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Research Article

Suppression of ryanodine receptor function prolongs Ca^{2+} release refractoriness and promotes cardiac alternans in intact hearts

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Beat-to-beat alternations in the amplitude of the cytosolic Ca^{2+} transient (Ca^{2+} alternans) are thought to be the primary cause of cardiac alternans that can lead to cardiac arrhythmias and sudden death. Despite its important role in arrhythmogenesis, the mechanism underlying Ca^{2+} alternans remains poorly understood. Here, we investigated the role of cardiac ryanodine receptor (RyR2), the major Ca^{2+} release channel responsible for cytosolic Ca^{2+} transients, in cardiac alternans. Using a unique mouse model harboring a suppression-of-function (SOF) RyR2 mutation (E4872Q), we assessed the effect of genetically suppressing RyR2 function on Ca^{2+} and action potential duration (APD) alternans in intact hearts, and electrocardiogram (ECG) alternans *in vivo*. We found that RyR2-SOF hearts displayed prolonged sarcoplasmic reticulum Ca^{2+} release refractoriness and enhanced propensity for Ca^{2+} alternans. RyR2-SOF hearts/mice also exhibited increased propensity for APD and ECG alternans. Caffeine, which enhances RyR2 activity and the propensity for catecholaminergic polymorphic ventricular tachycardia (CPVT), suppressed Ca^{2+} alternans in RyR2-SOF hearts, whereas carvedilol, a β-blocker that suppresses RyR2 activity and CPVT, promoted Ca^{2+} alternans in these hearts. Thus, RyR2 function is an important determinant of Ca^{2+}, APD, and ECG alternans. Our data also indicate that the activity of RyR2 influences the propensity for cardiac alternans and CPVT in an opposite manner. Therefore, overly suppressing or enhancing RyR2 function is pro-arrhythmic.

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Introduction

Cardiac alternans is a periodic, beat-to-beat alternation in the amplitude of contraction (mechanical alternans), a component of the electrocardiogram (ECG) waveform (e.g. T-wave alternans), the action potential duration (APD alternans), or the amplitude of the cytosolic Ca^{2+} transient (Ca^{2+} alternans). Cardiac alternans is frequently observed in various experimental settings and in patients with ischemic heart disease and heart failure [1–9], and is a well-recognized risk factor for ventricular fibrillation (VF) and sudden cardiac death [3,7,10–14]. Since its first description more than a century ago [15], cardiac alternans has been a subject of intensive investigation. However, despite its pivotal significance in arrhythmogenesis, the mechanisms underlying cardiac alternans remain poorly understood.

An increasing body of evidence supports the notion that among different forms of cardiac alternans, Ca^{2+} alternans plays a primary role. Chudin et al. [16] observed Ca^{2+} alternans in cardiomyocytes that were voltage-clamped, discarding APD alternans as a prerequisite for cardiac alternans. Furthermore, simultaneous recordings of membrane potential and Ca^{2+} transients in isolated

Biochemical Journal (2016) 473 3951–3964
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cardiomyocytes revealed that APD alternans did not occur without Ca^{2+} alternans, whereas Ca^{2+} alternans could be triggered in the absence of APD alternans [17]. Thus, APD alternans is secondary to and is not required for Ca^{2+} alternans. An increasing body of evidence indicates a primary role of Ca^{2+} dysregulation in cardiac alternans [4,9,16–23]. Therefore, understanding how Ca^{2+} alternans occurs is key to the understanding of the mechanisms of cardiac alternans.

In cardiac muscle cells, membrane depolarization triggers the release of Ca^{2+} from the sarcoplasmic reticulum (SR) via a mechanism known as Ca^{2+}-induced Ca^{2+} release (CICR) [24]. In this process, membrane depolarization activates the L-type Ca^{2+} channel, resulting in a small Ca^{2+} influx. This Ca^{2+} entry then opens the cardiac ryanodine receptor (RyR2), leading to a large SR Ca^{2+} release and subsequently muscle contraction. The amplitude of the Ca^{2+} transient (CICR) depends on (i) the L-type Ca^{2+} current (I_{Ca}) — the trigger for CICR, (ii) the SR Ca^{2+} content, and (iii) the activity of RyR2 [24]. In ventricular myocytes, it has consistently been shown that there are no beat-to-beat alternations in the peak I_{Ca} during Ca^{2+} alternans [9,25,26], and in human atrial myocytes, alternation in the peak I_{Ca} when present is a consequence rather than a cause of Ca^{2+} alternans [27,28], thus excluding I_{Ca} alternans as the cause of Ca^{2+} alternans. Alternation in SR Ca^{2+} content is also unlikely to be a primary cause of Ca^{2+} alternans, as Ca^{2+} alternans can occur with or without beat-to-beat alternations in SR Ca^{2+} content [19,26]. This leaves the activity of the RyR2 channel as a primary candidate responsible for Ca^{2+} alternans. It has been proposed that beat-to-beat alternations in the availability/recovery of functional RyR2s determine the propensity for Ca^{2+} alternans [29,30]. Consistent with this view, pharmacological interventions that alter the activity of RyR2 affect the propensity for Ca^{2+} alternans. For instance, inhibiting RyR2 activity by tetracaine, intracellular acidification, or metabolic inhibition (reducing ATP level) promotes Ca^{2+} alternans in single isolated cardiomyocytes [25,31,32]. These observations led to the proposition that depressed RyR2 function underlies Ca^{2+} alternans [23,26,32,33]. However, these pharmacological manipulations can affect many cellular targets and processes. Thus, direct evidence for the link between depressed RyR2 function and Ca^{2+} alternans is lacking. Furthermore, whether specifically suppressing RyR2 activity can lead to APD alternans and/or ECG alternans has yet to be demonstrated.

To determine the effect of specifically depressing RyR2 activity on cardiac alternans, we employed a genetically engineered mouse model harboring a suppression-of-function (SOF) RyR2 mutation E4872Q [34]. We have previously shown that the E4872Q mutation abolishes luminal Ca^{2+} activation, inhibits cytosolic Ca^{2+} activation, and markedly reduces the opening time and open probability of single RyR2 channels [34]. Here we carried out *in situ* laser-scanning confocal Ca^{2+} imaging in cardiomyocytes in intact wild-type (WT) and RyR2-SOF E4872Q mutant hearts (*ex vivo*). We found that RyR2-SOF prolongs the refractoriness of SR Ca^{2+} release and promotes Ca^{2+} alternans in intact hearts. We also showed, for the first time, that RyR2-SOF enhances the propensity for APD alternans in intact hearts and ECG alternans *in vivo*. Furthermore, caffeine, an agonist of RyR2 that can induce catecholaminergic polymorphic ventricular tachycardia (CPVT) [35,36], alleviates Ca^{2+} alternans in the RyR2-SOF hearts, whereas carvedilol, a β-blocker that suppresses RyR2 activity and CPVT [36], worsens it. Thus, although RyR2-SOF protects against CPVT [34], it enhances cardiac alternans. These results demonstrate that excessively suppressing or enhancing RyR2 activity is arrhythmogenic, and suggest that normalizing RyR2 function is key to minimize the propensity for CPVT and alternans-induced arrhythmias.

