To date, a considerable number of single-amino acid RyR2 mutations (>150) have been associated with the development of CPVT.[4 and (http://www.fsm.it/cardmoc)] Remarkably, the vast majority of these mutations[3] fall within three loci ("hot spots") termed CPVT-I, CPVT-II, and CPVT-III domains, of the RyR2 protein that are involved in several aspects of RyR2 regulation. Yet, most CPVT mutations characterized to date display a single mechanism of RyR2 dysfunction. [6-13] This stereotyped mechanism of dysfunction is puzzling given the multiple functional domains of the RyR2 protein potentially affected by each of the CPVT mutations. Moreover, CPVT episodes, by definition, occur after acute β-adrenergic stimulation but it is unknown whether phosphorylation of RyR2 channels is an obligatory step to elicit abnormal Ca^{2+} release in mutant channels or whether sympathetic stimulation only exacerbates the activity of a RyR2 channel already on the verge of ignition.

We performed an in-depth characterization of a novel CPVT-linked mutation, RyR2-V2475F, that was identified *post mortem* in a case of unexplained drowning of a young boy.[14] The mutation falls within the canonical CPVT-II domain, but unlike other RyR2 mutants, RyR2-V2475F displays *multiple* mechanisms of RyR2 dysfunction, including increased cytosolic Ca^{2+} sensitivity, altered $[Ca^{2+}]_{luminal}$ regulation and abnormal response to PKA phosphorylation. *In vivo*, the RyR2-V2475F mutation is highly arrhythmogenic. Thus, the RyR2-V2475F mutation produces a heterogeneous mechanism of RyR2 dysfunction.

METHODS

Recombinant mutant channels were generated by site-directed mutagenesis, expressed in HEK293 cells and purified as described before.[6] Mice with the RyR2-V2475F mutation were generated by homologous recombination. RyR2-V2475F$^{+/-}$ mice and age-matched wild-type (WT) littermates were maintained and studied according to the protocol approved by the Institutional Animal Care and Use Committees of the University of Wisconsin-Madison and the University of Michigan-Ann Arbor, and by the Association for Assessment and Accreditation of Laboratory Care International.

An expanded Materials and Methods section is available in the online data supplement.

RESULTS

Expression and characterization of recombinant RyR2-V2475F channels

RyR2 mutations associated with CPVT cluster in three well defined domains that control different aspects of channel function. Because these canonical domains are well conserved among species, we used site-directed mutagenesis to introduce in the mouse *RYR2* gene several mutations that are associated with CPVT in humans. The RyR2 mutations R176Q and R414C (pertaining to CPVT-I), V2475F (pertaining to CPVT-II), and G4671C (pertaining to CPVT-III) were expressed as described in Methods. We first tested their sensitivity to activation by Ca^{2+} using [^{3}H]ryanodine binding assays. [^{3}H]Ryanodine is a conformationally-sensitive ligand that binds to the open state of RyRs and may be used as a reliable index of the activity of the channel. Fig. 1*A* shows the Ca^{2+}-dependence of [^{3}H]ryanodine binding of purified WT (control) and mutant channels. For all channels, [Ca^{2+}] threshold for activation was above *p*Ca 8, and reached plateau at *p*Ca ~5. The activation was sigmoidal and could be fitted with a Hill equation with an EC_{50} = *p*Ca 6.3±0.12 for WT, R176Q, R414C and G4671 channels. Remarkably, only V2475F displayed significant difference with WT, with an EC_{50} = 6.8±0.15. At *p*Ca 7, activation of WT channels was only ~10% of normalized value, whereas in V2475F, activation exceeded ~40%. Since previous work has demonstrated that Ca^{2+} in the range of 100 nM (*p*Ca 7) to 10 μM (*p*Ca 5) binds to a high affinity Ca^{2+}-binding site in the cytosolic portion of the RyR protein,[2,6,7] the results suggest that the V2475F mutation confers RyR2 channels hypersensitivity to cytosolic Ca^{2+}. Mutations that alter RyR2 activity at *p*Ca 7 are especially interesting since this is the basal [Ca^{2+}] reached during diastole.

To directly test whether the V2475F mutation increased the sensitivity of the RyR2 channel to cytosolic Ca^{2+}, we reconstituted purified WT and V2475F channels in planar lipid bilayers.[15] Fig. 1*B*, left panels, shows single channel activity at the indicated *cis* (cytosolic) [Ca^{2+}]; the right panels are current histograms of cumulative recording performed in n=6 (WT) and n=4 (V2475F) channels. To exclude effects of $[Ca^{2+}]_{luminal}$ on the activity of the channel, [Ca^{2+}] in the *trans* side of the channel was intentionally low (nominally free [Ca^{2+}], ~ 3 μM) and current flowed from cytosolic to luminal sides of the channel. At cytosolic *p*Ca 7, the probability of the channel being open (P_o) was higher for V2475F compared to WT channels (P_o = 0.081±0.022 and 0.023±0.006, respectively). At higher [Ca^{2+}] (*p*Ca 5.3), P_o increased for both channels, as expected, but the activity of V2475F was still significantly higher than WT (P_o = 0.683±0.192 and 0.422±0.124, respectively). Overall, these results confirmed that the mutation alone (in the absence of other cofactors) increases the sensitivity of RyR2 channels to cytosolic Ca^{2+} and confers a “gain-of-function” phenotype.

PKA phosphorylation exacerbates the hypersensitivity of V2475F channels to cytosolic Ca^{2+}

Tachyarrhythmia in CPVT is triggered by physical exercise or anxiety, that is, during sympathetic stimulation.[3,5] Sympathetic stimulation of the heart triggers the β-adrenergic cascade, which activates protein kinases.[1,2] We tested the effect of PKA and CaMKII phosphorylation on recombinant WT and V2475F channels. Fig. 2*A* shows Western blots of WT and V2475F channels before ("Ctl.") and after ("+PKA") incubation with the catalytic subunit of PKA (1 μg/ml). Antibodies used were: anti-RyR2, which detected total protein, and the phosphorylation-sensitive anti-pS2808 (PKA and CaMKII site), anti-pS2030 (PKA site) and anti-pS2814 (CaMKII site).[16] As expected, PKA phosphorylation of WT and V2475F channels did not change the band intensity of anti-RyR2 or anti-pS2814 and increased the phospho-signal of anti-pS2808 and anti-pS2030. However, V2475F channels were less sensitive to S2808 phosphorylation, and more sensitive to S2030 phosphorylation with respect to WT (Fig. 2*B*). We next tested whether the differential phosphorylation of V2475F channels had a functional correlate. We repeated the Ca^{2+}-dependence of [^{3}H]ryanodine binding experiments using the PKA-treated WT and V2475F channels. PKA phosphorylation did not change significantly the sensitivity of WT channels (EC_{50} = 6.4±0.14) but exaggerated the already abnormal sensitivity of V2475F for cytosolic Ca^{2+} (EC_{50} = 7.1±0.16) (Fig. 2*C*). By contrast, we did not detect differential CaMKII phosphorylation of S2808 or S2814 (S2030 was not responsive to CaMKII, as expected), or changes in [^{3}H]ryanodine binding after CaMKII phosphorylation (Online Fig. I). These results suggest that PKA phosphorylation of V2475F channels, an almost obligatory condition during β-adrenergic stimulation of the heart, may be an exacerbating condition that favors uncontrollable Ca^{2+} release in CPVT episodes.

