# The Mechanism of Flecainide Action in CPVT Does Not Involve a Direct Effect on RyR2

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***Rationale:*** **Flecainide, a class 1c antiarrhythmic, has emerged as an effective therapy in preventing arrhythmias in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT) refractory to β-adrenergic receptor blockade. It has been proposed that the clinical efficacy of flecainide in CPVT is because of the combined actions of direct blockade of ryanodine receptors (RyR2) and $Na^+$ channel inhibition. However, there is presently no direct evidence to support the notion that flecainide blocks RyR2 $Ca^{2+}$ flux in the physiologically relevant (luminal-to-cytoplasmic) direction. The mechanism of flecainide action remains controversial.**

***Objective:*** **To examine, in detail, the effect of flecainide on the human RyR2 channel and to establish whether the direct blockade of physiologically relevant RyR2 ion flow by the drug contributes to its therapeutic efficacy in the clinical management of CPVT.**

***Methods and Results:*** **Using single-channel analysis, we show that, even at supraphysiological concentrations, flecainide did not inhibit the physiologically relevant, luminal-to-cytosolic flux of cations through the channel. Moreover, flecainide did not alter RyR2 channel gating and had negligible effect on the mechanisms responsible for the sarcoplasmic reticulum charge-compensating counter current. Using permeabilized cardiac myocytes to eliminate any contribution of plasmalemmal $Na^+$ channels to the observed actions of the drug at the cellular level, flecainide did not inhibit RyR2-dependent sarcoplasmic reticulum $Ca^{2+}$ release.**

***Conclusions:*** **The principal action of flecainide in CPVT is not via a direct interaction with RyR2. Our data support a model of flecainide action in which $Na^+$-dependent modulation of intracellular $Ca^{2+}$ handling attenuates RyR2 dysfunction in CPVT. (*Circ Res*. 2015;116:1324-1335. DOI: 10.1161/CIRCRESAHA.116.305347.)**

**Key Words:** antiarrhythmic drugs ■ cardiac arrhythmias ■ flecainide ■ polymorphic catecholergic ventricular tachycardia ■ ryanodine receptor calcium release channel

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a genetic condition characterized by an increased propensity to adrenergic-induced polymorphic or bidirectional ventricular tachycardia in structurally normal hearts. The autosomal dominant form (CPVT1) is caused by mutations in the cardiac ryanodine receptor (RyR2), whereas the recessive form (CPVT2) arises from mutations in cardiac calsequestrin.[1–3] Common to both CPVT1 and CPVT2 is dysfunctional sarcoplasmic reticulum (SR) $Ca^{2+}$ release during periods of increased adrenergic drive (eg, exercise or emotional stress) that triggers arrhythmogenic delayed afterdepolarizations via the sodium–calcium exchanger.[4]

**Editorial, see p 1284**
**In This Issue, see p 1277**

β-Adrenergic receptor blockers are the first line pharmacotherapy in the clinical management of CPVT but the approach of limiting cardiac excitability is suboptimal and some patients exhibit persistent tachyarrhythmias.[5,6] Flecainide, a class 1c antiarrhythmic and potent $Na^+$-channel blocker, has emerged as an effective alternative in patients with CPVT refractory to β-blockers.[7–9] However, enthusiasm for using flecainide is tempered by the incomplete understanding of its mechanisms of action in the context of CPVT. Investigations of flecainide in a mouse model of CPVT2 and in genotyped CPVT1 and 2 patients concluded that this drug's principal mechanism of action was direct block of the open RyR2 channel, which limited augmented SR $Ca^{2+}$ release through RyR2 and attenuated delayed afterdepolarization–mediated triggered activity.[8,10]

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| Nonstandard Abbreviations and Acronyms | |
|---|---|
| CPVT | catecholaminergic polymorphic ventricular tachycardia |
| PFR | pore-forming region |
| RyR2 | cardiac ryanodine receptor |
| SR | sarcoplasmic reticulum |

Although plausible based on the data reported, the biophysical characterization of blockade of sheep RyR2 by flecainide favored the net flow of ions through the RyR2 channel in the cytosolic-to-luminal direction, that is, the nonphysiological direction and did not establish whether flecainide inhibited the physiological (luminal-to-cytosolic) movement of cations through the channel.

Other groups have challenged the controversial assertion that RyR2 is the primary target of flecainide in CPVT. Liu et al[11] reported that the efficacy of flecainide in the R4496C$^{+/-}$ RyR2 mouse model of CPVT1 depended on its well-characterized action on $Na^+$ channels by increasing the threshold for triggered activity. Sikkel et al[12] proposed that the flecainide-induced attenuation of $Ca^{2+}$ wave generation was due predominantly to reduced cytosolic $Ca^{2+}$ ensuing from modulation of the sodium–calcium exchanger/$I_{Na}$ axis.[1–3] The proposed triple mode model of flecainide effect, which attempts to reconcile the observed actions of flecainide on RyR2, $I_{Na}$ and sodium–calcium exchanger, has at its core the direct block of RyR2 by flecainide as the major mechanism.[4,13]

An important step in resolving the controversy is to demonstrate the ability of flecainide to directly modulate RyR2-mediated luminal-to-cytosolic ion flux. Here, we report the detailed examination of the ability of flecainide to influence the conduction and gating properties of RyR2. At concentrations exceeding 50 μmol/L (ie, substantially higher than would be achieved in humans via clinical dosing regimens),[5,6,14] we demonstrate for the first time that cytosolic flecainide does not inhibit the physiologically relevant, luminal-to-cytosolic flux of cations through the RyR2 channel nor does it affect channel gating. Moreover, our data show that flecainide does not markedly inhibit essential charge-compensating monovalent cation counter currents through either the SR $K^+$ channel or the RyR2. Consistent with these findings, flecainide did not inhibit RyR2-mediated $Ca^{2+}$ release from the SR in permeabilized adult cardiac ventricular myocytes. Our data refute the proposal that the clinical effectiveness of flecainide in patients with CPVT is dependent on its ability to modulate SR $Ca^{2+}$ release by a direct action on RyR2.

## Methods

A detailed Methods section is available in the Online Data Supplement.

