Supplementary Information

Carvedilol and Its New Analogs Suppress Arrhythmogenic Store Overload-Induced Ca^{2+} Release

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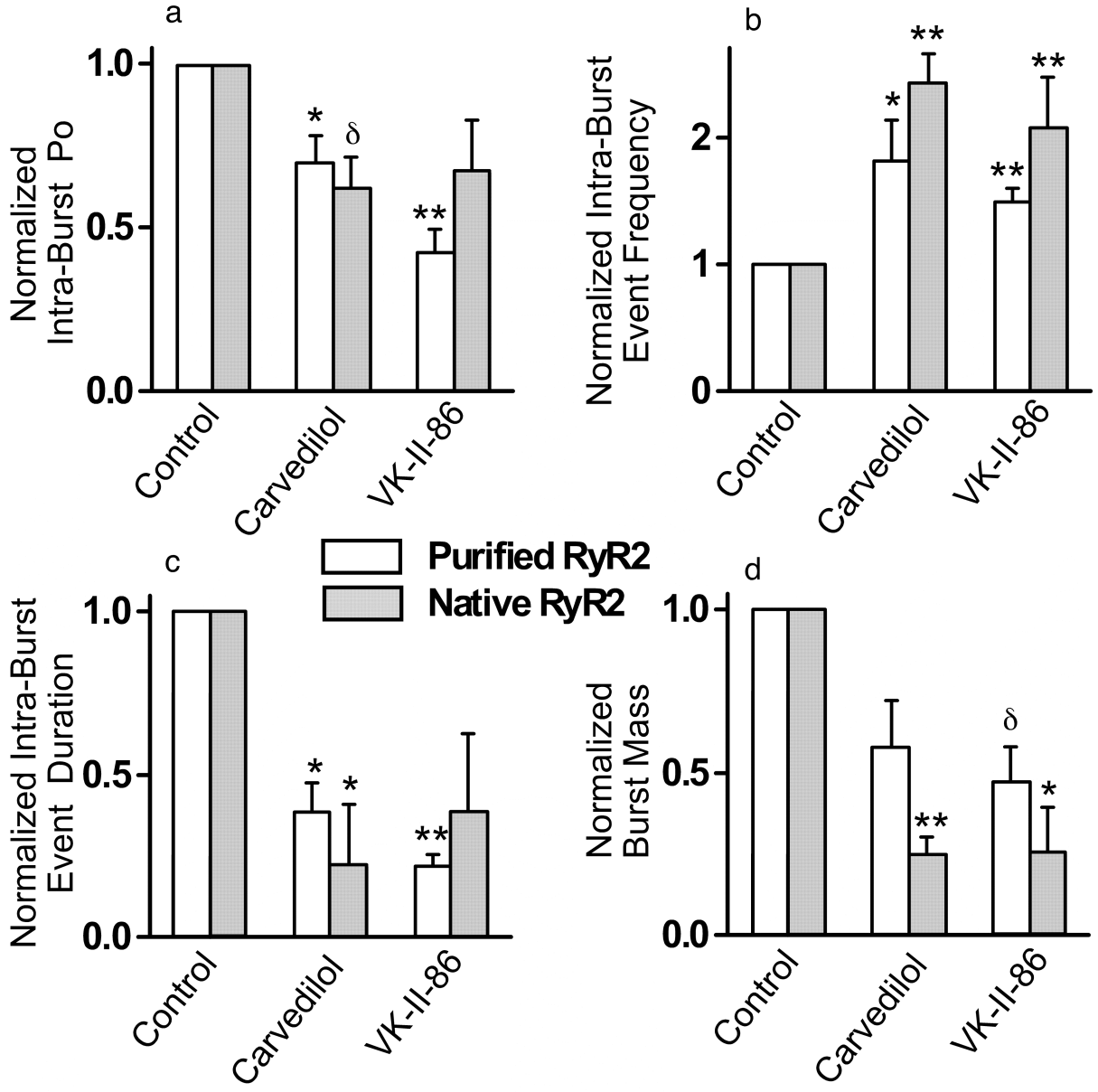
Supplementary Item & Number	Title or Caption
Supplementary Figure (Fig. S1)	Carvedilol and VK-II-86 alters the bursting activity of single purified and native RyR2 channels.
Supplementary Figure 2 (Fig. S2)	Generation and characterization of R4496C$^{+/-}$ knock-in mice.
Supplementary Figure 3 (Fig. S3)	Synthesis of carvedilol analogues, VK-II-86, CS-I-34, and CS-I-59.
Supplementary Figure 4 (Fig. S4)	Effect of VK-II-86 on SOICR and PKA-dependent phosphorylation of RyR2 in ventricular myocytes.
Supplementary Figure 5 (Fig. S5)	Beta-adrenergic receptor binding of carvedilol and carvedilol analogues, VK-II-86, CS-I-34, and CS-I-59.
Supplementary Figure 6 (Fig. S6)	Effect of VK-II-86 on single RyR2 channels.
Supplementary Figure 7 (Fig. S7)	Effect of carvedilol and VK-II-86 on CPVT in RyR2 R4496C$^{+/-}$ mutant mice.
Supplementary Figure 8 (Fig. S8)	Effect of VK-II-86, metoprolol, bisoprolol, and their combination on CPVT in RyR2 R4496C$^{+/-}$ mutant mice.
Supplementary Figure 9 (Fig. S9)	Effect of propranolol on SOICR and CPVT.
Supplementary Figure 10 (Fig. S10)	Effect of flecainide on SOICR and CPVT.
Supplementary Table 1	Beta-adrenergic Receptor Binding of Carvedilol and Carvedilol Analogues.

Supplementary Methods	

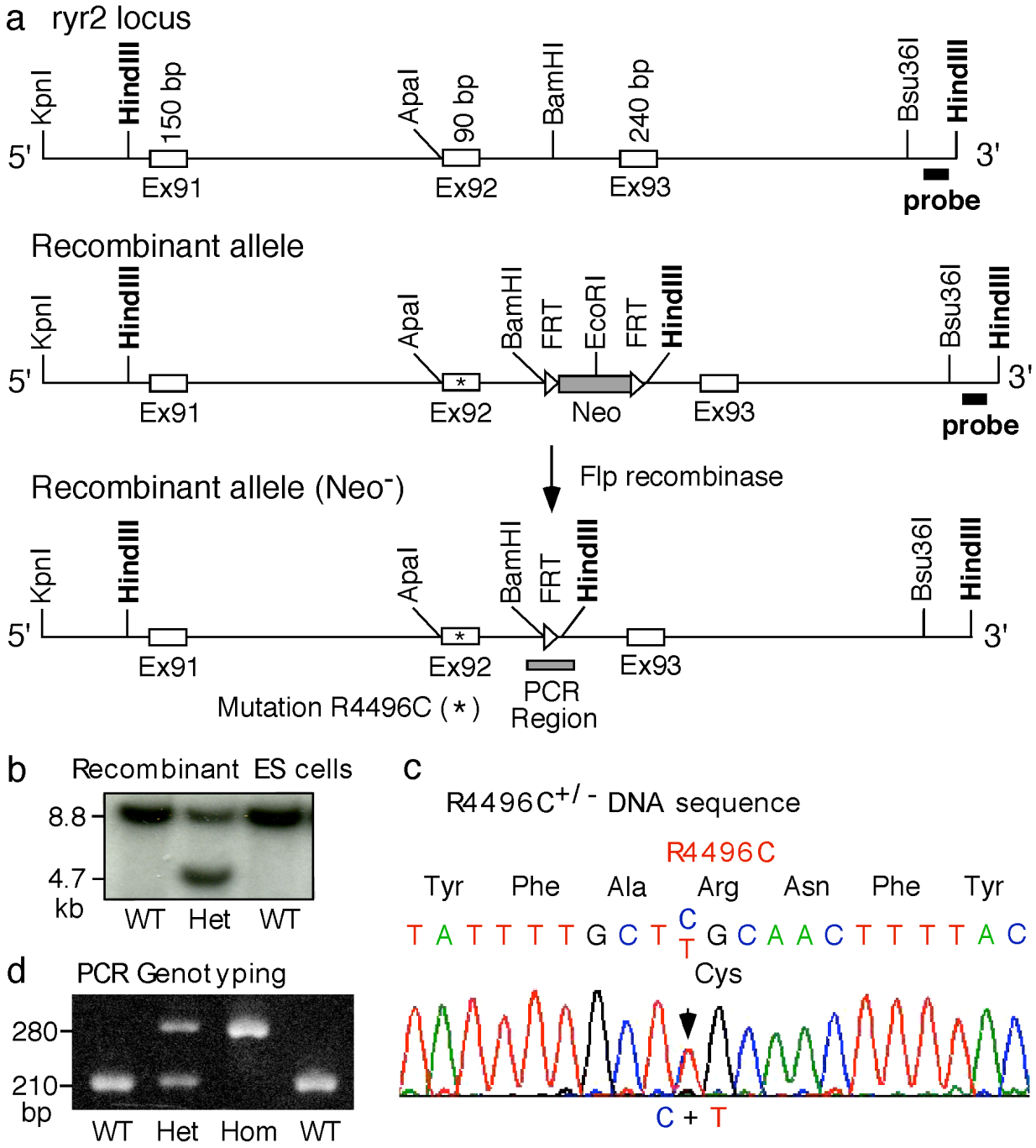
Supplementary Figure 1 (Fig. S1)



Supplementary Fig. S1: Carvedilol and VK-II-86 alters the bursting activity of single purified and native RyR2 channels Purified refers to recombinant CHAPS solubilized channels that were expressed in HEK293 cells and then incorporated into proteoliposomes before their insertion into the bilayer. Native refers to channels that were isolated from cardiac muscle and present in crude SR vesicles before their insertion into the bilayer. Solutions used in both types of channel studies are detailed in the Methods. The drugs (1 μM) were applied chronically as previously described. Bursts of single RyR2 openings are well known [1] and were evident in control conditions here. Bursts of 5 or more RyR2 openings

were quantified using the Poisson Surprise method with set inclusion and exclusion times (20 and 200 ms, respectively). Only bursts having a Poisson Surprise >10 were evaluated. Absolute (not normalized) control and post-drug results were statistically compared. Significant (* $p<0.05$; ** $p<0.01$) and nearly significant differences (δ indicating $p\leq0.07$) are marked. To facilitate graphic comparison, burst results were normalized to control values. Intra-burst Po (a) and intra-burst event frequency (b) of purified (open bars) and native (grey bars) are shown. Both drugs reduced intra-burst Po with the change attaining statistical significance in purified RyR2 data set. Both drugs also significantly increased intra-burst event frequency in all the data sets. The control purified and native RyR2 intra-burst Po were 0.69±0.05 and 0.71±0.13 ms, respectively. The control purified and native intra-burst event frequency were 73.98±7.81 and 52.05±13.88 s^{-1}, respectively. Intra-burst event duration (c) and burst mass (d) of purified and native RyR2s are shown. Both drugs significantly reduced intra-burst event duration in 3 of the 4 data sets. Both drugs also reduced burst mass with the change attaining statistical significance in the native RyR2 data set. Burst mass is the product of intra-burst Po and burst length. The control purified and native RyR2 intra-burst event durations were 9.01±1.69 and 21.25±10.05 ms, respectively. The control purified and native burst masses were 282±67 and 1035±166 ms, respectively.