V2475F channels display abnormal response to luminal Ca^{2+}

A prevalent mechanism believed to trigger CPVT episodes is increased channel sensitivity to luminal Ca^{2+}.[3] Recombinant WT and V2475F channels were reconstituted separately in planar lipid bilayers and the sensitivity of the channel to luminal Ca^{2+} was determined by clamping *cis* (cytosolic) Ca^{2+} to low levels (*p*Ca 7) and increasing Ca^{2+} gradually in the *trans* (luminal) side of the channel. Fig. 3*A* describes the sequential steps followed to generate the P_o vs. $[Ca^{2+}]_{luminal}$ plot of Fig. 3*B*. For each $[Ca^{2+}]_{luminal}$ tested, we recorded at least 2 min of continuous activity per channel. In WT and V2475F channels, an increase in $[Ca^{2+}]_{luminal}$ produced an increase in P_o, but the effect was more dramatic in the mutant channels (Fig. 3*B*). Mean open time was the main factor driving the increase in P_o for both groups of channels (Fig. 3*C*). For example, increasing $[Ca^{2+}]_{luminal}$ from 0.1 mM to 1 mM increased mean open time (τ_{open}) from 2.6±1.1 to 15.7±3.8 ms in WT channels, and from 21±6.4 to 103.5±14 in V2475F channels. Mean close time (τ_{close}) at the physiologically-relevant $[Ca^{2+}]$ of 1, 3 and 10 mM was only ~1.2-fold shorter for RyR2-V2475F. The frequency of openings was not directly related to $[Ca^{2+}]_{luminal}$, increasing only modestly for V2475F channels, and producing a biphasic response in WT channels (Fig. 3*D*). Thus, the negligible modification of τ_{close} and the extraordinary prolongation of τ_{open} by $[Ca^{2+}]_{luminal}$ are outstanding characteristics of the V2475F mutation.

Generation of RyR2-V2475F knock-in mice

We generated a knock-in mouse line harboring the RyR2-V2475F mutation. Fig. 4*A* shows the strategy used to generate the targeting vector and the resultant targeted allele. Fig. 4*B* and *C* show Southern blots and PCR that yield the expected amplification products. Fig. 4*D* shows that mice *heterozygous* for the mutation (RyR2-V2475F$^{-/+}$) have no gross anatomical or histological cardiac alterations, as expected from the clinical presentation of CPVT in humans. Furthermore, functional echocardiographic data (heart rate, ejection fraction, LVDP, etc) is normal for RyR2-V2475F$^{-/+}$ mice, resembling the clinical data (Fig. 4*E* and

4*F*, and Online Table I). However, heterozygotes propagate in a non-Mendelian fashion, generating offspring that is ~20% WT, ~80% heterozygotes (Fig. 4G). We were unable to detect homozygous embryos as early as 9 days of gestation. Therefore, although mice heterozygous for the mutation have no gross functional or structural alterations under basal conditions, the V2475F mutation appears too severe to be harbored in both alleles. Correspondingly, there are no known cases of CPVT patients homozygous for a given mutation and the heterozygous mice thus serve as excellent models to study this syndrome.

Variable P_o in RyR2 channels from RyR2-V2475F$^{-/+}$ heterozygous mice

The generation of the RyR2-V2475F$^{-/+}$ mice provided an excellent opportunity to study the effect of the mutation in a native environment. Fig. 5*A* shows aggregate data (P_o) from single channel recordings using purified recombinant WT channels ("RyR2-WT"), recombinant V2475F ("RyR2-V2475F"), and RyR2 channels obtained from cardiac SR of RyR2-V2475F$^{-/+}$ mice ("Het Mice"). Each bar represents an independent channel and all channels were recorded in the presence of 100 nM cytosolic [Ca^{2+}] and 1 mM $[Ca^{2+}]_{luminal}$ to maximize the phenotypic differences between WT and V2475F channels in their response to luminal Ca^{2+}. As expected from the results of Figs. 1 and 3, WT channels displayed a modest response to luminal Ca^{2+} which yielded low P_o (mean P_o = 0.06±0.02, n=11) (Fig. 5). V2475F channels, on the other hand, displayed a dramatic increase in P_o mainly due to an increase in mean open time, which yielded a P_o = 0.29±0.08 (n=10). By contrast, RyR2 channels from RyR2-V2475F$^{-/+}$ mice displayed widely variable P_o (Fig. 5*B*). Of the 10 recorded channels, 3 had the "signature effect" of the V2475F mutation (prolonged mean open time), which accounted for their high P_o. Thus, there is variability in channel activity that we suggest is attributed to the heterogeneous nature of the channel subunits contributing to the tetrameric RyR2 channel (Online Fig. V).

Pro-arrhythmic behavior in ventricular myocytes from V2475F$^{-/+}$ mice

With the molecular phenotype displayed by V2475F$^{-/+}$ mice in single channel experiments (Fig. 5), it was of interest to test whether their ventricular myocytes showed alterations in their intracellular Ca^{2+} signaling. Cells were subjected to a "stress" test to assess their capacity to handle SR Ca^{2+} load. Fluo-4 loaded ventricular myocytes were constantly perfused with Tyrode solution supplemented with Isoproterenol and then field-stimulated at 1 Hz during 5 sec to load the SR with Ca^{2+}. After stimulation, cells were "rested" and constantly monitored to measure spontaneous Ca^{2+} release (SCR) events. Fig. 6*A* shows representative examples of line-scan images (length vs. time) of WT and V2475F$^{-/+}$ cells subjected to the "stress" protocol. V2475F+/−cells subjected to this protocol had more SCRs, either partial or fully propagated (Fig. 6*A*). To determine the SR load at which SCR first occurs, WT or V2475F$^{-/+}$ cardiomyocytes were perfused with Tyrode solution and paced at 0.3 Hz for 30s. Caffeine (10 mM) was then applied to measure "basal SR load". Cells were then perfused with 100 nM Isoproterenol and continuously paced to gradually re-load the SR. This gradual re-loading of the SR with Ca^{2+} is reflected in the amplitude of the $[Ca^{2+}]_i$ transient (Fig. 6*B*), which is low immediately after caffeine and then increases as Ca^{2+} entry through L-type Ca^{2+} channels (I_{Ca}) fills the SR. To determine the SR load at which SCR occurs, a second caffeine pulse was applied immediately after the appearance of the first SCR. Under these conditions, the amplitude of this second caffeine-induced Ca^{2+} transient closely approximated the SR load threshold for SCR. The aggregate data for 25 cells is shown in Fig. 6*C*. "Basal" SR load was not significantly different between WT and V2475F$^{-/+}$ cells. On the other hand, the incidence (frequency) of SCR is higher in V2475F$^{-/+}$ cells (Fig. 6C, middle bars). Finally, the normalized SR load at which SCR first occurs is marginally higher in V2475F$^{-/+}$ cells (0.74 for WT and 0.89 for V2475F $^{-/+}$ of the caffeine-induced Ca^{2+} release; p=0.05).