Experimental procedures

Animal studies

All animal studies were approved by the Institutional Animal Care and Use Committees at the University of Calgary and the University of Iowa, and performed in accordance with the NIH guidelines. The RyR2 mutant mice harboring an SOF mutation E4872Q in RyR2 (RyR2-E4872Q) were generated using the knock-in (KI) approach as recently described [34]. Heterozygous E4872Q mice were bred with 129-E mouse strain to produce heterozygous E4872Q mice and their WT littermates. There are no homozygous RyR2-E4872Q mice as the homozygous RyR2-E4872Q mutation is embryonic lethal [34]. Adult heterozygous RyR2-E4872Q mutant mice and WT littermates (8–12 weeks) were used for all experiments.

Laser-scanning confocal Ca^{2+} imaging of intact hearts

Heterozygous RyR2-E4872Q mutant mice and their WT littermates were killed by cervical dislocation. Their hearts were quickly removed and loaded with 4.4 μM Rhod-2 AM (Biotium, Inc., Hayward, CA) in Ca^{2+}-free

Biochemical Journal (2016) 473 3951–3964
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oxygenated Tyrode's buffer (118 mM NaCl, 5.4 mM KCl, 25 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 11.1 mM glucose, 10 mM taurine, and 5 mM creatine, pH 7.4) via the retrograde Langendorff perfusion system at 25°C for 45 min [37]. Extracellular Ca^{2+} was stepwise increased to 0.25, 0.5, 1.0, and 1.8 mM and kept at 1.8 mM Ca^{2+} plus 5 μM blebbistatin (Toronto Research Chemicals, Toronto, ON) at 35°C throughout the experiment. The Langendorff-perfused hearts were placed on a recording chamber mounted onto the Nikon A1R microscope for *in situ* confocal imaging (line scan) of Ca^{2+} signals from epicardial ventricular myocytes. The pixel size of the resulting line-scan images ranged between 1.8 and 2 ms in the temporal dimension and between 0.1 and 0.4 μm in the spatial dimension. Ca^{2+} alternans in the WT and RyR2-E4872Q mutant hearts in the absence or presence of caffeine (1 mM) or carvedilol (3 μM) was induced by rapid electrical stimulation of the hearts at increasing frequencies (5–12 Hz, 6 V).

Determination of refractoriness of SR Ca^{2+} release

The refractoriness of Ca^{2+}-induced Ca^{2+} release from the SR was determined using the S1S2 stimulation protocol as described recently with some modifications [23]. Briefly, Ca^{2+} transients in a Rhod-2 AM-loaded heart were first induced at 5 Hz for 30 s (S1), followed by a single S2 stimulation at a specific interval. The heart was repeatedly stimulated by a series of S1S2 protocols with progressively decreased S1S2 intervals (from 200 to 40 ms). Ca^{2+} transients before and after S2 stimulation were continuously recorded using the line-scan mode in the Nikon-A1R confocal microscope.

Monophasic action potential recordings in Langendorff-perfused hearts

Hearts from heterozygous RyR2-E4872Q mutant mice and their WT littermates were perfused with oxygenated Tyrode's buffer with 95% O_2 and 1.8 mM Ca^{2+} at 35°C during the experiment. A monophasic action potential (MAP) electrode was placed against the left ventricles for epicardial MAP recording. Baseline MAP of sinus rhythm was recorded for 5–10 min, and the hearts were paced at the right atrium from 5 to10 Hz at 4–10 mA for >20 s to induce APD alternans. The total length of the recording was 30 min. MAP signal was amplified by Gould amplifiers (models 13-G 4615-58, 13-4615-50, and 13-4615-71) and acquired at 2000 Hz/channel using a Data Translation (DT 2821) analog and a digital input–output board. Analysis of MAP signals was performed using the Acknowledgment software (BIOPAC MP System, Goleta, CA).

Telemetric ECG recordings in conscious mice

An intraperitoneal implantation of the ECG telemetric transmitter (EA-F20, Data Sciences International, USA) into the abdomen of the RyR2-WT and RyR2-SOF (heterozygous E4872Q) mice was performed under general anesthesia (2% inhaled isoflurane in O_2) as described recently with some minor modifications [38]. The negative lead was placed in the right upper chest, and the positive lead in the left abdomen to form the lead II configuration. After the implantation and leads placement, the abdominal fascia and skin were sequentially closed with a 6-0 Prolene suture. A heating pad was used throughout the surgery to maintain the mouse's temperature. Telemetric ECG recordings started 2 days after the surgical implantation. After 5 days of continuous ECG recordings, isoproterenol (ISO; 0.5 mg/kg) was administrated intraperitoneally into RyR2-WT and RyR2-SOF mice to determine the impact of adrenergic stimulation on ECG alternans. A period (2 min) of ECG recordings 5 min before ISO injection and 8 min after ISO injection was used for alternans analysis before and after adrenergic stimulation, respectively.

ECG recordings and induction of ventricular tachyarrhythmias in anesthetized mice

Heterozygous RyR2-E4872Q mice and their WT littermates were assessed for their susceptibility to stress-induced ventricular tachyarrhythmias using ECG recordings as recently described [39]. WT and mutant mice were lightly anesthetized with isoflurane vapor (0.5–1%) and 95% O_2 on a heating pad (27°C). Two subcutaneous needle electrodes were inserted into the right upper limb and left lower abdomen for ECG recordings (BIOPAC MP System, Goleta, CA). The animals' ECG was continuously monitored under anesthesia until the heart rate became stable. Baseline ECG was recorded for 5–10 min. For the induction of ventricular tachyarrhythmia, mutant mice and their WT littermates were subjected to intraperitoneal injection of epinephrine (3.0 mg/kg) and caffeine (150 mg/kg). ECG was continuously recorded for 30 min after the infusion of epinephrine and caffeine.

Biochemical Journal (2016) 473 3951–3964
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Image and signal processing

The following signal and image processing methods were implemented using MATLAB (The Mathworks, Inc., Boston, MA). Line-scan fluorescence images were filtered using a median filter applied iteratively many times according to an estimation of the image noise variance. Noise variance was robustly estimated by means of a median absolute deviation of the image pixels. Identification of individual cells in the line scan was performed by manually labeling the cell regions. Average fluorescence signals of individual cells in each line scan were automatically obtained by spatial averaging of the pixels belonging to each marked cell. Average fluorescence signals of single cells were further filtered by applying a continuous wavelet transform of the signal with Gaussian wavelets of order 2. Zero-crossing of the derivative of the resulting wavelet transform was used to accurately locate peaks and valleys in the fluorescence signals. Peaks were then classified as either stimulated or spontaneous using cross-correlation with the stimulation pulse train. Peak amplitudes were defined as the difference between the peak and the corresponding previous valley. For each cell, alternans ratio was measured as the ratio of the absolute value of the difference in amplitude between two consecutive peaks over the amplitude of the largest peak. The presence of alternans periods was established by requiring at least six consecutive stimulated peaks presenting an alternans ratio above 0.05. For each cell, alternans duration was defined as the cumulative elapsed time of alternans periods over the total duration of the line scan. The average alternans duration was determined by averaging alternans durations of all cells in one scan area; and the average alternans ratio was determined by averaging alternans ratios of cells that displayed alternans in the same scan area. Analysis of S1S2 refractoriness was performed using Nikon-A1R analysis system and Image J.

Statistical analysis

All values shown are mean ± SEM unless indicated otherwise. To test for differences between groups, we used Student's *t*-test (two-tailed). A value of $P < 0.05$ was considered to be statistically significant.