### Conditions for Recording Single hRyR2 Channels

Single human RyR2 (hRyR2) channels were incorporated into bilayers formed using a suspension of phosphatidylethanolamine (Avanti Polar Lipids) in n-decane (35 mg/mL). Bilayers were formed in a solution containing 610 mmol/L KCl, 20 mmol/L HEPES (pH 7.4) in both (*cis* [0.5 mL] and *trans* [1 mL]) chambers. Channel incorporation from the *cis* chamber was facilitated by the introduction of an osmotic gradient (using 200 μL 3 mol/L KCl). On stirring, hRyR2 incorporates in a fixed orientation such that the *cis* chamber corresponds to the cytosolic side of the channel and the *trans* chamber to the luminal side. After channel incorporation, symmetrical ionic conditions were reinstated by perfusion of the *cis* chamber with a 610 mmol/L KCl and 20 mmol/L HEPES (pH 7.4) solution. All experiments were performed at room temperature (20–22°C). The effects of flecainide, propafenone, and tetracaine (Sigma) were determined after addition of the drug to either *cis* or *trans* chamber at concentrations indicated in the text.

### Ventricular Myocyte Isolation, Permeabilization, and Imaging of $Ca^{2+}$ Sparks and Waves

All animal surgical procedures and perioperative management were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th ed., 2011) under assurance number A5634-01. Imperial College Ethical Review Committee authorized the project license. Rats were euthanized by cervical dislocation after exposure to 5% isoflurane until righting reflex was lost. Cardiac myocytes were enzymatically isolated from the left ventricle of healthy adult male Sprague-Dawley rats by Langendorff perfusion. Cells were attached to coverslips using mouse laminin (Sigma-Aldrich) and superfused with a solution containing 90 mmol/L KCl, 10 mmol/L NaCl, 5 mmol/L (total) $K_2ATP$, 10 mmol/L creatine phosphate, 5.5 mmol/L (total) $MgCl_2$, 0.05 mmol/L $K_2EGTA$, and 0.02 mmol/L $CaCl_2$. The fluorophore used was 5 μmol/L fluo5F pentapotassium salt (LifeTech) for waves and fluo4 pentapotassium salt for sparks. Cells were permeabilized in this solution containing 0.1 mg/mL escin (Sigma) for ≈2 minutes, until $Ca^{2+}$ waves were observed (denoting permeabilization), whereupon cells were superfused with the original imaging solution containing varying concentrations of flecainide (0, 5, or 25 μmol/L). The same cell was imaged in the absence of drug (0), or after the addition of 5 and 25 μmol/L flecainide using a cross-over protocol.

## Results

### Concentration-Dependent Block of Cytosolic-To-Luminal Cation Flux by Flecainide

We have investigated the interactions of flecainide with individual purified, recombinant, hRyR2 channels reconstituted into planar phospholipid bilayers under voltage clamp conditions. The quality of channel tetramers was determined before and after density gradient centrifugation and function was confirmed using $Ca^{2+}$ activation (Online Figure I). As was the case in the original reports using native sheep cardiac RyR2,[7–10] we have used a monovalent cation ($K^+$) as the charge carrying species to maximize resolution of hRyR2 gating and block. With equal concentrations of $K^+$ on both sides of the bilayer the direction of net $K^+$ flux through the channel was determined by the holding potential applied across the bilayer. At positive holding potentials net cation flux is in the nonphysiological direction, from the cytosolic to the luminal side of the bilayer. At negative holding potentials net $K^+$ flux is from the luminal to the cytosolic side of the bilayer: equivalent to the physiological cation flux during $Ca^{2+}$ release from the SR. Because flecainide is reported to be a blocker of the open RyR2 channel,[8,10] in these experiments we maximized channel open probability (Po) by adding EMD 41000[11,15] to the solution at the cytosolic side of the channel.

Figure 1A shows current fluctuations of a representative hRyR2 channel at a holding potential of +40 mV (net $K^+$ flux from cytosol to lumen) before, and after, the addition of increasing concentrations of flecainide to the solution at the cytosolic side of the membrane. In agreement with earlier reports,[8,10] under these conditions flecainide induces short-lived,

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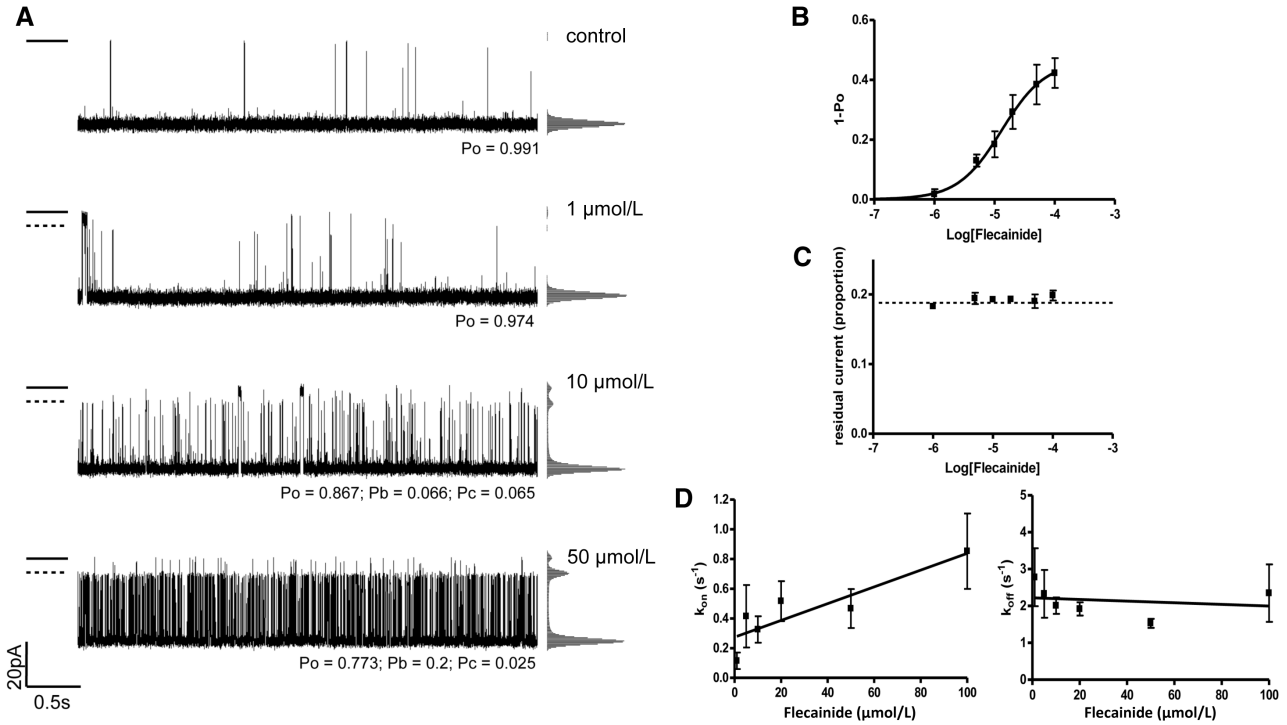


