HHS Public Access

Author manuscript

Biochem J. Author manuscript; available in PMC 2016 September 01.

Published in final edited form as:

Biochem J. 2015 September 1; 470(2): 233–242. doi:10.1042/BJ20150548.

Non-β-blocking *R*-carvedilol enantiomer suppresses Ca^{2+} waves and stress-induced ventricular tachyarrhythmia without lowering heart rate or blood pressure

Jingqun Zhang[*,1], **Qiang Zhou**[*,1], **Chris D. Smith**[†], **Haiyan Chen**[*], **Zhen Tan**[*], **Biyi Chen**[‡], **Alma Nani**[*], **Guogen Wu**[*], **Long-Sheng Song**[‡], **Michael Fill**[*,2], **Thomas G. Back**[†,2], and **S.R. Wayne Chen**[*,§,2,3]

[*]Department of Molecular Biophysics and Physiology, Rush University Medical Center, Chicago, IL 60612 U.S.A

[†]Department of Chemistry, University of Calgary, Calgary, Alberta, Canada, T2N 1N4

[‡]Division of Cardiovascular Medicine, Department of Internal Medicine, University of Iowa Carver College of Medicine, Iowa City, IA 52242, U.S.A

[§]The Libin Cardiovascular Institute of Alberta, Department of Physiology and Pharmacology, University of Calgary, Calgary, Alberta, Canada, T2N 4N1

Abstract

Carvedilol is the current β-blocker of choice for suppressing ventricular tachyarrhythmia (VT). However, carvedilol's benefits are dose-limited, attributable to its potent β-blocking activity that can lead to bradycardia and hypotension. The clinically used carvedilol is a racemic mixture of β-blocking *S*-carvedilol and non-β-blocking *R*-carvedilol. We recently reported that novel non-β-blocking carvedilol analogues are effective in suppressing arrhythmogenic Ca^{2+} waves and stress-induced VT without causing bradycardia. Thus, the non-β-blocking *R*-carvedilol enantiomer may also possess this favourable anti-arrhythmic property. To test this possibility, we synthesized *R*-carvedilol and assessed its effect on Ca^{2+} release and VT. Like racemic carvedilol, *R*-carvedilol directly reduces the open duration of the cardiac ryanodine receptor (RyR2), suppresses spontaneous Ca^{2+} oscillations in human embryonic kidney (HEK) 293 cells, Ca^{2+} waves in cardiomyocytes in intact hearts and stress-induced VT in mice harbouring a catecholaminergic polymorphic ventricular tachycardia (CPVT)-causing RyR2 mutation. Importantly, *R*-carvedilol did not significantly alter heart rate or blood pressure. Therefore, the non-β-blocking *R*-carvedilol enantiomer represents a very promising prophylactic treatment for Ca^{2+}-triggered arrhythmia

[2]Correspondence may be addressed to any of these authors (michael_fill@rush.edu, tgback@ucalgary.ca or swchen@ucalgary.ca).
[1]Alberta Innovates-Health Solutions (AIHS) Scientist.
[3]Present address: Department of Cardiology of Tongji Hospital, Tongji Medical School, Huazhong University of Science and Technology, Wuhan, China.

AUTHOR CONTRIBUTION

Jingqun Zhang, Qiang Zhou, Chris Smith, Long-Sheng Song, Michael Fill, Thomas Back and Wayne Chen designed the research. Jingqun Zhang, Qiang Zhou, Chris Smith, Haiyan Chen, Zhen Tan, Biyi Chen, Alma Nani and Guogen Wu performed the research. Jingqun Zhang, Qiang Zhou, Chris Smith, Thomas Back and Wayne Chen analysed the data. Jingqun Zhang, Chris Smith, Long-Sheng Song, Michael Fill, Thomas Back and Wayne Chen wrote the paper.

without the bradycardia and hypotension often associated with racemic carvedilol. Systematic clinical assessments of *R*-carvedilol as a new anti-arrhythmic agent may be warranted.

Keywords

β-blockers; carvedilol enantiomers; Ca^{2+} waves; Ca^{2+} -triggered arrhythmias; ryanodine receptor; sarcoplasmic reticulum

INTRODUCTION

Ventricular tachyarrhythmia (VT) is a leading cause of sudden cardiac death in patients with heart failure (HF). Despite an intense effort to suppress VT in HF, there are only a few anti-VT treatments that bring a significant survival benefit to HF patients [1–3]. Large clinical trials have shown that carvedilol, a non-selective β-blocker with α-blocking and antioxidant activities, substantially reduces VT and sudden death risk in HF patients [4–8]. However, the molecular mechanism underlying carvedilol's favourable anti-arrhythmic effect is unclear.

A major cause of VT in HF is abnormal diastolic intracellular Ca^{2+} handling [9,10]. It is well known that cardiac stress can lead to sarcoplasmic reticulum (SR) Ca^{2+} overload. Elevated SR Ca^{2+} load promotes cardiac ryanodine receptor (RyR2) opening, resulting in spontaneous SR Ca^{2+} release in the form of Ca^{2+} waves [9–11]. These Ca^{2+} waves can evoke delayed after depolarizations (DADs), triggered arrhythmia and sudden death [12–18]. Importantly, Ca^{2+} waves and DADs frequently occur in failing hearts [9,10]. Furthermore, naturally occurring mutations in RyR2 cause catecholaminergic polymorphic ventricular tachycardia (CPVT) by enhancing the propensity for Ca^{2+} waves and DADs [19–26]. Thus, spontaneous Ca^{2+} waves are a common cause of CPVT as well as VT in HF.

Suppressing abnormal RyR2-mediated Ca^{2+} waves would thus represent an effective strategy for preventing Ca^{2+} -triggered arrhythmias in both CPVT and HF. In support of the present view, we have recently shown that carvedilol is the only β-blocker tested that effectively suppresses spontaneous Ca^{2+} waves by directly modifying RyR2 gating, an action independent of its well-described β-blocking, α-blocking and antioxidant activities [27,28]. Racemic carvedilol, but not metoprolol, is also able to suppress CPVT in a RyR2 mutant mouse model [27]. This unique inhibitory action of racemic carvedilol on Ca^{2+} waves probably contributes to its favourable anti-arrhythmic benefits.

Racemic carvedilol is a potent β-blocker with an IC_{50} of ~1 nM [29]. Unfortunately, a substantially higher carvedilol concentration (~1 μM) is required to optimally suppress Ca^{2+} waves [27] and these higher doses would result in excessive β-blockade and the accompanying adverse effects, such as bradycardia and hypotension [30,31]. In other words, the anti-Ca^{2+} wave benefit of racemic carvedilol is dose-limited by the agent's potent β-blocking activity. This is indeed the case, as clinical studies show that high doses of racemic carvedilol produced better outcomes [32], but were associated with bradycardia and hypotension [31]. We have recently isolated the RyR2-targeted Ca^{2+} wave inhibition of carvedilol by synthesizing several carvedilol analogues that possess minimal β-blockade, but still inhibit Ca^{2+} waves [27,28]. These novel carvedilol analogues suppress CPVT in mice

without causing bradycardia [27]. Further development of these non-*β*-blocking carvedilol analogues with anti-Ca^{2+} wave action is likely to lead to a new class of RyR2-targeted anti-arrhythmic agents that can be used to specifically limit Ca^{2+} -triggered arrhythmias.

Today, the clinically used carvedilol is a racemic mixture composed of *R*- and *S*-carvedilol enantiomers [33–35]. Interestingly, unlike S-carvedilol, the *R*-carvedilol enantiomer does not have *β*-blocking activity [35–38]. This raises the exciting possibility that, like our novel non-*β*-blocking carvedilol analogues, the *R*-carvedilol enantiomer may provide carvedilol's favourable RyR2-targeted anti-arrhythmic action without the adverse effects of excessive *β*-blockade. To test this possibility, we synthesized *R*-carvedilol and assessed its effect on single RyR2 channels, Ca^{2+} waves in cells and stress-induced VT in mice. *R*-carvedilol inhibited Ca^{2+} waves in intact hearts by directly modifying the gating of RyR2 and suppressed VT in mice harbouring a CPVT-causing RyR2 mutation (R4496C). Importantly, *R*-carvedilol, unlike racemic carvedilol, did not cause bradycardia or hypotension in mice. These results indicate that *R*-carvedilol could be an effective anti-arrhythmic agent for limiting fatal Ca^{2+}-triggered arrhythmias without the bradycardia or hypotension associated with racemic carvedilol.

MATERIALS AND METHODS

Animal studies

All animal studies were approved by the Institutional Animal Care and Use Committees at Rush University Medical Center or the University of Calgary and were performed in accordance with NIH (National Institutes of Health) guidelines. The knockin mouse model harbouring the CPVT RyR2 mutation (R4496C) [27] and its wild-type littermates in the 129/SvImJ background were used in the present research.