Results

Genetically suppressing RyR2 activity increases the propensity for Ca^{2+} alternans in intact hearts

We have recently generated a KI mouse model, harboring an SOF RyR2 mutation (E4872Q) that markedly suppresses the activation of the RyR2 channel by luminal Ca^{2+} [34]. To determine the effect of genetically suppressing RyR2 function on cardiac alternans, we assessed the propensity for Ca^{2+} alternans in isolated Langendorff-perfused WT and heterozygous RyR2-E4872Q (SOF) mutant hearts. As shown in Figure 1, RyR2-WT hearts showed little or no beat-to-beat alternation in the amplitude of Ca^{2+} transients stimulated at 8 Hz (Figure 1A and Supplementary Figure S1), whereas at the same stimulation frequency, RyR2-SOF hearts displayed large beat-to-beat variations in the amplitude of the Ca^{2+} transient (Figure 1B). Both the average alternans duration (Figure 1C) and average alternans ratio (Figure 1D) were markedly increased in RyR2-SOF hearts when compared with those in the WT hearts ($P < 0.01$). The frequency dependence of Ca^{2+} alternans in WT and RyR2-SOF hearts is shown in Figure 2. Considerable Ca^{2+} alternans was detected in RyR2-SOF hearts at stimulation frequencies as low as 6–7 Hz (Figure 2A), while higher stimulation frequencies (9–10 Hz) were required to induce considerable Ca^{2+} alternans in WT hearts (Figure 2B). Furthermore, RyR2-SOF hearts displayed significantly higher alternans duration at each stimulation frequency between 7 and 10 Hz (Figure 2A; $P < 0.01$) and higher alternans ratio between 6 and 12 Hz compared with WT hearts (Figure 2B; $P < 0.05$). Collectively, these data indicate that genetically suppressing RyR2 activity markedly enhances the propensity for rapid stimulation-induced Ca^{2+} alternans in intact hearts.

Suppressing RyR2 activity increases the refractoriness of SR Ca^{2+} release in intact hearts

Prolonged refractoriness of SR Ca^{2+} release is believed to be the underlying mechanism of Ca^{2+} alternans [23,26,29,30]. To understand the mechanism by which suppressing RyR2 activity enhances Ca^{2+} alternans, we tested the hypothesis that suppressing RyR2 activity prolongs the refractoriness of SR Ca^{2+} release. To this end, we determined the refractoriness of SR Ca^{2+} release in isolated Langendorff-perfused WT and RyR2-SOF hearts. The heart was repeatedly stimulated by a series of S1S2 protocols with progressively decreased S1S2 intervals (from 200 to 40 ms; Figure 3). The amplitude of Ca^{2+} transients in both the WT and RyR2-SOF hearts was reduced when the S1S2 interval was progressively decreased (Figure 3A,B). However, the WT and

Biochemical Journal (2016) **473** 3951–3964
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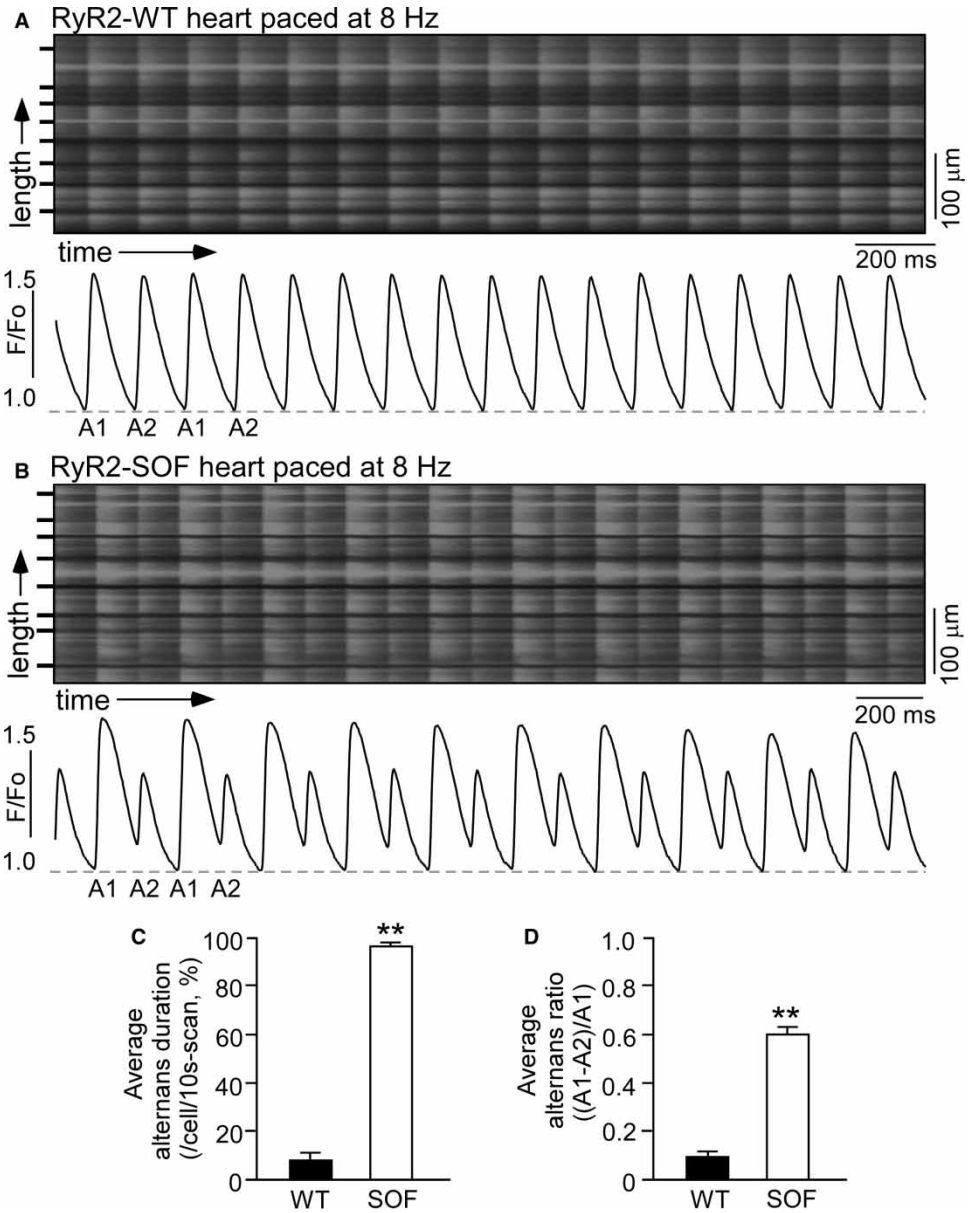

Figure 1. Ca^{2+} transient alternans in intact RyR2-WT and RyR2-SOF hearts.

Langendorff-perfused RyR2-WT (**A**) and RyR2-SOF mutant (**B**) hearts were loaded with Rhod-2 AM. Ca^{2+} transients were elicited by pacing at 8 Hz, and recorded using line-scanning confocal imaging. Cell boundaries were indicated by black bars. The F/F_0 traces depict the average fluorescence signal of the scan area. Alternans duration for each cell in the scan area and alternans ratio for each cell that displayed alternans in the same scan area were determined and averaged per cell to yield the average alternans duration (**C**) and average alternans ratio (**D**). Alternans duration is defined as the percentage of time in alternans over the 10-s scanning period, and alternans ratio is defined as the ratio of the difference in amplitude between the large and small Ca^{2+} transients over the amplitude of the large Ca^{2+} transient. Data shown are mean ± SEM ($n = 14$ scan areas from 5 RyR2-WT hearts and $n = 30$ scan areas from 12 RyR2-SOF hearts; $^{**}P < 0.01$).

