Supporting Information

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SI Materials and Methods

Generation of RyR2-A4860G Gene-Targeted Mice. The RyR2-A4860G knock-in targeting vector was constructed by using a combination of traditional cloning techniques and "recombineering," a highly efficient phage-based *Escherichia coli* homologous recombination system (1, 2). The targeting vector was electroporated into murine SV/129 AB2.2 ES cells (3). ES cells that integrated the targeting vector by homologous recombination or random integration were selected by growth on G418. The presence of ganciclovir (GANC) selected against clones that contain the HSV-TK cassette, thus enriching for clones that integrated the Neo cassette by homologous recombination. A total of 288 Neo^r, $GANC^r$ colonies were selected, replicated, and expanded. DNA was isolated from the 288 replica ES clones, digested with *BamH1* restriction endonuclease, electrophoresed on agarose gels, transferred to charged nylon membranes, and hybridized to radiolabeled 5′ probe. Twelve correctly targeted clones were identified by the appearance of a 6.9-kb band in addition to the 10.1-kb native band DNA from the 12 clones that appeared to be correctly targeted 5′p2 probe. The 12 clones that appeared to be correctly targeted on the 5′ side were digested with *BsteI*, electrophoresed on agarose gels, transferred to charged nylon membranes, and hybridized to radiolabeled 3′ probe. Six positive clones (1F, 1G1, 2G2, 2E11, 3B7, and 3D9) were thawed from the master plate and fully expanded. The remaining six clones (1A2,1E2, 2F5, 3B4, 3D8, and 3E12) were partially expanded and cryopreserved. The expanded clones were genotyped by using the 5′ and 3′ probes. Correctly targeted clones were identified by the presence of the 10.1-kb native band and a 6.6-kb altered band when hybridized to the 5′ probe and a 13.2-kb native band and a 6.8-kb altered band when hybridized with the 3′ probe. DNA sequence analysis was used to identify the correctly targeted clones and to confirm the presence of the floxed Neo cassette and the A-to-G substitution at position 4860. Chromosomes of three correctly targeted clones were counted. Two euploid clones, A4860-KI 3B7 and A4860-1F1, were microinjected into C57BL/6 blastocyst to produce chimeric founders, and the chimeric males were cross-bred with C57BL/6 females. Agouti pups carrying the RyR2-G4860 chromosome were identified by PCR. The floxed Neo cassette was excised by mating RyR2-$A4860G^{+/-}$ mice with EIIa-Cre transgenic mice.

Experimental Animals. RyR2-$A4860G^{+/-}$ mice and age-matched WT littermates were maintained and studied according to the protocol approved by the institutional animal care and use committees of the University of Michigan and by the Association for Assessment and Accreditation of Laboratory Care International.

Echocardiography. Transthoracic echocardiography was performed by using a Visual Sonics 770 ultrasonograph with a 30-MHz transducer (RMV 707B; Visual Sonics) as described previously (4). Mice were lightly anesthetized with isoflurane (1%) and maintained on a heated platform. Two-dimensionally guided M-mode images of the left ventricle and Doppler studies were acquired at the tip of the papillary muscles. Left ventricular mass-to-body weight ratio (LV/BW), left ventricle dimension in diastole (LVDd), thickness of the posterior wall in systole and diastole, and isovolumic contraction and relaxation time were recorded. Endocardial FS was calculated as (LVDd − LVDs)/LVDd × 100, where LVDs is left ventricle dimension in systole. All parameters were measured over at least three consecutive cycles.

Preparation of SR-Enriched Mouse Microsomes. SR-enriched mouse microsomes were isolated from the ventricles of hearts as previously described (5). Briefly, the ventricular tissue was homogenized and centrifuged at 8,000 × *g* for 20 min at 4 °C. The 8,000 × *g* supernatant was centrifuged at 100,000 × *g* in a Ti-70.1 rotor for 35 min. The pellets containing the SR-enriched microsomes were suspended in saline buffer plus 0.3 mol/L sucrose, and protein concentration was determined.

Cell Culture and Transfection. HEK293 cells were grown in DMEM with 10% (vol/vol) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin on 100-mm tissue culture dishes. Cells were transfected with RyR2 cDNA by using Xtreme-DNA (Roche Laboratories) according to the instructions of the manufacturer, and cell lysates were prepared from transfected cells as previously described (6). Briefly, HEK293 cells grown for 24 h after transfection were washed with PBS solution plus 2.5 mM EDTA and harvested in the same solution by centrifugation for 10 min at 700 × *g*. The cells were then solubilized in 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 protease inhibitors consisting of 1 mM benzamidine, 2 μg/mL leupeptin, 2 μg/mL pepstatin A, 2 μg/mL aprotinin, and 0.5 mM PMSF. The cells were lysed on ice for 1 h and lysates were obtained by centrifuging at 16,000 × *g* in a microcentrifuge at 4 °C for 10 min to remove insoluble material.