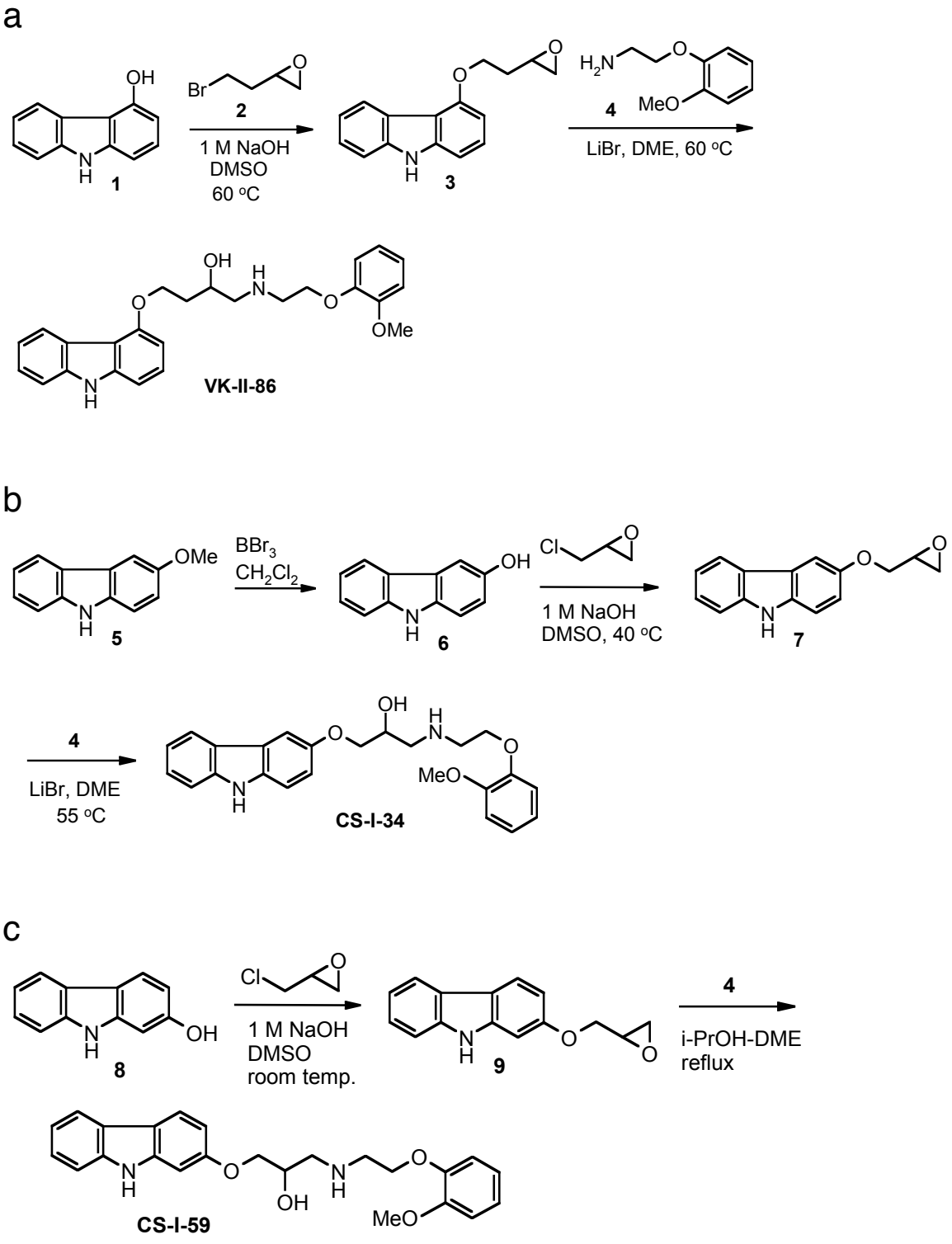
Supplementary Figure 2 (Fig. S2)



Supplementary Fig. S2: Generation and characterization of R4496C$^{+/-}$ knock-in mice (a) The mouse ryr2 locus encompassing Exons 91-93, the recombinant allele with the R4496C mutation in Exon 92 and the FRT-Neo-FRT cassette inserted after Exon 92, and the recombinant allele after removal of the Neo-gene by Flp recombinase are shown. (b) Southern blotting analysis of *Hind*III-digested genomic DNAs

isolated from recombinant ES cells using a DNA fragment near but outside the 3'end (*Bsu36*I) of the KI construct as a probe. (c) DNA sequencing analysis confirms the presence of the R4496C mutation in the R4496C$^{+/-}$ heterozygous mouse DNA. (d) PCR-based geneotyping of WT mice and heterozygous and homozygous R4496C mutant mice.

Supplementary Figure 3 (Fig. S3)

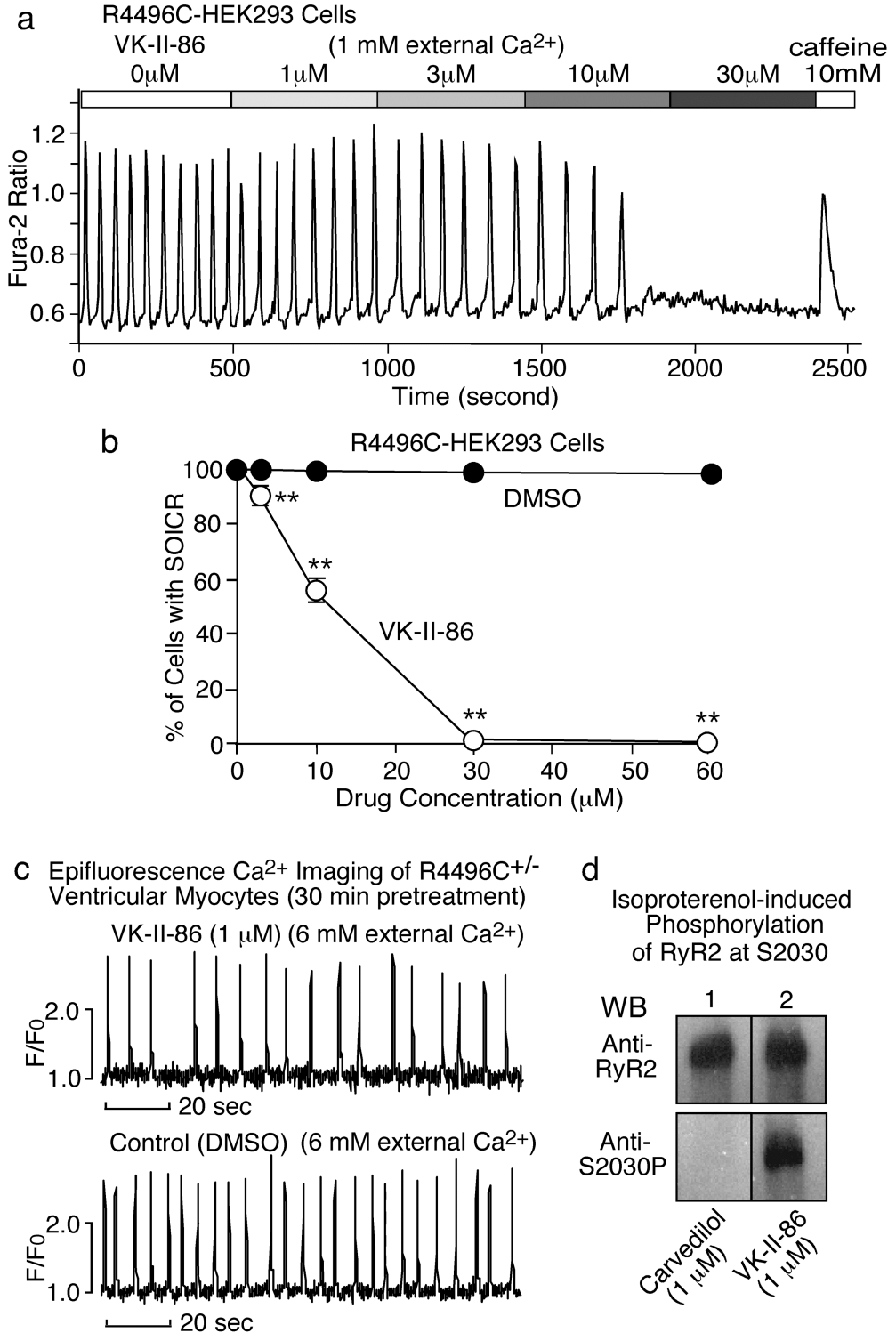


Supplementary Fig. S3: Synthesis of carvedilol analogues, VK-II-86, CS-I-34, and CS-I-59

(a) Alkylation of 4-hydroxycarbazole (**1**) with bromo epoxide **2**, in the presence of 1 M NaOH,

afforded intermediate epoxide **3**. LiBr-catalyzed epoxide ring-opening of **3** with amine **4** provided **VK-II-86**. (b) Demethylation of 3-methoxycarbazole (**5**) using BBr_3 gave 3-hydroxycarbazole (**6**). Alkylation of **6** with epichlorohydrin, in the presence of 1 M NaOH, afforded intermediate epoxide **7**. LiBr-catalyzed epoxide ring-opening of **7** with amine **4** provided **CS-I-34**. (c) Alkylation of 2-hydroxycarbazole (**8**) with epichlorohydrin, in the presence of 1 M NaOH, afforded intermediate epoxide **9**. Epoxide ring-opening of **9** with amine **4** in refluxing i-PrOH-DME provided **CS-I-59**.

