Flecainide exerts paradoxical effects on sodium currents and atrial arrhythmia in murine $RyR2-P2328S$ hearts


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Aims: Cardiac ryanodine receptor mutations are associated with catecholaminergic polymorphic ventricular tachycardia (CPVT), and some, including $RyR2-P2328S$, also predispose to atrial fibrillation. Recent work associates reduced atrial $Na_v1.5$ currents in homozygous $RyR2-P2328S$ ($RyR2^{SS}$) mice with slowed conduction and increased arrhythmogenicity. Yet clinically, and in murine models, the $Na_v1.5$ blocker flecainide reduces ventricular arrhythmogenicity in CPVT. We aimed to determine whether, and how, flecainide influences atrial arrhythmogenicity in $RyR2^{SS}$ mice and their wild-type (WT) littermates.

Methods: We explored effects of 1 $\mu M$ flecainide on WT and $RyR2^{SS}$ atria. Arrhythmic incidence, action potential (AP) conduction velocity (CV), atrial effective refractory period (AERP) and AP wavelength ($\lambda = CV \times AERP$) were measured using multi-electrode array recordings in Langendorff-perfused hearts; $Na_+^+$ currents ($I_{Na}$) were recorded using loose patch clamping of superfused atria.

Results: $RyR2^{SS}$ showed more frequent atrial arrhythmias, slower CV, reduced $I_{Na}$ and unchanged AERP compared to WT. Flecainide was anti-arrhythmic in $RyR2^{SS}$ but pro-arrhythmic in WT. It increased $I_{Na}$ in $RyR2^{SS}$ atria, whereas it reduced $I_{Na}$ as expected in WT. It increased AERP while sparing CV in $RyR2^{SS}$, but reduced CV while sparing AERP in WT. Thus, $RyR2^{SS}$ hearts have low $\lambda$ relative to WT; flecainide then increases $\lambda$ in $RyR2^{SS}$ but decreases $\lambda$ in WT.

Conclusions: Flecainide (1 $\mu M$) rescues the $RyR2-P2328S$ atrial arrhythmogenic phenotype by restoring compromised $I_{Na}$ and $\lambda$, changes recently attributed to increased sarcoplasmic reticular Ca$^{2+}$ release. This contrasts with the increased arrhythmic incidence and reduced $I_{Na}$ and $\lambda$ with flecainide in WT.

Keywords: atrial arrhythmia, conduction velocity, CPVT, flecainide, $Na_+^+$ currents, ryanodine receptor.

Atrial fibrillation (AF) is the most common sustained arrhythmia, predisposing to significant clinical morbidity and mortality (Benjamin et al. 1998, Stewart et al. 2002, Davis et al. 2012), yet its physiological mechanisms are incompletely understood. Nevertheless, acute atrial arrhythmogenesis may be related not only to cellular Ca$^{2+}$ homeostasis but also to altered action potential (AP) conduction and recovery (Zhang et al. 2011, King et al. 2013c).

Abnormal Ca$^{2+}$ release can arise from cardiac ryanodine receptor-2 (RyR2) mutations or a loss of calsequestrin-2 (CSQ2) (Priori & Chen 2011), potentially providing arrhythmic triggers (Mackenzie et al. 2001, 2004, Bootman et al. 2006, Zhang et al. 2010),...
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thereby leading to catecholaminergic polymorphic ventricular tachycardia (CPVT) (Priori & Chen 2011, Zhang et al. 2013a). Certain RyR2 mutations are also associated with AF (Bhuiyan et al. 2007, Sumitomo et al. 2007). The RyR2-P2328S mutation is associated with high incidences of both CPVT and atrial tachycardia (AT), despite normal cardiac structure (Swan et al. 1999, Laitinen et al. 2001). Murine hearts with a homozygotic RyR2-P2328S (RyR2S5S) mutation demonstrate both atrial and ventricular arrhythmic tendencies (Goddard et al. 2008, Zhang et al. 2011, 2013b, King et al. 2013b,c) providing a useful experimental model. Atrial RyR2S/S myocytes show diastolic elevations in intracellular [Ca2+] attributed to increased SR Ca2+ release (Zhang et al. 2011). This would be expected to increase Na+↔Ca2+ exchange (NCX) activity, accounting for delayed afterdepolarizations (DADs) causing triggered activity, implicated in the arrhythmic phenotype (King et al. 2013c).

It has recently been reported that flecainide exerts anti-arrhythmic effects in human CPVT (Watanabe et al. 2009, van der Werf et al. 2011). Flecainide reduced bigeminy and biventricular tachycardia, ECG features associated with human CPVT, in murine CSQ2−/− hearts. However, there is debate over the anti-arrhythmic mechanism of flecainide in CPVT. It has been suggested that flecainide directly reduces both RyR2-mediated Ca2+ release and the consequent triggering events (Watanabe et al. 2009, Hilliard et al. 2010, Hwang et al. 2011). Alternatively, anti-arrhythmic actions of flecainide may be attributed to inhibition of Na+1.5 function, thereby decreasing membrane excitability and the likelihood of triggered activity (Liu et al. 2011).

Further questions concerning the anti-arrhythmic mechanism of flecainide arise from reports implicating reduced conduction velocity (CV) in RyR2S5S atria relative to WT. These reports show that the impaired CV is secondary to reduced ISNA rather than abnormal fibrosis or structural remodelling (King et al. 2013b, c). Reduced CV has also been shown with other mutations associated with diastolic Ca2+ release and murine atrial arrhythmias including CREM-IbAC-X (Li et al. 2014) and CSQ2−/− (Glukhov et al. 2013). In each case, the resultant reduced AP wavelength (λ) would increase the likelihood of re-entrant arrhythmias (King et al. 2013a).