[^{3}H]Ryanodine Binding. [^{3}H]Ryanodine binding to cell lysates was carried out as previously described with modifications (5, 7). Briefly, a binding mixture of 300 μL contained 30 μL of cell lysate plus a standard mixture of 200 mM KCl, 25 mM Tris/50 mM Hepes (pH 7.4), 3 mM MgATP, 1 mM EGTA, 5 nM [^{3}H]ryanodine (68.4 Ci·mmol^{-1}; Dupont NEN), and $CaCl_2$ to set free [Ca^{2+}] from *p*Ca 8 to *p*Ca 3. Ca^{2+}/EGTA ratio was calculated by using MaxChelator (www.stanford.edu/~cpatton/maxc.html). For experiments in which there was maximal activation of RyR2s, binding medium contained 1 M KCl, 100 μM $CaCl_2$, and 20 mM Na·Hepes (pH 7.4). In both cases, the reaction was incubated for 2.5 h at 36 °C, filtered on GF/B glass filters (Whatman) presoaked with 1% polyethylenimine, and washed twice with 5 mL of distilled water by using an M24-R cell harvester (Brandel). Nonspecific binding was determined in the presence of 20 μM unlabeled ryanodine and has been subtracted from each sample.

Western Blots of RyR2. A total of 50 μg of HEK293 cell lysate or 50 μg of SR-enriched microsomes from WT and RyR2-$A4860g^{+/-}$ hearts was suspended in Laemmli buffer and separated by SDS/PAGE in 7.5% precast minigels (BioRad). The SDS/PAGE-resolved proteins were transferred to PVDF membranes at 30 V for 16–18 h at 4 °C. The membranes were subsequently blocked for 1 h at room temperature in PBS-T (in mmol/L, 3 KH_2PO_4, 10 Na_2HPO_4, 150 NaCl, pH 7.2–7.4, 0.1% Tween 20) plus 5% dried skim milk. Membranes were incubated for 2 h in PBS-T with an anti-RyR (34C) antibody (1:2,000; Thermo Scientific). Membranes were then washed three times for 10 min in PBS-T. After washing, membranes were incubated with a goat anti-mouse secondary antibody (IgG) conjugated to HRP in PBS-T for 40 min (1:5,000; Calbiochem). After washing three times for 10 min, protein–antibody reactions were detected by SuperSignal chemiluminescence (Pierce Biotechnology) and imaged by a ChemiDoz

MP imaging system (Bio-Lab). Densities of protein were measured by using Image Lab software (Bio-Lab).

Single RyR2 Channel Recordings. Recombinant WT and RyR2-A4860G proteins were partially purified from cell lysates by sucrose gradient centrifugation and incorporated into planar lipid bilayers as previously described (6, 8, 9). The *trans* (700 μL) and the *cis* (700 μL) chambers (corresponding to the luminal and cytoplasmic sides of the channel, respectively) contained a symmetrical solution of 250 mL KCl and 25 mM Hepes (pH 7.4). A phospholipid bilayer of phosphatidylethanolamine:phosphatidylserine (1:1 dissolved in N-decane to 20 mg/mL) was "painted" with a glass rod across an aperture of ~200 μm diameter in a Delrin cup. The *trans* chamber was the voltage control side connected to the head stage of a 200-A Axopatch amplifier, whereas the *cis* side was held at virtual ground. Recordings were performed in symmetrical 5 μM Ca^{2+} followed by subsequent recordings after raising luminal Ca^{2+} to 1 mM. Channel activity was filtered with an eight-pole low-pass Bessel filter set at 1.5 kHz and digitized at a rate of 4 kHz by using a Digidata 1200 AD/DA interface. Data acquisition and analysis were performed with Axon Instruments hardware and software (pClamp 8).

Mouse Ventricular Cell Isolation. Mice 16–20-wk of age from WT and RyR2-A4860G$^{+/-}$ groups were anesthetized by i.p. injection of 1 g/kg urethane. When the animal reached deep anesthesia, the heart was immediately excised and rinsed in Tyrode solution containing (in mM): 137 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 20 Hepes, 1.2 NaH_2PO_4, 10 glucose, and 10 taurine, pH 7.4 with NaOH. The heart was then cannulated to the Langendorff apparatus by the aorta and perfused through the coronary artery retrogradely with oxygenated Tyrode solution at 37 °C at a rate of 3 mL/min. The heart was then perfused with the Tyrode solution plus 200 U/mL collagenase II and 12.5 μM $CaCl_2$ (digestion solution) for digestion for approximately 10 min. After turning soft, the ventricles were cut off from the Langendorff apparatus and cut into pieces in the digestion solution. Tissue pieces were agitated gently in the digestion solution at 37 °C for approximately 3 min twice into single cells. Supernatant of single cells in the digestion solution was centrifuged at 500 rpm for 1 min, and the pellet was resuspended in Tyrode solution plus 4 mg/mL BSA and 50 μM $CaCl_2$. Calcium was reintroduced to 1.8 mM step by step.