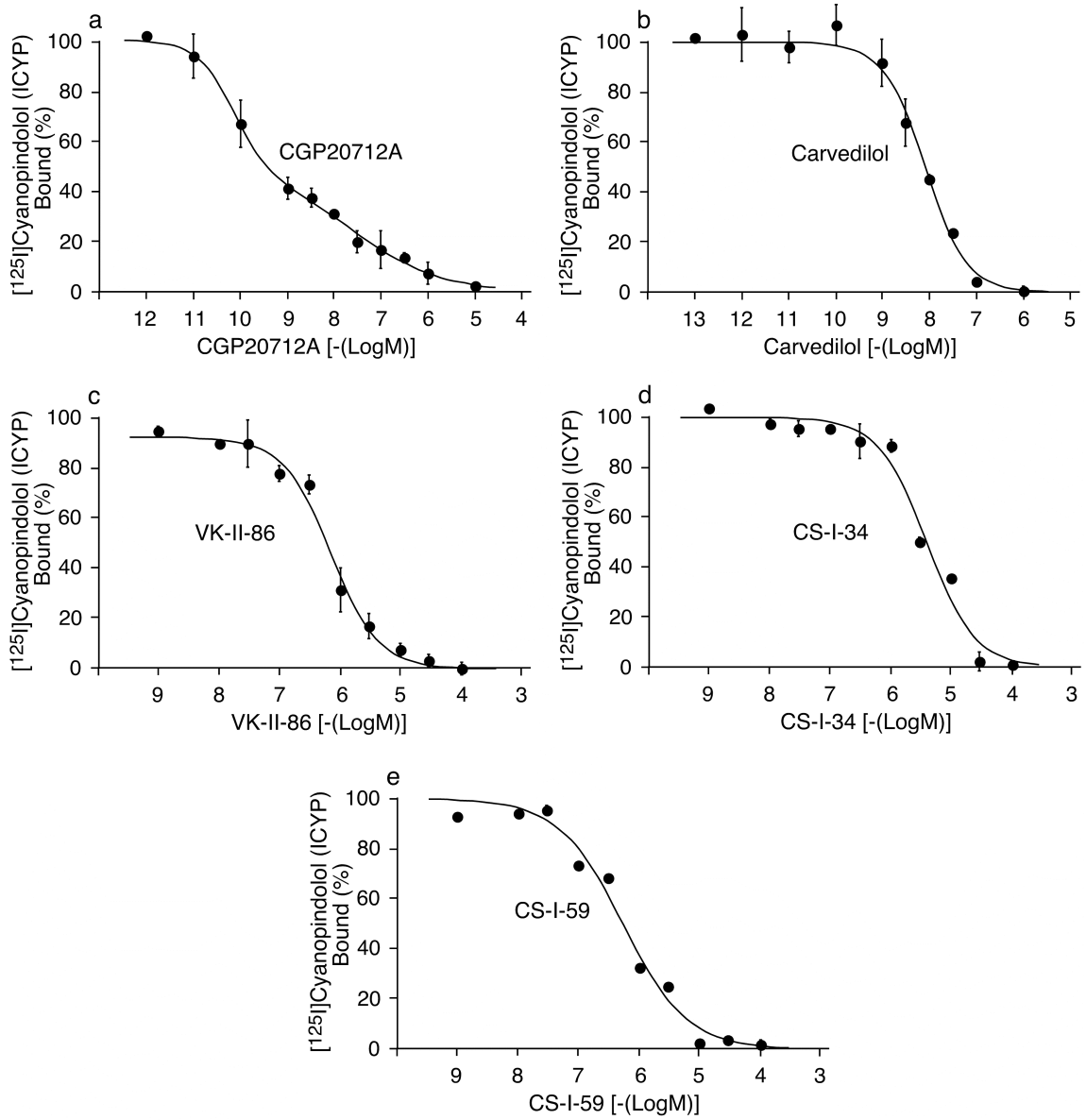
Supplementary Figure 4 (Fig. S4)



Supplementary Fig. S4: Action of VK-II-86 on SOICR and PKA-dependent phosphorylation of RyR2 in ventricular myocytes (a) Fura-2 ratios of representative RyR2-R4496C expressing HEK293 cells perfused with KRH buffer containing 1 mM external Ca^{2+} and increasing concentrations of VK-II-86,

followed by the addition of 10 mM caffeine. (b) The percentage of R4496C-HEK293 cells that display SOICR in the presence of various concentrations of VK-II-86 (white circles) or DMSO (black circles) (n=10) (**$P < 0.01$ vs DMSO). (c) Ventricular myocytes isolated from R4496C$^{+/-}$ mice and loaded with 5 μM fluo-4 AM were incubated with VK-II-86 (1 μM) or DMSO (control) for 30 min in KRH buffer containing 1 mM external Ca^{2+}. The external Ca^{2+} increased to 6 mM to induce SOICR in the continued presence of VK-II-86 or DMSO. Single cell epifluorescence imaging of SOICR in R4496C$^{+/-}$ ventricular myocytes was done after pre-incubation for 30 min with VK-II-86 or DMSO (control). Fluo-4 intensities of a small rectangular region of a representative cell treated with VK-II-86 or DMSO are shown. (d) Effect of carvedilol and VK-II-86 on isoproterenol-induced PKA phosphorylation of RyR2 at serine 2030. Rat cardiac myocytes were pre-treated with carvedilol (1μM) or VK-II-86 (1μM) for 5 min at 37 ^{o}C, followed by stimulation with 200 nM isoproterenol (Iso) in the continued presence of the drugs for 30 min at 37 ^{o}C. The beta-receptor blocking activity of carvedilol or VK-II-86 was then assessed by determining the level of phosphorylation of RyR2 using Western blotting of whole cell lysates. Top panels show the level of the RyR2 protein using the anti-RyR2 antibody and the bottom panels show the level of phosphorylation of RyR2 at the PKA-specific site (serine-2030) using a phospho-specific antibody[2, 3]. Since RyR2 is a target of beta--adrenergic receptor/PKA signalling, the level of PKA-dependent phosphorylation of RyR2 reflects the extent of Iso-induced beta-receptor activation. Note that carvedilol, but not VK-II-86, completely abolished the Iso-induced phosphorylation of RyR2 at serine-2030. Similar results were obtained from three separate experiments. The Western blot images with anti-RyR2 or anti-S2030P antibodies were from different areas of the same autoradiograph.

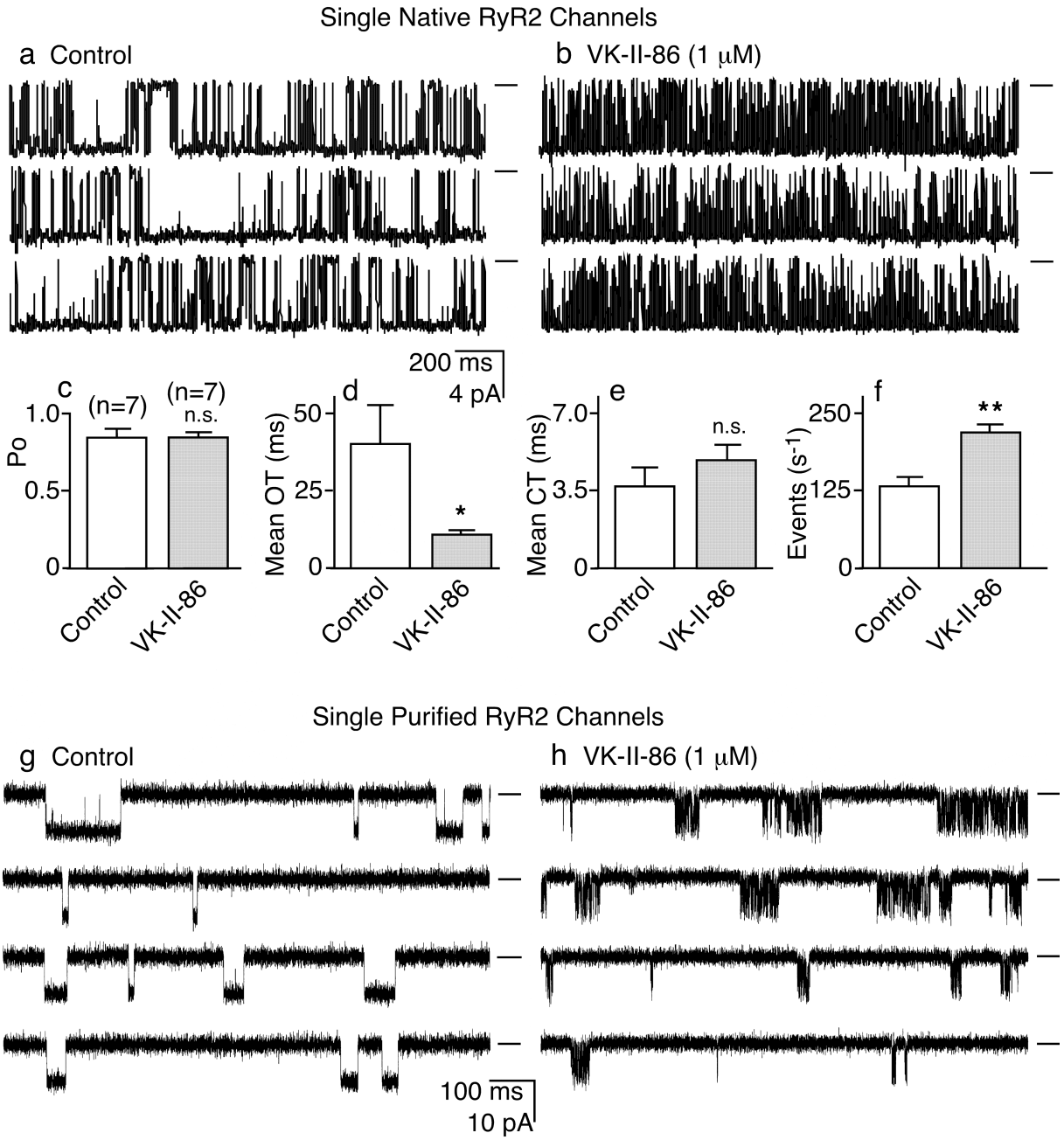
Supplementary Figure 5 (Fig. S5)