Na+1.5 inhibition by flecainide might be expected to further reduce CV and λ in RyR2S5S atria. Yet, Na+1.5 inhibition and consequent reduced Na+ entry might also increase forward-mode NCX activity (Liu et al. 2011, Sikkel et al. 2013), thus reducing diastolic Ca2+. Flecainide has also been shown to reduce RyR2-mediated Ca2+ release (Watanabe et al. 2009, Hilliard et al. 2010, Hwang et al. 2011). This study sought to assess whether, at the tissue level, there was a reduced arrhythmic tendency in the presence of flecainide in a system showing a RyR2 abnormality accompanied by compromised Na+ channel function and AP conduction velocity. We then investigated the alterations in arrhythmic tendency brought about by flecainide through an assessment of Na+ channel function, conduction velocity and recovery characteristics that might together rescue λ, otherwise compromised by the RyR2S5S mutation. This would establish a tissue-level significance of the previous cellular level results suggesting that altered Ca2+ homeostasis could affect Na+ channel function.

The experiments therefore test the influence of flecainide on arrhythmogenicity in RyR2S5S and WT atria and correlate this with its influence on ISNa, CV, AERP and λ. We thus complement a recent study reporting similar anti-arrhythmic inhibitory actions of another class Ic anti-arrhythmic agent, propafenone, on Ca2+-release events during atrial fibrillation in a CSQ2−/− model of CPVT (Faggioni et al. 2014), although ISNa and CV were not measured in that latter study.

Materials and methods

Experimental animals

All procedures were performed in licensed institutional premises under a UK Home Office project licence approved by a university ethics review board, under the UK Animals (Scientific Procedures) Act (1986), and conforming to European Parliament Directive 2010/63/EU. 3.5- to 11.5-month-old wild-type (WT, n = 22) and RyR2-P2328S (RyR2S5S, n = 23) inbred 129/Sv mice (Harlan, UK) were kept in plastic cages at room temperature in 12-h light/dark cycles. Mice had free access to sterile rodent chow and water. All chemical agents were purchased from Sigma-Aldrich (Poole, UK) except where otherwise indicated, with effects of flecainide studied at concentrations of 1 and 5 μM and dantrolene Na at 10 μM.

Experimental set-up in isolated Langendorff-perfused hearts

Mice were killed by cervical dislocation (Schedule 1: UK Animals (Scientific Procedures) Act 1986). Hearts were excised and placed in ice-cold bicarbonate-buffered Krebs-Henseleit solution (KH) containing (mM) NaCl 119, NaHCO3 25, KCl 4, KH2PO4 1.2, MgCl2 1, CaCl2 1.8, glucose 10 and Na-pyruvate 2; pH 7.4, 95% O2/5% CO2 (British Oxygen Company, Manchester, UK), then cannulated and perfused with KH as previously described (Zhang et al. 2010, 2011). After a 10- to 15-min stabilization period, hearts were
paced using an Ag/AgCl electrode at the epicardial surface of the right atrium. First, a regular pacing protocol imposed successive trains of 100 stimuli at frequencies of 5, 6.67, 8 and 10 Hz respectively. This was followed by a programmed electrical stimulation (PES) protocol which first paced at 10 Hz for 20 s. It then applied drive trains consisting of cycles of eight paced stimuli (S1), each followed by a single extra stimulus (S2). The S1–S2 interval was initially equal to the pacing interval, then reduced by 1 ms with each subsequent cycle until S1–S2 = 6 ms. Both the WT and RyR2<sup>SS</sup> hearts were stimulated using square-wave stimuli of 2 ms duration and amplitudes of twice diastolic excitation threshold (Sabir et al. 2007) (DS2A isolated constant voltage stimulator; Digitimer, Welwyn Garden City, Herts., UK). There was no significant difference in mean excitation threshold between WT and RyR2<sup>SS</sup> hearts (thresholds: WT, 1.68 ± 0.37 V (n = 9); RyR2<sup>SS</sup>, 1.75 ± 0.39 V (n = 17); P = 0.69). This protocol provided both arrhythmic incidences, defined as an occurrence of two or more non-stimulated atrial electrograms, and AERPs, defined as the period when the cell is refractory to the initiation of new APs, such that no atrial electrogram results from the S2 stimuli.

**Multi-electrode array recordings and conduction velocity vector analysis**

Multi-electrode array (MEA) recordings were made from the epicardial LA surface of both WT and RyR2<sup>SS</sup> hearts during stimulation protocols. Each MEA (ME32-FAI-System; Scientifica, Uckfield, UK) contained 32 recording electrodes of diameter 50 μm that were arranged in an array of successive rows of 4, 6, 6, 6, 6 and 4 electrodes within a 1.5 × 1.5 mm configuration with a 300-μm interelectrode distance as shown in Figure 1. Data were sampled at 10 kHz per channel. The positions of the stimulating electrode and the MEA were consistent throughout each experiment.

Local activation times (LATs) were calculated as the time from stimulation to the maximum negative rate of voltage change, (dV/dt)<sub>max</sub> of the extracellular atrial electrogram recording of the AP. The maximum negative deflection is a consistently identifiable feature of the waveform, corresponding to the intracellular AP peak, which has previously been employed to assess relative arrival times in extracellular multi-electrode recordings (Lambiasi et al. 2009, Zhang et al. 2014). It was then possible to determine the median LAT for each atrial electrogram (Fig. 1A). Relative LATs were then determined by subtracting the median LAT from the individual LATs for each atrial electrogram. Finally, the median relative LAT was found for each recording electrode over all the atrial electrograms at each recording frequency.

A velocity vector was calculated and attributed to the centre of these sites (Fig. 1B). Column (x) and row (y) time vector components were calculated from the median atrial electrogram LATs at four neighbouring recording sites (L1 to L4, ms) as $y = ((L1 + L2) - (L3 + L4))/2$ and $x = ((L1 + L4) - (L2 + L3))/2$ respectively. Velocity vector direction was calculated as $\theta = \arctan(2x/0.3, y/0.3)$, and its magnitude (mm ms<sup>−1</sup>) was calculated as

$$\sqrt{0.3 \cos \theta}^2 + 0.3 \sin \theta^2,$$

where 0.3 is the electrode spacing in mm.