RyR2-SOF hearts displayed significantly different relationship between Ca^{2+} transient amplitude and S1S2 interval (Figure 3C; $P < 0.05$). The RyR2-SOF hearts required a longer S1S2 interval to recover to a given Ca^{2+} transient amplitude when compared with WT hearts. Therefore, these data demonstrate that suppressing RyR2 activity prolongs the refractoriness of SR Ca^{2+} release, which would, in turn, contribute to the enhanced propensity for Ca^{2+} alternans.

Biochemical Journal (2016) 473 3951–3964
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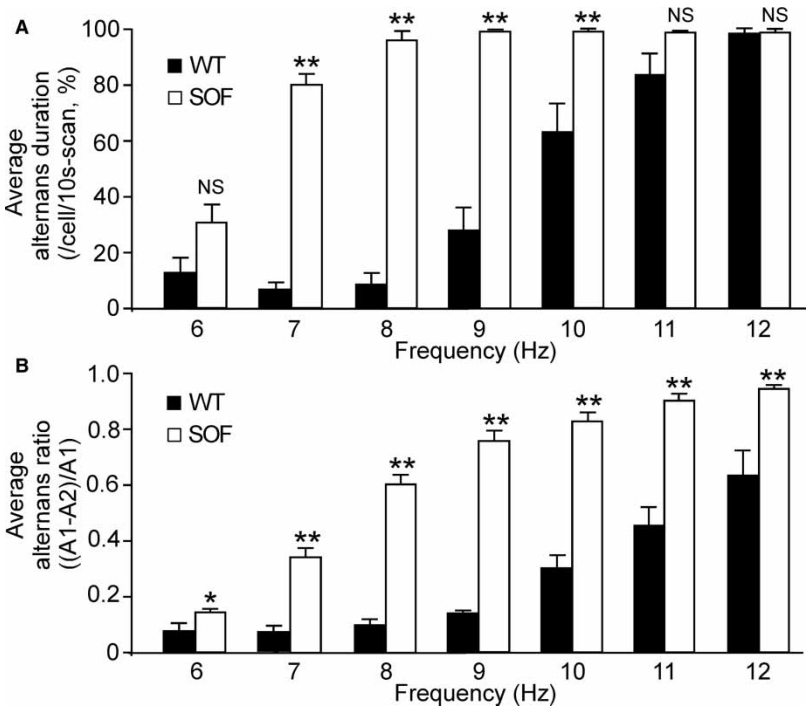

Figure 2. Average alternans duration and average alternans ratio at different pacing rates in intact RyR2-WT and RyR2-SOF hearts.

Ca^{2+} transients in intact Rhod-2 AM-loaded RyR2-WT and RyR2-SOF mutant hearts were elicited by pacing at different frequency (6–12 Hz), and recorded using the line-scan mode. (**A**) Average alternans duration (%) in cells in the scan area over the 10-s scan period. (**B**) Average alternans ratio in cells that displayed alternans in the scan area. Data shown are mean ± SEM ($n = 14$ scan areas from 5 RyR2-WT hearts and $n = 30$ scan areas from 12 RyR2-SOF hearts; $^{*}P < 0.05$ and $^{**}P < 0.01$).

Suppressing RyR2 activity enhances APD alternans in intact hearts

Given the close link between Ca^{2+} alternans and APD alternans [17], we next determined whether suppressing RyR2 activity also increases the propensity for APD alternans. To this end, we measured the MAP in isolated Langendorff-perfused WT and RyR2-SOF hearts stimulated at increasing frequencies (5–10 Hz). As shown in Figure 4, WT hearts showed no beat-to-beat alternation in the duration of AP (APD_{90}) at 8 Hz (Figure 4A), whereas significant beat-to-beat variation in APD_{90} was observed in RyR2-SOF hearts at the same stimulation frequency (8 Hz; Figure 4B; $P < 0.01$). Figure 4C shows the frequency dependence of APD alternans in WT and RyR2-SOF hearts. Considerable APD alternans was detected in RyR2-SOF hearts at as low as 6–7 Hz, while higher stimulation frequencies (9–10 Hz) were required to induce considerable APD alternans in WT hearts (Figure 4C). Furthermore, RyR2-SOF hearts displayed significantly higher APD alternans ratios at each stimulation frequency between 6 and 10 Hz compared with WT hearts (Figure 4C; $P < 0.05$). These results indicate that suppressing RyR2 activity enhances the propensity for APD alternans in intact hearts.

RyR2-SOF mice are prone to ISO-induced ECG alternans

The occurrence of APD alternans is expected to lead to ECG alternans. To test this possibility, we performed telemetric ECG recordings on conscious RyR2-WT and RyR2-SOF mutant mice at rest and after the injection of ISO, which was used to induce alternans [40,41]. As shown in Figure 5, RyR2-WT mice displayed little or no ECG alternans before or after the injection of ISO (0.5 mg/kg; Figure 5A). On the other hand, RyR2-SOF mice showed marked beat-to-beat alternations in the ECG waveform after, but not before, the injection of ISO (0.5 mg/kg; Figure 5B) in a manner similar to those described previously in mice [40]. In an attempt to quantify the magnitude of ECG alternans, we estimated the T-wave alternans ratio, using the method described previously [40,41]. We found that the T-wave alternans ratio was significantly higher in RyR2-SOF mice than that in RyR2-WT mice (Figure 5C,D). However, it should be noted that due to the presence of beat-to-beat

Biochemical Journal (2016) **473** 3951–3964
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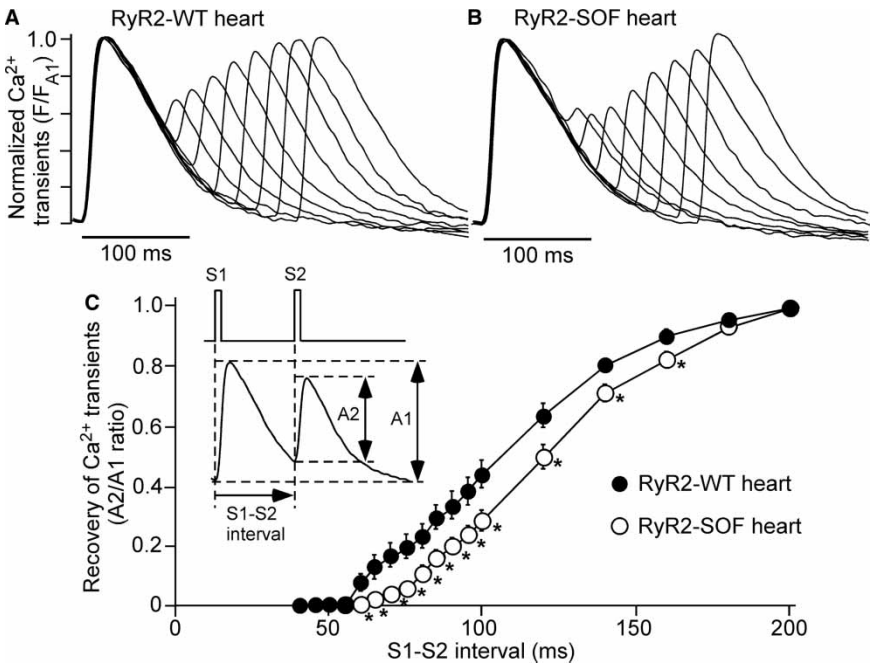

Figure 3. RyR2-SOF mutation prolongs the refractoriness of Ca^{2+} transients.