**Figure 1. Flecainide partially blocks a cytosolic-to-luminal current through human RyR2. A**, Representative single-channel traces recorded at +40 mV. Openings are downward from the closed level (black line). The increase in blocking events (marked with a dotted line) with increasing flecainide concentration is depicted in the frequency amplitude histograms and manifests as a decrease in Po. **B**, The effect of this block on channel Po (expressed here as 1-Po, rather than Pb to emphasize change in overall open probability) increases with concentration. **C**, Residual current is unchanged with increasing flecainide concentration. **D**, Rates of association ($k_{on}$) and dissociation ($k_{off}$) of flecainide. Data for (**B**) and (**D**) are from n=6 channels.

but well-resolved, transitions from the open state to a reduced conductance state, presumably reflecting partial occlusion of the RyR2 conduction pathway by flecainide. The probability of occurrence of these transitions increases as the concentration of flecainide is raised and with 50 µmol/L cytosolic flecainide, it is clear that during each blocking event some $K^+$ flux continues through the channel.

Parameters of cytosolic flecainide block of cytosolic-to-luminal cation flux are quantified in Figure 1B–1D. The probability of block is dependent on the concentration of cytosolic flecainide, with 50% of maximal occurrence of block at 13.1±1.9 µmol/L (where block is expressed as 1-Po, Figure 1B). Figure 1C demonstrates that the residual current, which continues to flow through RyR2 during flecainide block (19.2±0.6% of full conductance) is independent of flecainide concentration as would be expected if each blocking event occurs as the result of occupancy of a site within the conduction pathway of the channel by a single flecainide molecule. Consistent with this proposed mechanism, cytosolic flecainide's interaction with hRyR2 can be described by a simple 3 state scheme (Online Figure II), in which block occurs as the result of a bimolecular interaction between the open channel and flecainide:

$$\text{Closed} \rightleftarrows \text{Open} \underset{[\text{Flec}]}{\rightleftarrows} \text{Blocked} \quad (1)$$

As a consequence, the apparent rate constants for flecainide association with the open channel ($k_{on}$), and its dissociation from the channel ($k_{off}$), can be determined as the reciprocal of the mean dwell time in the open state and blocked state, respectively. It is clear from Figure 1D that, in agreement with Equation 1, the rate of association of flecainide is linearly dependent on its concentration, whereas flecainide concentration has no influence on the dissociation of bound flecainide.

The experiments presented in Figure 1 provide a detailed description of some of the mechanisms responsible for the partial block of cytosolic-to-luminal cation flux in hRyR2 by cytosolic flecainide, and confirm and extend the observations made in earlier investigations.[8,10] However, a rigorous assessment of the ability of flecainide to regulate RyR2-mediated $Ca^{2+}$ release from the SR to the cytosol requires considerably more information.

## Cytosolic Flecainide Does Not Block the Luminal-To-Cytosolic Flux of Ions Through hRyR2

We have investigated if the physiologically relevant flux of cations through RyR2 can be inhibited by cytosolic flecainide using different experimental approaches. The first of these was to monitor the action of cytosolic flecainide under conditions in which the net cation current through hRyR2 is driven luminal-to-cytosolic by the transmembrane holding potential. An example of data acquired at −40 mV is shown in Figure 2, where traces were obtained under conditions identical to those in Figure 1, with the holding potential reversed. Under these conditions, cytosolic flecainide, up to a concentration of 50 µmol/L, a concentration in excess of the therapeutically relevant range,[14] produced no significant reduction in channel Po.

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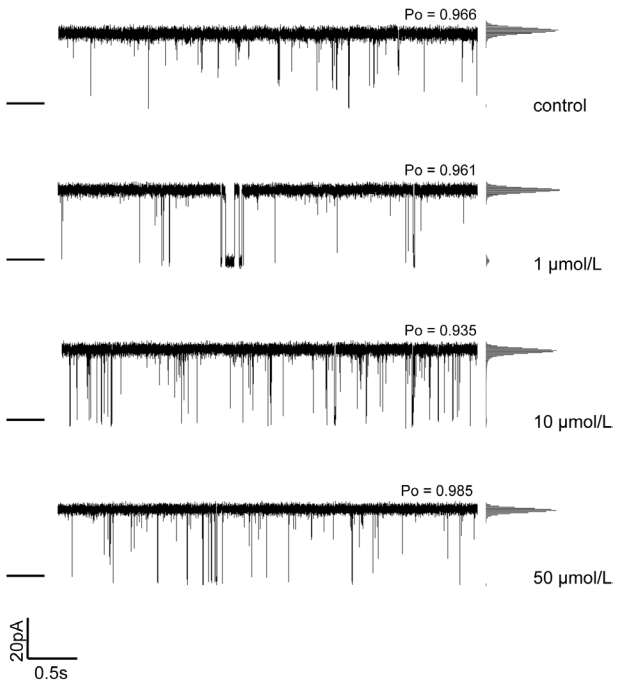


**Figure 2. Flecainide does not block voltage-driven luminal-to-cytosolic cation flux through human RyR2.** Representative single-channel traces, recorded at −40 mV (luminal-to-cytosolic current) in the presence of cytosolic flecainide. Openings are upward from the closed level (black line); no blocking events or significant changes in Po were observed (n=6).

A straightforward interpretation of this observation is that cytosolic flecainide does not block the physiologically relevant flux of cations through RyR2. However, in addition to determining the net flux of cations through RyR2, transmembrane holding potential may also directly influence the interaction of a blocking molecule with its binding site on the channel. Previous investigations have established that the effectiveness of blocking molecules, with similar modes of action to flecainide in RyR2, is increased at positive transmembrane potentials.[16–18] The probability of hRyR2 block by 50 µmol/L cytosolic flecainide was determined at holding potentials between ±70 mV (Figure 3A). These experiments demonstrate that the probability of block by cytosolic flecainide is influenced by transmembrane holding potential, with effective block observed only at positive potentials where net cation flux through hRyR2 is cytosolic-to-luminal. Measurements of rates of flecainide association and dissociation demonstrate that both are influenced by transmembrane holding potential (Figure 3B).

Voltage dependence of block arises either as a direct result of membrane voltage on the charged blocking molecule or as the result of interactions between the blocker and the permeant ions.[19–21] A direct influence of voltage on flecainide would require the blocker to be positioned within the voltage drop across the membrane (ie, within the selectivity filter). It is inconceivable that a molecule as large as flecainide could interact at a site within the selectivity filter of RyR2, and not fully occlude the pore (a residual current of ≈20% persists with flecainide bound (Figure 1C)). Indeed, a binding site outside the selectivity filter is supported by our demonstration (Online Figure III) of a direct competition between flecainide and tetrapentyl ammonium: a blocking molecule with an established binding site within the cytosolic vestibule of the RyR2 pore-forming region (PFR).[22] Therefore, it is logical to propose that the voltage dependence of RyR2 block by cytosolic flecainide arises from interactions with permeant ions within the pore. As a consequence, rather than blocking luminal-to-cytosolic cationic flux through RyR2, cytosolic flecainide, bound with relatively low affinity, will be destabilized by a net luminal-to-cytosolic flux of cations.