Supplementary Fig. S5: Beta-adrenergic receptor binding of carvedilol and carvedilol analogues, VK-II-86, CS-I-34, and CS-I-59 Beta-adrenergic receptor (AR) radioligand binding studies were performed using mouse ventricular membrane fractions and the beta-AR antagonist [^{125}I]-cyanopindolol as described previously [4]. [^{125}I]-cyanopindolol (ICYP) saturation binding experiments revealed a beta-AR density in mouse cardiac ventricular membranes of 35.3±2.3 fmol/mg protein with a K_D value of 80.5±5.8 pM (mean ± SD, n=4) and a beta1-AR/Beta2 AR ratio of 74.5/25.5. Representative [^{125}I]-

cyanopindolol displacement curves for CGP20712A (a), carvedilol (b), VK-II-86 (c), CS-I-34 (d), and CS-I-59 (e) are shown. Note that CGP20712A displayed a biphasic-binding curve with high- and low-affinity binding sites. The [^{125}I]-cyanopindolol bound to the mouse cardiac ventricular membranes were determined in the presence of a wide range of concentrations of the tested drugs. We then determined the IC_{50} value for each drug: 0.7 nM (pKi=9.16) and 2.6 μM (pKi=5.59) for the high and low affinity binding sites of CGP20712A; 0.5 nM (pKi=9.28) for carvedilol; 1.6 μM (pKi=5.80) for VK-II-86; 3.2 μM (pKi=5.49) for CS-I-34, and 1.3 μM (pKi=5.89) for CS-I-59. Thus, the IC_{50} of the beta-blocking activity of carvedilol analogues (VK-II-86, CS-I-34, and CS-I-59) was more than 2,000-fold greater than that of carvedilol. This directly illustrates that we have diminished the beta-AR blocking action of these agents and it is consistent with their minimal impact on Iso-stimulated and resting heart rates, as compared that of carvedilol.

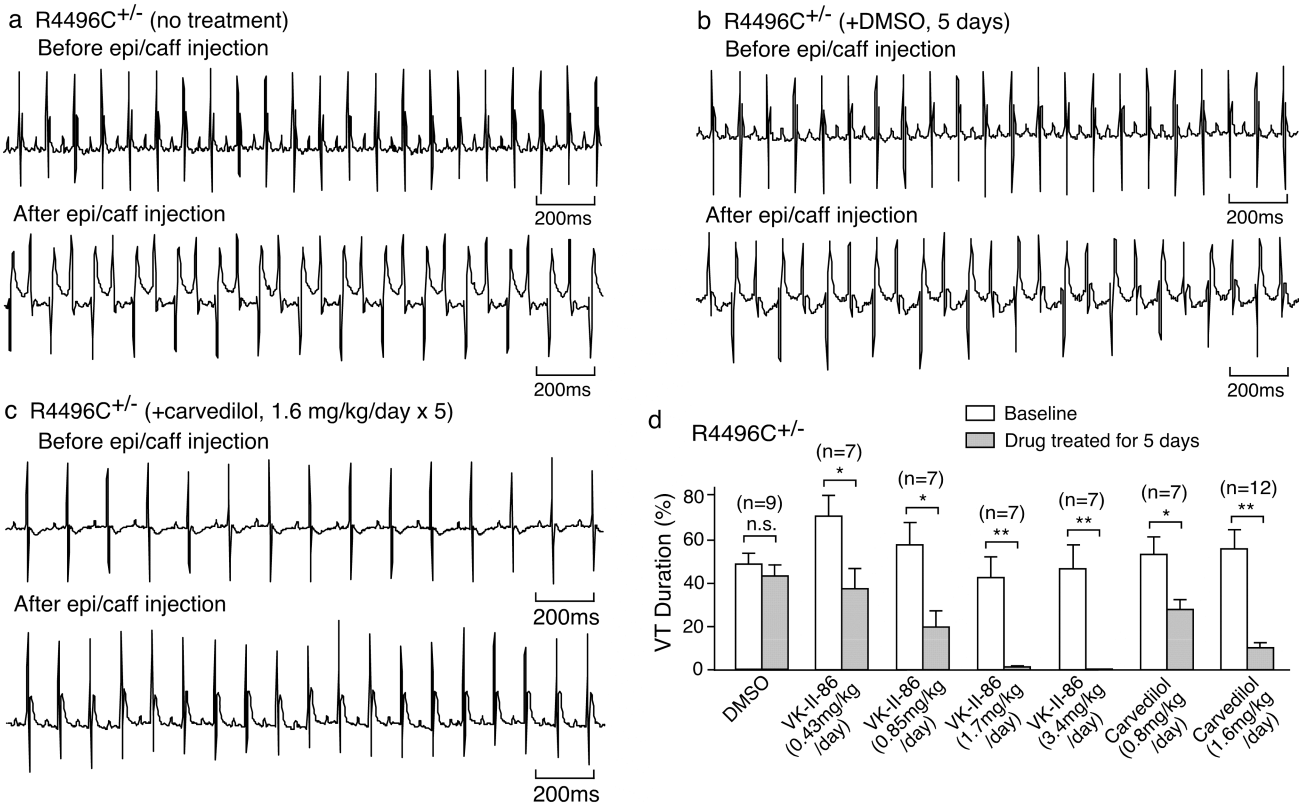
Supplementary Figure 6 (Fig. S6)



Supplementary Fig. S6: Action of VK-II-86 on single RyR2 channels Single native RyR2 channel activity was recorded in quasi-physiological salt solutions containing 120 mM HEPES-TRIS (pH 7.2), 50 μM free Ca^{2+} (0.5 mM EGTA), 1 mM free Mg^{2+} and 5 mM total ATP in the cytosolic solution, and 200 mM Cs-HEPES (pH 7.2), 1 mM free Mg^{2+} and 1 mM free Ca^{2+} in the luminal solution. SR microsomes

were pre-incubated for 1 hour without (a, control) or with (b) VK-II-86 (1 μM) before they were fused into the bilayer with the drug in both the cytosolic and luminal solutions. Single control (n=7) and VK-II-86-treated (n=7) channels were recorded at -40 mV. Openings are downward. Baselines are indicated (short bars). Open probability (Po) (c), mean open time (OT) (d), mean closed time (TC) (e), and event frequency (s^{-1}) (f) are shown in panels c-f (*$P < 0.05$; ** $P < 0.01$; vs control). Also shown are representative single channel current traces of recombinant RyR2-R4496C mutant channels purified from HEK293 cells pre-incubated without (g, control) or with VK-II-86 (1μM) (h) for 1 hour before being fused into the bilayers. Single control (n=8) and VK-II-86-treated (n=16) channels were recorded at -20 mV. Openings are downward. Baselines are indicated (short bars).

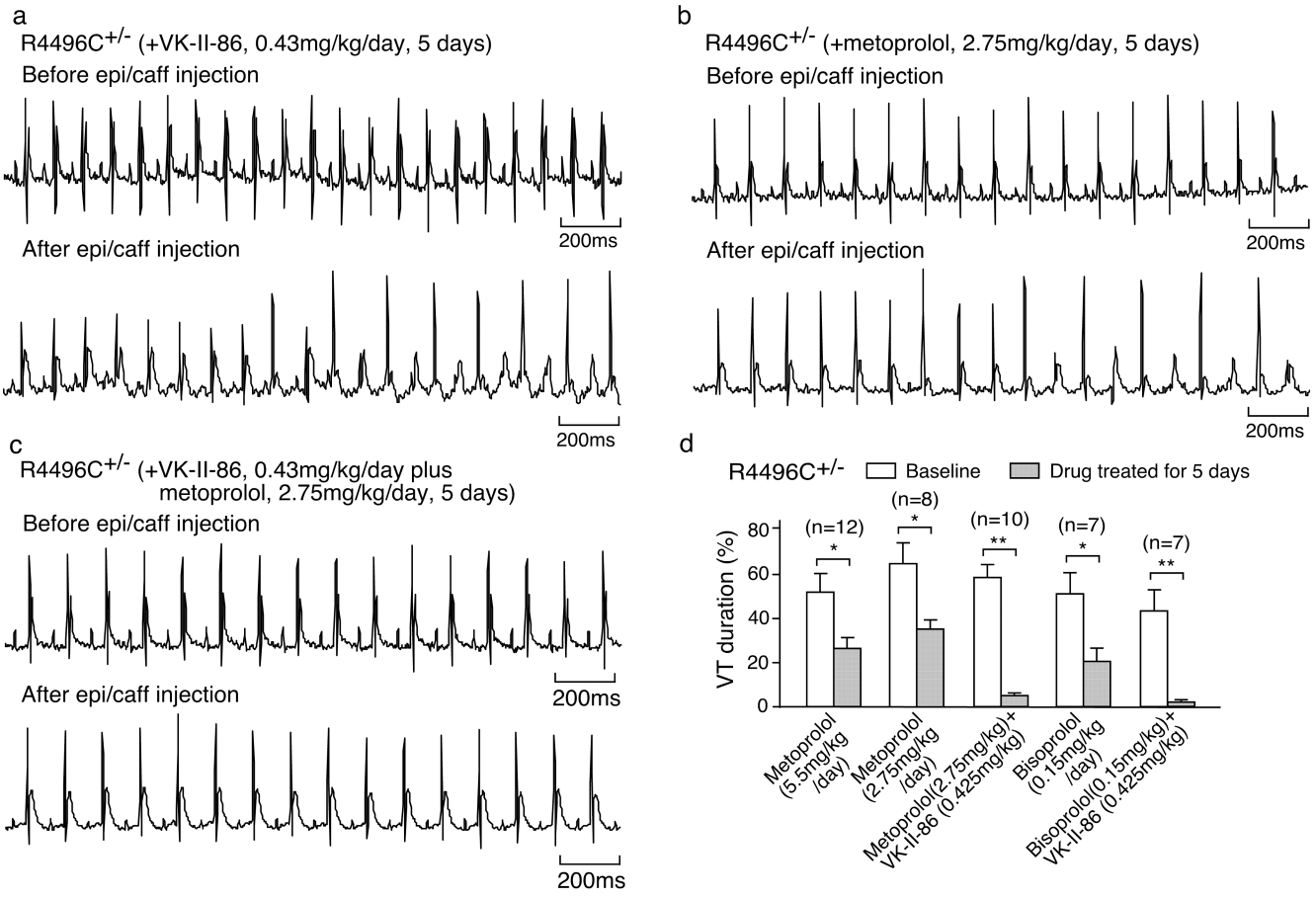
Supplementary Figure 7 (Fig. S7)