This was done for every interleaved combination of neighbouring recording sites, producing a 5 × 5 grid of CV vectors each spaced 0.3 mm apart (Fig. 1C). The vectors were then plotted and inspected for wave collisions and wave breaks that would break the necessary assumption of uniform conduction direction between adjacent pins (Fig. 1D); any such vectors were manually removed. Median velocity and standard deviation were calculated from the remaining vectors, yielding a single value of CV under each intervention and pacing rate for each of the hearts studied in each experimental group. $i$ was subsequently calculated in hearts for which both CV and AERP values were available (0 and 1 μm flecainide).

**Loose patch-clamp recording and assessment of Na<sup>+</sup> current**

Loose patch-clamp experiments were performed as previously described (King et al. 2013b). This technique was chosen to permit measurement of Na<sup>+</sup> currents in whole, perfused, freshly dissected atria, without the potential disruption of intracellular Ca<sup>2+</sup> homeostasis that might occur during cell isolation and preparation for a tight patch approach. The maintenance of intercellular connectivity allowed recording of Na<sup>+</sup> currents under similar experimental conditions to those employed in the CV experiments. Na<sup>+</sup> currents recorded from such experiments have been shown to be in agreement with those obtained from tight patch techniques (Eickhorn et al. 1990). Micropipettes were pulled from plain thick-walled borosilicate glass capillary (GC 150-10; Harvard Apparatus, Kent, UK) using a micropipette puller (Brown-Flaming Model P-97, Sutter Instrument Company, Novato, CA, USA). The pipette was held in a micromanipulator mounted on the stage of a compound microscope and scribed transversely at a point along its shaft.
where it was a little over 40 μm in diameter using a diamond knife under visual control at 250 × magnification. Transverse force applied to the distal tip caused the pipette to fracture at this point orthogonal to its axis. The pipette was then fire-polished using an electrically heated nichrome filament at 400 × magnification to produce a tip with an internal diameter of approx. 40 μm, previously shown to yield the most consistent Na+ currents in atrial patches (King et al. 2013b). Internal tip diameters were measured at 1000 × magnification using a calibrated eyepiece graticule. Pipettes were bent through an angle of about 45° approx. 1 mm from the tip, so that it approached the membrane vertically when mounted on the headstage of the recording amplifier.

The left atrium was mounted upon a Sylgard (Dow Chemical Company, Chicago, IL, USA) gel platform and placed in an actively grounded bath filled with KH buffer maintained at just above room temperature (25 ± 3 °C) using a heat exchanger and fluid circulator. The pipette was filled to two-thirds along its shaft with KH buffer; an air-filled line connected to the pipette holder allowed suction to be applied during loose patch formation. Electrical connections to bath and pipette were made with Ag/AgCl electrodes. Loose patch-clamp recordings were carried out using a custom-built amplifier designed to compensate for leakage current, series resistance errors and pipette capacitance (Stühmer & Almers 1982). The pipette was lowered until a resistance increase was observed, indicating contact with the atrial surface. Gentle suction was then applied to draw a patch of membrane into the pipette tip. Voltage-clamp steps were delivered under computer control; a negative-going clamp step represents a corresponding depolarization relative to the resting membrane potential.

Activation properties were investigated with a series of depolarizing test pulses of 75 ms duration, delivered 5 ms following the beginning of the sampling period using a P/4 pulse protocol (Bezanilla & Armstrong 1977). Although the P/4 protocol corrects for relative errors during the clamp step itself, it also adds baseline offsets during the correction procedure. The underlying drift in clamp voltage was <1 mV in all cases. The test voltage steps delivered single depolarizing voltage excursions from rest ranging from 20 mV to 120 mV, incremented by 10 mV between trials. The complete series of trials was bracketed by 80 mV depolarizing pulses to check patch stability. The depolarizing voltage steps elicited distinct inward currents which activated rapidly and then inactivated, thus closely resembling previous loose patch-clamp measurements (Almers et al. 1983, Roberts et al. 1986). These were often followed by increasing outward currents; nevertheless, the time course of peak I_{Na} remained clearly separable permitting an assessment of Na+ channel expression and activation. In addition, some activation experiments were performed in the presence of 10 μM dantrolene Na, as a specific RyR blocker, at the 80 mV voltage step used for testing patch stability. Inactivation properties were investigated by incorporating a depolarizing pre-pulse of 5 ms duration and variable amplitude immediately prior to the test pulse, which had a fixed magnitude of 100 mV from rest and a duration of 70 ms.

Clamp currents were filtered over the bandwidth DC-10 kHz (8-pole Bessel filter) and digitized at 50 kHz using custom written software. The resulting traces were not zeroed to the initial baseline to best display currents at each voltage step. Resting potential measurements were performed in a similar superfused atrial preparation using KCl-filled 10–20 MΩ glass microelectrodes in the isolated right atrial portions of the same hearts from which the left atria were obtained.
Table 1 Effects of flecainide in WT and RyR2\(^{SS}\) atria