An alternative approach is to investigate block under conditions where the driving force for ion movement through RyR2 is provided solely by an ionic gradient in the absence of a transmembrane holding potential. Under these conditions (210 mmol/L $K^+$ cytosolic and 850 mmol/L $K^+$ luminal), 50 µmol/L cytosolic flecainide produces no blocking events to a reduced conductance level (Figure 3C), and overall open probability is unchanged (Figure 3D). Taken together, these 2 experimental approaches indicate that flecainide at the cytosolic face of RyR2, even at a concentration 10-fold higher than that needed to reduce arrhythmic events in a mouse model of CPVT,[10] is incapable of blocking luminal-to-cytosolic cation flux through the channel.

## Flecainide Does Not Block hRyR2 From the Luminal Side of the Channel

Flecainide that has crossed the sarcolemma into the cytosol will probably further equilibrate with the SR lumen (see Online Methods section). Could luminal flecainide inhibit hRyR2-mediated movement of cations from the SR lumen to the cytosol? Figure 4A shows current fluctuations of a representative channel before and after the addition of 50 µmol/L flecainide to the solution at the luminal side of the channel at a holding potential of −40 mV, so that net $K^+$ flux is luminal-to-cytosolic. Under these conditions, no blocking events were observed. Figure 4B demonstrates that neither 5 nor 50 µmol/L luminal flecainide produced a significant reduction in hRyR2 open probability or current amplitude. These experiments establish that flecainide cannot enter the RyR2 PFR and block permeant cation flux from the luminal side of the channel.

## Flecainide Does Not Affect hRyR2 Gating

The data presented above demonstrate that the therapeutic action of flecainide in the treatment of CPVT does not involve block of luminal-to-cytosolic cation flux through the open channel. However, the physiologically relevant RyR2-mediated release of $Ca^{2+}$ from the SR could be inhibited if flecainide reduced RyR2 open probability by an action on channel gating. We have tested this hypothesis by monitoring hRyR2 gating in the presence of a range of concentrations of cytosolic flecainide. In these experiments, hRyR2 channels were activated solely by the physiological regulator of open probability, cytosolic $Ca^{2+}$, and gating was monitored at −40 mV so that net $K^+$ flux was luminal-to-cytosolic. Under these conditions, flecainide produces no open-channel blocking events and channels simply fluctuate between open and closed gating conformations. Figure 5 demonstrates that

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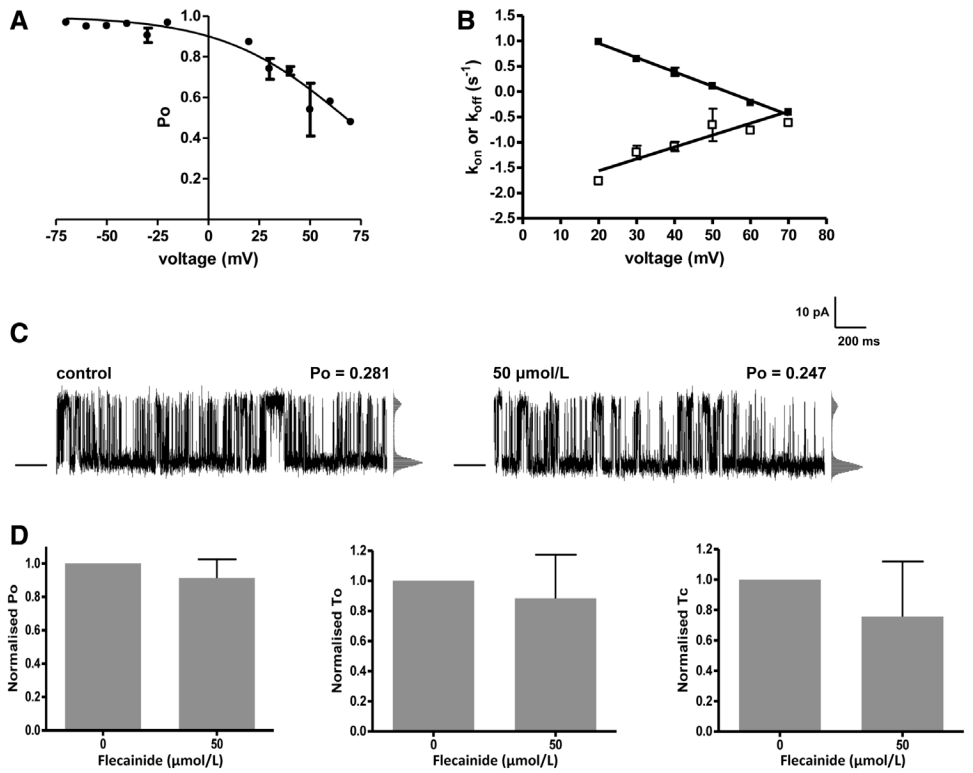


**Figure 3. Block is voltage dependent and does not occur when the current is driven by an ionic gradient in the luminal-to-cytosolic direction. A**, Po of human RyR2 channels (n=6) in the presence of 50 µmol/L cytosolic flecainide determined at holding potentials between ±70 mV. **B**, Rates of association ($k_{on}$, open squares) and dissociation ($k_{off}$, solid squares) are voltage dependent. **C**, Representative single-channel traces, recorded at 0 mV, with a luminal-to-cytosolic current provided by an ionic gradient. Openings are upward from the closed level (black line). **D**, Po, mean open time (To) and mean closed time (Tc) were not significantly altered by 50 µmol/L cytosolic flecainide under these conditions (n=3).

cytosolic flecainide, up to a concentration of 500 µmol/L, has no significant influence on channel open probability, mean open time, mean closed time, or single-channel current amplitude.