Supplementary Fig. S7: Effect of carvedilol and VK-II-86 on CPVT in RyR2 R4496C$^{+/-}$ mutant mice

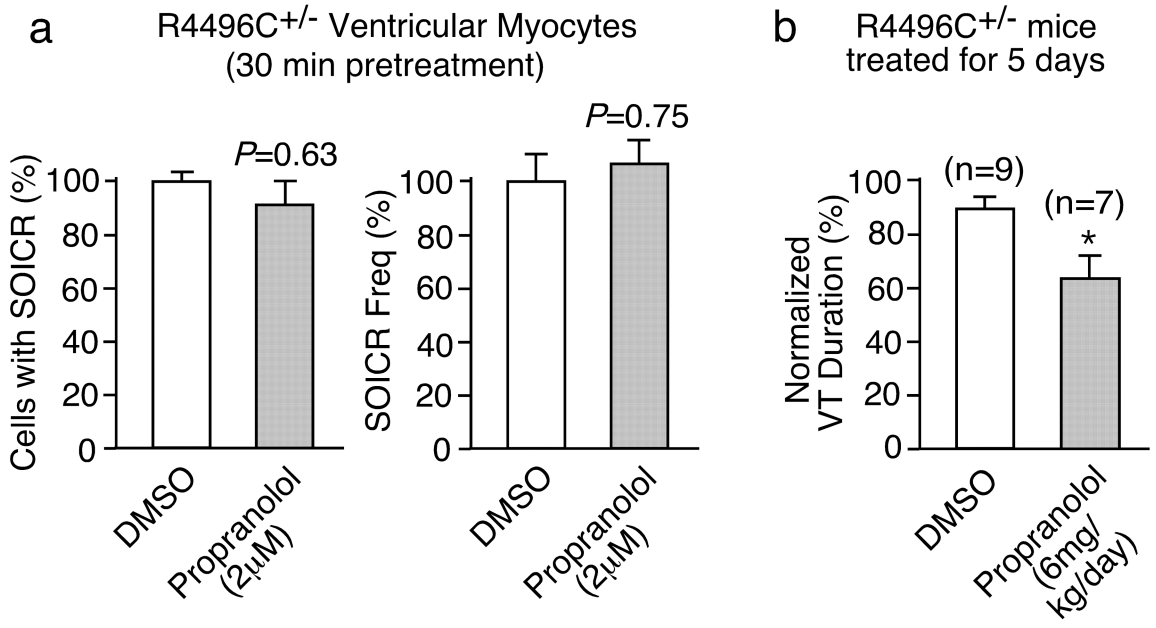
(a) Representative ECG recordings of R4496C$^{+/-}$ mutant mice without drug treatment before and after i.p. injection of epinephrine (1.6 mg/kg) and caffeine (120 mg/kg). (b) Representative ECG recordings of R4496C$^{+/-}$ mutant mice treated (i.p. injection) with DMSO for 5 days before and after injection of epinephrine and caffeine. (c) Representative ECG recordings of R4496C$^{+/-}$ mutant mice treated with carvedilol (1.6mg/kg/d) before and after injection of epinephrine and caffeine. (d) VT duration (%, per 30 min) in RyR2 R4496C$^{+/-}$ mutant mice before treatment (baseline, white bars) and after treatment (drug treated, gray bars) with DMSO, different doses of VK-II-86 or carvedilol for 5 days (n=7-12) (** $P < 0.01$ vs baseline).

Supplementary Figure 8 (Fig. S8)



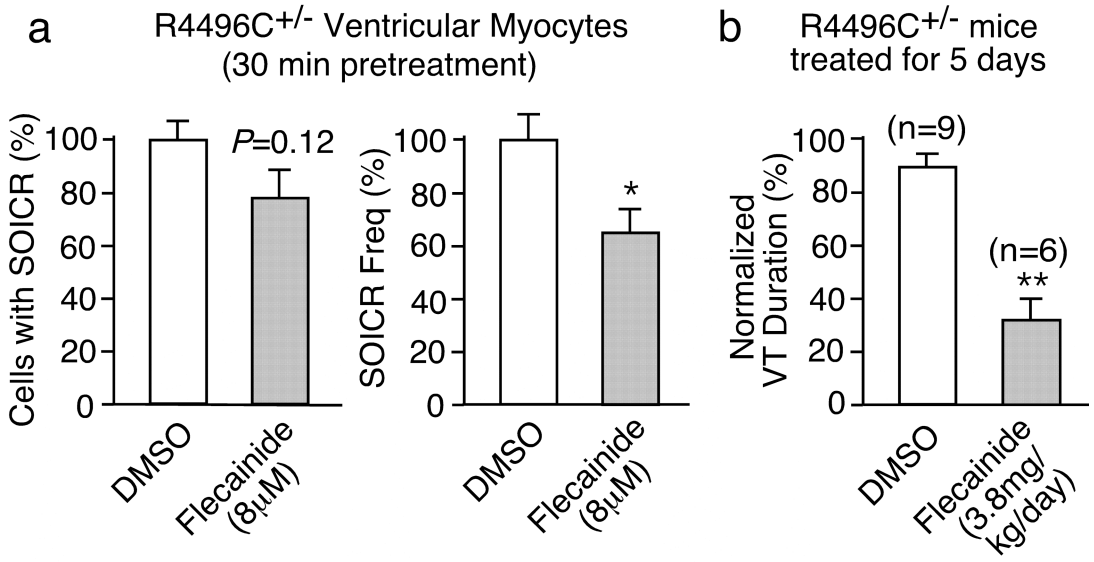
Supplementary Fig. S8: Effect of VK-II-86, metoprolol, bisoprolol, and their combination on CPVT in RyR2 R4496C$^{+/-}$ mutant mice (a) Representative ECG recordings of R4496C$^{+/-}$ mutant mice treated (i.p. injection) with VK-II-86 for 5 days before and after injection (i.p.) of epinephrine and caffeine. (c) Representative ECG recordings of R4496C$^{+/-}$ mutant mice treated with metoprolol for 5 days before and after injection of epinephrine and caffeine. (c) Representative ECG recordings of R4496C$^{+/-}$ mutant mice treated with VK-II-86 plus metoprolol for 5 days before and after injection of epinephrine and caffeine. (d) VT duration (%, per 30 min) in RyR2 R4496C$^{+/-}$ mutant mice before treatment (baseline, white bars) and after treatment (drug treated, gray bars) with DMSO, 2 doses of metoprolol, VK-II-86 plus metoprolol, bisoprolol, or VK-II-86 plus bisoprolol for 5 days (n=6-9) (** $P < 0.01$ vs baseline).

Supplementary Figure 9 (Fig. S9)



Supplementary Fig. S9: Effect of propranolol on SOICR and CPVT Bankston and Kass[5] showed that carvedilol and propranolol, but not metoprolol, inhibit Na channel currents. To assess if Na channel inhibition suppresses SOICR, we tested propranolol action on SOICR. (a) The percentage of RyR2-R4496C$^{+/-}$ ventricular myocytes that display SOICR and the SOICR frequency in cells treated with propranolol (2 μM) or DMSO (control) normalized to that in the presence of DMSO (n=3). The total numbers of cells analyzed were 137 for DMSO and 93 for propranolol. (b) VT duration (%) in the 30-min period of ECG recordings in R4496C$^{+/-}$ mutant mice treated with (i.p.) DMSO (control) or propranolol (6 mg/kg/day) for 5 days normalized to the animal's own baseline before drug treatment (n=7) (*$P < 0.05$ vs DMSO). We found that propranolol (2 μM) had no effect on SOICR. We also tested the action of propranolol on CPVT in R4496C$^{+/-}$ mice. A 5-day treatment with propranolol (6 mg/kg/day) reduced the VT duration by ~35%. However, a 5-day treatment with carvedilol (1.6 mg/kg/day) reduced VT significantly more (~80%; $P<0.002$). These results indicate that carvedilol's Na current block does not contribute substantially to its inhibitory action on SOICR or SOICR-evoked CPVT.

Supplementary Figure 10 (Fig. S10)



Supplementary Fig. S10: Action of flecainide on SOICR and CPVT (a) The percentage of RyR2-R4496C$^{+/-}$ ventricular myocytes that display SOICR and the SOICR frequency in cells treated with flecainide (8 μM) or DMSO (control) normalized to the DMSO value (n=4-6) (*$P < 0.05$, **$P < 0.01$ vs DMSO). The total numbers of cells analyzed were 141 for DMSO and 141 for flecainide. (c) VT duration (%) in the 30-min period of ECG recordings in R4496C$^{+/-}$ mutant mice treated with (i.p.) DMSO (control) or flecainide (3.8 mg/kg/day) for 5 days normalized to the animal's own baseline value before drug treatment (n=6-9) (**$P < 0.01$ vs DMSO).

Supplementary Table 1

Supplementary Table 1: Beta-adrenergic Receptor Binding of Carvedilol and Carvedilol Analogues
The average pKi and n_H values for CGP20712A, carvedilol, and carvedilol analogues are shown.

Drugs	pKi High (pKi) / Low (pKi)		High affinity fraction (%)	*Slope* (n_H)
Carvedilol (n=4)	9.282±0.312			0.985±0.123
CGP20712A (n=3)	9.156±0.193	5.59±0.175	72.5±2.6	0.525±0.107*
VK-II-86 (n=3)	5.797±0.267			0.885±0.142
CS-I-34 (n=3)	5.494±0.243			1.271±0.321
CS-I-59 (n=3)	5.894±0.324			0.899±0.175

*P<0.01; mean ± SD

Supplementary Methods

Single-cell Ca^{2+} imaging of HEK293 cells: Stable, inducible HEK293 cells expressing a CPVT-causing RyR2 mutant, R4496C, display robust spontaneous Ca^{2+} oscillations (SOICR), but parental HEK293 cells do not[6, 7]. These RyR2-R4496C cells were used to assess the impact of beta-blockers on SOICR. For screening, a relatively high concentration (30 μM) of beta-blockers was used to maximize detection of SOICR inhibition by beta-blockers. SOICR was measured using single-cell Ca^{2+} imaging and the fluorescent Ca^{2+} indicator dye fura-2/AM (Invitrogen) as described previously[6, 7]. 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 (Krebs–Ringer–Hepes) buffer (125 mM NaCl, 5 mM KCl, 1.2mM KH_2PO_4, 6 mM glucose, 1.2 mM $MgCl_2$ 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 Ca^{2+} concentration was then stepped to 0.5 mM for 5 min before increasing to 1 mM. The cells were continuously perfused with KRH buffer containing 1 mM $CaCl_2$ and the various drugs for 8-10 min. For testing the action of prazosin (30μM), phentolamine (30μM), N-(2-mercaptopropionyl)-glycine (MPG, 1mM), α-tocopherol (600μM), or DMSO (control) on SOICR, RyR2-R4496C HEK293 cells were incubated with the drug for 30 min. Caffeine (10 mM) was applied at the end of each experiment to verify the presence of functioning 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 that responded to caffeine. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise specified.