Patch Clamp. Whole-cell patch-clamp experiments were performed by using an Axopatch 200B amplifier and a Digidata 1322A digitizer (both from Axon Instruments) at room temperature. For AP recording, cells were bathed in a solution containing (in mM): 135 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 1.2 NaH_2PO_4, and 10 glucose, pH 7.4 with NaOH. The pipette internal solution contains (in mM): 120 K aspartate, 20 KCl, 1 $MgCl_2$, 4 Na_2ATP, 0.1 Na_2GTP, 10 Hepes, and 10 glucose, pH 7.2 with KOH. Cells were current-clamped, and the APs were triggered by DS8000 Digital Stimulator (World Precision Instruments) at 1 Hz 20 times. For Ca^{2+} current recording, cells were bathed in the bath solution plus 30 μM TTX and 4 mM 4-aminopyridine. The pipette internal solution contains (in mM): 110 CsCl, 6 $MgCl_2$, 5 Na_2ATP, 0.3 Na_2GTP, 10 Hepes, and 15 TEA·Cl, pH 7.2 with CsOH. Cells were voltage-clamped at −70 mV, depolarized from −60 mV to +70 mV at 10-mV increments for 300 ms, and repolarized to −70 mV between sweeps. For catecholaminergic challenge, we applied 300 nM ISO in the bath solution. For NCX inhibition, we applied 300 nM or 3 μM CB-DMB in the bath solution as indicated. For EGTA chelation of intracellular Ca^{2+}, 10 mM EGTA was added in the internal solution for AP recording. The liquid junction potential between bath solution and pipette solution was calculated with pClamp and corrected after experiments.

Confocal Ca^{2+} Imaging. Ca^{2+} transients triggered by APs or Ca^{2+} currents were recorded at the same time by an LSM510 Meta inverted confocal microscope (Carl Zeiss) with an argon laser of 488 nm excitation and a magnification of 40× with a 1.2 N.A. water-immersion objective. The Ca^{2+} indicator Fluo-4 pentapotassium salt (0.2 mM; Invitrogen) was added to the internal solution before the experiment. All images were acquired along the long axis of the cell, avoiding the nucleus area, by line-scan mode at the scanning rate of 3.84 ms per line. For Ca^{2+} imaging without patch clamp, cells were incubated in bath solution with 10 μM Fluo-4 AM as previously described (10). After the incubation, cells were washed with fresh bath solution. Image acquirement parameters are the same as described earlier. For SR Ca^{2+} load measurement, cells were field-stimulated at 1 Hz with 50 V and 2 ms stimulus. Bath solution with 20 mM caffeine was perfused immediately after 20 pulses pacing to the cell for SR Ca^{2+} load measurement.

Ventricular Optical Mapping Experiments. Mice were heparinized (0.5 U/g i.p.) and then euthanized by i.p. injection of 1 g/kg urethane. When the animal reached deep anesthesia, the heart was immediately excised and placed in ice-cold cardioplegic solution, then retrogradely perfused through the cannulated aorta with warm (36.8 ± 3 °C), oxygenated (100% O_2) Tyrode solution containing (in mM) 130 NaCl, 1.2 NaH_2PO_4, 1.0 $MgCl_2$, 4.0 KCl, 1.8 $CaCl_2$, 5.6 glucose, and 25.0 Hepes, pH 7.4. The preparation was allowed to equilibrate for 15 min. Volume-conducted ECGs (pseudo lead 2) were acquired and digitized at 1 KHz (MiniDigi 1A digitizer and AxoScope software; Axon Instruments). The preparation was then perfused with 7 μM Blebbistatin (Cayman Chemical), a mechanical uncoupler, to immobilize the heart. The hearts were then stained with 100 μM Di-4-ANEPPS (Life Technologies), a voltage-sensitive fluorescent dye. High-resolution optical mapping studies were performed by using an 80 × 80 pixel CCD camera (SciMeasure) coupled to a SMZ-1000 dissection microscope (Nikon), imaging a 6.4 × 6.4-mm area at 800 frames per second, providing a spatial resolution of 80 μm per pixel. The visualized area included the anterior ventricular epicardium (left ventricle, septum, right ventricle, and apex). Optical movies (4 s) were acquired under 532-nm fluorescence excitation (argon laser, 1 W) and 640 ± 20 nm emission. Optical movies were recorded at baseline and at progressively shorter basic cycle lengths (150 ms to 100 ms) during RV free wall pacing. Hearts were then stressed by perfusing Tyrode solution containing ISO (200 nM) and caffeine (1 mM) or high extracellular calcium (3.6 mM) for 20 min to induce arrhythmias. Analysis was performed by using custom-made software. Briefly, movies were spatiotemporally filtered, background fluorescence was subtracted, and averaged activation maps were constructed under steady-state baseline and paced conditions. Phase maps were generated for arrhythmia visualization as described elsewhere (11).