Due to the possibility that residual Ca^{2+} and/or caffeine from the first Caffeine-evoked Ca^{2+} transient affected the estimation of the threshold for SCR, we performed an independent test using HEK293 cells transfected with WT and V2475F channels. In this system, the main determinant of SOICR is assumed to be the intrinsic channel's sensitivity for luminal Ca^{2+}.[6] Online Fig. III shows a similar threshold for store overload-induced Ca^{2+} release (SOICR) between WT- and V2475F-expressing cells. These results suggest that increased sensitivity to $[Ca^{2+}]_{luminal}$ in single RyR2-V2475F channels does not manifest as a decrease in the threshold for SCR.

Arrhythmic behavior in whole hearts and in intact V2475F$^{-/+}$ mice

Having demonstrated that V2475F$^{-/+}$ cardiomyocytes display a pro-arrhythmic substrate in the form of higher frequency of SCR, we next tested whether whole hearts from the same mice showed propensity to develop arrhythmias under β-adrenergic stimulation. We performed experiments in freshly explanted hearts from V2475F$^{-/+}$ mice using the Langendorff system and with simultaneous recording of electrograms, left ventricular (LV) pressure, temperature, and coronary flow. Fig. 7*A* (WT) and 7*B* (V2475F$^{+/-}$) show electrogram (top panel) and LV pressure (middle panel), expanded from the bottom panel. Hearts were paced at 400-600 bpm with an electrode placed in the apex. In the WT group, perfusion with Tyrode solution supplemented with 1 μM isoproterenol increased LV pressure as expected and had no significant arrhythmic behavior. In contrast, the same maneuver performed in V2475F$^{-/+}$ hearts frequently produced arrhythmic episodes characterized by premature ventricular complexes (PVC) (Fig. 7) and ventricular bigeminy (not shown). In extreme cases we observed ventricular fibrillation (Fig. 7*B*). The type of arrhythmia, incidence, and duration were also quantified by electrocardiography in anesthetized WT and V2475F$^{-/+}$ mice after intraperitoneal injection of Epinephrine (2 mg/kg) and caffeine (120 mg/kg) (Fig. 7*C*). This cocktail had minimal effect on WT mice (6.5±2.4 and 2.2±0.6 non-sustained and sustained arrhythmias, respectively, during a 30-min recording), but was highly arrhythmogenic in V2475F$^{-/+}$ mice (59±28 and 14.8±6.0 non-sustained and sustained arrhythmias, respectively, during a 30-min recording period). Most of the arrhythmias were identified as Premature Ventricular Complexes (PVC), bigeminy or tachyarrhytmias (Fig. 7*C*). Bidirectional ventricular tachycardia (BVT), was not present in WT mice but present in 2 out of 6 V2475F$^{-/+}$ mice (Fig. 7*C* "VF-1" and 7*D* right panel).

Optical mapping reveals multiple ventricular foci of arrhythmia

We mapped the anterior ventricular epicardium of V2475F$^{-/+}$ and WT hearts. Two mutant and 2 WT hearts were perfused with 200 nM isoproterenol (0.2 mg/ml) for 10 minutes. Afterwards, the Ca^{2+} concentration was doubled (from 1.8 to 3.6 mM). A bolus of isoproterenol (0.2 mg/ml) plus caffeine (100 mM) was applied 10 and 20 min later. Long lasting arrhythmias (> 1 minute) were elicited in both mutant hearts. One WT heart had no arrhythmias, and the second heart underwent a short episode of VT (about 10 seconds). The spontaneous arrhythmias that occurred in the mutant hearts included bigeminy, short bursts of non-sustained VT and sustained polymorphic VT. Impulse propagation during polymorphic VT is described in more detail in the legend of Fig. 8.

DISCUSSION

In this study, we used a multidisciplinary approach encompassing molecular, cellular, whole heart and intact animal experiments to elucidate the mechanisms by which a novel RyR2 mutation, V2475F, causes RyR2 dysfunction, abnormal $[Ca^{2+}]_i$ handling, and ventricular tachyarrhythmias. The mutation distinguished itself among other normally "silent" CPVT mutations by displaying a strong phenotype even in the absence of exacerbating factors (Fig. 1). Heterozygous mice (RyR2-V2475$^{+/-}$) faithfully recapitulate the cardinal signs of CPVT,

including normal functional echocardiogram and electrocardiogram at rest, as well as absence of structural cardiac abnormalities (Fig. 4). However, when challenged with an arrhythmogenic cocktail that activates the β-adrenergic cascade, RyR2-V2475F$^{+/-}$ mice display a highly arrhythmogenic phenotype (Figs. 7, 8).

Recombinant RyR2-V2474F channels exhibit three different functional defects

To date, most CPVT mutations have been functionally segregated into distinct groups affecting either luminal Ca^{2+} sensitivity,[3,6,7] FKBP12.6 association,[8,9] interdomain interactions,[10,11] or Ca^{2+}-dependent inactivation.[12,13] This categorization provides a "clean" operational sorting of CPVT mutations but it appears counterintuitive based on the structural complexity of the RyR2 protein. Actually, it would appear logical that a given CPVT mutation affected more than one aspect of RyR2 regulation since multiple functional domains have been mapped to regions where each mutation falls.[27] We present evidence here that the V2475F mutation affects cytosolic Ca^{2+} sensitivity (Fig. 1), PKA phosphorylation (Fig. 2), and luminal Ca^{2+} modulation (Fig. 3), three modes of RyR2 regulation highly relevant during a CPVT episode. Multiple modes of dysfunctional Ca^{2+} release for a single CPVT mutation has been suggested,[5,13] but it has not been demonstrated. This is the first documented case of heterogeneous RyR2 dysfunction caused by a CPVT-associated mutation.

In simplified solutions containing Ca^{2+} as the only relevant agonist of RyRs, recombinant RyR2-V2475F channels displayed an increased sensitivity to activation by cytosolic [Ca^{2+}] that was most conspicuous at *p*Ca 7 (100 nM [Ca^{2+}], Fig. 1). The increased Ca^{2+} sensitivity of V2475F was noted in a range of [Ca^{2+}] sufficiently low (*p*Ca 8 – *p*Ca 6) to safely ascertain the exclusive participation of cytosolic activation sites, inasmuch as luminal Ca^{2+} sites require greater [Ca^{2+}] for activation (tens to hundreds μmol/L, Fig. 2 and refs. 2,6,7). Furthermore, V2475F channels activated by cytosolic *p*Ca 7 without $[Ca^{2+}]_{luminal}$ displayed an increased frequency of openings without significant alteration of their mean close time (Fig. 1), suggesting that the mutation does not destabilize the channel's close state as proposed for other CPVT mutations.[8-11] Although other CPVT mutations have been shown to alter cytosolic Ca^{2+}sensitivity, this phenotype becomes apparent only after PKA dissociation of FKBP12.6.[8,9] In the case of V2475F, this is a defect intrinsic to the channel protein. At face value, this alteration portends significant diastolic Ca^{2+} leak in intact cardiomyocytes and is conceivable that it contributed to the embryonic lethality of the RyR2-V2475F homozygous mice.