Synthesis of the (*R*)-(+)-carvedilol enantiomer

Commercially available (*R*)-glycidol was converted into its (*R*)-*o*-nitrobenzenesulfonate (nosylate) by the method of Shiratsuchi et al. [39]. The (*R*)-glycidyl nosylate (9.60 g, 37.0 mmol) in 35 ml of N,N-dimethylformamide (DMF) was added drop-wise to a cooled (0°C) solution of 4-hydroxycarbazole (6.90 g, 37.7 mmol) and sodium hydroxide (1.55 g, 38.7 mmol) in 100 ml of DMF and 1 ml of water. Stirring was continued for 5 h at 0°C and then at room temperature overnight. The mixture was diluted with brine, extracted with ethyl acetate and the combined organic layers were washed with saturated aqueous sodium bicarbonate, 1 M sodium hydroxide and brine. The resulting solution was dried over anhydrous sodium sulfate, concentrated under vacuum and subjected to flash chromatography over silica gel (elution with 2–4% ethyl acetate/toluene) to afford 7.95 g (90%) of the corresponding 4-[(*R*)-1-oxiranylmethoxy]-9*H*-carbazole as a white solid, melting point (mp) 159–160°C, with ^{1}H and ^{13}C NMR spectra identical to those of the racemic material.

2-(2-Methoxyphenoxy)ethylamine (7.00 g, 41.9 mmol) in 15 ml of propan-2-ol was added drop-wise to the above product (5.49 g, 22.9 mmol) in 35 ml of propan-2-ol. The mixture was refluxed for 1.5 h. The solvent was evaporated and the product was purified by flash

chromatography over silica gel (elution with 3–7% of methanol–dichloromethane) to provide 6.20 g (67%) of (*R*)-(+)-carvedilol as a white solid foam, mp 115–116°C; $[\alpha]_D^{21}$ + 17.3°C (c 1.0, acetic acid); lliterature mp 121–123°C; $[\alpha]_D^{20}$ + 18.4° (c 1, acetic acid) [40]. Elemental analysis calculated for $C_{24}H_{26}N_2O_4$: C 70.93, H 6.45, N 6.89; found: C 70.75, H 6.67, N 6.85. The product gave IR, ^1H and ^{13}C NMR spectra identical to those of authentic racemic carvedilol.

Single-cell Ca^{2+} imaging of HEK293 cells

Stable, inducible human embryonic kidney (HEK) 293 cells expressing the CPVT-causing RyR2 mutant, R4496C, display robust spontaneous Ca^{2+} oscillations when perfused with 1 mM Ca^{2+} in Krebs–Ringer–HEPES (KRH) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 6 mM glucose, 1.2 mM $MgCl_2$ and 25 mM HEPES, pH 7.4). These RyR2-R4496C cells were used to assess the impact of *R*-carvedilol on spontaneous Ca^{2+} oscillations. Ca^{2+} oscillations were measured using single-cell Ca^{2+} imaging and the fluorescent Ca^{2+} indicator dye fura 2 acetoxymethyl ester (fura 2/AM) (Invitrogen) as described previously [27]. Briefly, cells grown on glass coverslips for 20–24 h after induction by 1 μg/ml tetracycline were loaded with 5 μM fura 2/AM in KRH buffer 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 on an inverted microscope (Nikon TE2000-S) and perfused with KRH buffer containing $CaCl_2$ stepped from 0.1 mM, 0.5 mM to 1 mM for 5 min each. The cells were then continuously perfused with KRH buffer containing 1 mM $CaCl_2$ and *R*-carvedilol or DMSO (control) stepped from 0 μM to 3 μM, 10 μM and 30 μM for 8 min each. Caffeine (15 mM) was applied at the end of each experiment to verify the presence of functional RyR2 channels. Time-lapse images (0.25 frame/s) were captured and analysed with Nikon NIS-Elements software. Fluorescence intensities were measured from regions of interest centred on individual cells that responded to caffeine. All chemicals were obtained from Sigma unless otherwise specified.

Confocal Ca^{2+} imaging of intact hearts

Excised hearts were loaded with Rhod-2 AM (rhodamine 2 acetoxymethyl ester; 3–4 μM; Biotium) in Krebs–Henseleit (KH) solution (120 mM NaCl, 24 mM $NaHCO_3$, 11.1 mM glucose, 5.4 mM KCl, 1 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 10 mM taurine and 5 mM creatine, oxygenated with 95% O_2 and 5% CO_2) at room temperature for 40 min via a retrograde Langendorff perfusion system, as described previously [41]. After Rhod-2 AM loading, hearts were attached to a confocal microscope system and perfused sequentially with 5 ml of KH solution containing 0 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM Ca^{2+} and then continuously with 6 mM Ca^{2+} (37°C) to induce Ca^{2+} waves. *In situ* confocal line-scan imaging of Ca^{2+} signals arising from epicardial myocytes was performed and acquired at a rate of 1.93 ms per line. To minimize motion artefacts during Ca^{2+} imaging, blebbistatin (5–10 μM) was added to the perfusion solution. To eliminate the intrinsic sinus rhythm interrupting Ca^{2+} wave measurements, as well as to help standardize SR Ca^{2+} load, the arterioventricular (AV) node was ablated by electro-cautery. The AV-node-ablated hearts were then paced at 6 Hz for at least 30 s before being switched to 1 Hz to promote Ca^{2+} waves [42]. The frequency of Ca^{2+} waves per 100 μm line was measured.

Single-channel recordings in planar lipid bilayers

Heavy SR microsomes were prepared from rat ventricular muscle using the method described by Chamberlain et al. [43]. 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 10-μm thick teflon partition separating two compartments. The solution on one side of the bilayer (*cis*) was virtually grounded and initially contained 250 mM HEPES and 120 mM Tris/HCl (pH 7.4). The solution on the other side initially contained 250 mM HEPES and 10 mM $CaCl_2$ (pH 7.4). Heavy SR microsomes (5–15 μg) were added to the *cis* solution along with 500 mM CsCl and 2 mM $CaCl_2$ to promote microsome–bilayer fusion. SR vesicles were pre-incubated for 1 h with the tested drugs (1 μM) before their addition to the *cis* solution. After microsome fusion, the cytosolic side of the RyR2 channel faced the *cis* compartment [44,45] and solutions in both compartments were exchanged for cell-relevant solutions. The cell-relevant cytosolic solution contained 120 mM HEPES/Tris/HCl (pH 7.2), 50 μM free Ca^{2+} (0.5 mM EGTA), 1 mM free Mg^{2+} and 5 mM total ATP. The cell-relevant luminal solution contained 200 mM Cs-HEPES (pH 7.2), 1 mM free Mg^{2+} and 1 mM free Ca^{2+}. Solutions on both sides of the bilayer (before and after SR microsome fusion) contained 1 μM of the tested drug. Recipes for the Ca^{2+} buffer solutions were generated using WinMAXC 2.05 and verified by a Ca^{2+} electrode. Single RyR2 recordings were made at room temperature (20–22°C) with net current in the luminal-to-cytosolic direction. Analysis was done using pCLAMP9 software (Molecular Devices). Currents were sampled at 50 μs/pt and filtered (4-pole Bessel) at 1 kHz for display.

ECG recordings and induction of VTs in anaesthetized mice

RyR2-R4496C heterozygous mutant mice (RC) readily display polymorphic VT as well as bidirectional VT when challenged by an intraperitoneal injection of epinephrine (adrenaline) (1.2 mg/kg of body weight) and caffeine (100 mg/kg of body weight) cocktail (referred to as epi/caff) [27]. A 3–10 min baseline ECG (electrocardiogram; heart rate stable) was established before epi/caff injection. Following epi/caff injection, the ECG was recorded for 30 min (continuously). Briefly, mice were lightly anaesthetized with isoflurane vapour (0.5%) and 95% O_2. Anaesthetized mice were placed on a heating pad (27°C) and needle electrodes (BIOPAC MP System) were inserted subcutaneously into the right-upper limb and left-lower abdomen. The VT duration (as percentages of time) in each of ten consecutive 3-min periods or over the entire 30-min period post-injection was determined. VT was defined as three or more consecutive ectopic beats.

Drug treatments of RyR2-R4496C$^{+/-}$ mice

RyR2-R4496C$^{+/-}$ mice were treated with different doses of *R*-carvedilol (0.4, 0.8, 1.6 or 3.2 mg/kg/day) or DMSO of the same volume according to the body weight via intraperitoneal injection for five consecutive days. ECG recordings were performed on day five as described previously [27]. Heart rate before epi/caff injection and the VT duration after epi/caff challenge in control and drug-treated mice were determined.

Measurement of isoproterenol-stimulated heart rates

Drug (racemic carvedilol, *R*-carvedilol or DMSO) action on isoprenaline (also known as isoproterenol) (Iso)-stimulated heart rate was determined in anaesthetized wild-type mice [27]. Briefly, mice were lightly anaesthetized with isoflurane vapour (0.5%) and then placed on a heating pad (27°C) where needle electrodes were applied subcutaneously into the right-upper limb and left-lower abdomen for ECG recordings (BIOPAC MP System). The animals' ECGs were continuously monitored under anaesthesia until the heart rate stabilized. The heart rates were continuously monitored via ECG recordings. Iso (0.8 mg/kg) was then intraperitoneally injected to increase the heart rate. Three minutes later, various drugs were intraperitoneally injected to evaluate their action on the Iso-stimulated heart rate, which were continuously monitored via ECG recordings.