## Flecainide Does Not Block the Physiologically Relevant Flux of $Ca^{2+}$ Through hRyR2

The experiments reported to this point have been performed using $K^+$ as the permeant cation in hRyR2. The use of $K^+$ is

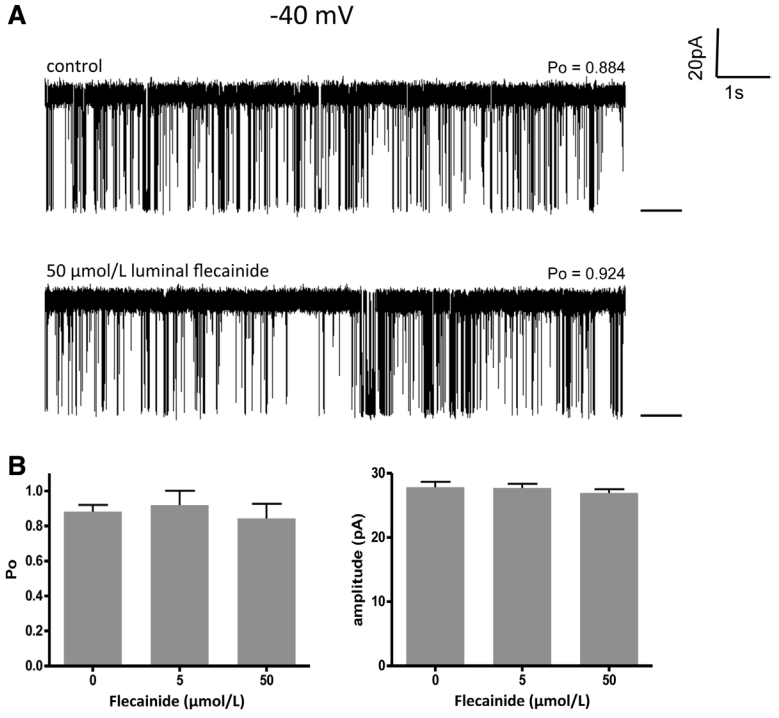


**Figure 4. Luminal flecainide does not block a luminal-to-cytosolic cation flux through human RyR2. A**, Representative single-channel traces recorded at −40 mV. Openings are upward from the closed level (black line). **B**, No significant changes in Po or current amplitude were observed in the presence of 5 µmol/L (n=3) or 50 µmol/L (n=5) luminal flecainide.

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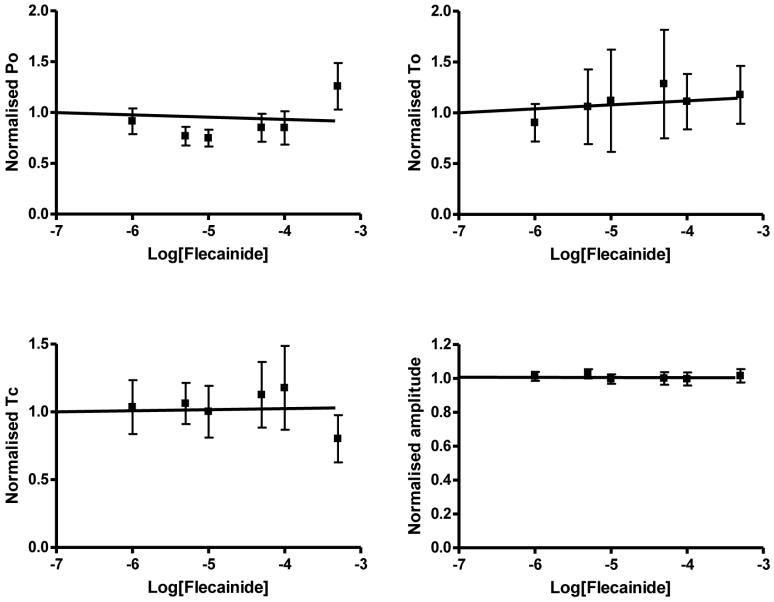


**Figure 5. Flecainide does not affect human RyR2 gating**. Luminal-to-cytosolic flux (at –40 mV) was measured with $Ca^{2+}$ as the sole activating ligand. Open probability, mean open and closed durations, and current amplitude were not significantly altered by cytosolic flecainide (n=4–8).

valuable because (1) it maximizes the resolution of hRyR2 gating and block and (2) it allows manipulation of the direction of net current flow through the open channel by varying either transmembrane holding potential or ionic gradient, and hence provides insights into the mechanisms underlying the actions of flecainide in RyR2. However, in the intact cardiac muscle cell, the driving force for luminal-to-cytosolic $Ca^{2+}$ flux is provided by the $Ca^{2+}$ gradient across the SR membrane and during this electrogenic process the trans-SR membrane potential is maintained at 0 mV by charge-compensating movements of monovalent ions through various SR membrane channels. It is possible to recreate the cytosolic and SR luminal ionic environments in our experiments, and to regulate RyR2 gating by including appropriate activating and inhibitory ligands.[23,24] Figure 6A shows representative current fluctuations of a single hRyR2 channel under these conditions. A driving force for $Ca^{2+}$ movement is provided by a 100-fold (luminal-to-cytosolic) gradient and the transmembrane potential is clamped at 0 mV. Channel Po is regulated by the inclusion of cytosolic $Ca^{2+}$, $Mg^{2+}$, and ATP (see Online Methods section for details). The addition of 50 μmol/L cytosolic flecainide produces no noticeable blocking events (Figure 6A), change in current amplitude (Figure 6B), or significant reduction in Po (Figure 6C).

## Flecainide Will Not Limit SR $Ca^{2+}$ Release by Inhibiting the Charge-Compensating Counter Current

If flecainide does not influence $Ca^{2+}$ release from the SR by modulating RyR2 function, could it affect SR $Ca^{2+}$ release indirectly by reducing or preventing an essential charge-compensating counter current?

Rapid, regulated, release of $Ca^{2+}$ from the cardiac SR store is such a key component of cardiac muscle function that it is