<table>
<thead>
<tr>
<th>Genotype, flecainide ((\mu)M)</th>
<th>WT, 0</th>
<th>WT, 1</th>
<th>WT, 5</th>
<th>RyR(^{SS}), 0</th>
<th>RyR(^{SS}), 1</th>
<th>RyR(^{SS}), 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arrhythmic incidence</td>
<td>1.1 ± 0.22, 10</td>
<td>2.56 ± 0.453, 9*</td>
<td>N/A</td>
<td>2.65 ± 0.377, 17(^{**})</td>
<td>1.59 ± 0.385, 17(^{**})</td>
<td>N/A</td>
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<tr>
<td>(mean events per heart)</td>
<td></td>
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<tr>
<td>(I_{Na}) activation properties</td>
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<tr>
<td>(I_{Na,max}) (nA)</td>
<td>-14.36 ± 0.30, 7</td>
<td>-9.62 ± 0.21, 7(^{***})</td>
<td>7.98± ± 1.94, 7</td>
<td>-9.29 ± 0.51, 6(^{***})</td>
<td>-12.14 ± 0.31, 6(^{***})</td>
<td>-9.81 ± 0.90, 6</td>
</tr>
<tr>
<td>(k) (mV)</td>
<td>19.15 ± 1.32, 7</td>
<td>14.67 ± 1.49, 7</td>
<td>7.62 ± 2.35, 7</td>
<td>13.15 ± 3.07, 6</td>
<td>11.97 ± 1.98, 6</td>
<td>26.3 ± 6.7, 6</td>
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<tr>
<td>(V^*) (mV)</td>
<td>65.86 ± 1.06, 7</td>
<td>59.12 ± 1.50, 7*</td>
<td>7.63 ± 2.35, 7</td>
<td>43.48 ± 7.51, 6</td>
<td>48.42 ± 2.50, 6(^{**})</td>
<td>61.44 ± 4.98, 6</td>
</tr>
<tr>
<td>(I_{Na}) inactivation properties</td>
<td></td>
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<tr>
<td>(I_{Na,max}) (nA)</td>
<td>-11.51 ± 0.15, 7</td>
<td>-8.48 ± 0.06, 7(^{***})</td>
<td>-6.53 ± 0.37, 7(^{***})</td>
<td>-9.58 ± 0.15, 6(^{***})</td>
<td>-12.01 ± 0.20, 6(^{***})</td>
<td>-7.7 ± 0.1, 6(^{***})</td>
</tr>
<tr>
<td>(k) (mV)</td>
<td>16.51 ± 0.83, 7</td>
<td>14.76 ± 0.44, 7</td>
<td>24.24 ± 3.75, 7</td>
<td>12.96 ± 0.81, 6</td>
<td>14.58 ± 0.97, 6</td>
<td>16.4 ± 0.82, 6</td>
</tr>
<tr>
<td>(V^*) (mV)</td>
<td>56.69 ± 0.75, 7</td>
<td>56.69 ± 0.41, 7</td>
<td>53.1 ± 2.51, 7</td>
<td>49.98 ± 0.76, 6(^{***})</td>
<td>53.64 ± 0.88, 6(^{**})</td>
<td>53.88 ± 0.71, 6(^{**})</td>
</tr>
<tr>
<td>Resting potential (mV)</td>
<td>-70.92 ± 0.65, 16</td>
<td>-72.49 ± 0.56, 15</td>
<td>-70.43 ± 0.51, 16</td>
<td>-71.89 ± 0.51, 15</td>
<td>-70.67 ± 0.58, 15</td>
<td>-71.44 ± 0.28, 15</td>
</tr>
<tr>
<td>Conduction Velocity (mV)</td>
<td>0.326 ± 0.018, 9</td>
<td>0.216 ± 0.019, 9(^{***})</td>
<td>0.168 ± 0.012, 8(^{***})</td>
<td>0.313 ± 0.026, 9</td>
<td>0.306 ± 0.018, 10(^{***})</td>
<td>0.22 ± 0.22, 9*</td>
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<tr>
<td>At 6 Hz (m s(^{-1}))</td>
<td>0.381 ± 0.024, 15</td>
<td>0.216 ± 0.018, 10(^{***})</td>
<td>0.143 ± 0.016, 7(^{***})</td>
<td>0.275 ± 0.021, 14(^{***})</td>
<td>0.279 ± 0.019, 10</td>
<td>0.188 ± 0.02, 9*</td>
</tr>
<tr>
<td>At 8 Hz (m s(^{-1}))</td>
<td>0.332 ± 0.020, 10</td>
<td>0.215 ± 0.021, 9(^{**})</td>
<td>0.164 ± 0.008, 4(^{***})</td>
<td>0.263 ± 0.026, 11</td>
<td>0.275 ± 0.020, 9</td>
<td>0.17 ± 0.21, 7*</td>
</tr>
<tr>
<td>AERP (ms)</td>
<td>24.36 ± 1.47, 9</td>
<td>27.67 ± 2.917, 9</td>
<td>N/A</td>
<td>23 ± 1.77, 17</td>
<td>34 ± 3.9, 17(^{**})</td>
<td>N/A</td>
</tr>
<tr>
<td>Wavelength (cm)</td>
<td>0.869 ± 0.072, 8</td>
<td>0.672 ± 0.086, 8*</td>
<td>N/A</td>
<td>0.627 ± 0.078, 10</td>
<td>0.738 ± 0.083, 10*</td>
<td>N/A</td>
</tr>
</tbody>
</table>

All results shown are means ±SEMs, sample size (n). The sample size indicates the number of hearts used for arrhythmic incidence, conduction velocity, AERP, wavelength, \(I_{Na}\) activation properties and \(I_{Na}\) inactivation properties. For resting potential, the sample size indicates the number of patches.

N/A, unavailable data due to loss of excitability. AERP, atrial effective refractory period; \(k\), steepness factor describing the currents’ dependence upon voltage; \(V^*\), half-maximal voltage describing the voltage excursion corresponding to half-maximal current.

*Significant effects of 1 or 5 \(\mu\)M flecainide compared to 0 \(\mu\)M.

†Differences between RyR2\(^{SS}\) and WT genotypes at the same flecainide concentration. Single, double and triple symbols denote \(P < 0.05\), \(P < 0.01\) and \(P < 0.001\) respectively.

‡A maximal recorded value, or unavailable data, where a Boltzmann function did not fit.

Variations in \(I_{Na}\) were measured with the loose patch-clamp method before and following alterations in the solution bathing the external face of the membrane. These involved raising the patch pipette, exchanging the solution in the bath and then re-establishing the patch at the same membrane location. \(I_{Na}\) records obtained in response to progressively increasing depolarizing steps from the resting potential gave virtually superimposable and thus reproducible current–voltage relationships before and following withdrawal and restoration of the patch pipette in a WT left atrium. Similar electrode withdrawals and reapplications during which external [\(Na^+\)] was first reduced from 146 to 39 mM and then returned to 146 mM produced fully reversible reductions in the observed currents. The kinetics of these inward currents were similar to those described previously (Lemoine et al. 2011, King et al. 2013b) consistent with their representing \(I_{Na}\). The resting membrane potential measurements obtained independently using sharp intracellular microelectrodes from \(n = 15\) or 16 cells of 3 or 4 hearts in the presence and absence of flecainide ranged from \(-70.43 ± 0.51\) mV to \(-72.49 ± 0.56\) mV (Table 1), a variation which was not significantly different and is within the error of tip potential recordings (Adrian 1956). This represented a consistent baseline voltage from which voltage excursions (V) could be imposed.