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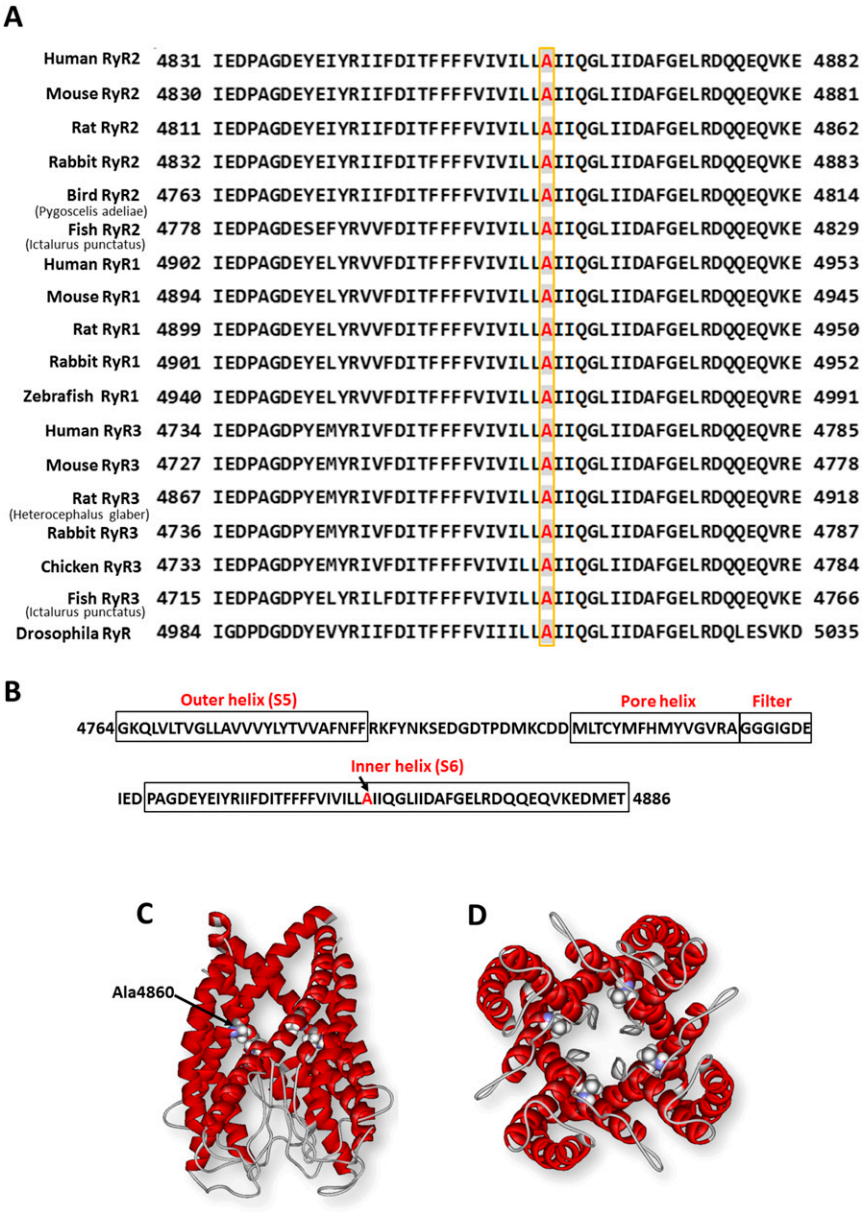

Fig. S1. RyR2 residue A4860 is highly conserved across species and RyR isoforms and forms part of a transmembrane domain supporting the channel's pore. (*A*) Amino acid sequence of the RyR segment that forms the inner helix of the channel's pore. Alanine 4860 (human sequence) is boxed and in red. (*B*) RyR2 amino acid residues that form part of the outer helix, pore helix, selectivity filter, and inner helix of the channel's pore. (*C* and *D*) Schematic ribbon diagram of the pore-forming region of RyR2 seen laterally (*C*) or from the SR lumen (*D*). Ala4860 is depicted as a CPK representation of the Ala residue (arrow, *C*), and there are four Ala4860 residues per channel.