Langendorff-perfused RyR2-WT (**A**) and RyR2-SOF (**B**) mutant hearts were loaded with Rhod-2 AM. Hearts were first stimulated at 5 Hz for 30 s (S1), followed by a single S2 stimulation. A series of S1S2 stimulations are repeatedly applied with progressively reduced S1S2 interval from 200 to 40 ms. The amplitude of Ca^{2+} transients was recorded using the line-scan mode. (**C**) The relationship between S2/S1 ratio of Ca^{2+} transient amplitude and S1S2 interval is shown. Data shown are mean ± SEM ($n = 6$ for RyR2-WT hearts and $n = 8$ for RyR2-SOF hearts; $^*P < 0.05$).

alternations in other components of ECG (Figure 5B), it is difficult to specifically and accurately quantify the T-wave alternans ratio. Thus, the above estimation of the T-wave alternans ratio is intended to show the presence of ECG alternans, rather than the quantification of the extent of T-wave alternans. Nevertheless, these data clearly indicate that suppressing RyR2 activity enhances the propensity for ISO-induced ECG alternans *in vivo*.

Caffeine suppresses Ca^{2+} alternans in RyR2-SOF hearts

We have previously shown that caffeine enhances RyR2 activity by preferentially sensitizing the channel to luminal Ca^{2+} activation [42], an effect opposite to that of the RyR2-E4872Q mutation [34]. Thus, caffeine may be able to inhibit Ca^{2+} alternans in the heterozygous RyR2-SOF E4872Q mutant hearts by normalizing the effect of the RyR2-E4872Q mutation. To test this hypothesis, we determined the effect of caffeine on Ca^{2+} alternans in isolated Langendorff-perfused RyR2-SOF hearts. As shown in Figure 6, caffeine (1.0 mM) significantly reduced both the alternans duration and alternans ratio in RyR2-SOF mutant hearts ($P < 0.01$). Caffeine also shortened the refractoriness of SR Ca^{2+} release (Supplementary Figure S2). These data indicate that although caffeine increases the propensity for CPVT, it can rescue Ca^{2+} alternans associated with suppressed RyR2 activity.

Carvedilol worsens Ca^{2+} alternans in RyR2-SOF hearts

We have previously demonstrated that carvedilol, a nonselective β-blocker, suppresses RyR2 activity, the occurrence of spontaneous Ca^{2+} waves, and wave-evoked ventricular tachyarrhythmias (VTs) in a mouse model of CPVT [36]. These effects of carvedilol are similar to those of the RyR2-SOF mutation (E4872Q) [34]. This inhibitory effect of carvedilol raises an important question of whether carvedilol promotes Ca^{2+} alternans as the E4872Q mutation does. To address this question, we assessed the impact of carvedilol on Ca^{2+} alternans in the RyR2-SOF hearts. As shown in Figure 7 and Supplementary Figure S3, carvedilol (3 μM) increased both the alternans duration and alternans ratio ($P < 0.01$). Therefore, although carvedilol suppresses CPVT, it

Biochemical Journal (2016) **473** 3951–3964
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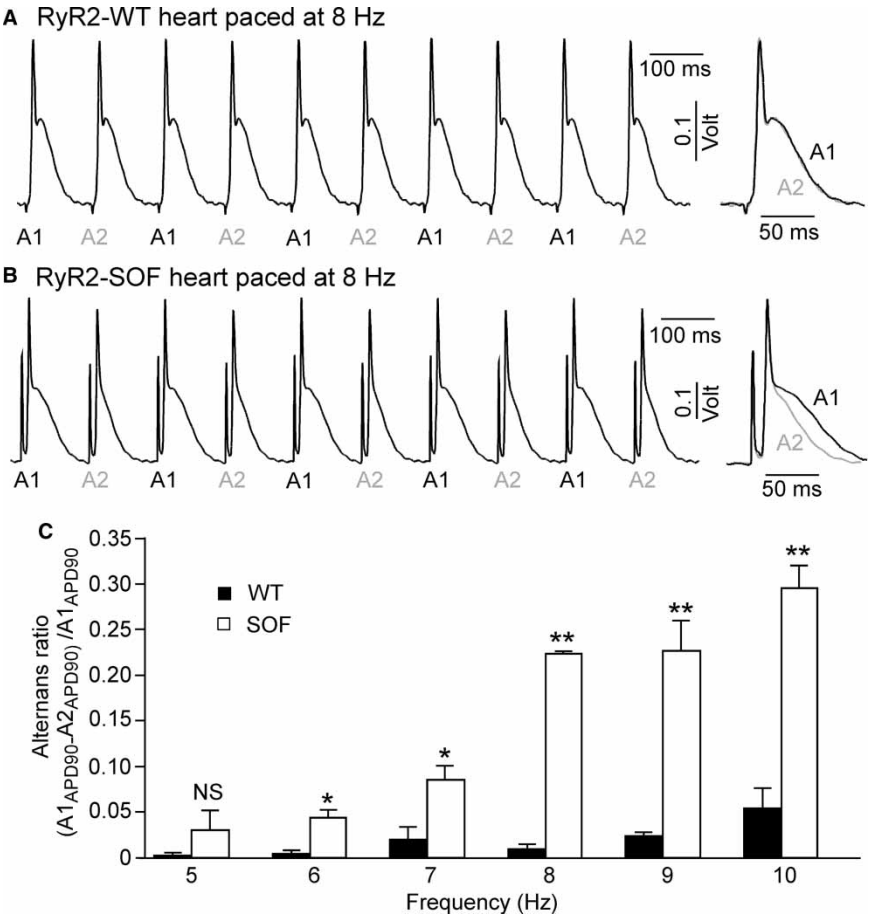

Figure 4. RyR2-SOF mutant mice are more susceptible to APD alternans.

Hearts from the RyR2-WT and RyR2-SOF mutant mice were perfused with oxygenated Tyrode's buffer containing 1.8 mM Ca^{2+}. After the heart rate became stable, rapid pacing was applied to trigger APD alternans. Representative traces of MAP recordings from RyR2-WT (**A**) and RyR2-SOF heart (**B**) paced at 8 Hz are shown. Panel (**C**) shows APD alternans in RyR2-WT and RyR2-SOF hearts at different pacing frequencies (5–10 Hz). Data shown are mean ± SEM ($n = 3$ hearts for each group; $^{*}P < 0.05$ and $^{**}P < 0.01$) NS, not significant.

worsens Ca^{2+} alternans in the setting of suppressed RyR2 activity. This observation is consistent with our previous finding that the SOF RyR2-E4872Q mutation protects against CPVT, but it enhances cardiac alternans (Figures 2–5).

Discussion

In the present study, we employed a KI mouse model harboring an SOF RyR2 mutation (E4872Q) [34] to determine the effect of genetically suppressing RyR2 activity on cardiac alternans. We demonstrate, for the first time, that genetically suppressing RyR2 function promotes Ca^{2+} and APD alternans in intact hearts and ECG alternans *in vivo*. We also show that agents that suppress or enhance RyR2 activity and CPVT exert an opposite impact on Ca^{2+} alternans. These observations suggest that the activity of RyR2 inversely determines the propensity for cardiac alternans and CPVT. These data have important implications for the treatment of RyR2-associated cardiac arrhythmias.