Blood pressure measurements

R-carvedilol (1.6 mg/kg/day), racemic carvedilol (1.6 mg/kg/day) or DMSO of the same volume according to the body weight was administered to 129/SvImJ wild-type mice by intraperitoneal injection for five consecutive days. Blood pressure before and after the 5-day treatment was measured by using the CODA™ 8-Channel High Throughput Non-Invasive tail-cuff blood pressure system (Kent Scientific) following the manufacturer's instructions. Briefly, mice were allowed to enter holders freely. The holders were placed on an infrared warming platform (25°C). A blood pressure cuff was then placed on the mouse's tail. Recordings were performed with five acclimation cycles, 20 test cycles, 5 s between cycles, a deflation time of 20 s, a maximum occlusion pressure of 250 mmHg and a minimum volume of 15 μl. Systolic, diastolic and mean blood pressures were determined using the CODA program.

Statistical analysis

All values shown are means ± S.E.M. unless indicated otherwise. To test for differences between groups, we used Student's *t* test (two-tailed) or one-way ANOVA with post-hoc test. The paired *t* test was used to compare blood pressures before and after drug treatment. Statistical analyses were performed using the SPSS V.15.0 (SPSS). A *P*-value <0.05 was considered statistically significant. To make *a priori* statistical power calculations, we used G*Power software to predict the needed number of experiments to detect a significant difference.

RESULTS

R-carvedilol suppresses spontaneous Ca^{2+} oscillations in HEK293 cells

To determine whether the non-β-blocking *R*-carvedilol enantiomer is able to suppress spontaneous Ca^{2+} release, we synthesized *R*-carvedilol (Figure 1A) and assessed its effect on spontaneous Ca^{2+} oscillations in HEK293 cells expressing a CPVT-causing RyR2 mutation (R4496C). As shown in Figure 1(B), RyR2-R4496C-expressing HEK293 cells displayed spontaneous Ca^{2+} oscillations in the absence of *R*-carvedilol (control). Application of *R*-carvedilol inhibited these Ca^{2+} oscillations in a concentration-dependent manner (Figures 1C and 1D), similar to that of racemic carvedilol [27]. Thus, like racemic

carvedilol, *R*-carvedilol is also able to suppress spontaneous RyR2-mediated Ca^{2+} release in HEK293 cells.

R-carvedilol directly modifies the gating of the RyR2 channel

We have shown that racemic carvedilol suppresses spontaneous Ca^{2+} release by directly altering RyR2 channel function [27]. To determine whether *R*-carvedilol directly modifies the function of the RyR2 channel, we assessed the effect of *R*-carvedilol on single RyR2 channels in lipid bilayers. We have previously shown that with longer drug incubation time, lower carvedilol concentrations were able to modify the gating of single RyR2 channels and suppress spontaneous Ca^{2+} release in cardiomyocytes [27]. Hence, we pre-incubated SR microsomes with *R*-carvedilol (1 μM) for 1 h prior to their incorporation into lipid bilayers. We found that *R*-carvedilol (1 μM) significantly reduced the mean open time (OT; 40.8 ± 12.9–9.1 ± 3.6 ms; $P < 0.05$) and increased the event frequency (133 ± 15–257 ± 6 s^{-1}; $P < 0.001$) of single RyR2 channels (Figure 2). The open probability (P_o; 0.84 ± 0.06 compared with 0.57 ± 0.16) and mean closed time (CT; 3.7 ± 0.8 compared with 9.1 ± 6.1 ms) were not significantly altered by *R*-carvedilol (Figure 2).

The possibility that the drug-treated channels are displaying an increased frequency of sub-conductance states was evaluated by plotting amplitude frequency (all points) histograms (Figure 2). The control and drug-treated histograms each had two distinct peaks (closed and full open). There was no distinct peak associated with a sub-conductance state in the drug-treated case. Note that the full-open peak is skewed but this skewing is equal in both the control and drug-treated histograms. Thus, there was no evidence that *R*-carvedilol evokes a sub-conductance state.

We have also analysed the open and closed time distribution (Supplementary Figure S1). The control and *R*-carvedilol-treated open and closed time histograms were best fitted by assuming three open-state components. *R*-carvedilol reduced OT and narrowed the overall open time range. This smaller open time range is consistent with the smaller (compared with control) error level for the *R*-carvedilol-treated OT bar in (Figure 2). In contrast, *R*-carvedilol increased CT and widened the overall closed time range. This larger closed time range is consistent with the larger (compared with control) error level for the *R*-carvedilol-treated CT bar in Figure 2. These results demonstrate that, like racemic carvedilol [27], *R*-carvedilol directly modifies the gating of RyR2.

R-carvedilol suppresses spontaneous Ca^{2+} waves in intact hearts

To determine whether *R*-carvedilol suppresses spontaneous Ca^{2+} release in the context of cardiac cells and intact hearts, we assessed the effect of *R*-carvedilol on spontaneous Ca^{2+} waves in ventricular myocytes in intact hearts expressing the wave-promoting RyR2 mutation (R4496C). Spontaneous Ca^{2+} waves were induced by elevating the extracellular Ca^{2+} concentration to 6 mM and monitored by using confocal line-scan Ca^{2+} imaging of intact hearts (*ex vivo*) [42]. To eliminate the influence of the intrinsic sinus rhythm on Ca^{2+} wave, we ablated the AV node by electro-cautery [42]. AV-node-ablated hearts were electrically paced at 6 Hz to standardize SR Ca^{2+} loading before the pace was reduced to 1.0 Hz. As shown in Figure 3, spontaneous Ca^{2+} waves occurred in 89.3 ± 4.7% of cells with a

frequency of 3.3 ± 0.4 Hz/100 μm during the 1.0 Hz pacing period (Figure 3A) under control conditions (DMSO). As in our single RyR2 channel studies, these intact hearts were pre-incubated with *R*-carvedilol (1 μM) for 1 h, as this would allow carvedilol to accumulate in the tissue [46–51]. We found that *R*-carvedilol (1 μM) significantly reduced the occurrence (51.1 ± 7.9%) and frequency (0.6 ± 0.1 Hz/100 μm) of Ca^{2+} waves (i.e. the Ca^{2+} release that occurred between electrical stimulations), as compared with control ($P < 0.001$) (Figures 3B and 3C). We previously showed that racemic carvedilol also inhibits spontaneous Ca^{2+} waves in cardiomyocytes [27]. Thus, like racemic carvedilol, *R*-carvedilol suppresses spontaneous Ca^{2+} waves in cardiomyocytes.

R-carvedilol does not alter depolarization-induced Ca^{2+} transients in intact hearts

We also examined the action of *R*-carvedilol on depolarization-induced Ca^{2+} transients. The amplitude and dynamics of Ca^{2+} transients evoked by depolarization were measured using confocal line-scan Ca^{2+} imaging of intact hearts *ex vivo*. We found that *R*-carvedilol (1 μM) had no significant impact on the amplitude ($\Delta F/F_0$) (*R*-carvedilol: 1.64 ± 0.09 compared with DMSO: 1.86 ± 0.19), time to peak (*R*-carvedilol: 36.0 ± 0.7 ms compared with DMSO: 35.4 ± 1.0 ms) or time to 50% decay (T50); *R*-carvedilol: 58.0 ± 1.4 ms compared with DMSO: 60.0 ± 2.8 ms) of depolarization-evoked Ca^{2+} transients (Figure 4). Thus, *R*-carvedilol suppressed spontaneous Ca^{2+} waves that occur between electrical stimulations (Figure 3), but did not significantly alter the depolarization-induced Ca^{2+} transients.

R-carvedilol suppresses CPVT in RyR2-R4496C mutant mice

CPVT is caused by spontaneous Ca^{2+} waves. Our finding that *R*-carvedilol suppresses Ca^{2+} waves would suggest that *R*-carvedilol may also limit CPVT. To test this idea, we employed RyR2-R4496C mutant mice that are known to develop Ca^{2+} wave-evoked CPVT [27]. As shown in Figure 5, long-lasting VTs (66.4 ± 4.4%) including bidirectional VTs were readily induced in R4496C mice by the injection of caffeine and epinephrine (Figures 5A and 5C). Pre-treating R4496C mice with *R*-carvedilol daily for 5 days suppressed CPVT in a dose-dependent manner. At a dose of 1.6 mg kg/day, *R*-carvedilol reduced VT duration by ∼70% (to 20.8 ± 4.2%; $P < 0.001$) (Figures 5B and 5D). Since *R*-carvedilol was given daily for 5 days, it is possible that metabolites of *R*-carvedilol produced during the 5-day treatment may contribute to the observed effects of *R*-carvedilol, either by directly interacting with the RyR2 channel or via potential long-term effects on, e.g., gene expression or membrane properties. We have previously shown that racemic carvedilol (1.6 mg/kg/day for 5 days) reduces VT duration in RyR2-R4496C mice by ∼80% [27]. Thus, like racemic carvedilol, Ca^{2+} -wave-inhibiting *R*-carvedilol is also effective at suppressing CPVT.