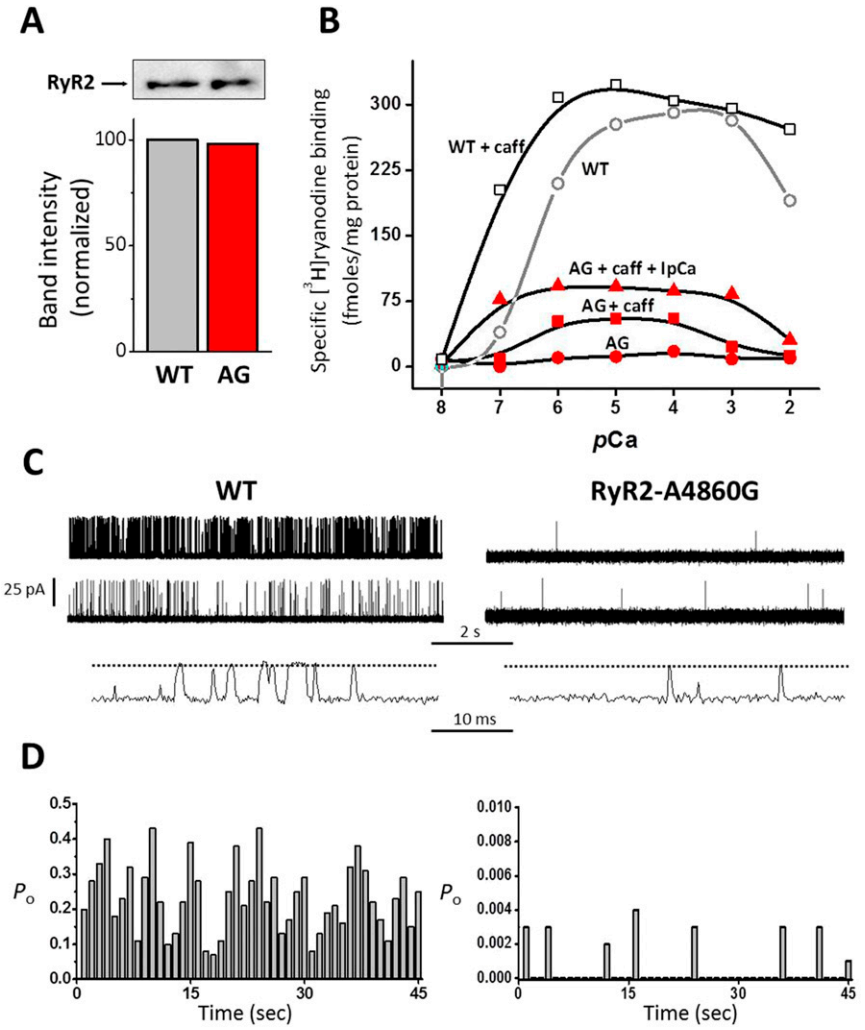

Fig. S2. Expression and functional characterization of the RyR2-A4860 mutation. (*A*) HEK293 cells expressed RyR2-A4860 (WT) and RyR2-G4860 equally effectively, as determined by Western blots of RyR2. (*B*) [^{3}H]Ryanodine binding to HEK293 cell lysates expressing WT and RyR2-G4860 (AG) channels at the specified free [Ca^{2+}] and in the presence of caffeine (caff, 10 mM) or caffeine plus imperacalcin (caff + IpCa, 10 mM and 1 μM, respectively). Imperacalcin (formerly imperatoxin) is a high-affinity (K_d ~5 nM) peptide agonist of RyRs that induces a long-lasting subconductance state, but, unlike ryanodine, this effect is fast and reversible (1). In [^{3}H]ryanodine binding assays, imperacalcin increases the binding of [^{3}H]ryanodine by increasing channel activity without interacting with the ryanodine binding site (2). As the effect of imperacalcin is synergized by caffeine (3), we used these two agonists in tandem in an attempt to potentiate their activating effect. Data points are the mean of four experiments, which yielded <10% error. (*C*) Single-channel activity of recombinant RyR2 channels reconstituted in lipid bilayers and corresponding diary of activity (*D*). Traces and diary are representative of the activity of 12 WT and 4 RyR2-G4860 channels recorded under 300-mM cesium methanesulfonate (symmetrical) and nominally free Ca^{2+} (~5 μM free [Ca^{2+}] determined with a Ca^{2+} electrode).