A large number of mutations in RyR2 have been linked to CPVT [43]. Most of the CPVT-associated RyR2 mutations enhance the channel activity and increase the propensity for spontaneous SR Ca^{2+} release, Ca^{2+} waves, and triggered arrhythmia [43]. Thus, a reasonable and logical strategy to combat CPVT is to suppress the activity of RyR2. In support of this strategy, we showed that carvedilol, a β-blocker that is able to reduce

Biochemical Journal (2016) **473** 3951–3964
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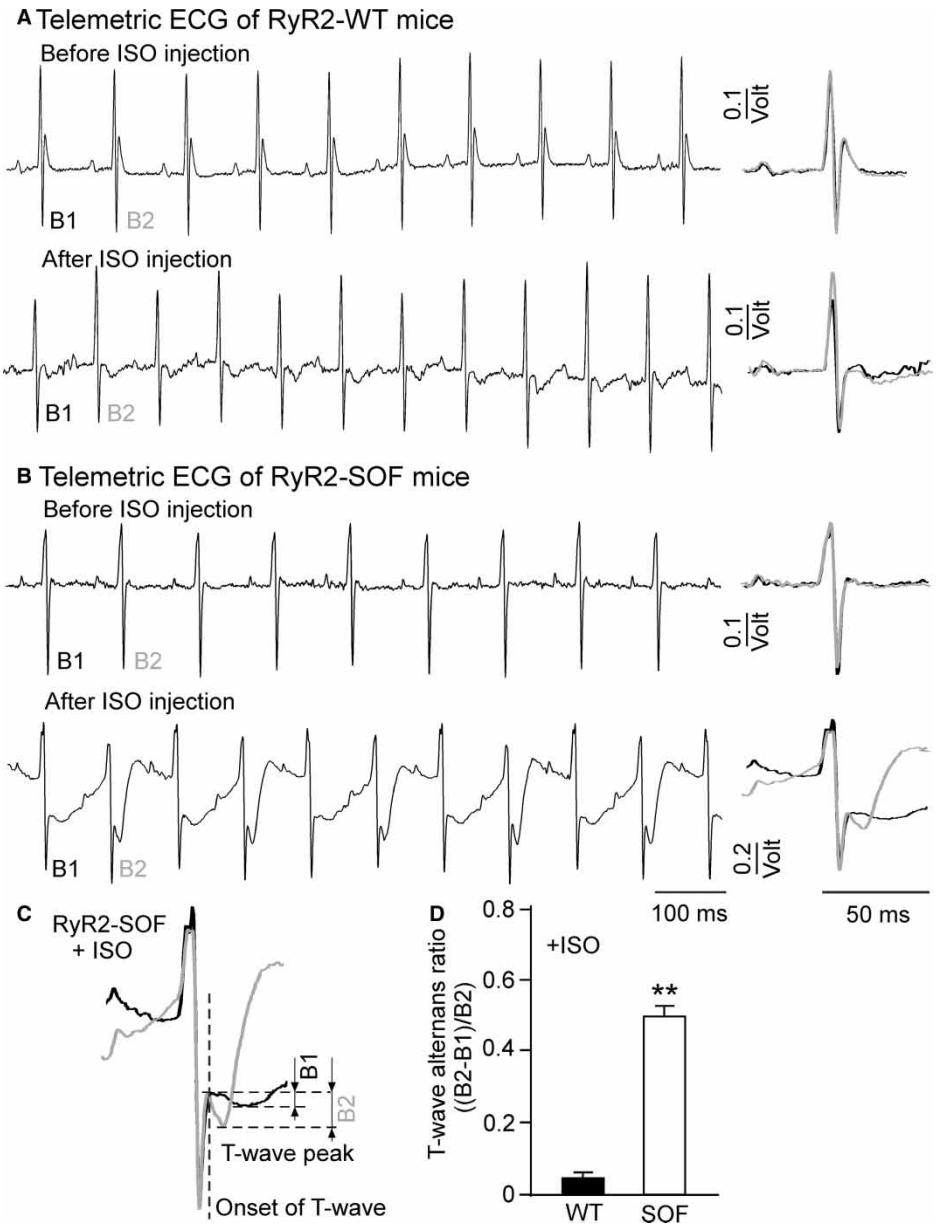

Figure 5. RyR2-SOF mice are highly susceptible to ISO-induced ECG alternans.

Telemetric ECG recordings were carried out on conscious RyR2-WT and RyR2-SOF mice before and after the administration (i.p.) of ISO (0.5 mg/kg). Representative ECG traces from RyR2-WT (**A**) and RyR2-SOF mice (**B**) 5 min before and 8 min after the injection of ISO are shown. (**C**) ECG waveforms of two consecutive beats are superimposed to depict ECG alternans. (**D**) Summarized data of T-wave alternans ratio defined as the ratio of the difference in the T-wave peak amplitude between the large and small T-waves over the large T-wave peak amplitude. Data shown are mean ± SEM ($n = 9$ for RyR2-WT mice and $n = 9$ for RyR2-SOF mice; $^{**}P < 0.001$).

the open duration of single RyR2 channels and inhibit the occurrence of spontaneous Ca^{2+} waves, effectively suppresses stress-induced VT in a CPVT mouse model harboring a gain-of-function (GOF) RyR2 mutation R4496C [36]. Similarly, we found that genetically suppressing the activity of RyR2 by knocking in an SOF RyR2 mutation E4872Q completely protects RyR2-R4496C mutant mice from CPVT [34]. It should be noted that the RyR2-E4872Q mutation abolishes luminal Ca^{2+} activation of single RyR2 channels and RyR2-mediated spontaneous Ca^{2+} release in HEK293 cells and in ventricular myocytes isolated from heterozygous