R-carvedilol does not alter heart rate or blood pressure

Racemic carvedilol is a potent β-blocker that lowers both heart rate and blood pressure [35–38]. Since *R*-carvedilol has little β-blocking activity, *R*-carvedilol would be expected to have minimal impact on heart rate or blood pressure. Indeed, we found that *R*-carvedilol (1.6 mg/kg) had no effect on Iso-induced heart rate increase, whereas racemic carvedilol significantly suppressed it (Figure 6A). Pre-treating mice with racemic carvedilol (1.6 mg/kg/day) for 5 days also decreased heart rate [racemic carvedilol: 420 ± 8 beats per minute (bpm) compared with DMSO: 659 ± 9 bpm; $P < 0.001$], but pretreatment with *R*-

carvedilol (1.6 mg/kg/day) for 5 days did not (639 ± 9 bpm; Figure 6B). We also assessed the action of *R*-carvedilol on blood pressure. Pre-treating mice with racemic carvedilol (1.6 mg/kg/day) for 5 days significantly reduced the systolic, diastolic and mean blood pressure, whereas pre-treatment with *R*-carvedilol (1.6 mg/kg/day) for 5 days had no significant effect on any of these blood pressure measurements (only mean blood pressure shown; *R*-carvedilol: from 98.6 ± 4.5 mmHg to 96.4 ± 3.6 mmHg; compared with DMSO: from 98.4 ± 3.1 mmHg to 95.6 ± 3.0 mmHg; compared with racemic carvedilol: from 100.1 ± 2.6 mmHg to 83.3 ± 2.5 mmHg; Figure 6C). This is consistent with previous reports [35–38]. Taken together, our data indicate that, unlike racemic carvedilol, the non-*β*-blocking *R*-carvedilol enantiomer suppresses stress-induced VT without lowering heart rate or blood pressure.

DISCUSSION

The major finding of the present study is that the non-*β*-blocking *R*-carvedilol enantiomer suppresses arrhythmogenic spontaneous Ca^{2+} waves and stress-induced VT in mice without significantly lowering heart rate or blood pressure. We have shown that the clinically used racemic carvedilol also suppresses Ca^{2+} waves and stress-induced VT, but also causes bradycardia [27]. Indeed, bradycardia and hypotension are two major adverse effects of carvedilol attributable to this agent's potent *β*-blocking activity [30,31]. Therefore, *R*-carvedilol's capacity to suppress Ca^{2+} waves provides a promising new anti-arrhythmic prophylactic option for treating Ca^{2+} -triggered arrhythmias without the adverse effects of bradycardia and hypotension currently associated with racemic carvedilol.

Spontaneous SR Ca^{2+} release in the form of Ca^{2+} waves in cardiac cells can lead to DADs, which in turn can cause triggered activities, cardiac arrhythmias and sudden death [9–11]. These arrhythmogenic Ca^{2+} waves result from abnormal opening of RyR2 channels by elevated SR luminal Ca^{2+} during SR Ca^{2+} overload [21,22,26,52–56]. Many conditions such as excessive *β*-adrenergic receptor stimulation (as during emotional or physical stress) can lead to SR Ca^{2+} overload and store-overload-induced Ca^{2+} waves [12–18]. These Ca^{2+} waves and subsequent DADs frequently occur in diseased hearts [9,10]. Spontaneous Ca^{2+} waves are thus a common pathological entity. Therapeutically targeting spontaneous Ca^{2+} waves and especially the RyR2 channel may represent an effective and attractive approach to suppressing dangerous Ca^{2+} -wave-evoked VT in various pathological settings. Indeed, we have demonstrated that racemic carvedilol directly modifies the gating of single RyR2 channels and effectively suppresses Ca^{2+} wave and wave-evoked CPVT [27]. Additionally, we reported that a carvedilol analogue (VK-II-86) with minimal *β*-blocking activity also alters RyR2 gating and suppresses Ca^{2+} waves and CPVT [27]. In the present study, we demonstrate that the non-*β*-blocking *R*-carvedilol enantiomer reduces the duration of RyR2 channel openings and inhibits spontaneous Ca^{2+} waves and CPVT. Taken together, these observations support the practicality and effectiveness of this therapeutic strategy for preventing Ca^{2+} -mediated arrhythmias by modifying the gating of the RyR2 channel [27,56].

Our studies showed that *R*-carvedilol and novel carvedilol analogues (e.g. VK-II-86) have limited *β*-blocking activity, but still suppress Ca^{2+} waves and wave-evoked VT. This

indicates that β-blockade is not required for inhibiting Ca^{2+} waves and wave-evoked VT in our experimental setting. However, blocking β-adrenergic receptor signalling does suppress the stress-induced SR Ca^{2+} overload that promotes Ca^{2+} waves. Hence, β-blockade will reduce the likelihood of spontaneous Ca^{2+} waves. Indeed, clinical studies have consistently demonstrated the benefit of β-blockade in reducing the occurrence of VT and risk of sudden death [57–59]. Therefore, although excessive β-blockade can lead to adverse effects, adequate (well-managed) β-blockade is clearly valuable and beneficial. In this regard, combining the benefit of the non-β-blocking *R*-carvedilol with the benefit of a well-managed β-blockade regimen might provide a promising new approach to preventing Ca^{2+} -triggered arrhythmia with optimal control of heart rate and blood pressure.

We have recently produced and characterized a large number of carvedilol analogues that are capable of suppressing RyR2-mediated spontaneous Ca^{2+} release [27,28]. Developing these novel compounds into clinically useful anti-arrhythmic agents will require years of efforts. However, the *R*-carvedilol enantiomer is already present in racemic carvedilol that is being used clinically today. In other words, *R*-carvedilol is currently being used in humans along with the *S*-carvedilol enantiomer. More encouragingly, *R*-carvedilol alone has already been applied to healthy human volunteers without major adverse effects [35]. Thus, an important and exciting future effort is to systematically assess the safety and efficacy of *R*-carvedilol in human patients. Given that abnormal intracellular Ca^{2+} handling commonly occurs in many cardiac settings, *R*-carvedilol with its anti-Ca^{2+} wave action may provide a promising and effective treatment for CPVT as well as for VT in HF and other heart conditions associated with SR Ca^{2+} mishandling.

In summary, the clinically used carvedilol is a racemic mixture of the β-blocking *S*-carvedilol and non-β-blocking *R*-carvedilol. We show that *R*-carvedilol inhibits spontaneous Ca^{2+} waves by directly modifying the gating of RyR2 and suppresses Ca^{2+} wave-evoked VT without significantly lowering heart rate or blood pressure. Thus, *R*-carvedilol can limit Ca^{2+} -triggered arrhythmias without the unwanted bradycardia and hypotension that are typically associated with racemic carvedilol. Given our results in the present study and this agent's current clinical application, large-scale clinical assessments of *R*-carvedilol as a novel anti-arrhythmic agent may be warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING

This work was supported by the U.S. National Institutes of Health [grant numbers R01HL075210 (to M.F., T.G.B. and S.R.W.C.), R01HL057832 (to M.F.) and R01HL090905 (to L.S.S.)]; the Canadian Institutes of Health Research the Heart and Stroke Foundation of Alberta, the Canada Foundation for Innovation; and the Heart and Stroke Foundation/Libin Cardiovascular Institute Professorship in Cardiovascular Research (to S.R.W.C.).

Abbreviations

AV	arterioventricular

bpm	beats per minute
CPVT	catecholaminergic polymorphic ventricular tachycardia
CT	mean closed time
DAD	delayed after depolarization
DMF	dimethylformamide
ECG	electrocardiogram
epi/caff	epinephrine (adrenaline) and caffeine
fura 2/AM	fura 2 acetoxymethyl ester
HEK	human embryonic kidney
HF	heart failure
Iso	isoprenaline
KH	Krebs–Henseleit
KRH	Krebs–Ringer–HEPES
mp	melting point
OT	mean open time
P_o	open probability
Rhod-2 AM	rhodamine 2 acetoxymethyl ester
RyR2	cardiac ryanodine receptor
SR	sarcoplasmic reticulum
VT	ventricular tachyarrhythmia

References

1. Waldo AL, Camm AJ, deRuyter H, Friedman PL, MacNeil DJ, Pauls JF, Pitt B, Pratt CM, Schwartz PJ, Veltri EP. Effect of d-sotalol on mortality in patients with left ventricular dysfunction after recent and remote myocardial infarction. The SWORD investigators Survival with oral d-sotalol. Lancet. 1996; 348:7–12. [PubMed: 8691967]
2. Miller L. Limitations of current medical therapies for the treatment of heart failure. Rev Cardiovasc Med. 2003; 4(Suppl 2):S21–S29. [PubMed: 12776010]
3. Kamath GS, Mittal S. The role of antiarrhythmic drug therapy for the prevention of sudden cardiac death. Prog Cardiovasc Dis. 2008; 50:439–448. [PubMed: 18474286]
4. Poole-Wilson PA, Swedberg K, Cleland JG, Di Lenarda A, Hanrath P, Komajda M, Lubsen J, Lutiger B, Metra M, Remme WJ, et al. Comparison of carvedilol and metoprolol on clinical outcomes in patients with chronic heart failure in the carvedilol or metoprolol european trial (COMET): randomised controlled trial. Lancet. 2003; 362:7–13. [PubMed: 12853193]
5. Stroe AF, Gheorghiade M. Carvedilol: Beta-blockade and beyond. Rev Cardiovasc Med. 2004; 5(Suppl 1):S18–S27. [PubMed: 15184840]