Still, the most dramatic effect of the V2475F mutation was its remarkable hypersensitivity to luminal Ca^{2+} activation (Fig. 3*A*). The exaggerated response of V2475F channels to $[Ca^{2+}]_{luminal}$ essentially dwarfed the otherwise modest response displayed by WT channels (Fig. 3*B*). Since the number of openings was actually decreased in the V2475F channels with respect to WT in the luminal Ca^{2+} titration curve (Fig. 3*D*), this dysfunction is at variance with altered cytosolic Ca^{2+} activation (Fig. 1) and strongly suggests the participation of luminal Ca^{2+} sites as determinants of this molecular phenotype. Also, we used purified RyR2 channels and thus, the easiest explanation for this effect is that Ca^{2+} acted directly on the RyR2 channel and not on accessory proteins, as postulated for some forms of luminal Ca modulation.[21] Therefore, an extraordinary luminal Ca^{2+}-induced increase in mean open time by direct interaction of Ca^{2+} ions with the channel is the most distinctive phenotype of this mutation (Fig. 4*A*). This "signature effect" was later used to estimate the approximate proportion of dysfunctional channels in heterozygous RyR2-V2475F$^{+/-}$ hearts.

By definition, arrhythmic episodes in CPVT are triggered by sympathetic stimulation. Others and we have previously shown that RyR2 channels are among the first proteins to

undergo metabolic phosphorylation in hearts perfused with β-adrenergic agonists.[15,22] A relevant question, therefore, is whether phosphorylation worsens the molecular phenotype of RyR2 channels harboring CPVT mutations. We found that PKA phosphorylation modestly but significantly increased the cytosolic Ca^{2+}-dependence of activation of V2475F, but not WT channels (Fig. 2*C*). Specific phospho-sites were differentially phosphorylated in V2475F channels, resulting in decreased Ser2808 phosphorylation and increased Ser2030 phosphorylation (both PKA sites). The CaMKII site Ser2814 was not differentially affected. Thus, although the functional role of each of these sites is still controversial,[22,23] there was uneven efficacy of PKA on the distinct phospho-sites, and this likely contributed to intensify the molecular phenotype of RyR2-V2475F channels. This is a novel aspect of RyR2 modulation in CPVT mutations. By contrast, CaMKII phosphorylation was not differentially affected by the V2475F mutation (Online Fig. I).

Severe arrhythmic phenotype in mice harboring the V2475F mutation

Given the strong molecular phenotype of V2475F channels, we expected a correspondingly severe phenotype in mice harboring this mutation. Indeed, the mutation appears embryonic lethal in homozygous mice (Fig. 4*G*), and triggers life-threatening arrhythmias in heterozygous mice (RyR2-V2475F$^{+/-}$). However, given that the mutation confers RyR2 channels a gain of function at baseline (*i.e*, in the absence of sympathetic stimulation, Figs. 1 and 3), it is surprising that RyR2-V2475F$^{+/-}$ hearts are functionally and structurally indistinguishable from WT under basal conditions. We believe that at least two factors restrict the effects of the mutation at baseline. First, if we assume that mutant and WT alleles contribute equally and randomly to the formation of a tetrameric channel, then up to ~94% of RyR2s channels would be phenotypically altered in RyR2-V2475F$^{+/-}$ mice if only one subunit was necessary to confer the gain of function (dominant-positive mutation, please see Online Fig. V). However, only ~30% of RyR2-V2475F$^{+/-}$ channels reconstituted in lipid bilayers exhibited dramatically prolonged mean open time in the presence of 1 mM $[Ca^{2+}]_{luminal}$ (Fig. 5). This fact alone strongly suggests that multiple subunits are necessary to confer the phenotype, thus decreasing the incidence of abnormal channels in the heterozygous population. Second, ventricular myocytes have powerful auto-regulatory mechanisms that control the activity of “leaky” RyR2s in the long run: Eisner’s group has shown that increasing RyR2 activity (by Ca^{2+}-sensitization, phosphorylation, etc) induces more Ca^{2+} release in the first few beats, but this drives greater Ca^{2+} extrusion by the NCX and progressively decreases SR load, reducing the Ca^{2+} available for release in subsequent beats. Despite the reduced SR Ca^{2+} content, intracellular $[Ca^{2+}]$ transients remain relatively constant due to higher fractional release by the sensitized RyR2s, and a new steady-state is established in which Ca^{2+} influx and efflux are re-balanced.[24] Thus, auto-regulatory mechanisms can effectively preclude *tonic* hyperactivity of RyR2s, at least within a defined range of SR loads. In the RyR2-V2475F$^{+/-}$ mouse, the basal hyperactivity of the relatively small pool of abnormal channels may be controlled by this mechanism.

In β-adrenergic stimulated ventricular myocytes, dysfunctional RyR2-V2475F$^{+/-}$ channels clearly contribute to increased propensity for spontaneous Ca^{2+} release (SCR) (Fig. 6*A*, 6*C*) despite the auto-regulatory mechanisms mentioned above. Interestingly, the $[Ca^{2+}]_{luminal}$ necessary to provoke SCR is similar for V2475F$^{+/-}$ and WT cardiomyocytes (Fig. 6*B*), and in HEK293 cells, the threshold for SOICR was also similar for WT and RyR2-V2475F channels (Online Fig. I). These findings may appear counterintuitive given the increased P_o of RyR2-V2475F as a function of $[Ca^{2+}]_{luminal}$ (Fig. 3*B*), but it is possible that other processes such as cytosolic Ca^{2+} sensitivity and phosphorylation (both of which are altered in V2475F channels) take precedence over luminal $[Ca^{2+}]$ to determine the likelihood of SCR. Also, while most CPVT-linked mutations that display decreased threshold for SOICR also display dramatic decrease of their mean close time (τ_{close} = 7- to 20-fold faster than

WT),[6,30] the V2475F mutation displays a τ_{close} that is only ~1.2-fold faster than WT at physiologically-relevant [Ca^{2+}] (Online Fig. II), clearly, a marginal alteration in the context of other CPVT mutations. If we make the reasonable assumptions that SOICR is a good surrogate for SCR, and that τ_{close} is a critical determinant of the stability of the channel's closed state, we would expect then that mutations that decrease τ_{close} also lower the threshold for SCR. This is not the case for the V2475F mutation. Thus, we find it unlikely that the arrhythmogenic Ca^{2+} release in the RyR2-V2475F$^{+/-}$ cardiomyocytes is solely determined by their threshold for SCR. Instead, we believe that the increased sensitivity to luminal Ca^{2+}, concomitantly with elevated cytosolic Ca^{2+} sensitivity and RyR2 phosphorylation, all converge to fasten RyR2 restitution, thus shortening Ca^{2+} release refractoriness and increasing the incidence of SCR. In support of this hypothesis, the *latency* for SCR, a direct index of RyR2 restitution, was substantially faster in RyR2-V2475F$^{-/+}$ than WT cardiomyocytes (Online Fig. VI). The dissociation of $[Ca^{2+}]_{luminal}$ from SCR likelihood is not unprecedented: in a canine model of ventricular fibrillation, the increased rate of arrhythmogenic SCR was not determined by $[Ca^{2+}]_{luminal}$; instead, RyR2 oxidation and phosphorylation shortened RyR2 refractoriness.[31]

In Langendorff-perfused hearts and in intact animals, β-adrenergic stimulation brings about discrete arrhythmic behavior in RyR2-V2475F$^{+/-}$ mice. The demonstration of tachyarrhythmias in normally arrhythmia-resilient mice[26] is especially remarkable and underscores the importance of RyR2 dysfunction as arrhythmogenic trigger. Optical mapping revealed the multi-focal origin of such events (Fig. 8), compatible with triggered activity at various myocardial layers. All these events suggest active participation of ventricular cells as generators of arrhythmias, however, bidirectional ventricular tachycardia (BVT), a "pathognomonic" event of digitalis intoxication[19] and CPVT[17] that reflects alternating firing by the His-Purkinje system[18] was also observed in intact mice. The remarkable electrocardiographic similarity across species caused by Ca^{2+}-triggered arrhythmias suggests common pathways regardless of the molecular mechanisms. Induction of DADs is possibly the converging point.