Single channel recordings in lipid bilayers: Recombinant RyR2-R4496C channels were purified from cell lysate prepared from HEK293 cells transfected with the R4496C cDNA by sucrose density

gradient centrifugation as described previously[8, 9]. Heart phosphatidylethanolamine (50%), brain phosphatidylserine (35%), and heart phosphatidylcholine (15%) (Avanti Polar Lipids), dissolved in chloroform, were combined and dried under nitrogen gas and resuspended in 30 μl of *n*-decane at a concentration of 12 mg lipid per ml. 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) with or without carvedilol (1 μM) or VK-II-86 (1 μM) was used for all recordings, unless indicated otherwise. A 4-μl aliquot (≈ 1 μg of protein) of the sucrose density gradient-purified recombinant RyR2-R4496C mutant channels was added to the cis chamber and pre-incubated in the presence of the drug for 1 hour before they were fused into the lipid bilayers. Spontaneous channel activity was always tested for sensitivity to EGTA and Ca^{2+}. The chamber to which the addition of EGTA inhibited the activity of the incorporated channel presumably corresponds to the cytosolic side of the Ca^{2+} release channel. The holding potential was -20 mV. The direction of single channel currents was always measured from the luminal to the cytosolic side of the channel, unless mentioned otherwise. The cytosolic and luminal free Ca^{2+} concentrations were then adjusted to 45 nM and 2.5 mM, respectively. Recordings were filtered at 2,500 Hz. Data analyses were carried out using the pclamp 8.1 software package (Axon Instruments). Free Ca^{2+} concentrations were calculated using the computer program of Fabiato and Fabiato [10]. For single channel recordings of native RyR2 channels, heavy SR microsomes were prepared from rat ventricular muscle using the method described by Chamberlain et al. [11]. Planar lipid bilayers were composed of a 5:4:1 mixture (50 mg/ml in *n*-decane) of bovine brain phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine. Bilayers were formed across a 100 μm diameter hole in a Teflon partition separating two compartments. The cytosolic solution contained 120 mM HEPES-TRIS (pH 7.2), 50 μM free Ca^{2+} (0.5 mM EGTA), 1 mM free Mg^{2+} and 5 mM total ATP. The luminal solution contained 200 mM Cs-HEPES (pH 7.2), 1 mM free Mg^{2+} and 1 mM free Ca^{2+}. SR vesicles were pre-incubated for 1 hour with the tested drug (1 μM) before they were fused into the

bilayer with the same drug in both the cytosolic and luminal solutions. Channel incorporation always resulted in the cytosolic side of the RyR2 channel facing the cis compartment [12-14]. This compartment is referred to as cytosolic and the other as luminal. Immediately upon observing single channel activity, the solutions were replaced to establish the test conditions described in the figure legends. Recipes for the Ca^{2+} buffer solutions used were generated using WinMAXC 2.05 (Stanford University, CA) and verified by Ca^{2+} electrode. Recordings were made at room temperature (20-22 °C). Analysis was done using pCLAMP9 software (Molecular Devices, Sunnyvale, CA). Currents were sampled at 50 μs/pt and filtered for display at 0.75 kHz (4-pole Bessel).

Immunoblotting analysis: Immunoblotting analysis was carried out using the method described previously[2, 3]. Rat cardiac myocytes were isolated and pre-treated with carvedilol or VK-II-86 for 5 min at 37 ^{o}C, followed by stimulation with 200 nM isoproterenol (Iso) in the continued presence of the drugs for 30 min at 37 ^{o}C. Myocytes were then centrifuged at 15,000 x g for 30 s. Cell pellets were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4) and 2% SDS. The resulting cell lysates were used for Western blotting studies. Briefly, the SDS-PAGE (6% acrylamide) resolved proteins were transferred to nitrocellulose membranes at 45 V for 18-20 h at 4 ^{o}C in the presence of 0.01% SDS according to the method of Towbin et al.[15]. The nitrocellulose membranes containing transferred proteins were blocked for 30 min with PBS containing 0.5% Tween-20 and 5% skim milk powder. The blocked membrane was incubated with the anti-RyR2 (1:1000) or anti-S2030(PO_3) (1:1000) antibody, and washed 3 times for 15 min each, with PBS containing 0.5% Tween-20. The membrane was then incubated with the secondary anti-mouse or anti-rabbit IgG (H&L) antibodies conjugated to horseradish peroxidase (1:20,000) for 30 min. After washing 3 times for 15 min each, the bound antibodies were detected by an enhanced chemiluminescence kit from Thermo Scientific.

Beta-adrenergic receptor radioligand binding: Mouse hearts were rapidly removed and tissues isolated from the left and right ventricle. Cleaned and minced myocardium was homogenized in ice-cold homogenates buffer (mmol/L: sucrose 250, Tris 5, $MgCl_2$ 1, EDTA 1, dithiothreitol 1, phenylmethylsulfonyl fluoride [PMSF] 0.01, pH 7.4) with a Polytron homogenizer (Brinkmann) at a setting of 9, two times for 10 seconds and once for 5 seconds at 4°C. The homogenate was filtered through three layers of cheesecloth and centrifuged at 500*g* for 10 minutes at 4°C. The supernatant, after being filtered through two layers of cheesecloth, was centrifuged at 45 000*g* for 30 minutes at 4°C to yield membranes. The pellet was resuspended in cold binding buffer (incubation buffer, mmol/L: Tris 50, $MgCl_2$ 10, EDTA 1, dithiothreitol 1, PMSF 0.01, pH 7.4) and centrifuged at 45 000g for 30 minutes. The final plasma membrane pellet was resuspended to give a final concentration of 1 mg/ml protein. The protein concentration was determined by the method of Lowry et al. [16] using bovine albumin serum as the standard. For saturation binding experiments, membrane preparations (~100 μg protein) were incubated with [^{125}I]-cyanopindolol (ICYP) (1 to 300 pmol/L) in the incubation buffer either alone or with propranolol (20 μmol/L), which was used for determining the nonspecific binding. The incubation was carried out at room temperature (25°C) for 2 hours in a total volume of 250 μL. The reaction was terminated by the addition of ice-cold incubation buffer and by a rapid vacuum filtration through type A/C glass fiber filters (Brandel. Inc). The density (B_{max}) and affinity (K_d) of binding sites for ICYP were determined by linear regression analysis of saturation isotherm data, linearly transformed according to the method of Scatchard. The proportions of beta1- and beta2-adrenoceptor subtypes were determined from displacement curves of ICYP binding (50 pmol/L) by CGP20712A (a beta1-selective antagonist, 10 pmol/L to 10 μmol/L). Competition experiments of ICYP binding with increasing concentrations of carvedilol (0.1 pmol/L-1.0 μmol/L) and its analogues (1 nmol/L to 100 μmol/L) were performed. All experiments were carried out in duplicate.

Generation of knock-in mouse model harboring the CPVT RyR2 mutation, R4496C: A genomic DNA phage clone containing part of the mouse cardiac ryanodine receptor gene was isolated from the

lambda mouse 129-SV/J genomic DNA library (Stratagene) and used to construct the RyR2 R4496C knock-in (KI) targeting vector. This genomic DNA fragment (about 15 kb) was released from the lambda vector by Not I, and was subcloned into pBluescript to form the plasmid, RyR2-R4496-BS. PCR-based site-directed mutagenesis was performed to generate a 924 bp DNA fragment containing the R4496C mutation in exon 92 using the RyR2-R4496-BS plasmid as a template. This DNA fragment was then subcloned into the targeting vector that contains a neomycin selection cassette flanked by FRT sites using XhoI and BamHI. The 4211 bp KpnI-ApaI genomic DNA fragment containing exon 91 was isolated from the RyR2-R4496-BS plasmid and inserted into the targeting vector via KpnI and ApaI sites. The 1200 bp SalI-NotI genomic DNA fragment was generated by PCR from the RyR2-R4496-BS plasmid, and was inserted into the targeting vector via the SalI and Not I sites. The DNA sequences of all exons and PCR fragments used for constructing the targeting vector were confirmed by DNA sequencing. The targeting vector was linearized with KpnI and subsequently electroporated into R1 embryonic stem (ES) cells. G418-resistant ES clones (480 clones) were screened for homologous recombination by Southern blotting using an external probe. Briefly, genomic DNA was extracted from G418-resistant ES cell clones. ES cell DNA was digested using HindIII, separated on a 1% (wt/vol) agarose gel, and subsequently blotted onto a nitrocellulose membrane. The DNA probe, a 720 bp DNA fragment, was generated by PCR from mouse genomic DNA and using the specific primers, forward: 5'-GCCAATTGTCTTAGTCAGGGT-3'; and reverse: 5'-CTCCAACTTCCGTGCCACTTC-3'. The PCR product was subsequently radiolabeled using [^{32}P]dCTP by random priming (Invitrogen). DNA blots were hybridized with the radiolabeled probe and visualized by autoradiography. Two of the eight independent homologous recombinant ES clones were microinjected into blastocysts from C57BL/6J mice to generate male chimeras at the Mouse Facility of UCSD. Male chimeras were bred with female Black Swiss mice to generate germline transmitted heterozygous R4496C-neo knock in mice. RyR2-R4496C-neo male mice were bred with female mice that express Flp recombinase to remove the selectable marker (neo). The genotypes from F1 generation without neo were determined by PCR using

DNA from tail biopsy specimens using the DNeasy Tissue Kit from Qiagen and the DNA primers, forward: 5'-CCCAGTGTTCCTAGGACGATTCAGT-3'; reverse: 5'-GCACTTGCTGCCTCACACACACAGT-3'.