6. Greenberg B. Nonselective versus selective beta-blockers in the management of chronic heart failure: clinical implications of the carvedilol or metoprolol european trial. Rev Cardiovasc Med. 2004; 5(Suppl 1):S10–S17. [PubMed: 15184835]
7. Remme WJ. Which beta-blocker is most effective in heart failure? Cardiovasc Drugs Ther. 2010; 24:351–358. [PubMed: 20596764]
8. DiNicolantonio JJ, Hackam DG. Carvedilol: A third-generation beta-blocker should be a first-choice beta-blocker. Expert Rev Cardiovasc Ther. 2012; 10:13–25. [PubMed: 22149523]
9. Bers DM. Calcium and cardiac rhythms: Physiological and pathophysiological. Circ Res. 2002; 90:14–17. [PubMed: 11786512]
10. Pogwizd SM, Bers DM. Cellular basis of triggered arrhythmias in heart failure. Trends Cardiovasc Med. 2004; 14:61–66. [PubMed: 15030791]
11. Engelhardt S, Hein L, Dyachenkow V, Kranias EG, Isenberg G, Lohse MJ. Altered calcium handling is critically involved in the cardiotoxic effects of chronic beta-adrenergic stimulation. Circulation. 2004; 109:1154–1160. [PubMed: 14967726]
12. Kass RS, Tsien RW. Fluctuations in membrane current driven by intracellular calcium in cardiac Purkinje fibers. Biophys J. 1982; 38:259–269. [PubMed: 6809065]
13. Orchard C, Eisner D, Allen D. Oscillations of intracellular Ca^{2+} in mammalian cardiac muscle. Nature. 1983; 304:735–738. [PubMed: 6888540]
14. Stern M, Kort A, Bhatnagar G, Lakatta E. Scattered-light intensity fluctuations in diastolic rat cardiac muscle caused by spontaneous Ca^{2+} -dependent cellular mechanical oscillations. J Gen Physiol. 1983; 82:119–153. [PubMed: 6886671]
15. Wier W, Kort A, Stern M, Lakatta E, Marban E. Cellular calcium fluctuations in mammalian heart: direct evidence from noise analysis of aequorin signals in Purkinje fibers. Proc Natl Acad Sci USA. 1983; 80:7367–7371. [PubMed: 6580652]
16. Marban E, Robinson SW, Wier WG. Mechanisms of arrhythmogenic delayed and early afterdepolarizations in ferret ventricular muscle. J Clin Invest. 1986; 78:1185–1192. [PubMed: 3771791]
17. Schlotthauer K, Bers DM. Sarcoplasmic reticulum Ca^{2+} release causes myocyte depolarization. underlying mechanism and threshold for triggered action potentials. Circ Res. 2000; 87:774–780. [PubMed: 11055981]
18. Xie LH, Weiss JN. Arrhythmogenic consequences of intracellular calcium waves. Am J Physiol Heart Circ Physiol. 2009; 297:H997–H1002. [PubMed: 19561309]
19. Priori SG, Napolitano C, Tiso N, Memmi M, Vignati G, Bloise R, Sorrentino VV, Danieli GA. Mutations in the cardiac ryanodine receptor gene (hRyR2) underlie catecholaminergic polymorphic ventricular tachycardia. Circulation. 2001; 103:196–200. [PubMed: 11208676]
20. Priori SG, Napolitano C, Memmi M, Colombi B, Drago F, Gasparini M, DeSimone L, Coltorti F, Bloise R, Keegan R, et al. Clinical and molecular characterization of patients with catecholaminergic polymorphic ventricular tachycardia. Circulation. 2002; 106:69–74. [PubMed: 12093772]
21. Jiang D, Xiao B, Yang D, Wang R, Choi P, Zhang L, Cheng H, Chen SRW. RyR2 mutations linked to ventricular tachycardia and sudden death reduce the threshold for store-overload-induced Ca^{2+} release (SOICR). Proc Natl Acad Sci USA. 2004; 101:13062–13067. [PubMed: 15322274]
22. Jiang D, Wang R, Xiao B, Kong H, Hunt DJ, Choi P, Zhang L, Chen SRW. Enhanced store overload-induced Ca^{2+} release and channel sensitivity to luminal Ca^{2+} activation are common defects of RyR2 mutations linked to ventricular tachycardia and sudden death. Circ Res. 2005; 97:1173–1181. [PubMed: 16239587]
23. Cerrone M, Colombi B, Santoro M, di Barletta MR, Scelsi M, Villani L, Napolitano C, Priori SG. Bidirectional ventricular tachycardia and fibrillation elicited in a knock-in mouse model carrier of a mutation in the cardiac ryanodine receptor. Circ Res. 2005; 96:e77–e82. [PubMed: 15890976]
24. Liu N, Colombi B, Memmi M, Zissimopoulos S, Rizzi N, Negri S, Imbriani M, Napolitano C, Lai FA, Priori SG. Arrhythmogenesis in catecholaminergic polymorphic ventricular tachycardia: Insights from a RyR2 R4496C knock-in mouse model. Circ Res. 2006; 99:292–298. [PubMed: 16825580]

25. Cerrone M, Noujaim SF, Tolkacheva EG, Talkachou A, O'Connell R, Berenfeld O, Anumonwo J, Pandit SV, Vikstrom K, Napolitano C, Priori SG, Jalife J. Arrhythmogenic mechanisms in a mouse model of catecholaminergic polymorphic ventricular tachycardia. Circ Res. 2007; 101:1039–1048. [PubMed: 17872467]
26. Priori SG, Chen SR. Inherited dysfunction of sarcoplasmic reticulum Ca2+ handling and arrhythmogenesis. Circ Res. 2011; 108:871–883. [PubMed: 21454795]
27. Zhou Q, Xiao J, Jiang D, Wang R, Vembaiyan K, Wang A, Smith CD, Xie C, Chen W, Zhang J, et al. Carvedilol and its new analogs suppress arrhythmogenic store overload-induced Ca^{2+} release. Nat Med. 2011; 17:1003–1009. [PubMed: 21743453]
28. Smith CD, Wang A, Vembaiyan K, Zhang J, Xie C, Zhou Q, Wu G, Chen SR, Back TG. Novel carvedilol analogues that suppress store-overload-induced Ca^{2+} release. J Med Chem. 2013; 56:8626–8655. [PubMed: 24124794]
29. Yao A, Kohmoto O, Oyama T, Sugishita Y, Shimizu T, Harada K, Matsui H, Komuro I, Nagai R, Matsuo H, Serizawa T, Maruyama T, Takahashi T. Characteristic effects of alpha1-beta1,2-adrenergic blocking agent, carvedilol, on [Ca2+]i in ventricular myocytes compared with those of timolol and atenolol. Circ J. 2003; 67:83–90. [PubMed: 12520158]
30. Packer M, Bristow MR, Cohn JN, Colucci WS, Fowler MB, Gilbert EM, Shusterman NH. The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. U.S carvedilol heart failure study group. N Engl J Med. 1996; 334:1349–1355. [PubMed: 8614419]
31. Ko DT, Hebert PR, Coffey CS, Curtis JP, Foody JM, Sedrakyan A, Krumholz HM. Adverse effects of {beta}-blocker therapy for patients with heart failure: A quantitative overview of randomized trials. Arch Intern Med. 2004; 164:1389–1394. [PubMed: 15249347]
32. Bristow MR, Gilbert EM, Abraham WT, Adams KF, Fowler MB, Hershberger RE, Kubo SH, Narahara KA, Ingersoll H, Krueger S, et al. Carvedilol produces dose-related improvements in left ventricular function and survival in subjects with chronic heart failure. Circulation. 1996; 94:2807–2816. [PubMed: 8941106]
33. van Zwieten PA. Pharmacodynamic profile of carvedilol. Cardiology. 1993; 82(Suppl 3):19–23. [PubMed: 8106159]
34. Frishman WH. Carvedilol. N Engl J Med. 1998; 339:1759–1765. [PubMed: 9845712]
35. Stoschitzky K, Koshucharova G, Lercher P, Maier R, Sakotnik A, Klein W, Liebmann PM, Lindner W. Stereoselective effects of (R)- and (S)-carvedilol in humans. Chirality. 2001; 13:342–346. [PubMed: 11400186]
36. Nichols AJ, Sulpizio AC, Ashton DJ, Hieble JP, Ruffolo RR Jr. The interaction of the enantiomers of carvedilol with alpha 1- and beta 1-adrenoceptors. Chirality. 1989; 1:265–270. [PubMed: 2577144]
37. Bartsch W, Sponer G, Strein K, Muller-Beckmann B, Kling L, Bohm E, Martin U, Borbe HO. Pharmacological characteristics of the stereoisomers of carvedilol. Eur J Clin Pharmacol. 1990; 38(Suppl 2):S104–S107. [PubMed: 1974497]
38. Hanada K, Asari K, Saito M, Kawana J, Mita M, Ogata H. Comparison of pharmacodynamics between carvedilol and metoprolol in rats with isoproterenol-induced cardiac hypertrophy: effects of carvedilol enantiomers. Eur J Pharmacol. 2008; 589:194–200. [PubMed: 18534575]
39. Shiratsuchi M, Kawamura K, Akashi T, Ishihama H, Nakamura M, Takenaka F. Synthesis and activity of optical isomers of nipradilol. Chem Pharm Bull. 1987; 35:3691–3698. [PubMed: 2893666]
40. Leinert, H. Process for the preparation of optically-active carbazole derivatives, new R- and S-carbazole derivatives and pharmaceutical compositions containing these compounds. US Pat 4,697,022. 1987.
41. Wei S, Guo A, Chen B, Kutschke W, Xie YP, Zimmerman K, Weiss RM, Anderson ME, Cheng H, Song LS. T-tubule remodeling during transition from hypertrophy to heart failure. Circ Res. 2010; 107:520–531. [PubMed: 20576937]
42. Zhang J, Chen B, Zhong X, Mi T, Guo A, Zhou Q, Tan Z, Wu G, Chen AW, Fill M, et al. The cardiac ryanodine receptor luminal Ca^{2+} sensor governs Ca2+ waves, ventricular tachyarrhythmias and cardiac hypertrophy in calsequestrin-null mice. Biochem J. 2014; 461:99–106. [PubMed: 24758151]