CONCLUSIONS

Experiments *in vitro* showed that RyR2 mutations linked to CPVT, ARVD, or hypertrophic cardiomyopathy each exhibit distinct molecular phenotypes.[13,25,28] Here, we demonstrate for the first time that a CPVT-linked mutation can simultaneously induce multiple molecular alterations in the RyR2 channel, yet retain the distinctive traits of the disease at the whole heart and intact animal levels. Given that various regulatory regions may potentially be affected by a point mutation in the RyR2, we find it surprising that this variegated molecular dysfunction had not been detected before. We hypothesize that multidisciplinary screening strategies in a larger number of mutations will show even more complex mechanisms of CPVT. Unveiling these mechanisms is not merely an academic exercise but a task with relevant clinical implications. For example, it is plausible that the location and the mechanisms of arrhythmia determine the severity of the phenotype or condition the efficacy of antiarrhythmic drugs, especially those that directly target the RyR2. Thus, identifying the mutations and their mechanisms could serve as a valuable tool for risk stratification or therapy individualization in CPVT patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

SOURCES OF FUNDING

This work was supported by National Institutes of Health grants PO1-HL094291 and RO1-HL055438 (to HHV), by National Institutes of Health grants P01-HL039707 and P01-HL087226 (to JJ), by the Leducq Foundation and Centro Nacional de Investigaciones Cardiovasculares, Spain (to JJ), and by National Institutes of Health grant K99-HL105574 to (SNJ). RL was supported by an AHA pre-doctoral fellowship.

Non-Standard Abbreviations

BDVT	Bidirectional Ventricular Tachycardia
CaMKII	Calcium/Calmodulin-dependent Protein Kinase II
CPVT	Catecholaminergic Polymorphic Ventricular Tachycardia
LV	Left Ventricle
LVDP	Left Ventricle Developed Pressure
PKA	Protein Kinase A
P_o	Open Probability
PVC	Premature Ventricular Complex
RyR2	Cardiac Ryanodine Receptor
RyR2-V2475F+/−	Heterozygous mutant RyR2 carrying the V2475F substitution
SCR	Spontaneous Calcium Release
SOICR	Store Overload-Induced Calcium Release
τ_{close}	Mean Close Time
τ_{open}	Mean Open Time
VT	Ventricular Tachycardia
V2475F	substitution of Valine to Phenylalanine in the position 2475 of RyR2 linear sequence
WT	Wild Type
$[Ca^{2+}]$	Calcium concentration
$[Ca^{2+}]_{luminal}$	Sarcoplasmic Reticulum Calcium Concentration
$[^3H]$ryanodine	Tritiated Ryanodine

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Novelty and Significance

What Is Known?

- Mutations in *RYR2*, the gene encoding the cardiac Ca^{2+} release channel/ryanodine receptor (RyR2), are associated with Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT), a syndrome characterized by exercise- or stress-induced tachyarrhythmias.
- In murine models of CPVT, most RyR2 mutations associated with CPVT are postulated to cause one distinct defect in RyR2 function.
- Since CPVT point mutations often map to regions of the channel that have been implicated in multiple regulatory interactions, categorization of the molecular phenotype into distinct functional groups may be counterintuitive.

What New Information Does This Article Contribute?

- RyR2-V2475F, a previously uncharacterized mutation associated with CPVT in humans, produces not a single, but multiple, forms of RyR2 dysfunction.
- Despite the heterogeneity of RyR2 dysfunction generated by the RyR2-V2475F mutation, the murine model retains the distinctive electrocardiographic traits of the disease, i.e., adrenergically-triggered premature ventricular complexes and bidirectional ventricular tachycardia.

CPVT is a rare, but highly malignant, arrhythmogenic syndrome characterized by exercise- or stress-induced tachyarrythmias in the absence of structural cardiac alterations. Mutations in *RYR2* underlie a great portion of CPVT cases in humans, but the functional alterations caused by the mutations remain incompletely understood. Untimely Ca^{2+} release (i.e., diastolic or spontaneous sarcoplasmic reticulum Ca^{2+} release) by mutant RyR2 seems to be the primary event that generates arrhythmias in CPVT. Each CPVT-linked RyR2 mutation characterized to date was thought to cause spontaneous Ca^{2+} release by altering a single mechanism of RyR2 function, i.e., regulation of channel activity by luminal Ca^{2+}, association with the accessory protein FKBP12.6, or channel inter-domain interactions. The novel CPVT-linked mutation RyR2-V2475F displays multiple mechanisms of RyR2 dysfunction, including increased cytosolic Ca^{2+} sensitivity, altered regulation by luminal $[Ca^{2+}]$, and abnormal PKA phosphorylation, three modes of channel regulation highly relevant in CPVT episodes. Given that various regulatory regions may potentially be affected by a point mutation in the RyR2, a variegated molecular dysfunction may be the norm in all CPVT mutations. Multidisciplinary screening strategies in a larger number of mutations may show even more complex mechanisms of CPVT and serve as a valuable tool for therapy individualization in CPVT patients.

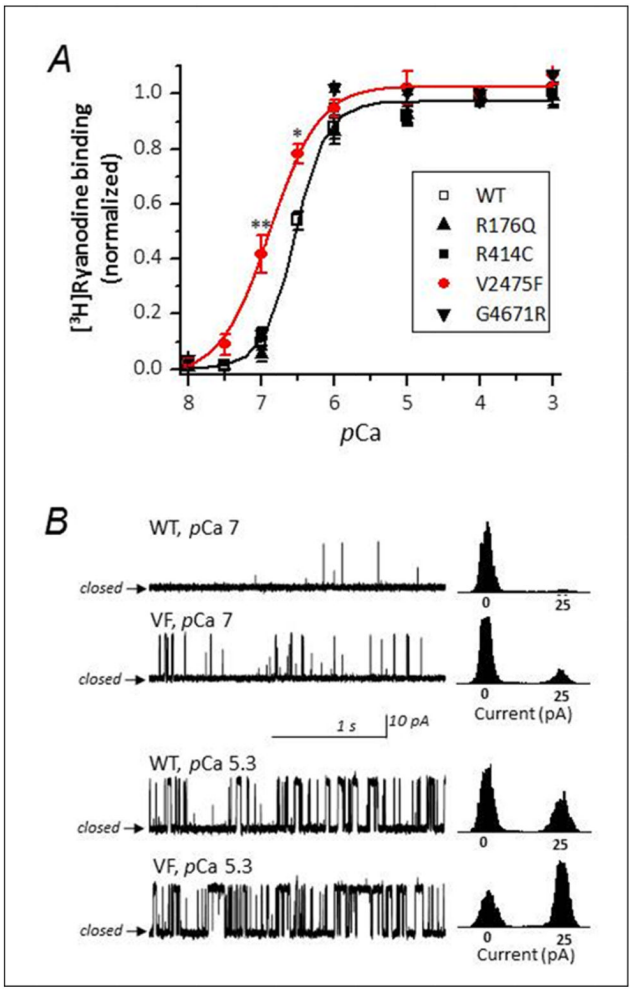

Figure 1. RyR2-V2475F channels display higher sensitivity to cytosolic Ca^{2+} than WT channels