Single-cell Ca^{2+} imaging of mouse ventricular myocytes: Mouse hearts were mounted on the Langendorff apparatus and single ventricular myocytes were isolated by using the collagenase type II (Worthington Biochem) and protease method described previously[17]. Ventricular myocytes were placed on laminin coated glass coverslips and loaded with 5 μM Fluo-4 AM (Invitrogen) plus 0.02% pluronic F-127 in KRH buffer (125 mM NaCl, 5 mM KCl, 1.2mM KH_2PO_4, 11 mM glucose, 1.2 mM $MgCl_2$ and 15 mM Hepes, pH 7.4) containing 1 mM Ca^{2+} and carvedilol (1 μM), metoprolol (2 μM), VK-II-86 (1 μM) or DMSO (control) for 30 minutes at 23 °C. Myocytes were washed for 10 minutes with KRH solution containing 1 mM Ca^{2+} and the drug. The Ca^{2+} concentration in the KRH solution was then stepped to 2mM for 6min and 3 mM for 6 min before increasing it to 6 mM to induce SOICR (perfusion buffer also contains the drug). The cells were then continuously perfused with KRH buffer containing 6 mM Ca^{2+} in the continued presence of the drug. In studies with low doses of drugs, ventricular myocytes were pre-incubated with metoprolol (0.6 μM), carvedilol (0.3 μM), VK-II-86 (0.3 μM), prazosin (0.6 μM), vitamine E (6 μM), or DMSO (control) for 3 hours in a 1.5ml microcentrifuge tube. The cells were then transferred onto a perfusion chamber and loaded with 5 μM Fluo-4 AM plus 0.02% pluronic F-127 in KRH buffer containing 1 mM Ca^{2+} and the drug for 30 minutes at 23 °C. Myocytes were washed for 10 minutes with KRH solution containing 1 mM Ca^{2+} and the drug. The Ca^{2+} concentration of the KRH solution was then stepped to 2mM for 6 min and 3 mM for 6 min before increasing it to 6 mM to induce SOICR (perfusion buffer also contains the drug). The cells were then continuously perfused with KRH buffer containing 6 mM Ca^{2+} in the continued presence of the drug. SOICR occurred as propagating global Ca^{2+} waves. The '% of cells with SOICR' was determined by calculating the percentage of cells that display Ca^{2+} waves after a drug treatment. This percentage was then normalized to that of cells treated with DMSO. The SOICR frequency was determined by calculating the average number of Ca^{2+} waves per minute and the result was also

normalized to values in DMSO treated cells. Time-lapse images were captured at 10 frames/s and analyzed with the Compix Simple PCI 6 software. Fluorescence intensities were measured from small rectangular regions of interest in each cell.

Laser scanning confocal imaging: Mouse ventricular myocytes isolated from the R4496C$^{+/-}$ mice were placed on laminin coated glass coverslips and loaded with 5 μM Fluo-4 AM (Invitrogen) plus 0.02% pluronic F-127 in Tyrode solution containing 1 mM Ca^{2+} and carvedilol (1 μM), metoprolol (2 μM), VK-II-86 (1 μM) or DMSO (control) for 30 minutes at 23 °C. Myocytes were washed for 10 minutes with Tyrode solution containing 1 mM Ca^{2+} and the drug. The Ca^{2+} concentration in the Tyrode solution was then stepped to 2 mM for 6 min and 3 mM for 6 min before increasing it to 6 mM to induce SOICR (perfusion buffer also contains the drug). The cells were then continuously perfused with Tyrode buffer containing 6 mM Ca^{2+} in the continued presence of the drug. The SR Ca^{2+} content was determined by measuring the amplitude of caffeine (15 mM) induced Ca^{2+} transients after each treatment. Ca^{2+} images were recorded using a confocal microscope (LSM510, Carl Zeiss MicroImaging Inc., Germany) as described previously[18]. Only myocytes that were quiescent in the presence of 1mM external Ca^{2+} were used for Ca^{2+} imaging.

Design and synthesis of carvedilol analogues: It has been demonstrated that hydrogen-bonding interactions involving both of the amino and hydroxyl group of carvedilol play key roles in β-blockade[19]. Moreover, recent evidence indicates that the lipophilic carbazole moiety of carvedilol becomes embedded in the cell membrane of the target cells[20]. Thus, we postulated that the hydroxyl and amino groups interact with a nearby membrane-bound receptor and that the spacing and orientation between the carbazole moiety and the key functional groups on the linker chain joining it to the 2-methoxyphenyl substituent are critical for the drug's β-blocking activity. Following this logic, three compounds (VK-II-86, CS-I-34 and CS-I-59) were designed and synthesized as follows.

Synthesis of VK-II-86: Bromo epoxide **2** (0.546 g, 2.90 mmol) and 1 M NaOH solution (0.097 g, 2.42 mmol) were added to a solution of 4-hydroxycarbazole (**1**) (0.300 g, 2.42 mmol) in DMSO (1 mL). The reaction mixture was stirred for 24 h at 60° C. It was then cooled to 20 °C, diluted with 25 mL of water and extracted with ethyl acetate (3 × 20 mL). The collected organic layers were washed with brine, dried (Na_2SO_4) and concentrated. The residue was purified by flash chromatography on silica-gel (EtOAc-hexanes) to afford epoxide **3** (0.254 g, 61%) as an oil. To a solution of epoxide **3** (253 mg, 1.00 mmol) in anhydrous 1,2-dimethoxyethane (DME, 6 mL) were added amine **4** (334 mg, 2.00 mmol) and LiBr (catalytic). The reaction mixture was stirred for 24 h at 60° C. The solvent was removed under vacuum, the residue was taken up in ether (10 mL) and washed with water and brine, dried over Na_2SO_4, evaporated under reduced pressure and the residue was purified by flash chromatography on silica-gel (EtOAc-hexanes) to obtain 307 mg (73%) of VK-II-86; mp 61-63 ^oC; ^1H NMR (300 MHz, $CDCl_3$) δ 8.28 (d, J = 7.6 Hz, 1 H), 8.08 (s, 1 H), 7.42–7.30 (m, 3 H), 7.22 (t, J = 6.7 Hz, 1 H), 7.05 (d, J = 8.0 Hz, 1 H), 6.95–6.87 (m, 4 H), 6.71 (d, J = 8.0 Hz, 1 H), 4.43 (t, J = 6.0 Hz, 2 H), 4.13 (t, J = 5.0 Hz, 2 H), 4.10-4.02 (m, 1 H), 3.84 (s, 3 H), 3.10-2.99 (m, 3 H), 2.74 (t, J = 6.0 Hz, 1 H), 2.20–2.07 (m, 2 H), 1.65 (s, 1 H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.4, 149.5, 148.1, 141.0, 138.8, 126.7, 124.9, 122.9, 122.6, 121.7, 121.0, 119.5, 114.0, 112.6, 111.9, 110.1, 103.6, 101.1, 68.5, 66.9, 64.9, 55.8, 55.1, 48.4, 34.6; mass spectrum (*m/z*, %) 420 (M^+, <1), 297 (17), 209 (63), 182 (100). HRMS calc'd for $C_{25}H_{28}N_2O_4$: 420.2049; found: 420.2066.

Synthesis of CS-I-34: A solution of 3-methoxy-9*H*-carbazole (**5**) (1.20 g, 6.06 mmol) in dry dichloromethane (50 mL) was cooled in an ice bath and 19.5 mL of a 1.0 M solution of BBr_3 in dichloromethane was added dropwise. After 1 h, the reaction was allowed to warm to room temperature and stirred an additional 2 h. The reaction was cooled in an ice bath and slowly quenched with water (50 mL) and diluted further with ethyl acetate (250 mL). The organic phase was separated and washed with water (30 mL) and brine (2 × 25 mL), dried over $MgSO_4$ and concentrated in vacuo to give the crude product as a grey solid. Purification by flash chromatography on silica gel using a gradient elution of 30-40% ethyl acetate in hexanes afforded 3-hydroxy-9*H*-carbazole (**6**) (1.00 g, 90%) as a yellow solid: mp 184-186 °C; ^1H NMR

(300 MHz, DMSO-d_6) δ 10.87 (s, 1 H), 8.90 (s, 1 H), 7.97 (d, *J* = 7.7 Hz, 1 H), 7.25-7.41 (m, 4 H), 7.05 (dd, *J* = 7.6, 7.2 Hz, 1 H), 6.88 (dd, *J* = 8.6, 2.2 Hz, 1 H); ^{13}C NMR (75 MHz, d_6-DMSO) δ 150.3, 140.4, 133.7, 125.1, 123.0, 122.2, 120.0, 117.7, 115.0, 111.2, 110.8, 104.8. To a solution of **6** (201 mg, 1.09 mmol) in DMSO (1.0 mL) at room temperature was added 1 M NaOH (1.1 mL) followed by epichlorohydrin (0.13 mL, 1.6 mmol). The reaction was stirred at 45 °C for 16 h. The reaction was cooled to room temperature, treated with water (25 mL), and extracted with chloroform (4 × 15 mL). The combined organic extracts were dried over $MgSO_4$ and concentrated in vacuo to give a beige solid. Purification by flash chromatography on silica gel (5% ethyl acetate-toluene) afforded epoxide **7** (150 mg, 58%) as a white solid: mp 124-126 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.0 Hz, 1 H), 7.92 (s, 1 H), 7.57 (d, *J* = 2.4 Hz, 1 H), 7.36-7.40 (m, 2 H), 7.31 (d, *J* = 8.7 Hz, 1 H), 7.16-7.20 (m, 1 H), 7.08 (dd, *J* = 8.7, 2.4 Hz, 1 H), 4.32 (dd, *J* = 11.0, 3.2 Hz, 1 H), 4.07 (dd, *J* = 11.0, 5.6 Hz, 1 H), 3.40-3.45 (m, 1 H), 2.93 (dd, *J* = 4.7, 4.4 Hz, 1 H), 2.80 (dd, *J* = 4.9, 2.6 Hz, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.9, 140.4, 134.9, 126.1, 123.9, 123.4, 119.3, 115.8, 111.5, 110.9, 104.9, 70.1, 50.6, 45.0. Amine **4** (126 mg, 0.754 mmol), followed by a catalytic amount of LiBr, were added to a solution of the epoxide **7** (120 mg, 0.502 mmol) in anhydrous DME (2.0 mL). The reaction was heated to 55 °C and stirred for 24 h. The reaction was cooled to room temperature and concentrated in vacuo to give a residue that was partitioned between water and chloroform. The aqueous phase was separated and extracted with chloroform (3 × 15 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo to give a yellow oil. Purification by flash chromatography on silica gel using a gradient elution of 5-7% methanol-dichloromethane afforded CS-I-34 as a white solid (100 mg, 49%): mp 146-147 °C; IR (KBr) 3458, 3378, 2922, 2834, 1507, 1452, 1253, 1222, 1186, 1121, 745 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (dd, *J* = 7.8, 0.7 Hz, 1 H), 7.93 (s, 1 H,), 7.56 (d, *J* = 2.5 Hz, 1 H), 7.35-7.39 (m, 2 H), 7.30 (dd, *J* = 8.8, 0.2 Hz, 1 H), 7.19-7.16 (m, 1 H), 7.06 (dd, *J* = 8.8, 2.5 Hz, 1 H), 6.94-6.86 (m, 4 H), 4.16-4.08 (m, 5 H), 3.82 (s, 3 H), 3.09 (t, *J* = 5.2 Hz, 2 H), 2.99 (dd, *J* = 12.1, 3.7 Hz, 1 H), 2.89 (dd, *J* = 12.4, 7.3 Hz, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.1, 150.0, 148.4, 140.5, 134.8, 126.0, 124.0, 123.5, 121.9, 121.2, 120.5, 119.3, 115.7, 114.6, 112.1, 111.4, 110.9, 104.7, 71.7,