**Statistical analysis**

Data are expressed as means ± SEM. Different experimental genotype groups of unpaired data were
compared using two-way ANOVA followed by Bonferroni-corrected t-tests if significant differences were found (GraphPad Prism v.6; GraphPad Software, Inc., La Jolla, CA, USA). Twenty-two WT and 26 RyR2SS hearts were used in the experiments – 7 WT and 6 RyR2SS for the loose patch experiments, with the remainder paced at 6.67, 8 and 10 Hz and then taken through the PES protocol, allowing the determination of CV, arrhythmogenicity, AERP and wavelength in the same hearts when all protocols were successfully completed; ‘n’ denotes the number of hearts successfully studied in each case. Statistical significance was defined as $P < 0.05$.

**Results**

The experiments determined the arrhythmic incidence during electrical pacing in both RyR2SS and WT hearts in the presence and absence of 1 and 5 μM flecainide. These were then correlated with $I_{\text{Na}}$, CV, AERP and $\lambda$, reductions which have been previously associated with arrhythmic substrate.

**Flecainide increases arrhythmic incidence in WT atria yet is anti-arrhythmic in RyR2SS**

As summarized in Table 1, incidences of atrial arrhythmia, classified as an occurrence of two or more non-stimulated atrial electrograms, were greater in untreated RyR2SS than WT as previously reported (King et al. 2013c). Flecainide (1 μM) exerted anti-arrhythmic effects in RyR2SS in contrast to pro-arrhythmic effects in WT (Fig. 2). Two-way ANOVA demonstrated strong interactions ($P = 0.0032$; $F = 9.644$) between the effects of flecainide and genotype upon arrhythmic incidence. Post hoc testing demonstrated that RyR2SS hearts showed significantly higher incidences of arrhythmia than WT before flecainide challenge ($t = 3.018; P < 0.05$). Application of 1 μM flecainide significantly increased arrhythmic incidence in WT ($t = 2.584; P < 0.05$) but significantly decreased it in RyR2SS ($t = 2.520; P < 0.05$). Following application of 1 μM flecainide to both genotypes, there was no significant difference in arrhythmic incidence between WT and RyR2SS ($t = 1.826; P > 0.05$).

Five micromolar flecainide was also tested; however, over a third of hearts (10 of 27) then became unresponsive to stimulation during either or both regular pacing (particularly at the higher frequencies) and PES.

**Flecainide increases Na$^+$ currents in RyR2SS in contrast to decreasing Na$^+$ currents in WT**

Activation and inactivation curves were obtained by plotting peak inward currents, $I_{\text{Na(max)}}$ against $V$ (Figs 3 and 4 respectively). These could be fitted to Boltzmann functions to provide empirical indications of maximum peak currents ($I_{\text{Na(max)}}$), steepness factors ($k$) describing their dependence upon voltage and the voltage excursions corresponding to half-maximal current ($V^*$). Such optimizations were possible for all activation and inactivation data apart from WT atria studied in 5 μM flecainide. Both protocols demonstrated that RyR2SS had a significantly reduced maximal inward Na$^+$ current ($I_{\text{Na(max)}}$) compared to WT (Fig. 3, activation: $t = 8.48; P < 0.001$; Fig. 4, inactivation: $t = 8.42; P < 0.001$), but, whereas 1 μM flecainide reduced $I_{\text{Na(max)}}$ in WT (activation: $t = 12.53, P < 0.001$, inactivation; $t = 17.88, P < 0.001$), it paradoxically increased such inward currents in RyR2SS (activation: $t = 4.38, P < 0.01$, inactivation: $t = 8.84, P < 0.001$). Five micromolar flecainide decreased $I_{\text{Na(max)}}$ in both RyR2SS and WT (inactivation: $t = 9.42, P < 0.001$ and $t = 11.47, P < 0.001$ respectively).

Similarly contrasting effects on $I_{\text{Na(max)}}$ were obtained with the specific RyR blocker dantrolene (10 μM) (Fig. 3, inset). Thus, in response to 80 mV voltage steps, dantrolene produced a net decrease in $I_{\text{Na(max)}}$ in WT atria (−15.08 ± 1.68 vs. −10.89 ± 1.42, $n = 7$, $t = 3.05; P < 0.05$), but increased $I_{\text{Na(max)}}$ in RyR2SS atria (−8.04 ± 1.19 vs. −11.54 ± 1.00, $n = 7, t = 4.26; P < 0.01$).

The $k$ of the activation curves were indistinguishable between both genotypes and through all flecainide concentrations (Fig. 3, $P > 0.05$). The $k$ of the inactivation curves were similar, though smaller in the RyR2SS than the WT atria in the absence of flecainide (Fig. 4, $t = 2.79, P < 0.05$). This difference was abolished by 1 μM flecainide ($t = 0.16, P > 0.05$). Additional increases in flecainide concentration to 5 μM further increased $k$ in both RyR2SS and WT ($t = 2.73, P < 0.05$ and $t = 1.86, P > 0.05$) compared with 0 μM flecainide, and compared with 1 μM flecainide in WT ($t = 2.32, P < 0.05$).