43. Chamberlain BK, Volpe P, Fleischer S. Calcium-induced calcium release from purified cardiac sarcoplasmic reticulum vesicles: general characteristics. J Biol Chem. 1984; 259:7540–7546. [PubMed: 6736018]
44. Tu Q, Velez P, Cortes-Gutierrez M, Fill M. Surface charge potentiates conduction through the cardiac ryanodine receptor channel. J Gen Physiol. 1994; 103:853–867. [PubMed: 8035165]
45. Qin J, Valle G, Nani A, Nori A, Rizzi N, Priori SG, Volpe P, Fill M. Luminal Ca2+ regulation of single cardiac ryanodine receptors: Insights provided by calsequestrin and its mutants. J Gen Physiol. 2008; 131:325–334. [PubMed: 18347081]
46. Hjalmarson A. Cardioprotection with beta-adrenoceptor blockers. does lipophilicity matter? Basic Res Cardiol. 2000; 95(Suppl 1):I41–I45. [PubMed: 11192352]
47. Caron G, Steyaert G, Pagliara A, Reymond F, Crivori P, Gaillard P, Carrupt PA, Avdeef A, Comer J, Box KJ, et al. Structure-lipophilicity relationships of neutral and protonated beta-blockers. Helv Chim Acta. 1999; 82:1211–1222.
48. Neugebauer G, Akpan W, von Mollendorff E, Neubert P, Reiff K. Pharmacokinetics and disposition of carvedilol in humans. J Cardiovasc Pharmacol. 1987; 10(Suppl 11):S85–S88. [PubMed: 2454375]
49. Doze P, Elsinga PH, Maas B, Van Waarde A, Wegman T, Vaalburg W. Synthesis and evaluation of radiolabeled antagonists for imaging of beta-adrenoceptors in the brain with PET. Neurochem Int. 2002; 40:145–155. [PubMed: 11738481]
50. Fujimaki M. Stereoselective disposition and tissue distribution of carvedilol enantiomers in rats. Chirality. 1992; 4:148–154. [PubMed: 1586585]
51. Stahl E, Mutschler E, Baumgartner U, Spahn-Langguth H. Carvedilol stereopharmacokinetics in rats: affinities to blood constituents and tissues. Arch Pharm. 1993; 326:529–533.
52. Bassani JW, Yuan W, Bers DM. Fractional SR Ca release is regulated by trigger Ca and SR Ca content in cardiac myocytes. Am J Physiol. 1995; 268:C1313–C1319. [PubMed: 7762626]
53. Shannon TR, Ginsburg KS, Bers DM. Potentiation of fractional sarcoplasmic reticulum calcium release by total and free intra-sarcoplasmic reticulum calcium concentration. Biophys J. 2000; 78:334–343. [PubMed: 10620297]
54. Diaz ME, Trafford AW, O'Neill SC, Eisner DA. Measurement of sarcoplasmic reticulum Ca^{2+} content and sarcolemmal Ca^{2+} fluxes in isolated rat ventricular myocytes during spontaneous Ca^{2+} release. J Physiol. 1997; 501:3–16. [PubMed: 9174989]
55. Eisner DA, Kashimura T, Venetucci LA, Trafford AW. From the ryanodine receptor to cardiac arrhythmias. Circ J. 2009; 73:1561–1567. [PubMed: 19667488]
56. Chen W, Wang R, Chen B, Zhong X, Kong H, Bai Y, Zhou Q, Xie C, Zhang J, Guo A, et al. The ryanodine receptor store-sensing gate controls Ca2+ waves and Ca2+ -triggered arrhythmias. Nat Med. 2014; 20:184–192. [PubMed: 24441828]
57. Anh D, Marine JE. Beta blockers as anti-arrhythmic agents. Heart Fail Rev. 2004; 9:139–147. [PubMed: 15516862]
58. Zicha S, Tsuji Y, Shiroshita-Takeshita A, Nattel S. Beta-blockers as antiarrhythmic agents. Handb Exp Pharmacol. 2006; 171:235–266. [PubMed: 16610347]
59. Adamson PB, Gilbert EM. Reducing the risk of sudden death in heart failure with beta-blockers. J Card Fail. 2006; 12:734–746. [PubMed: 17174236]

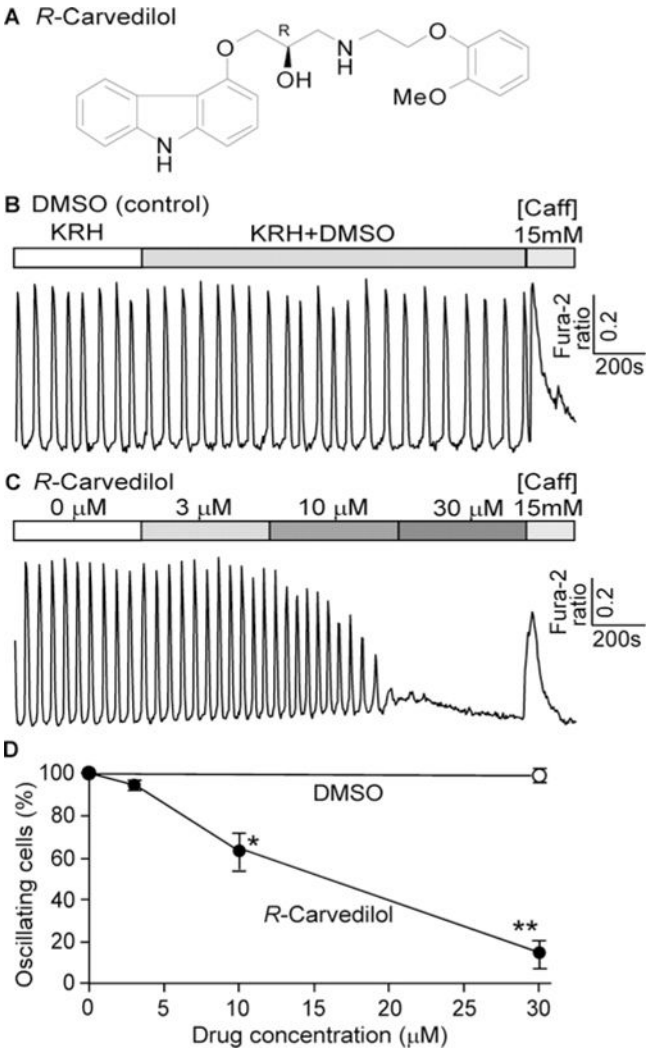

Figure 1. *R*-carvedilol inhibits spontaneous Ca^{2+} release in HEK293 cells

(**A**) Chemical structure of *R*-carvedilol. (**B** and **C**) Stable inducible HEK293 cells expressing the RyR2-R4496C mutant were loaded with 5 μM fura2/AM in KRH buffer. Representative traces of fura 2 ratios in HEK293 cells (~197–419) perfused with 1 mM extracellular Ca^{2+} in KRH buffer containing DMSO (**B**) or various concentrations of *R*-carvedilol (0, 3, 10 and 30 μM) are shown. (**C**) Percentage of cells showing spontaneous Ca^{2+} oscillations in cells treated with DMSO (control) or *R*-carvedilol. Data shown are means ± S.E.M. (n=5–7; * P < 0.05, ** P < 0.001 compared with DMSO).