69.0, 68.6, 56.0, 51.9, 48.9; mass spectrum (*m/z*, %) 406 (2), 183 (100), 154 (10), 109 (11). HRMS calc'd for $C_{24}H_{26}N_2O_4$: 406.1893; found: 406.1889. Anal. calc'd for $C_{24}H_{26}N_2O_4$: C, 70.92; H, 6.45; N, 6.89; found: C, 70.70; H, 6.37; N, 6.74.

Synthesis of CS-I-59: A mixture of 2-hydroxy-9*H*-carbazole (**8**) (0.720 g, 3.91 mmol) in DMSO (2.0 mL) and 1 M NaOH (4.0 mL) was stirred for 15 min. at room temperature, after which epichlorohydrin (0.50 mL, 6.3 mmol) was added and the reaction mixture was stirred for 16 h. The precipitate was filtered and washed with water (20 mL). The isolated solid was partitioned between water (40 mL) and ethyl acetate (75 mL), the aqueous phase was separated and extracted with ethyl acetate (30 mL) and chloroform (2 × 25 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo to give a copper-brown solid. Purification by flash chromatography on silica gel using a gradient elution of 35-45% ethyl acetate-hexanes afforded epoxide **9** (445 mg, 48%) as an off-white solid: mp 185-187 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.12 (s, 1 H), 7.94-7.99 (m, 2, H), 7.42 (d, *J* = 8.0 Hz, 1 H), 7.29 (dd, *J* = 7.6, 7.6 Hz, 1 H), 7.11 (dd, *J* = 7.6, 7.2 Hz, 1 H), 6.99 (d, *J* = 2.0 Hz, 1 H), 6.80 (dd, *J* = 8.4, 2.0 Hz, 1 H,), 4.41 (dd, *J* = 11.2, 2.4 Hz, 1 H), 3.92 (dd, *J* = 11.2, 6.4 Hz, 1 H), 3.35-3.43 (m, 1 H), 2.87 (t, *J* = 4.6 Hz, 1 H), 2.77 (dd, *J* = 5.0, 2.6 Hz, 1 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 157.3, 140.9, 139.7, 124.2, 122.5, 120.9, 119.2, 118.5, 116.4, 110.6, 107.9, 95.3, 69.2, 49.8, 43.7. The epoxide **9** (0.408 g, 1.71 mmol) was added to a solution of the amine **4** (0.484 g, 2.90 mmol) in isopropanol (2.5 mL) and DME (0.5 mL), and the resultant mixture was refluxed for 3 h. After cooling to room temperature, the reaction was concentrated in vacuo to give a cream-cloured solid that was purified by flash chromatography on silica gel using a gradient elution of 3-7% methanol-dichloromethane to afford CS-I-59 (321 mg, 46%) as a white solid: mp 146-148 °C; IR (KBr) 3552, 3395, 3057, 2923, 2833, 1628, 1609, 1500, 1457, 1252, 742 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.07 (s, 1 H), 7.97 (d, *J* = 7.8 Hz, 1 H), 7.94 (d, *J* = 8.6 Hz, 1 H), 7.41 (d, *J* = 8.0 Hz, 1 H), 7.26 (td, *J* = 7.2, 1.2 Hz, 1 H), 7.09 (td, *J* = 7.5, 1.0 Hz, 1 H), 7.01-6.82 (m, 5 H), 6.78 (dd, *J* = 8.5, 2.2 Hz, 1 H), 5.07 (d, *J* = 4.6 Hz,1 H), 4.07-3.93 (m, 5 H), 3.74 (s, 3 H), 2.92 (t, *J* = 5.5 Hz, 2 H), 2.85-2.65 (m, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 157.8, 149.1, 148.0, 141.0, 139.7, 124.0, 122.6, 121.0, 120.8, 120.7, 119.1,

118.4, 116.1, 113.6, 112.2, 110.5, 108.0, 95.2, 70.9, 68.3, 68.2, 55.4, 52.4, 48.4; mass spectrum (*m/z*, %) 406 (M^+, 56), 183 (100), 180 (78), 154 (20); HRMS calc'd for $C_{24}H_{26}N_2O_4$: 406.1893; found: 406.1879. Anal. calc'd for $C_{24}H_{26}N_2O_4$: C, 70.92; H, 6.45; N, 6.89; found: C, 70.65; H, 6.49; N, 6.80.

Animal studies: All animal studies were approved by the applicable Institutional Animal Care and Use Committees, and performed in accordance with NIH guidelines. Adult R4496C mutant mice and wild type control littermates (2-4 months old) were used for all experiments. The RyR2 R4496C mutant mice consistently develop VTs after catecholamine and caffeine challenge.

ECG recordings and induction of ventricular tachycardia in anesthetized mice: The effects of carvedilol, metoprolol and other compounds on ventricular tachycardia induced by epinephrine and caffeine were studied in anesthetized R4496C$^{+/-}$ or R4496C$^{+/+}$ mutant mice. Briefly, mice were lightly anesthetized with isoflurane vapor (0.5-1%). Anesthetized mice were placed on a heating pad (28 °C) and subcutaneous needle electrodes were applied to the left, right upper limb and right lower limb for ECG recording (BIOPAC MP System, Goleta, CA). The ECG was continuously monitored under anesthesia until heart rate stabilized. Baseline ECG was recorded for 5 minutes, followed by the intraperitoneal injection of epinephrine (1.6 mg/kg) and caffeine (120 mg/kg). ECG was then continuously recorded for another 30 minutes. ECG recordings were analyzed using the AcqKnowledge 3.9.1 program (BIOPAC, Goleta, CA).

Drug tests in RyR2-R4496C$^{+/-}$ heterozygous mutant mice: We found that carvedilol at 1.6 mg/kg/day for 5 days significantly suppressed CPVT in our RyR2 R4496C$^{+/-}$ mutant mouse model. This dose of carvedilol was therefore used as a guide to titrate the dose of metoprolol to achieve equivalent beta-blockade and heart rate reduction. We found that metoprolol at 5.5 mg/kg affected the Iso-enhanced heart rate and non-stimulated heart rate to an extent similar to that observed with carvedilol at 1.6mg/kg. This dose of metoprolol (5.5mg/kg) was therefore chosen for studying its impact on CPVT. It is important to

know that in the COMET trial [21], the mean dose of metoprolol was 85 mg, which is ~2 fold higher than that of carvedilol (42mg). In the MERIT-HF trial[22], the mean dose of metoprolol was 159 mg, which is 3.78 fold higher than that of carvedilol (42 mg) used in the COMET trial. The dose of metoprolol used in our mouse studies was 5.5 mg/kg, which is 3.44 fold higher than that of carvedilol (1.6 m/kg). In cellular studies, we used 2μM metoprolol and 1μM carvedilol, which yield a metoprolol/carvedilol dose ratio of 2 (in μM) and 3.44 (in mg/kg). Thus, the dose of metoprolol used in our studies is appropriate compared to those used in the clinical trials. The duration of VT in RyR2 R4496C$^{+/-}$ mutant mice vary widely. To minimize mouse-to-mouse variations, we determined the duration of VT (defined as >3 consecutive ventricular beats) in the absence of drugs to establish a baseline for each mouse. The mice were then treated with various drugs for 5 days and the duration of VT was determined and compared with each animal's baseline level. The RyR2 R4496C$^{+/-}$ mutant mice used here had strong CPVT phenotypes with VT durations ≥15% of each 30 min recording. An epinephrine plus caffeine challenge was performed twice in the same R4496C$^{+/-}$ mutant mouse at an interval of 7 days. Animals were randomized into different groups and treated with various drugs by intraperitoneal injection for 5 days. The final dose of drug was administered 30 minutes before ECG testing. The percentage of time that the mice were in VT, including sustained VTs and non-sustained VTs, within a 30 min recording period was determined and compared.

Acute drug tests in RyR2 R4496C$^{+/+}$ homozygous mutant mice: To evaluate the acute effect of different drugs on CPVT, the R4496C$^{+/+}$ mutant mice monitored continuously with ECG recordings were intraperitoneally injected with epinephrine (1.6 mg/kg) and caffeine (120 mg/kg) to induce ventricular tachycardia (CPVT). A single dose of each of the different drugs was then intraperitoneally injected into the mice 12 minutes after the injection of epinephrine and caffeine. ECG was continuously recorded for additional 21 minutes.

Heart rate measurements: The effect of drugs on heart rate was determined in anesthetized RyR2 R4496C mutant mice or wild type littermates. Briefly, mice were lightly anesthetized with isoflurane vapor (0.5-1%). Anesthetized mice were placed on a heating pad (28 °C) and subcutaneous needle electrodes were applied to the left, right upper limb and right lower limb for ECG recordings (BIOPAC MP System, Goleta, CA). The animals' ECG was continuously monitored under anesthesia until the heart rate became stabilized. Isoproterenol (Iso, 0.4mg/kg) was then intraperitoneally administered into the mice to increase the heart rate. Three minutes later, various drugs were intraperitoneally administered to assess their impact on Iso-stimulated heart rate. The Iso-stimulated heart rate was continuously monitored via ECG recordings.

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* test. Statistical analyses were performed using the SPSS V.15.0 (SPSS, Chicago, IL). A P value <0.05 was considered to be statistically significant.

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