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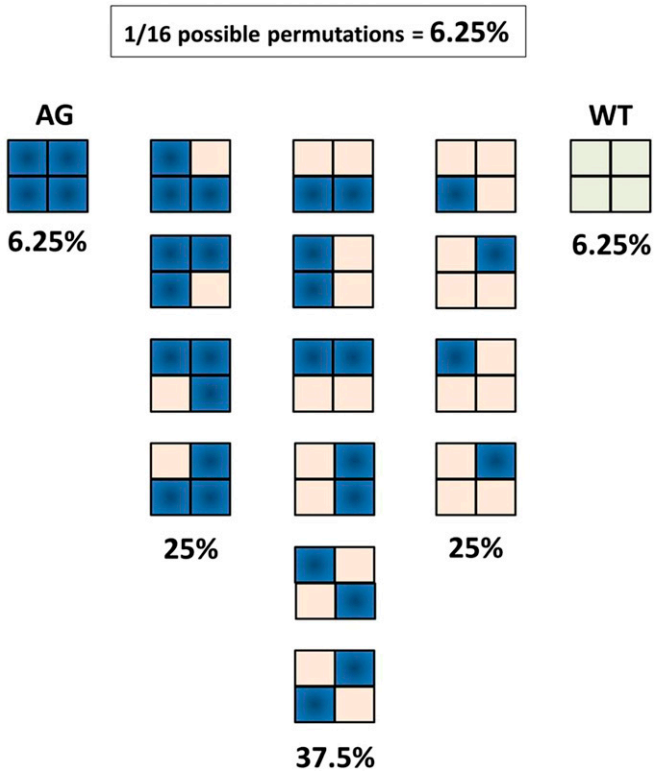


Fig. S3. Probabilities of harboring a given number of mutant (AG) or WT subunits in a tetrameric channel. Permutations are based on the assumption that both alleles contribute subunits that assemble equally and randomly to form the tetrameric channel.