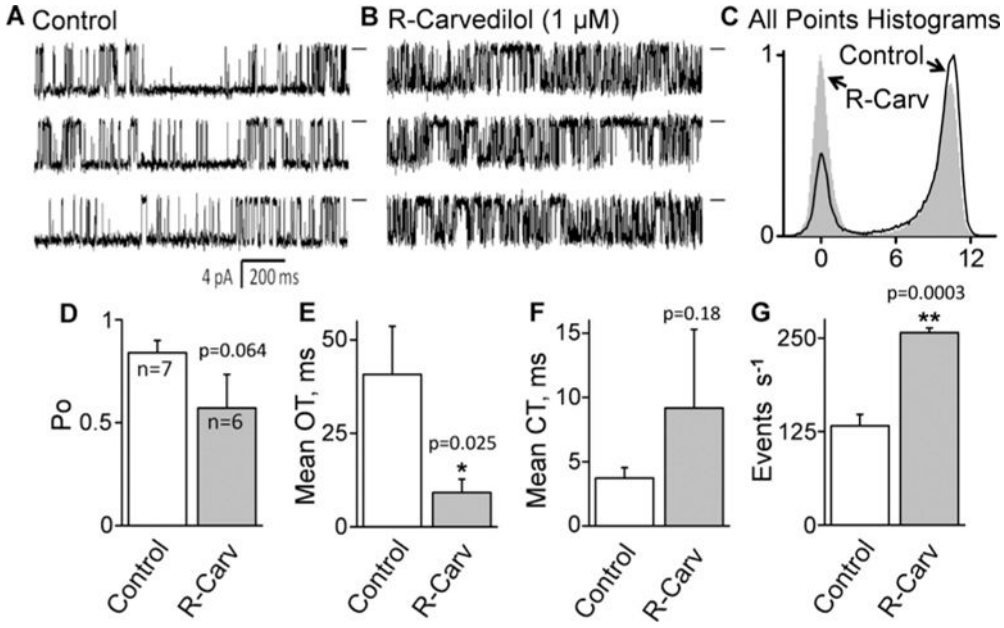

Figure 2. *R*-carvedilol modifies the gating of single RyR2 channels

Single rat RyR2 channels were recorded in quasi-physiological salt solutions. SR microsomes were pre-incubated for 1 h without (control) (**A**) or with (1 μM) *R*-carvedilol (**B**) before they were fused into the lipid bilayer with the drug in both the cytosolic and luminal solutions. Single control (n=7) and *R*-carvedilol-treated (n = 6) channels were recorded at −40 mV. Openings are downward. Baselines are indicated (short bars). All-point histograms (**C**), P_o (**D**), OT (**E**), CT (**F**) and event frequency (s^{-1}) (**G**) are shown (*$P < 0.05$; **$P < 0.01$ compared with control).

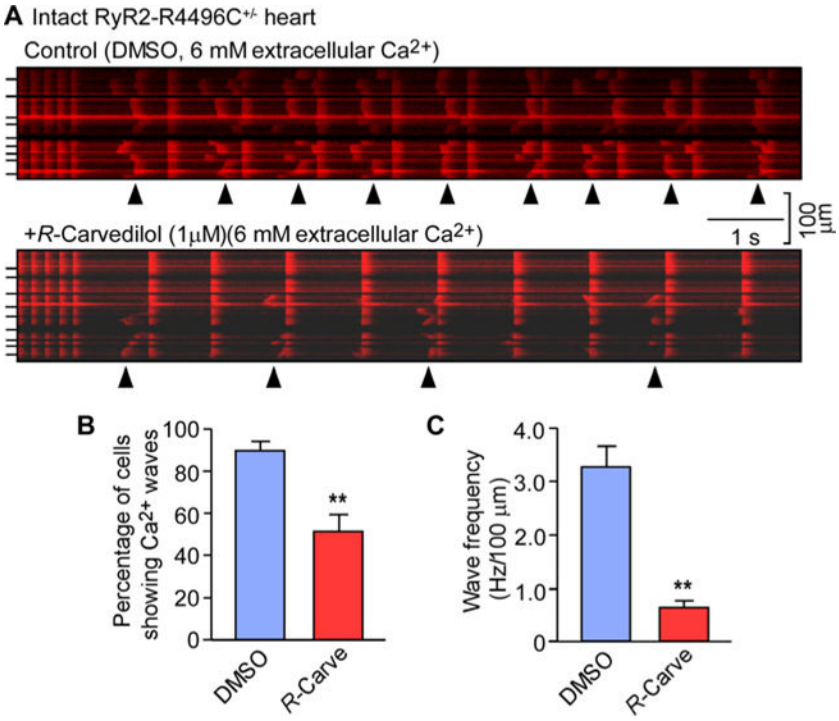

Figure 3. *R*-carvedilol suppresses spontaneous Ca^{2+} waves in intact hearts

Intact hearts isolated from RyR2-R4496C$^{+/-}$ mice were loaded with Rhod-2 AM and Langendorff-perfused with KH solution containing 6 mM extracellular Ca^{2+} to induce SR Ca^{2+} overload. Blebbistatin (10 μM) was used to inhibit muscle contraction. The AV node was ablated by electro-cautery and the hearts were paced at 6 Hz first and then the pace was switched to 1 Hz. Intracellular Ca^{2+} dynamics in epicardial ventricular myocytes in intact hearts was monitored by using line-scan confocal Ca^{2+} imaging. (**A**) Representative line-scan images of Ca^{2+} dynamics in hearts treated with DMSO or *R*-carvedilol (1 μM). (**B**) Percentage of cells showing Ca^{2+} waves. (**C**) Frequency of spontaneous Ca^{2+} waves. Arrowheads show the occurrence of Ca^{2+} waves. Short bars to the left indicate cell boundaries within the intact heart. Data shown are means ± S.E.M. from 27–34 areas of four hearts for each group (**P <0.001 compared with DMSO).

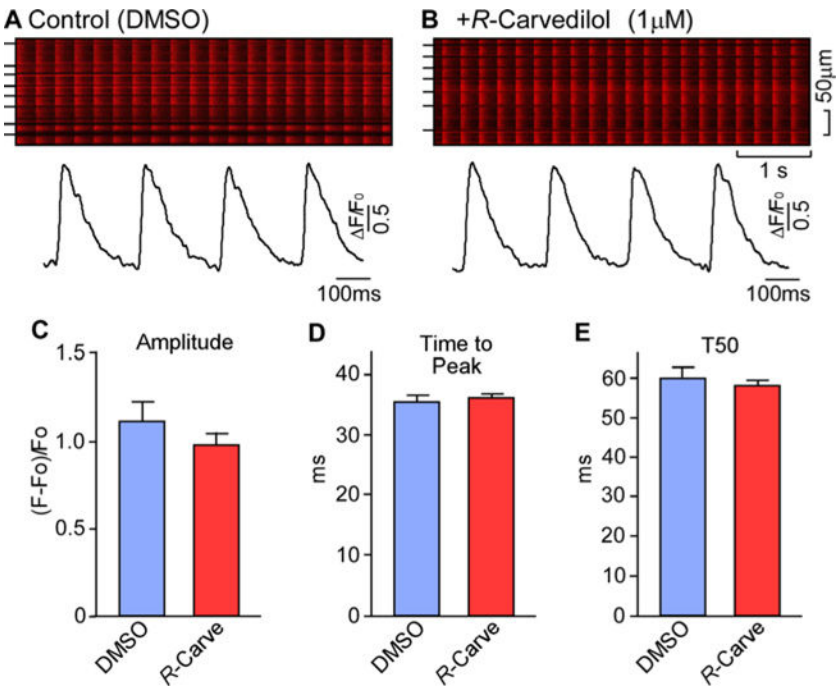

Figure 4. *R*-carvedilol does not markedly affect depolarization-induced Ca^{2+} transients in intact hearts

Intact hearts isolated from RyR2-R4496C$^{+/-}$ mice were loaded with Rhod-2 AM in KH solution and Langendorff-perfused with blebbistatin (10 μM) to inhibit muscle contraction. The perfused heart was paced at 4 Hz and Ca^{2+} transients were monitored by line-scan confocal Ca^{2+} imaging. Representative images/traces in hearts treated with DMSO (**A**) or with *R*-carvedilol (1 μM) (**B**). Short bars to the left indicate cell boundaries within the intact heart. The amplitude (**C**), time to peak (**D**) and time to 50% decay (T50) (**E**) of Ca^{2+} transients in hearts treated with DMSO or *R*-carvedilol are shown. Data shown are means ± S.E.M. from six or seven areas of three or four hearts for each group.