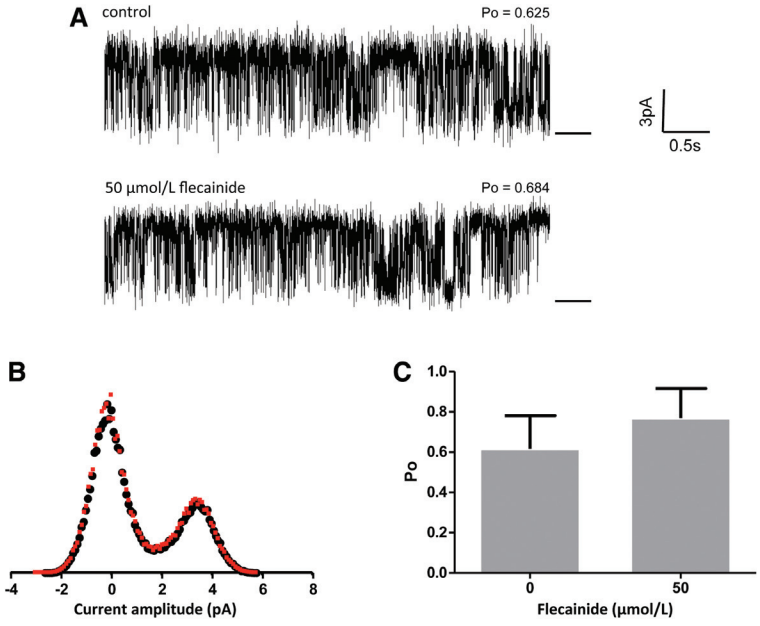


**Figure 6. Flecainide does not block the physiologically relevant $Ca^{2+}$ flux through human RyR2**. **A**, Representative single-channel traces recorded at 0 mV where the driving force for $Ca^{2+}$ movement is provided by a luminal-to-cytosolic chemical gradient. Openings are upward from closed level (black line). **B**, Current amplitude histogram before (black) and after (red) addition of 50 μmol/L flecainide. **C**, Open probability is not significantly altered in the presence of 50 μmol/L flecainide under these conditions (n=3).

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not surprising that the SR membrane is over endowed with charge-compensating systems. Two of these involve the movement of $K^+$ from the cytosol into the SR store in response to the luminal-to-cytosol flux of $Ca^{2+}$[23] and are hence potential targets for block by flecainide. In addition to the SR $K^+$ channel, now identified as the trimeric intracellular cation channel (TRIC),[25] RyR2 can carry a $K^+$ counter current during $Ca^{2+}$ release.[23,26] The relative contribution of these 2 SR charge-compensating mechanisms during RyR2-mediated $Ca^{2+}$ release and sarco/endoplasmic reticulum calcium ATPase (SERCA2a)-dependent $Ca^{2+}$ uptake is a topic of much interest and debate.[26,27]

We investigated the potential actions of flecainide on the SR $K^+$ channel by incorporating isolated rat cardiac low-density SR membrane vesicles into planar bilayers using methods developed in our group.[28] Current fluctuations of individual channels were monitored at ±40 mV with flecainide present in the solutions at both sides of the bilayer. Figure 7A shows single-channel current traces from a representative SR $K^+$ channel at a holding potential of +40 mV. The channel shows long-lasting open events, which are unaffected by the presence of either 5 or 50 µmol/L flecainide. Subsequent addition of 2.5 mmol/L succinyl choline, an established blocker of the cardiac SR $K^+$ channel,[29] leads to the occurrence of characteristic blocking events and a reduction in channel Po (Figure 7B). Because it is not possible to unequivocally determine the orientation of the SR $K^+$ channel after reconstitution of isolated SR vesicles into the bilayer, we reversed net $K^+$ current through the channel by imposing a holding potential of −40 mV. As was the case at +40 mV, flecainide produced no discernable block under these conditions (data not shown). Flecainide, even at supraphysiological concentrations, does not inhibit $K^+$ movement through this charge-compensating pathway.

In contrast, flecainide does reduce the charge-compensating $K^+$ counter current through RyR2. As established by the data presented in Figures 1–3, flecainide, present at the cytosolic face of RyR2, is a concentration- and voltage-dependent partial blocker of the cytosolic-to-luminal flux of $K^+$ through the open RyR2 channel. Would block of this form be sufficient to reduce

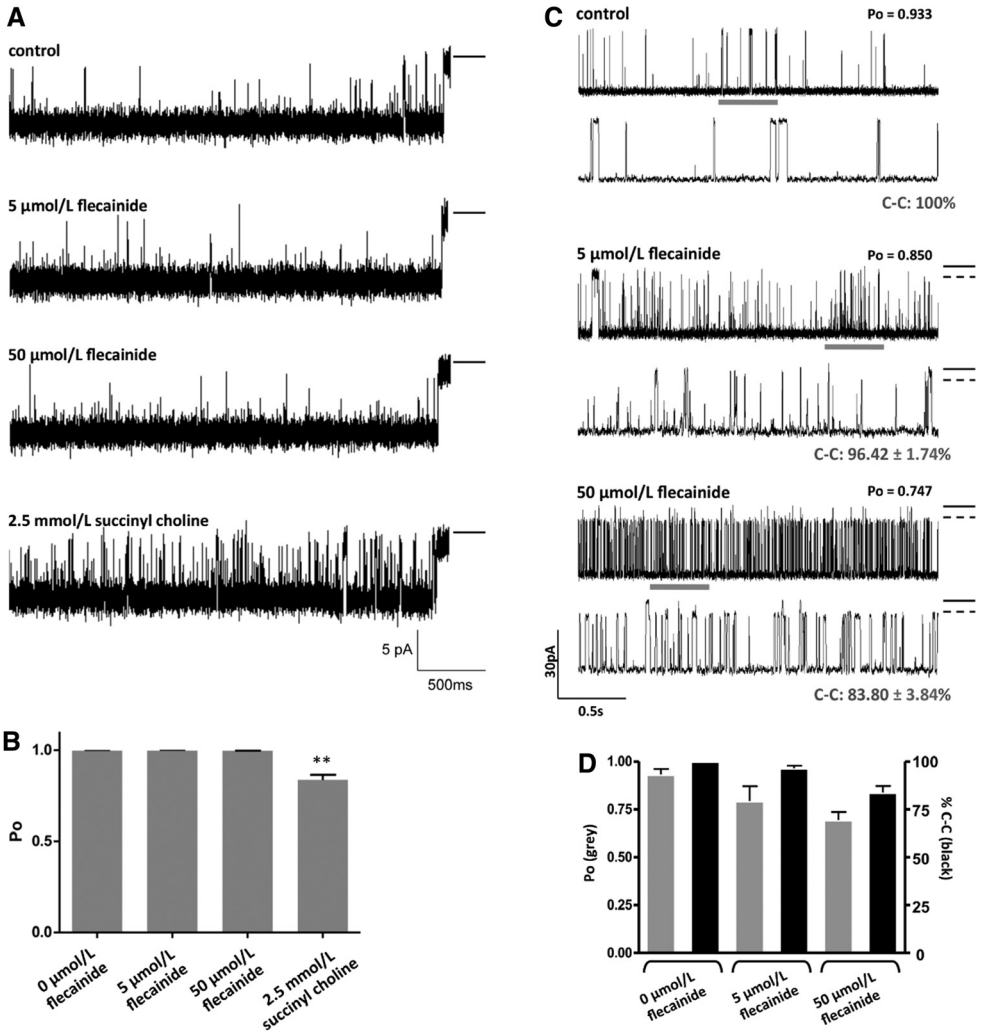


**Figure 7. Flecainide does not markedly inhibit the charge-compensating counter current. A**, Representative single sarcoplasmic reticulum (SR) potassium channel traces recorded at +40 mV. **B**, Flecainide does not block the SR potassium channel, but succinyl choline demonstrates characteristic block (n=5). **C**, Representative single human RyR2 (hRyR2) traces recorded at +40 mV, showing partial block of the cytosolic-to-luminal flux of ions where the reduction in counter current (C–C) has been calculated (the portion of trace underlined is expanded below). Openings are downward from the closed level (solid line) **D**, Reduction in Po and C–C resulting from the partial block of hRyR2 (n=3–6).