A, Ca^{2+}-dependence of [^{3}H]ryanodine binding curves performed in parallel using the solubilized and purified channels indicated in the inset. Asterisk indicates $p<0.05$. *B*, Single channel recordings of WT and V2475F channels reconstituted in lipid bilayers. Left panels show 2-sec segments of representative single channel activity at the indicated *cis* (cytosolic) Ca^{2+} and the right panels are current histograms of at least 5 min of cumulative recording performed in n=6 (WT) and n=4 (V2475F) channels

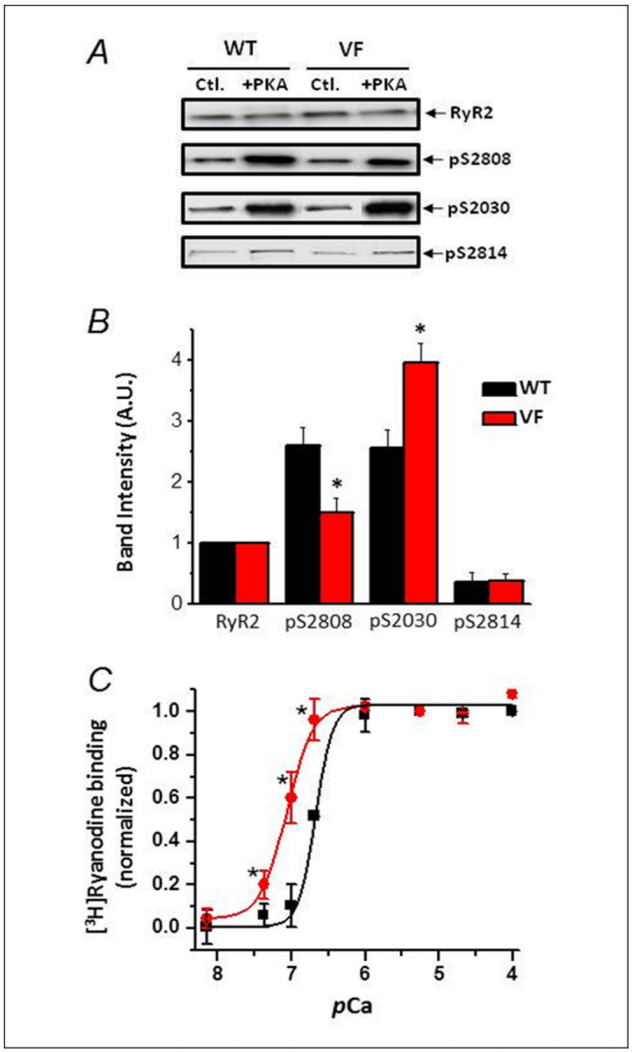

Figure 2. PKA phosphorylation increases the sensitivity of V2475F channels to cytosolic Ca^{2+}

A, Western blots of WT and V2475F channels before ("Ctl.") and after ("+PKA") incubation with the catalytic subunit of PKA. Antibodies used were: anti-RyR2 (1:3000), anti-pS2809 (1:10000), anti-pS2030 (1:10000) and anti-pS2814 (1:10000). *B*, Average band intensity (normalized to total RyR2 protein) of n=4 independent determinations with the indicated antibodies. *C*, Ca^{2+}-dependence of [^{3}H]ryanodine binding using PKA-treated solubilized WT and V2475F channels. PKA phosphorylation did not change significantly the sensitivity of WT channels (EC_{50} = 6.4±0.14) but increased the already abnormal sensitivity of V2475F for cytosolic calcium (EC_{50} = 7.1±0.16, n=5). Asterisk indicates $p<0.05$.

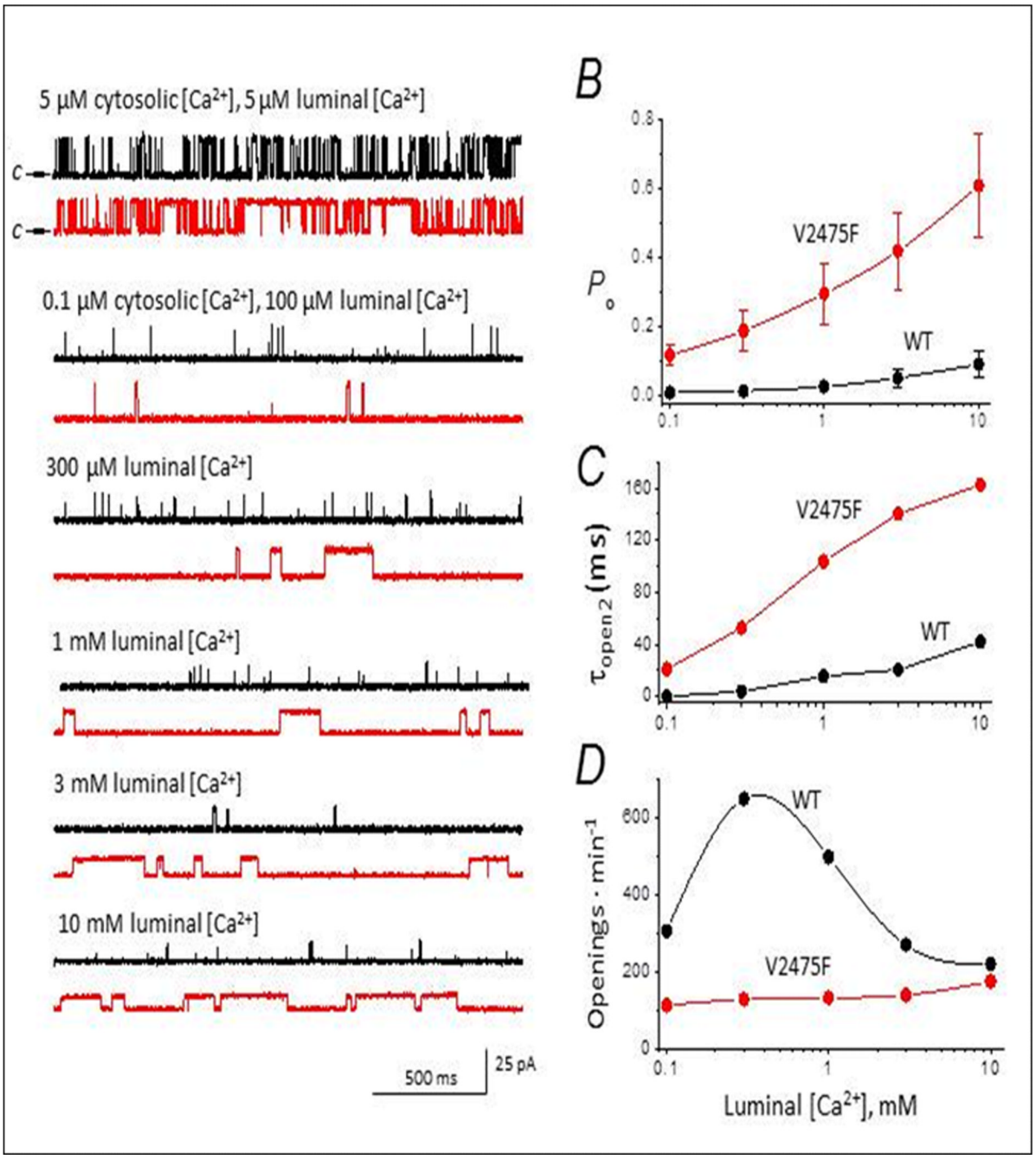

Figure 3. Increased sensitivity of V2475F channels to luminal calcium

A, Representative single channel recordings (2-s traces) of WT (black) and V2475F (red) recombinant RyR2 channels, in the presence of the indicated calcium concentrations in the *cis* (cytosolic) and *trans* (luminal) sides of the channel. "c→" in the top traces corresponds to closed state of the channel, and this polarity is the same for all traces. Average P_o (*B*), t_{open} (*C*), and rate of openings (*D*) are plotted for WT and V2475F channels (n=6 and 5 channels, respectively) at the indicated luminal [Ca^{2+}]. Cytosolic [Ca^{2+}] was kept constant at 0.1 μmol/L throughout the titration.