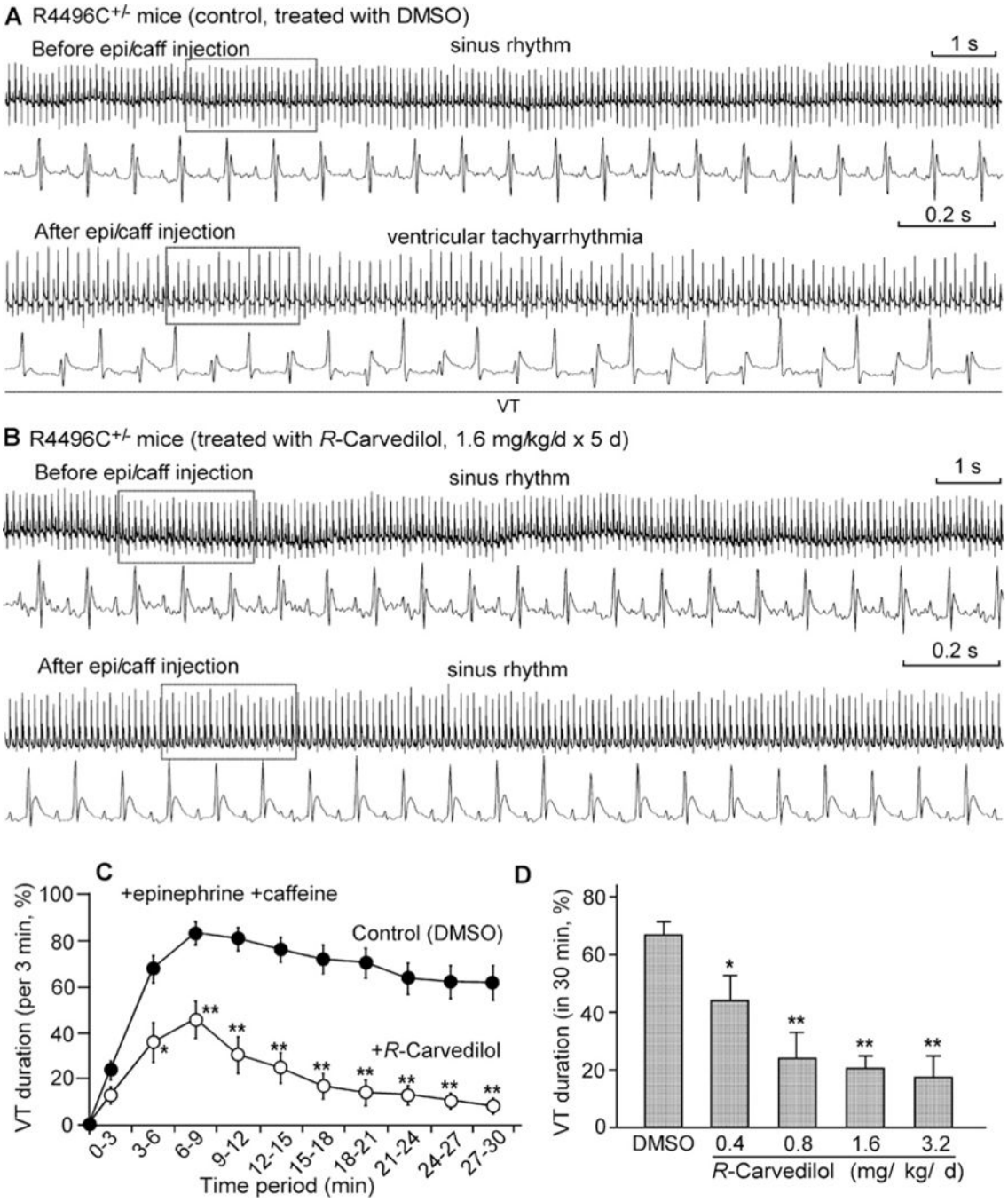

Figure 5. *R*-Carvedilol suppresses CPVT in RyR2-R4496C$^{+/-}$ mutant mice

Representative ECG recordings (15 s-trace at top, 2 s-trace at bottom) of RyR2-R4496C$^{+/-}$ mice treated with DMSO (control) (**A**) or with *R*-carvedilol (1.6 mg/kg/day for 5 days) (**B**) before (top panels) and 3–6 min after (bottom panels) intraperitoneal injection of epinephrine (1.2 mg/kg) and caffeine (100 mg/kg). VT occurred intermittently and percentage time in VT (VT duration) in mice treated with DMSO (control) or R -carvedilol (1.6 mg/kg/day) was measured in sequential 3-min periods post-injection (**C**). Average time in VT (VT duration) in mice treated with DMSO or various doses of *R*-carvedilol (0.4, 0.8,

1.6 and 3.2 mg/kg/day) was measured (post-injection) over the entire 30-min recording period (**D**). The time scale bar sets for ECG traces before and after injection of epinephrine and caffeine are the same. Data shown are means ± S.E.M. from 12–38 mice for each group (*P <0.05, **P <0.001 compared with DMSO).

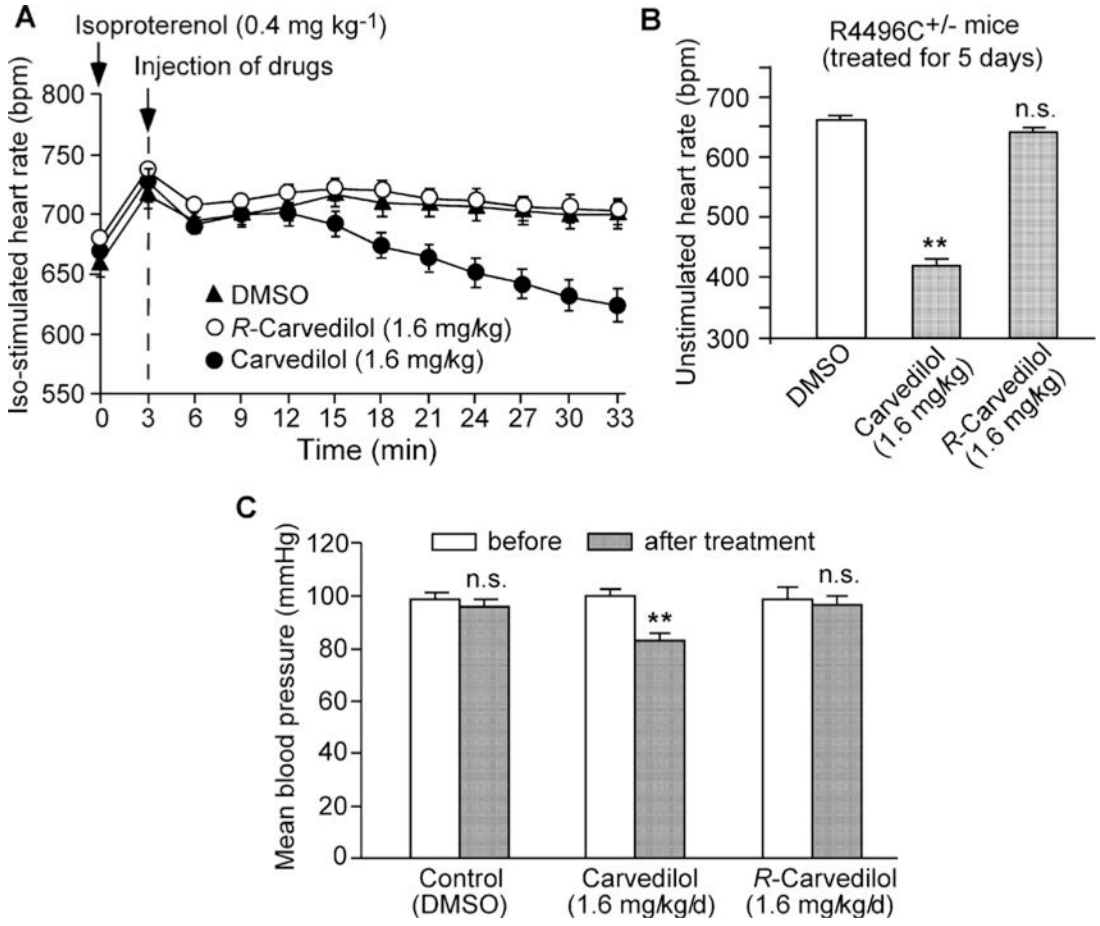

Figure 6. Effect of *R*-carvedilol on heart rate and blood pressure

(**A**) Iso-stimulated heart rate was monitored by ECG recordings in R4496C$^{+/-}$ mice (n=6 for each group) after the injection of Iso (0.8 mg/kg), followed by the treatment with DMSO (control), *R*-carvedilol (1.6 mg/kg) or racemic carvedilol (1.6 mg/kg). (**B**) Unstimulated heart rate was determined by ECG recordings in R4496C$^{+/-}$ mice (n = 18–38 for each group) after the treatment with DMSO (control), *R*-carvedilol (1.6 mg/kg/day) or racemic carvedilol (1.6 mg/kg/day) for 5 days. (**C**) Mean blood pressure was determined by measuring the systolic and diastolic blood pressure using the CODA™ high-throughput non-invasive tail-cuff blood pressure system in wild-type mice (n=7–11 for each group) treated with DMSO (control), *R*-carvedilol (1.6 mg/kg/day) or racemic carvedilol (1.6 mg/kg/day) for 5 days. Data shown are means ± S.E.M. (**P<0.001; compared with DMSO).