Biochemical Journal (2016) **473** 3951–3964
DOI: 10.1042/BCJ20160606

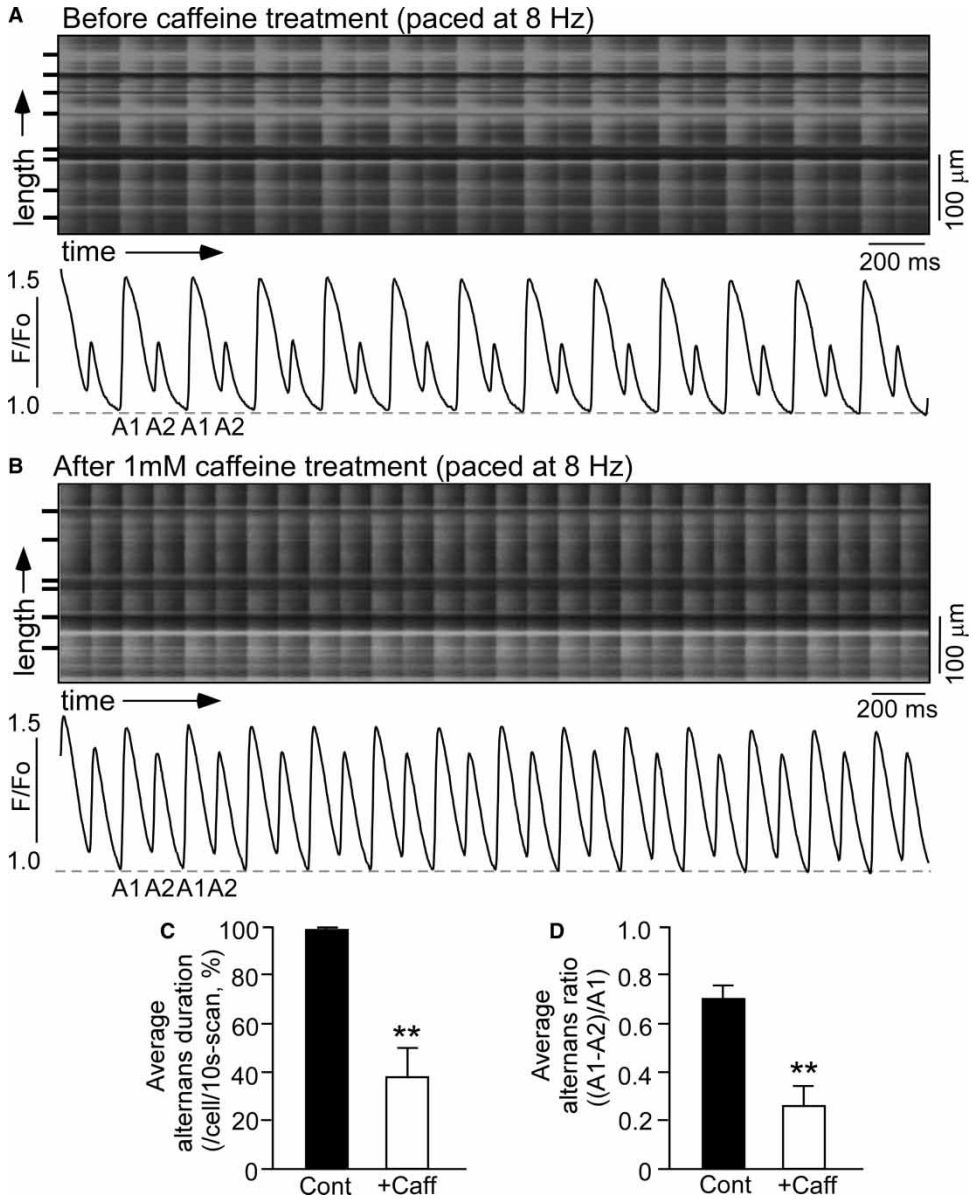

Figure 6. Caffeine suppresses Ca^{2+} alternans in intact RyR2-SOF hearts.

Langendorff-perfused RyR2-SOF mutant hearts were loaded with Rhod-2 AM. Ca^{2+} transients were elicited by pacing at 8 Hz, and recorded before (**A**) and after (**B**) the application of caffeine (1 mM) using the line-scan mode. Cell boundaries were indicated by the black bars. The F/F_0 traces depict the average fluorescence signal of the scan area. (**C**) Average alternans duration (%) in cells in the scan area over the 10-s scan period. (**D**) Average alternans ratio in cells that displayed alternans in the scan area. Data shown are mean ± SEM ($n = 10$ scan areas for each group from 5 RyR2-SOF hearts; $^{**}P < 0.01$).

RyR2-E4872Q mutant mice and in intact E4872Q mutant hearts. Furthermore, homozygous RyR2-E4872Q mutation is embryonic lethal, indicating the severe impact of the E4872Q mutation on RyR2 channel function in the E4872Q mutant KI mice [34]. Therefore, pharmacologically or genetically suppressing the activity of RyR2 limits CPVT. However, loss-of-function (LOF) or SOF RyR2 mutations have also been associated with idiopathic ventricular fibrillation [44,45], suggesting that overly suppressing RyR2 is also pro-arrhythmic. Indeed, we found that the SOF RyR2-E4872Q mutation enhances cardiac alternans, a well-known risk factor for re-entrant arrhythmias, VF, and sudden cardiac death. Moreover, carvedilol, which suppresses RyR2 and CPVT, exacerbates Ca^{2+} alternans in RyR2-SOF (E4872Q) mutant hearts. On the other hand, caffeine, which

Biochemical Journal (2016) **473** 3951–3964
DOI: 10.1042/BCJ20160606

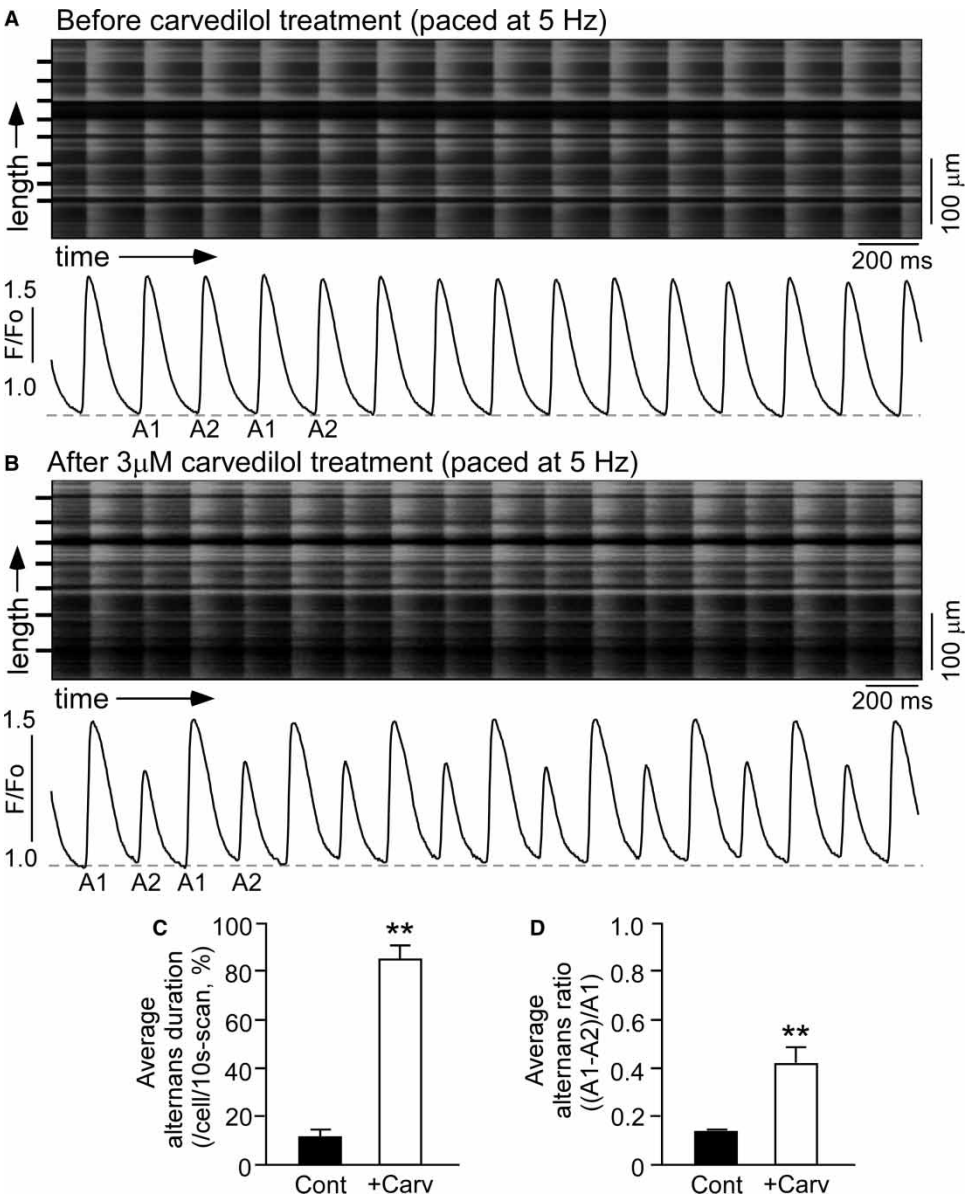

Figure 7. Carvedilol promotes Ca^{2+} alternans in intact RyR2-SOF hearts.