Finally, $V^*$ of activation was consistently smaller in RyR2SS than in WT atria whether in 0 ($t = 2.92; P < 0.05$) or 1 μM flecainide ($t = 3.48; P < 0.01$, Fig. 3). Flecainide (1 μM) decreased $V^*$ in WT ($t = 3.39; P < 0.01$), but not RyR2SS ($t = 0.57, P > 0.05$). The $V^*$ of inactivation was similarly reduced in untreated RyR2SS compared to WT ($t = 5.75; P < 0.001$, Fig. 4). However, 1 μM flecainide increased $V^*$ in RyR2SS ($t = 2.87, P < 0.05$) but not WT ($t = 0, P > 0.05$), with RyR2SS showing a smaller $V^*$ than WT ($t = 3.02, P < 0.05$). Increases in flecainide concentration to 5 μM similarly increased $V^*$ in RyR2SS ($t = 3.43, P < 0.01$) relative to findings with 0 μM flecainide. It left $V^*$ in WT close to that
Figure 2 Contrasting actions of flecainide on arrhythmic incidence in RyR2<sup>S/S</sup> and WT. (a) Illustration of the S1S2 stimulation protocol, consisting of repeated cycles of 8 S1 stimuli, each followed by a single extrasystolic S2 stimulus imposed at successively shorter S1S2 intervals. The first and last few cycles of the protocol are shown, with the intervening cycles omitted (dashed lines). The protocol was terminated when an S2 either failed to elicit an AP, as observed by a missing atrial electrogram, or produced an arrhythmia. Thus, panel (a) depicts the penultimate stimulus cycle, whose S2 stimulus successfully elicited conducting electrical activity (a), followed by the final cycle that induced either arrhythmia or refractoriness. Typical traces obtained from (b) WT and (c) RyR2<sup>S/S</sup> before (i) and following (ii) introduction of 1 µM flecainide were obtained from the last stimulus cycle whose S2 stimulus successfully elicited electrical activity (left panels) and the final cycle which induced either arrhythmia or refractoriness (right panels) as described above. The filled arrowheads indicate timings of regular (S1) stimulation, and the filled arrows indicate the resulting S1 atrial electrogram. The open arrowheads indicate the timing of the extrasystolic (S2) stimuli, and the open arrows indicate the resulting S2 atrial electrogram. The arrowheads are directly below the stimulus artefact, and the arrows are directly above the resulting atrial electrogram. Note that atrial electrogram conduction from the point of stimulation to the point of recording is slow relative to conduction of the stimulus artefact, such that the S2 stimulus artefacts can appear within the preceding S1 waveform at the recording site despite occurring after the atrial electrogram at the stimulus site. Panel (d) depicts the results of applying the PES protocol to 10 WT and 17 RyR2<sup>S/S</sup> hearts to assess the incidence of arrhythmic events normalized to the number of hearts studied in each group. * denotes a difference (P < 0.05) at 0 and 1 µM flecainide within a genotype. † denotes a difference (P < 0.05) between RyR2<sup>S/S</sup> and WT genotypes at the same flecainide concentration.
obtained at 0 μM flecainide (t = 1.26, P > 0.05) as well as the corresponding result in the RyR2<sup>S/S</sup> (t = 0.20; P > 0.05). Thus, both k and V* values in the activation and inactivation characteristics in the atria of RyR2<sup>S/S</sup> and WT mice showed consistent patterns with the addition of 0, 1 and 5 μM flecainide.
WT, and full reversibility in the RyR2\textsuperscript{S/S}, at all the investigated pacing rates.

Two-way ANOVA demonstrated significant effects of flecainide on CV at all pacing frequencies (6 Hz: $F = 20.11$, $P < 0.001$; 8 Hz: $F = 25.72$, $P < 0.0001$; 10 Hz: $F = 13.88$, $P < 0.0001$), while genotype differences were significant only at 6 Hz ($F = 7.067$, $P = 0.0106$, 8 Hz: $F = 0.001364$, $P = 0.9707$, 10 Hz: $F = 0.004279$, $P = 0.9481$). Nevertheless, the interaction between genotype and flecainide was significant at all frequencies (6 Hz: $F = 3.456$, $P = 0.0396$; 8 Hz: $F = 8.383$, $P = 0.0006$; 10 Hz: $F = 3.375$, $P = 0.0433$), indicating that the effect of flecainide is dependent upon both its concentration and genotype. Thus, a full pairwise comparison between all factors was permitted.

At 6 Hz pacing, post hoc tests demonstrated significant differences between WT and RyR2\textsuperscript{S/S} when both were treated with 1 $\mu$M flecainide ($t = 3.316$, $P < 0.001$). One micromolar flecainide significantly reduced CV only in the WT ($t = 3.943$, $P < 0.001$), while 5 $\mu$M reduced CV in both the WT ($t = 5.471$, $P < 0.001$) and RyR2\textsuperscript{S/S} ($t = 3.343$, $P < 0.01$).

At 8 Hz pacing, post hoc tests demonstrated that CV was significantly slower in the untreated RyR2\textsuperscript{S/S} relative to the untreated WT ($t = 3.952$, $P < 0.001$). Similar to 6 Hz, 1 $\mu$M flecainide significantly reduced CV only in the WT ($t = 5.604$, $P < 0.001$) while 5 $\mu$M reduced CV in both the WT ($t = 7.206$, $P < 0.001$) and RyR2\textsuperscript{S/S} ($t = 2.826$, $P < 0.05$).

At 10 Hz pacing, post hoc tests demonstrated that flecainide significantly reduced CV only in the WT at 1 $\mu$M ($t = 3.748$, $P < 0.01$) and in both the WT and the RyR2\textsuperscript{S/S} at 5 $\mu$M ($t = 4.179$, $P < 0.001$ and $t = 2.824$, $P < 0.05$ respectively).

**RyR2\textsuperscript{S/S} AERP increases with flecainide treatment, while WT AERP remains unchanged**

Individual and mean (±SEM) AERPs from WT and RyR2\textsuperscript{S/S} before and following addition of 1 $\mu$M flecainide are shown in Figure 6 and Table 1. To a first approximation, AERP would be expected to depend mainly on action potential duration (APD) and Na\textsuperscript{+} channel availability in the final repolarization phase. However, two-way ANOVA empirically demonstrated that flecainide increased AERP only with flecainide intervention ($F = 4.761$, $P < 0.05$). It did so with application of flecainide (1 $\mu$M) in RyR2\textsuperscript{S/S} ($t = 3.82$, $P < 0.01$) but not in WT. Basal AERP values were indistinguishable between untreated WT and RyR2\textsuperscript{S/S} in agreement with previous reports (King et al. 2013c). Previous studies suggest that these changes likely take place in an absence of changes in APD in the RyR2\textsuperscript{S/S} system.
Furthermore, it has been shown in the Scn5a+/-C0 system, in which there was a loss of Na+ channel function, that flecainide produced a shortening of the APD in the face of a lengthening VERP (Martin et al. 2011).