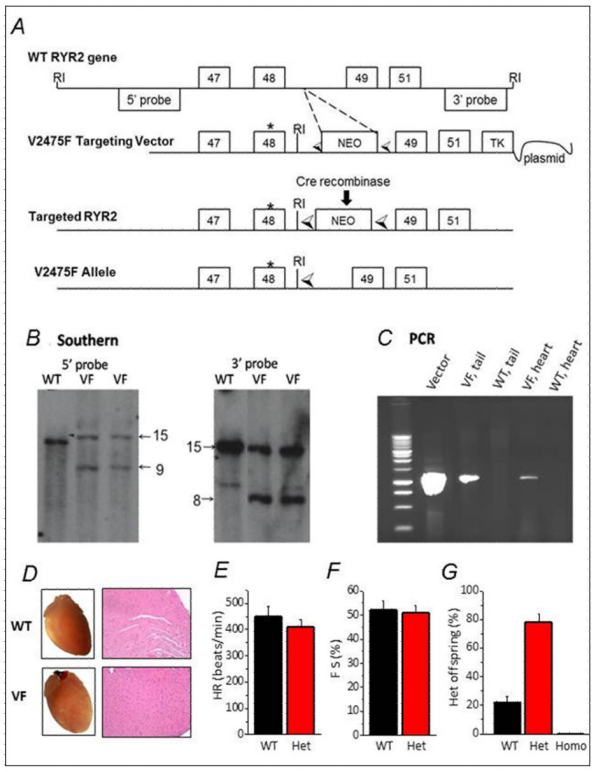

Figure 4. Generation of the RyR2-V2475F mouse

A, Strategy for generation of the transgenic mice by homologous recombination. **A**, (first line) The region of the WT RYR2 gene containing exons 47, 48, 49 and 51 (numbered boxes). (Second line) the RyR2-V2475F targeting vector containing the V2475F substitution (*), a translationally silent Mlu I restriction site (M), the loxP flanked pGK promoter/EM7 promoter-NEO-pGHpA cassette (NEO), the location of the EcoRI restriction sites (R1) used in genotyping and the MC1-HSV-Tk cassette (TK). (Third line) Homologous recombination between the endogenous RyR2 locus and the V2475F targeting vector resulted in a chromosome carrying the V2475F substitution and the loxP flanked Neo cassette. (Last line): the V2475F allele after Cre excision of the Neo cassette. **B**, The 5′ and 3′ Southern blot probes used in genotyping the NeoR ES cells. **C**, PCR confirmation of WT and heterozygous V2475F mice. The expected 1Kb band is seen in the heterozygotes. The targeting vector was used as positive control. *D*, H&E-stained hearts indicate no structural alterations in V2475F heterozygous mice compared to WT. *E*, heart rate, and *F*, fractional shortening, were not significantly different between WT and V2475F heterozygous mice. *G*, Non-Mendelian propagation of V2475F heterozygous mice yields ~20% WT, ~80% heterozygotes, and 0% homozygotes.

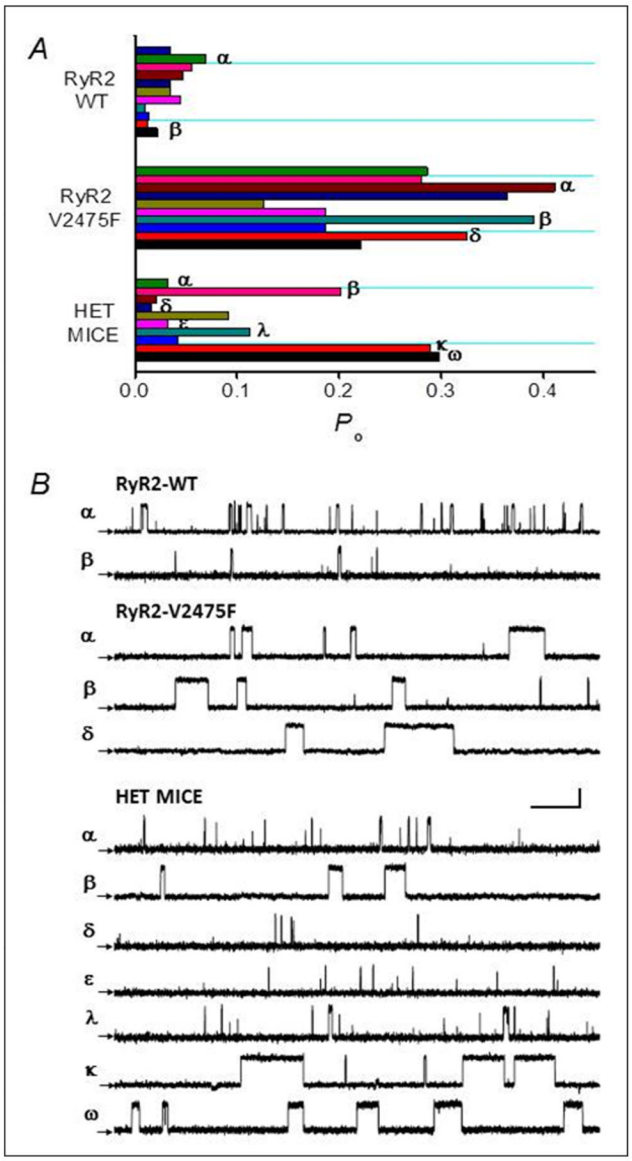

Figure 5. Variable P_o in RyR2 channels from V2475F heterozygous mice

Purified WT ("RyR2 WT") and V2475F ("RyR2 V2475F") recombinant RyR2 channels show distinctive P_o when recorded in the presence of 0.1 μmol/L *cis* (cytosolic) Ca^{2+} and 1 mmol/L *trans* (luminal) Ca^{2+}. By contrast, RyR2 channels obtained from $V2475F^{+/-}$ heterozygous mice ("HET MICE") display variable P_o under the same recording conditions. Each bar represents the average P_o of independently-recorded individual channels. Mean P_o for $V2475F^{+/-}$ channels were: 0.034, 0.016, 0.033 and 0.112 (α, δ, ε, and λ, respectively) and 0.214, 0.289, and 0.297 (β, κ, and ω, respectively). *B*, Representative 2-s recordings from 2 min of continuous activity of the channels correspondingly labeled with the same Greek character in *A*. Scale bars represent 15 pA (vertical) and 250 ms (horizontal).