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the overall charge-compensating capacity of the SR and inhibit RyR2-mediated $Ca^{2+}$ release? Leaving aside the fact that, in addition to a counter current through RyR2, the SR membrane system has other viable charge-compensating pathways, flecainide's inhibition of the RyR2 counter current is not limiting. Flecainide does not fully occlude the RyR2 conduction pathway so that during each blocking event ≈20% of the unblocked $K^+$ current continues to flow (Figure 1C). We have calculated the reduction in RyR2-mediated $K^+$ counter current resulting from block by cytosolic flecainide (Figure 7C and 7D). At +40 mV, 50 μmol/L flecainide reduces RyR2 Po to ≈0.75 but has a much smaller influence on total counter current, reducing it by only ≈16%. Overall, it is clear that flecainide will not produce a meaningful reduction in charge-compensating counter current during RyR2-mediated Ca2+ release from the cardiac SR.

### Flecainide Does Not Block SR $Ca^{2+}$ Release in Permeabilized Rat Cardiac Myocytes

Measurements of the actions of flecainide on the cation conduction and gating properties of individual hRyR2 and SR $K^+$ channels indicate that flecainide will not inhibit the physiologically relevant release of $Ca^{2+}$ from cardiac SR as the result of a direct block of RyR2, or by the inhibition of charge-compensating $K^+$ currents through SR $K^+$ channels and RyR2.

We have tested this proposal by monitoring $Ca^{2+}$ sparks and waves in individual adult rat cardiac myocytes, permeabilized with escin, an experimental system in which the barrier to flecainide's access to the cytosol is removed, but the SR membrane network remains intact. We consider this to be the most reliable way of assessing SR $Ca^{2+}$ release in a cell system, while ensuring that there is no contribution from the sarcolemma, any effects seen will arise directly from an action of flecainide on the SR. Flecainide has no measureable effect on $Ca^{2+}$ sparks (Figure 8A and 8B) and waves (Figure 8C and 8D) determined under these experimental conditions.

## Discussion

Flecainide, a class 1c antiarrhythmic local anesthetic, is a well-characterized use-dependent blocker of sarcolemmal $Na^+$ channels that has, in recent years, been identified as a novel and effective tool in the treatment of patients with CPVT1 and 2. However, its mechanism of action in CPVT remains controversial. The original reports in which the therapeutic potential of flecainide in the treatment of CPVT was demonstrated proposed that its primary target is the SR $Ca^{2+}$ release channel, RyR2. Flecainide was reported to inhibit arrhythmogenic $Ca^{2+}$ waves because of its ability to modulate SR $Ca^{2+}$ release by blocking the open channel.[8,10] As outlined in the introduction, subsequent studies have highlighted alternative mechanisms of action for flecainide that are focused on its proven ability to block $Na^+$ channels. In response to these investigations,[11,12] it has been proposed[13] that the clinical efficacy of flecainide in patients with CPVT involves suppression of $Ca^{2+}$ waves due a triple mode of action comprising (1) a direct action of flecainide on RyR2,[8,10] (2) a $I_{Na}$-dependent reduced probability of delayed afterdepolarization triggered action potentials as observed by Liu et al[11] and (3) a reduction in cytosolic $Ca^{2+}$

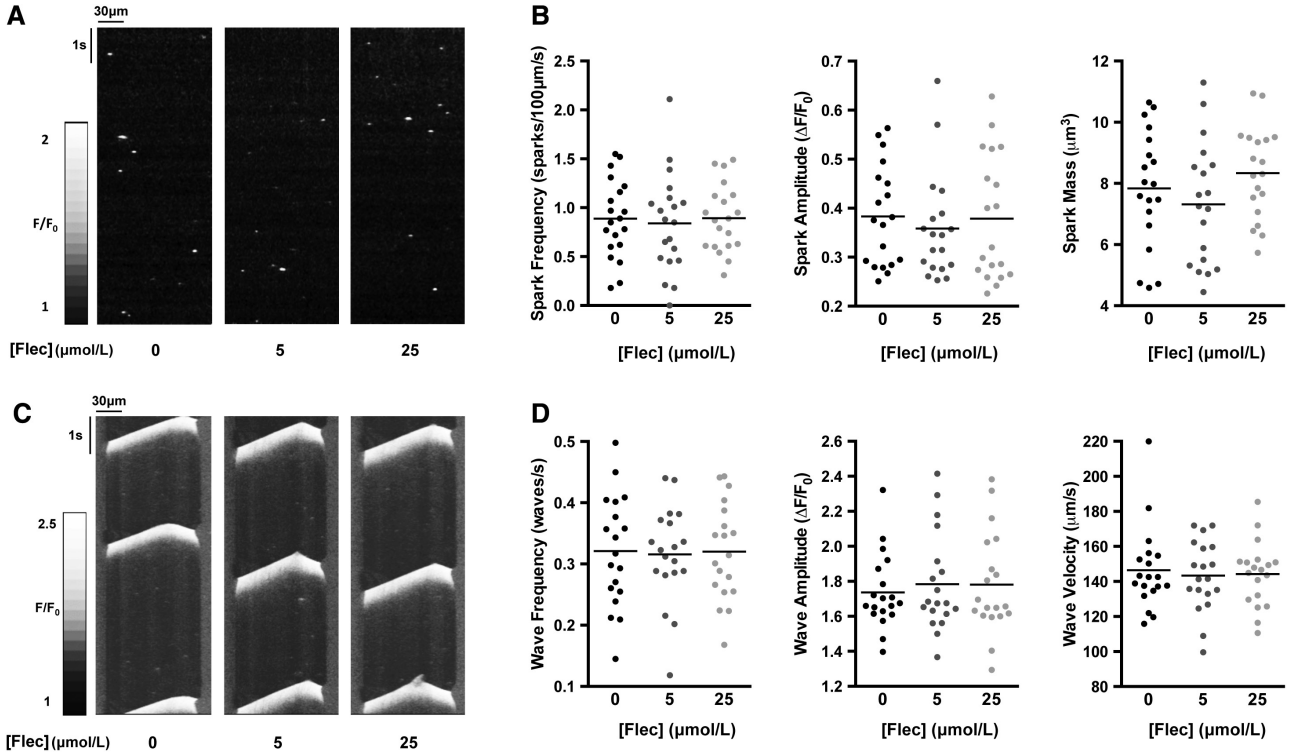


**Figure 8. Flecainide does not affect sarcoplasmic reticulum $Ca^{2+}$ release in permeabilized rat cardiac myocytes.** Representative confocal line-scans of (**A**) $Ca^{2+}$ sparks and (**C**) $Ca^{2+}$ waves in escin-permeabilized rat cardiac myocytes after the addition of flecainide at 5 and 25 μmol/L. Quantitative analysis of (**B**) $Ca^{2+}$ sparks and (**D**) $Ca^{2+}$ waves revealed the lack of flecainide effect on any parameter. There were no statistically significant differences between group mean as determined by ANOVA (n=19 cells).