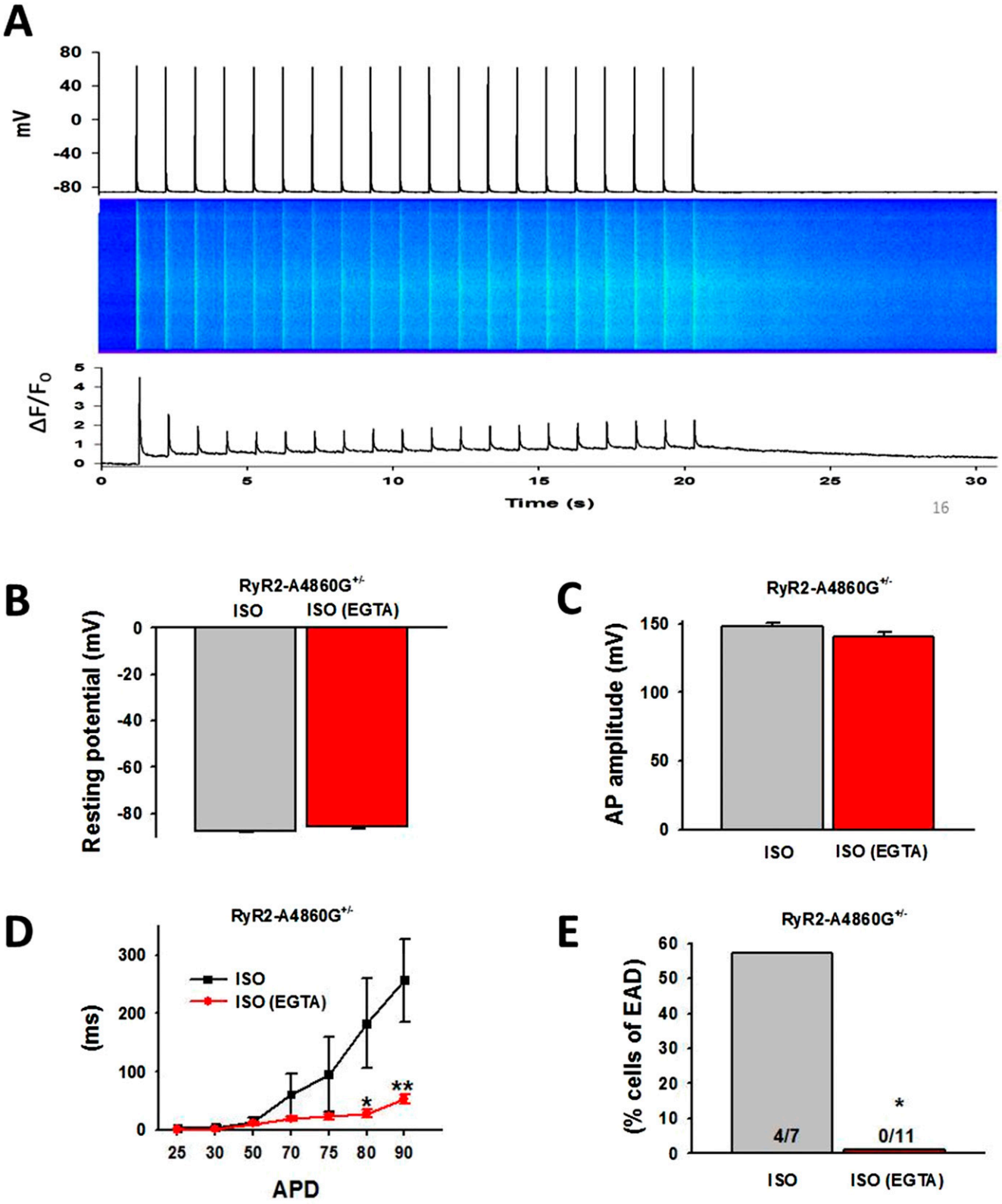


Fig. S4. Shortening of the AP and abolition of EADs by intracellular EGTA. (*A*) Representative APs (*Upper*), Ca^{2+} imaging (*Middle*), and fluorescence intensity profile (*Lower*) of current-clamped RyR2-A4860G$^{+/-}$ ventricular myocytes stimulated at 1 Hz with 300 nM ISO shortly after breaking into the cell as in Fig. 3 but including in the pipette the slow Ca^{2+} chelator EGTA (10 mM). Resting potential (*B*) and AP amplitude (*C*) were not modified by EGTA, but APD (*D*) was significantly shortened (*$P < 0.05$; **$P \leq 0.01$). (*E*) Incidence of EADs in RyR2-A4860G$^{+/-}$ cells containing EGTA was 0% (0 of 11 cells), compared with 55% (4 of 7 cells) without EGTA in identical protocols. Data are from five mice.

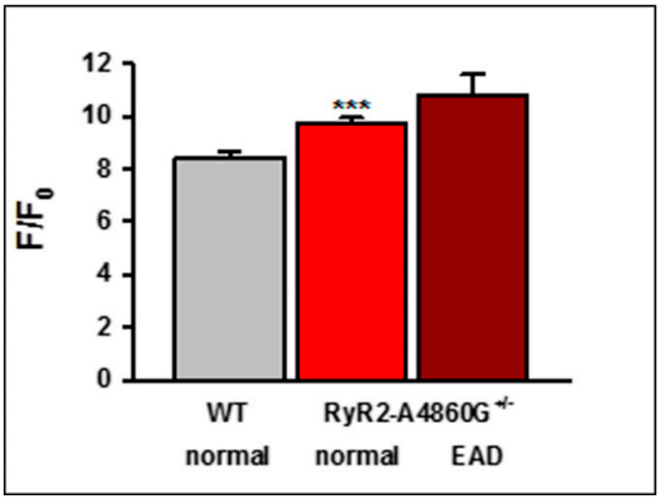


Fig. S5. SR Ca^{2+} load was higher in RyR2-A4860G$^{+/-}$ cells at basal condition (n = 39, n = 56, and n = 7 from n = 5 WT and n = 7 RyR2-A4860G$^{+/-}$ mice, respectively). ***$P \leq 0.001$ vs. WT.

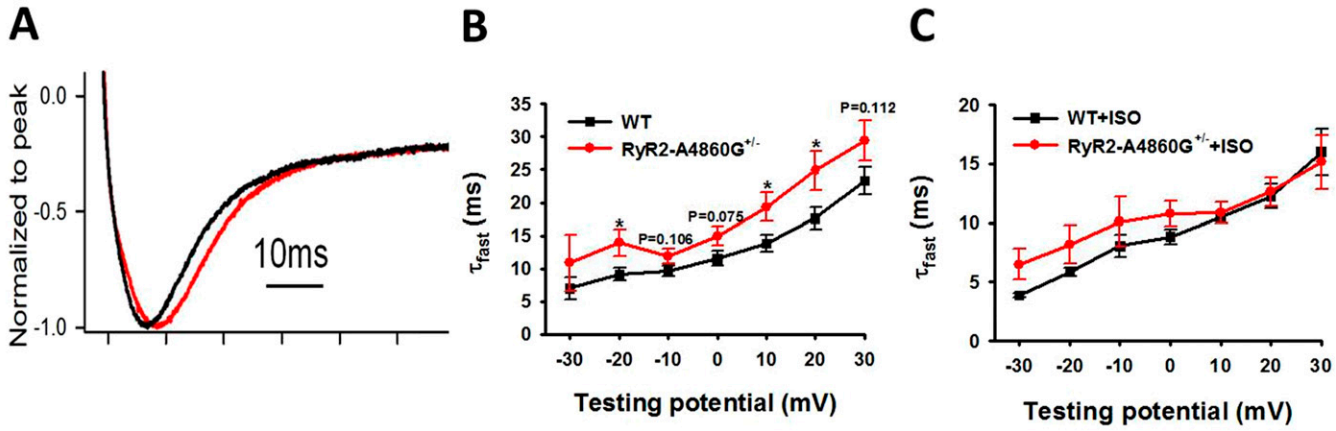
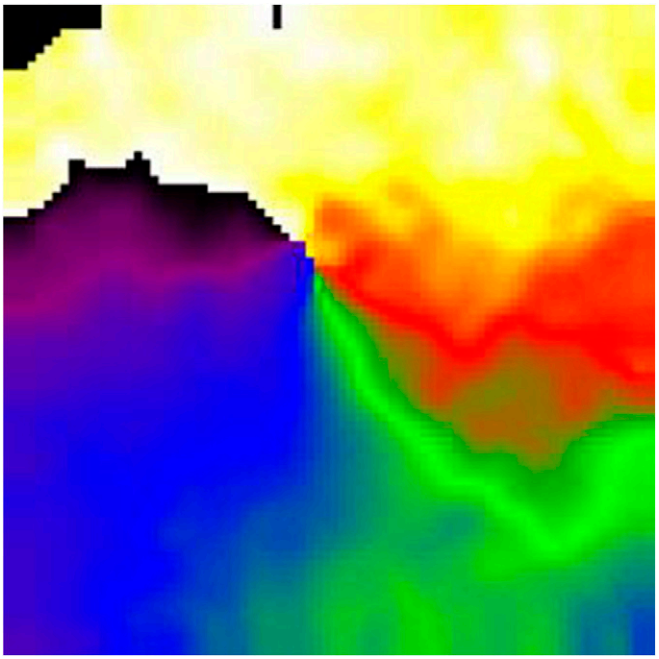