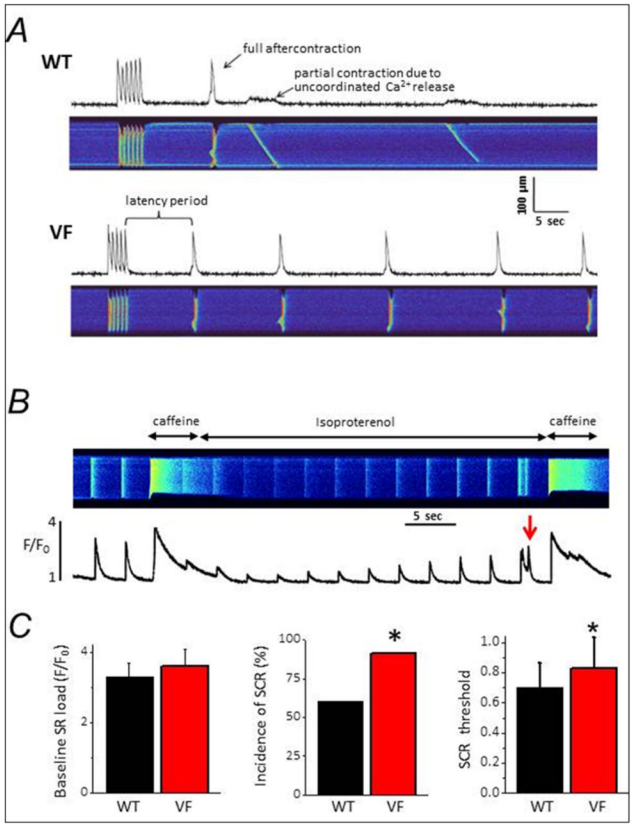

Figure 6. Pro-arrhythmic behavior in ventricular myocytes from V2475F heterozygous mice

A, Confocal line-scan images of $[Ca^{2+}]_i$ dynamics in ventricular myocytes of WT and $V2475F^{+/-}$ hearts. Cells were constantly perfused with Tyrode solution supplemented with Isoproterenol and then stimulated at 1 Hz during 5 sec to load the SR with Ca^{2+}. Cells were then "rested" and the latency and number of spontaneous calcium release (SCR) events measured (Fig. S6). *B*, Protocol to measure SR load threshold for SCR in WT and V2475F cells. Please see text for details. *C*, Combined data from at least n= 25 cells/genotype. Asterisk (*) indicates $p \leq 0.05$.

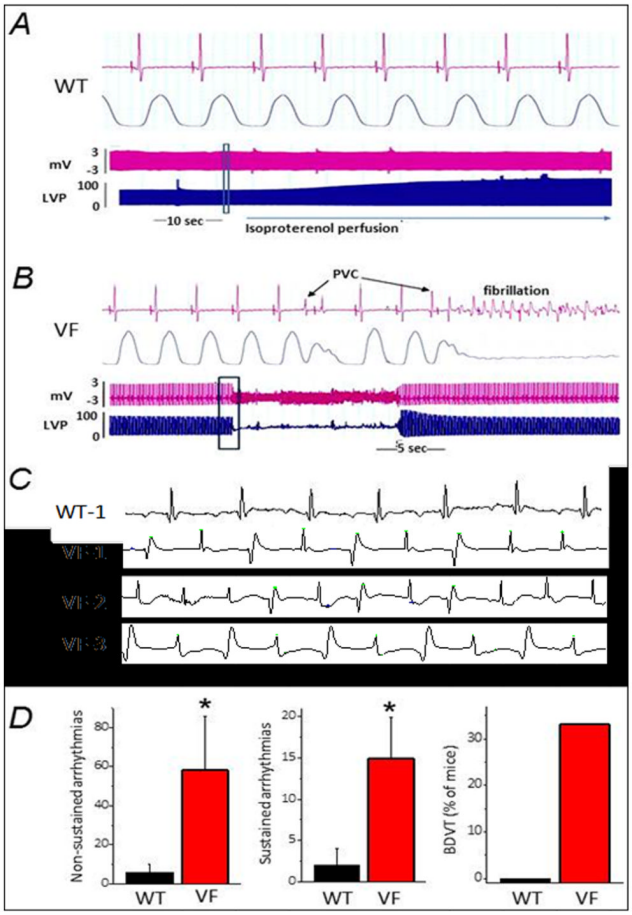

Figure 7. Arrhythmic behavior in Langendorff-perfused whole hearts and in intact V2475F$^{-/+}$ heterozygous mice

A and *B*, Electrogram (red trace) and left ventricular pressure (blue trace) from WT and V2475F hearts. Freshly-explanted hearts were Langendorff-perfused with Tyrode solution without and then with 300 nM Isoproterenol, as indicated in the bottom panel of *A*. Top two traces were taken at the time indicated by the rectangular box in the bottom two traces. *C*, Surface electrocardiograms (1s duration) in anesthetized WT and V2475F mice after intraperitoneal injection of Epinephrine (2 mg/kg) and caffeine (120 mg/kg). *D*, Average data obtained in n=6 WT and n=6 V2475F mice over a constantly monitored period of 30 min. Sustained arrhythmias are arrhythmias lasting more than 5s. BDVT = bidirectional ventricular tachycardia.

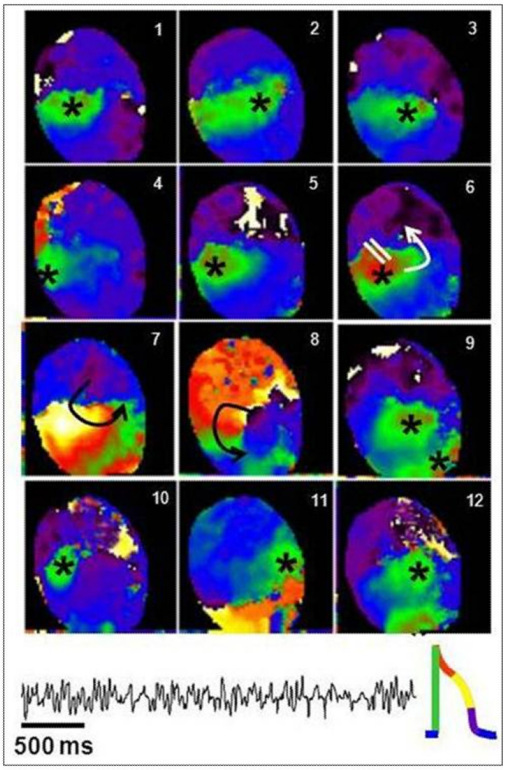

Figure 8. Focal origin of polymorphic ventricular tachycardia

Snapshots from a phase movie showing 12 consecutive beats in which breakthrough patterns emerged at different locations (asterisk), demonstrating the multifocal origin of polymorphic VT in a heterozygous V2475F heart. The volume-conducted ECG of the arrhythmia episode is shown below the snapshots. The colors denote the phases of the action potential, with green as upstroke (action potential insert at the bottom of the figure). The asterisks show the breakthrough sites, where the waves of depolarization (green) originated. During beat 6 a breakthrough occurs, however, unlike the other beats, unidirectional propagation block develops (white parallel lines), leading to the generation of a short-lived reentry (panels 7 and 8), after which, a pattern of focal tachycardia resumed.