**Figure 5** Paradoxical actions of flecainide on conduction velocities in RyR2S/S and WT. Three-dimensional representations of local activation times (LATs) each accompanied by matrices representing the calculated velocity vectors in WT (a, n = 15) and RyR2S/S hearts (b, n = 14) in 0, 1 and 5 μm flecainide. Mean (±SEM) epicardial conduction velocities for WT (clear bars) and RyR2S/S (black bars) in 0, 1, 5 and following subsequent return to 0 μm flecainide during regular 6.67, 8 and 10 Hz pacing (c). *denotes a difference arising from use of 1 μm flecainide within a genotype compared to the respective control (0 μm flecainide). †denotes a difference between RyR2S/S and WT genotypes with the same concentrations of flecainide. In each case, single, double and triple symbols denote P < 0.05, P < 0.01 and P < 0.001 respectively.

**Figure 6** Paradoxical actions of flecainide on AERP in RyR2S/S and WT. Individual paired and mean (±SEM) AERPs in 0 and 1 μm flecainide for WT (n = 9) and RyR2S/S (n = 17) hearts. ** denotes a difference (P < 0.01) arising from use of 1 μm flecainide within a genotype compared to the respective control (0 μm flecainide).

Action potential wavelengths correlate with arrhythmic incidence in both RyR2S/S and WT

Two-way ANOVA demonstrated strong interactions (P = 0.0021; F = 13.72) between the effects of flecainide and genotype upon \( \lambda \), indicating that the effect of flecainide is different in WT compared to RyR2S/S. Flecainide decreased \( \lambda \) in WT (t = 2.39, P < 0.05) while increasing it in RyR2S/S atria (t = 2.42, P < 0.05).

We then correlated CV, AERP and \( \lambda \) with arrhythmic incidences before and following application of 1 μm flecainide (Fig. 7). As indicated above, flecainide significantly reduced CV in WT but not RyR2S/S, directly correlating with the increased arrhythmic incidence in WT but not the decreased incidence of arrhythmia in RyR2S/S (Fig. 7A). In contrast, flecainide significantly increased AERP in RyR2S/S but not WT atria directly correlating with the decreased arrhythmic incidences in RyR2S/S but not the increased arrhythmic incidences in WT (Fig. 7B). However, flecainide decreased \( \lambda \) in WT but increased \( \lambda \) in RyR2S/S (Fig. 7C). In contrast to CV and AERP, changes in \( \lambda \) therefore correlated with alterations in arrhythmia in both RyR2S/S and WT. This implicates \( \lambda \) as the
primary predictor for arrhythmic incidences rather than either CV or AERP alone, in both RyR2SS and WT.

Discussion

The present study demonstrates a novel paradoxical effect of the \( I_{\text{Na}} \) blocker flecainide on arrhythmic incidence and \( I_{\text{Na}} \) in RyR2-P2328S and WT atria. It follows directly from evidence for its anti-arrhythmic effects in human CPVT (Pott et al. 2011, van der Werf et al. 2011, Watanabe et al. 2013). Its findings complement a recent report that the alternative class Ic anti-arrhythmic agent, propafenone, similarly exerted anti-arrhythmic actions during atrial fibrillation in a CSQ2/−/− model of CPVT (Faggioni et al. 2014). Although \( I_{\text{Na}} \) and CV were not measured in that latter study, the two reports converge upon common arrhythmic mechanisms through differing measurements and experimental systems. It also reconciles several previous studies at the cellular as opposed to tissue level. These attributed the anti-arrhythmic effects of flecainide to a range of factors. First, flecainide was suggested to reduce triggered activity arising from DADs (Liu et al. 2011). This could result from direct actions inhibiting spontaneous RyR2-mediated SR Ca\(^{2+}\) release implicated in such DADs: flecainide blocks RyR2-Ca\(^{2+}\) release channel open states, thereby reducing Ca\(^{2+}\) wave frequency in CSQ2/−/− mice and rat myocytes (Watanabe et al. 2009, Hilliard et al. 2010, Galimberti & Knollmann 2011). However, a subsequent study reported that whereas flecainide prevented isoproterenol-induced CPVT, it did not exert major effects on Ca\(^{2+}\) homeostasis in RyR2-R4496C hearts (Liu et al. 2011). This suggested that flecainide increases the threshold for triggered activity by directly inhibiting Na\(_a\)1.5 function (Liu et al. 2011). Second, reductions in Na\(^+\) entry could reduce intracellular [Na\(^+\)], thereby increasing forward-mode NCX activity, in turn reducing intracellular [Ca\(^{2+}\)] (Sikkel et al. 2013). The alternative \( I_{\text{Na}} \) blockers, tetrodotoxin, propafenone or lidocaine similarly reduced Ca\(^{2+}\) spark and wave frequency, and wave velocity in WT rat myocytes, doing so only before \( I_{\text{Na}} \) inactivation brought about by alterations in holding voltage. Flecainide also increased NCX-mediated Ca\(^{2+}\) efflux, an effect reversed by reducing extracellular [Na\(^+\)] (Sikkel et al. 2013).