Langendorff-perfused RyR2-SOF mutant hearts were loaded with Rhod-2 AM. Carvedilol (3 μM) was continuously applied to the heart. Ca^{2+} transients were elicited in intact RyR2-SOF hearts by pacing at 5 Hz, and recorded before (**A**) and after (**B**) the application of carvedilol using the line-scan mode. Cell boundaries were indicated by the black bars. The F/F_0 traces depict the average fluorescence signal of the scan area. (**C**) Average alternans duration (%) in cells in the scan area over the 10-s scan period. (**D**) Average alternans ratio in cells that displayed alternans in the scan area. Data shown are mean ± SEM ($n = 10$ scan areas for each group from 4 RyR2-SOF hearts; $^{**}P < 0.01$).

enhances RyR2 activity and induces CPVT, suppresses Ca^{2+} alternans in these hearts. Suppression of Ca^{2+} alternans by caffeine has also been observed in isolated cardiomyocytes [5,25,31,32]. These observations indicate that suppressing RyR2 activity, although it can protect against CPVT, could increase the propensity for cardiac alternans in already depressed hearts. Thus, although carvedilol is the β-blocker of choice in preventing cardiac arrhythmias, using carvedilol to treat patients with LOF/SOF RyR2 function might be inappropriate or even harmful. Therefore, it is imperative to understand the exact functional impact of each disease-causing RyR2 mutation and to develop personalized treatments by normalizing the functional defect of RyR2.

Biochemical Journal (2016) 473 3951–3964
DOI: 10.1042/BCJ20160606

Recent studies have convincingly demonstrated that rapid pacing-induced Ca^{2+} alternans results from beat-to-beat alternations in the refractoriness of CICR from the SR [23,26]. This refractoriness of CICR is largely determined by (1) the recovery of the CICR trigger (i.e. the activity of the L-type Ca^{2+} channel), (2) the SR Ca^{2+} load, and (3) the activity of RyR2. While there is little evidence that I_{Ca} is the critical determinant of Ca^{2+} alternans [9,23,25,26], it has been shown that the I_{Ca} amplitude can modulate the frequency dependency of Ca^{2+} alternans in human cardiomyocytes [27]. Ca^{2+} alternans has been reported in myocytes with and without concurrent alternation in the SR Ca^{2+} load [19,26]. When present, alternation in the SR Ca^{2+} load can amplify Ca^{2+} alternans [26], but it may not necessarily be the cause of Ca^{2+} alternans [46]. Consistent with the view that recovery/refractoriness of RyR2 activity constitutes a critical determinant of Ca^{2+} alternans [23,26], we found that genetically suppressing RyR2 function significantly prolongs the refractoriness of CICR in intact hearts. These data suggest that suppressed RyR2 activity may delay the recovery of RyR2 from inactivation and prolong the refractoriness of SR Ca^{2+} release, thereby promoting Ca^{2+} alternans. However, despite its significance, the mechanism underlying RyR2 refractoriness remains undefined. Cytosolic Ca^{2+}-dependent inhibition of RyR2, SR luminal Ca^{2+} depletion-induced desensitization of RyR2, and post-translational modulations of RyR2 have been proposed to contribute to the refractoriness of RyR2 [14]. It is of interest to point out that enhanced RyR2 activity in a mouse model harboring a GOF CPVT RyR2 mutation P2328S has also been shown to result in APD alternans [47]. However, whether the RyR2-P2328S mutation causes Ca^{2+} alternans in intact hearts is unclear. Nevertheless, these observations suggest that both suppressed and enhanced RyR2 activity could lead to alternans, but the exact mechanisms underlying alternans associated with suppressed or enhanced RyR2 function have yet to be determined.

It has recently been shown that Langendorff-perfused hearts, isolated from a KI mouse model harboring a VF-associated RyR2-SOF mutation (A4860G), display VF in the presence of ISO and elevated extracellular Ca^{2+} [48]. The RyR2-A4860G mutation was found to decrease the amplitude of CICR, leading to progressively increased SR Ca^{2+} content as a result of reduced excitation–contraction coupling gain. The resultant SR Ca^{2+} overload then causes bursts of prolonged SR Ca^{2+} release, which in turn enhances the propensity for early after-transients and early after-depolarizations (EADs) [48]. Hence, RyR2-SOF could lead to EADs and EAD-triggered cardiac arrhythmias. Unlike in the RyR2-SOF A4860G mutant heart, we detected little or no spontaneous SR Ca^{2+} release events (early or delayed after-transients) in the RyR2-SOF E4872Q mutant heart [34]. Furthermore, we detected no arrhythmic events at rest or after the injection of ISO or a mixture of caffeine and epinephrine in the RyR2-SOF E4872Q mice. The reasons for these differences between the A4860G and E4872Q mutant mice are unknown. One possibility is that the RyR2-A4860G mutation may suppress RyR2 activity to a smaller extent than does the RyR2-E4872Q mutation, such that the less suppressed RyR2-A4860G mutant is more likely to be activated during SR Ca^{2+} overload, leading to EADs and EAD-evoked VT/VF. On the other hand, such an SR Ca^{2+} overload may not be high enough to activate the strongly suppressed RyR2-E4872Q mutant. Hence, no EADs could be triggered. Instead, the strongly suppressed RyR2-E4872Q mutation prolongs the refractoriness of SR Ca^{2+} release and promotes cardiac alternans (Figures 1–5), which would enhance the propensity for alternans-induced re-entrant arrhythmias. However, based on the critical mass theory [49], it would be difficult to induce sustained re-entrant arrhythmias in the very small and thin mouse hearts. This may account, in part, for the lack of inducibility of ventricular arrhythmias in the RyR2-SOF E4872Q mutant hearts/mice (Figure 5) [34]. Nevertheless, these findings suggest that depending on the degree of inhibition, suppressing RyR2 activity could lead to EADs or cardiac alternans.

In summary, we have demonstrated for the first time that genetically suppressing RyR2 activity prolongs the refractoriness of SR Ca^{2+} release and promotes Ca^{2+} and APD alternans in intact hearts and ECG alternans *in vivo*. Pharmacologically suppressing or enhancing RyR2 activity promotes or rescues Ca^{2+} alternans. Our data demonstrate that the RyR2 activity inversely determines the propensity for cardiac alternans and CPVT. Hence, excessively suppressing or enhancing RyR2 activity is arrhythmogenic. Therefore, normalizing RyR2 activity is key to the treatment of RyR2-associated cardiac arrhythmias.

Abbreviations

APD, action potential duration; Ca^{2+} alternans, cytosolic Ca^{2+} transient; CICR, Ca^{2+}-induced Ca^{2+} release; CPVT, catecholaminergic polymorphic ventricular tachycardia; EAD, early after-depolarization; ECG, electrocardiogram; GOF, gain of function; ISO, isoproterenol; KI, knock-in; LOF, loss of function; MAP, monophasic action potential; RyR2, cardiac ryanodine receptor; SOF, suppression of function; SR, sarcoplasmic reticulum; VF, ventricular fibrillation; VT, ventricular tachyarrhythmia; WT, wild type.

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Author Contribution

X.Z., B.S., R.W., A.G., H.J.D., A.M.G., L.-S.S., L.H.-M., R.B., and S.R.W.C. designed research; X.Z., B.S., T.M., W.G., and R.W. performed research; X.Z., B.S., A.V., T.M., W.G., M.N., L.H.-M., R.B., S.R.W.C. analyzed data; and X.Z., B.S., A.V., R.W., A.M.G., L.-S.S., L.H.-M., R.B., and S.R.W.C. wrote the paper.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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