Fig. S6. Inactivation of L-type Ca channel current (I_{CaL}) in WT and RyR2-A4860G$^{+/-}$ ventricular myocytes. (*A*) Representative I_{CaL} from WT (black) and RyR2-A4860G$^{+/-}$ (red) cells after a voltage step from –70 to 0 mV. The kinetics of I_{CaL} inactivation were fitted with an exponential formula containing two time constants, τ_{fast} and τ_{slow}, in cells not treated (*B*) or treated (*C*) with 300 nM ISO (*$P < 0.05$ between groups of cells; n = 10 WT and n = 10 RyR2-A4860G$^{+/-}$ cells from n = 8 mice in each group).

Table S1. Echocardiographic parameters of WT and RyR2-A4860G$^{+/-}$ mice

Parameter	WT	RyR2-A4860G$^{+/-}$
Age, mo	4.0 ± 0.0	4.0 ± 0.0
BW, g	23.8 ± 1.9	24.5 ± 0.7
LV mass, mg	110.08 ± 7.89	120.84 ± 10.11
LV/BW	4.70 ± 0.26	4.91 ± 0.33
HR, bpm	488.6 ± 14.5*	442.5 ± 14.8*
IVCT, ms	17.16 ± 0.96	17.76 ± 1.92
IVRT, ms	16.00 ± 0.99	16.80 ± 1.21
IVS_d, mm	0.81 ± 0.04	0.84 ± 0.02
LVD_d, mm	3.90 ± 0.10	3.86 ± 0.15
LVD_s, mm	2.70 ± 0.19	2.73 ± 0.11
PW_d, mm	0.76 ± 0.04	0.84 ± 0.04
PW_s, mm	1.12 ± 0.04	1.24 ± 0.06
EF%	54.09 ± 2.98	55.88 ± 3.03
FS%	26.53 ± 1.66	28.96 ± 2.21
LV Vol_d, μL	66.58 ± 4.06	65.67 ± 6.46
LV Vol_s, μL	30.78 ± 3.12	28.40 ± 2.59

Structural and functional echocardiographic parameters for WT (n = 8) and RyR2-4860G$^{+/-}$ (n = 8) mice. bpm, beats per minute; BW, body weight; EF, ejection fraction; HR, heart rate; IVCT, isovolumic contraction time; IVRT, isovolumic relaxation time; IVS_d, interventricular septal end diastolic dimension; LV, left ventricular; LV Vol_d, left ventricular volume in diastole; LV Vol_s, left ventricular volume in systole; LVD_s, left ventricular end-systolic dimension; PW_d, posterior wall thickness in diastole; PW_s, posterior wall thickness in systole.
*Significant difference between groups at $P \leq 0.05$.



Movie S1. Ventricular epicardial optical mapping reveals complex patterns of AP wave propagation during spontaneous VF in RyR2-A4860G$^{+/-}$ heart under adrenergic stress and high extracellular Ca^{2+}. Phase map movie shows 4 s of real-time activity (3,200 total frames acquired at 800 frames per second with a 30-ms delay set between each frame) during VF.

Movie S1