Figure 7: Paradoxical actions of flecainide on CV, AERP and \( \lambda \) and their correlations with arrhythmic incidence. Left panels: comparison of CV (a), AERP (b) and \( \lambda \) (c) in WT (open bars, \( n = 8 \)) and RyR2SS (filled bars, \( n = 10 \)) hearts in 0 and 1 \( \mu \text{M} \) flecainide. These are correlated with incidences of atrial tachyarrhythmias (AT) (a–c, right panels). * denotes a difference arising from use of 1 \( \mu \text{M} \) flecainide within a genotype compared to the respective control (0 \( \mu \text{M} \) flecainide). † denotes a difference between RyR2SS and WT genotypes under the same concentration of flecainide. In each case, single, double and triple symbols denote \( P < 0.05, P < 0.01 \) and \( P < 0.001 \) respectively.
However, recent findings also associated both catecholamine-induced ventricular arrhythmia (Zhang et al. 2013b) and atrial arrhythmogenesis with reductions in CV also associated with RyR2S/S (King et al. 2013a,b,c). RyR2S/S atria showed reduced \( I_{\text{Na}} \) compared to WT. Increased AF susceptibility in association with conduction abnormalities has also been observed in other models of altered Ca\(^{2+}\) homeostasis, including murine CREM-1bAC-X AF (Li et al. 2014) and CSQ2\(^{−/−}\) hearts (Glukhov et al. 2013). In WT, elevating extracellular Ca\(^{2+}\) and manipulating cellular Ca\(^{2+}\) homeostasis using caffeine or cyclopiazonic acid acutely replicated these effects (Zhang et al. 2011).

These findings suggest that RyR2-mediated Ca\(^{2+}\) release in RyR2S/S results in inhibition of \( I_{\text{Na}} \) reducing CV, thus producing a re-entrant, arrhythmic substrate. Inhibition of RyR2-mediated Ca\(^{2+}\) release by flecainide should then paradoxically restore \( I_{\text{Na}} \) and rescue both the compromised CV and arrhythmic phenotype. Our findings confirm this prediction: untreated murine RyR2S/S atria were more arrhythmic than WT, confirming recent findings (King et al. 2013a,b,c), and at the cellular level showed reduced \( I_{\text{Na}} \) compared to the corresponding WT. Flecainide (1 \( \mu \)M) was anti-arrhythmic in RyR2S/S despite being pro-arrhythmic in WT. These findings were concordant with findings at the cellular level in which untreated RyR2S/S showed reduced \( I_{\text{Na}} \) compared to the corresponding WT. Flecainide then reduced \( I_{\text{Na}} \) in WT while increasing it in RyR2S/S. The use of an alternative more specific RyR blocker, dantrolene (10 \( \mu \)M), similarly reduced \( I_{\text{Na}} \) in WT atria while increasing it in RyR2S/S atria. Dantrolene has previously been shown to reduce Ca\(^{2+}\) spark frequency and arrhythmogenicity in induced pluripotent stem cells derived from a CPVT patient carrying a RyR2 S406L mutation (Jung et al. 2012). The pro-arrhythmic action of flecainide in the WT may appear surprising due to its clinical utility for atrial tachycardia without structural abnormality. However, flecainide has proved pro-arrhythmic in various models (Brugada et al. 1991, Stokoe et al. 2007) and most notoriously in the cardiac arrhythmia suppression trial (CAST 1989). It has been proposed that this may result from effects on cardiac repolarization, and indeed, there is evidence for reduced \( I_{\text{Kr}} \) in cardiac cells (Follmer & Colatsky 1990, Wang et al. 1996), prolonged QT interval in human patients (Katritsis et al. 1995, Sarubbi et al. 1998), and repolarization abnormalities and increased arrhythmic incidences in perfused guinea-pig hearts (Osadchii 2012). The present results additionally suggest that reduction in \( I_{\text{Na}} \), CV and \( \lambda \) may contribute to the pro-arrhythmic effects of flecainide. Thus, at the tissue level, untreated RyR2S/S showed reduced CVs compared to WT, despite similar AERPs. Flecainide decreased CV but conserved AERP in WT, whereas it spared CV and increased AERP in RyR2S/S. Nevertheless, \( \lambda \) derived from the product CV \( \times \) AERP correlated directly with arrhythmic tendency in both the RyR2S/S and WT under conditions of either 0 or 1 \( \mu \)M flecainide.

These electrophysiological findings in intact atria are compatible with previous evidence for interactions between Ca\(^{2+}\) homeostasis and Na\(_{1.5}\) expression and function in WT myocytes at the cellular level. Increases in pipette Ca\(^{2+}\) concentration reduced \( I_{\text{Na}} \) density and (dV/dt)\(_{\text{max}}\) in patch-clamped WT myocytes (Casini et al. 2009). The Ca\(^{2+}\) channel blocker verapamil and the Ca\(^{2+}\) ionophore calcimycin, respectively, increased and decreased Na\(_{1.5}\) mRNA and Na\(_{1.5}\) protein expression in rat cardiomyocytes (Oxford & Catterall 1989, Taouis et al. 1991, Duff et al. 1992). Increased extracellular [Ca\(^{2+}\)] and BAPTA-AM, respectively, expected to increase and decrease intracellular [Ca\(^{2+}\)] and correspondingly increased and decreased \( I_{\text{Na}} \) density in cultured neonatal rat myocytes (Chiamvimovvat et al. 1995).

The findings also agree with previous evidence for mechanisms linking Ca\(^{2+}\) homeostasis to Na\(_{1.5}\) at the molecular level. Na\(_{1.5}\) is a major calmodulin kinase II (CaMKII) target. Such phosphorylation shifts the voltage dependence of inactivation to negative potentials without affecting channel activation. This slows recovery from inactivation, enhances Na\(_{1.5}\) transitions into slower forms of inactivation and increases late \( I_{\text{Na}} \) (Wagner et al. 2011, Grandi & Herren 2014). However, in the present study, although RyR2S/S was associated with a negative shift in inactivation, activation properties were similarly affected. RyR2S/S showed a similar AERP as WT in the absence of flecainide.

The findings together demonstrate contrasting anti- and pro-arrhythmic actions of the Na\(_{1.5}\) channel blocker flecainide in murine RyR2S/S and WT atria respectively. They attribute these to corresponding changes in \( I_{\text{Na}} \), \( \lambda \) and therefore arrhythmic substrate while not excluding involvement of triggered activity in initiating arrhythmia with either genotype. This could involve a mechanism consistent with previously reported suggestions at the cellular level of interactions between cellular Ca\(^{2+}\) homeostasis and Na\(_{1.5}\) function.

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Conflict of interest
None.

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