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concentration resulting from the change in $Na^+/Ca^{2+}$ homeostasis because of $I_{Na}$ block as reported by Sikkel et al.[12]

Given the importance of flecainide in the treatment of CPVT, and in an attempt to clarify the role of RyR2 in its action, we have tested the hypothesis that flecainide's therapeutic action in patients with CPVT is dependent on its ability to directly modulate RyR2-mediated release of $Ca^{2+}$ from the cardiac SR. Our data demonstrate that, while high concentrations of cytosolic flecainide can, to some extent, block cytosolic-to-luminal flux of monovalent cations through RyR2, this local anesthetic cannot inhibit the physiologically relevant, luminal-to-cytosolic flux of cations through the channel or affect channel gating. Neither is luminal-to-cytosolic cation flux affected by high concentrations of flecainide at the luminal face of the channel. Moreover, flecainide does not significantly inhibit the essential charge-compensating monovalent cation counter current carried by the SR $K^+$ channel and RyR2. Consistent with these findings, flecainide does not inhibit RyR2-mediated $Ca^{2+}$ release from the SR in permeabilized adult cardiac ventricular myocytes.

These conclusions are based principally on observations of single hRyR2 function after reconstitution into planar bilayers and are, therefore, dependent on the orientation of the reconstituted channels. RyR channels show marked structural asymmetry with clearly defined cytosolic and luminal domains.[30,31] Given this, it is inconceivable that, during reconstitution, the massive, hydrophilic, cytoplasmic domain would cross the membrane in preference to insertion of the much smaller, hydrophobic, transmembrane domain into the membrane. As a consequence, in our investigations, the cytosolic face of hRyR2 channels will be exposed to the *cis* chamber and the luminal face to the *trans* chamber. RyR2 is also functionally asymmetrical and experiments confirming the orientation of reconstituted channels in our study are presented. We show hRyR2 activation by $Ca^{2+}$ in the nmol/L to μmol/L range (Online Figure I), and block by tetrapentyl ammonium (Online Figure III), both of which define the cytosolic side of the channel.[16,32]

## Why Doesn't Flecainide Block the Physiologically Relevant Movement of Cations Through RyR2?

Our data establish that interactions between flecainide and RyR2 only occur when the local anesthetic is added to the solution at the cytosolic side of the channel. When bound, the flecainide molecule adds a barrier to the RyR2 conduction pathway that may be either steric or electrostatic or both. This barrier reduces the rate of cation translocation from the cytosolic side of the channel to the lumen, that is, in the nonphysiological direction. The rate at which flecainide associates with its binding site is dependent on its concentration and, consistent with a simple bimolecular interaction, cytosolic flecainide concentration does not influence its rate of dissociation.

Both rates of association and dissociation of cytosolic flecainide are influenced by transmembrane potential. The voltage drop across membrane channels is concentrated in a short, narrow, region of the PFR within which discrimination between ions occurs, that is, the selectivity filter. Focusing of the voltage drop is made possible by intrusion of the cytosolic solution into the transmembrane region within the large cytosolic vestibule of the channel,[33] known to form part of $K^+$ and $Na^+$ channel architecture[34,35] with equivalent structures predicted in RyR1 and RyR2.[36,37] Therefore, for transmembrane potential to directly influence the binding site for flecainide, it is logical to assume that this site would need to be within the region of RyR2 equivalent to the selectivity filter in $K^+$ and $Na^+$ channels. Modeling studies suggest that while this region of RyR2 is unlikely to be the sole site of ion discrimination in the channel,[36] it is still the narrowest section of the conduction pathway and will be the region where the voltage drop occurs. Given this, it is highly improbable that a molecule as large as flecainide could bind within the RyR2 selectivity filter and not fully occlude the pore. Significant residual current in the blocked state (Figure 1C) and direct competition between flecainide and tetrapentyl ammonium (Online Figure III) indicate that flecainide, entering the PFR from the cytosolic side of the channel, is bound within the cytosolic vestibule of RyR2 and in this location does not fully occlude the pore. Our conclusion that the blocking flecainide molecule binds outside the voltage drop across the channel means that the observed dependence of block on transmembrane potential must be because of interactions between flecainide and permeant cations within the PFR. This conclusion is consistent with the observation that, in the absence of an applied transmembrane potential, cytosolic flecainide cannot block the luminal-to-cytosolic flux of either $K^+$ (Figure 3C and 3D) or $Ca^{2+}$ (Figure 6) through RyR2. With net luminal-to-cytosolic cation flux, the affinity with which flecainide is bound in the cytosolic vestibule is insufficient to prevent it being displaced by interactions with permeant cations—a phenomenon first reported by Armstrong in $K^+$ channels[19] and recently explored in more detail.[20,21] This, together with the observations that no blocking site for flecainide exists at the luminal face of RyR2 and that flecainide has no effect on RyR2 gating, demonstrates why flecainide cannot inhibit the physiologically relevant movement of cations through this channel.

The original proposal that the efficacy of flecainide in the treatment of CPVT resided in its ability to block the open RyR2 channel[8,10] was bolstered by the demonstration that another 1c antiarrhythmic, propafenone, was clinically effective in CPVT and blocked cytosolic-to-luminal cation flux in individual RyR2 channels in a manner equivalent to flecainide.[38] However, as demonstrated in Online Figure IV, and consistent with our observations with flecainide, propafenone does not block the physiologically relevant, luminal-to-cytosol movement of cations through RyR2. In contrast, tetracaine (100–1000 μmol/L), a local anesthetic that is a well-characterized inhibitor of $Ca^{2+}$ release from the SR,[39–41] decreases channel Po irrespective of the direction of current flow, at concentrations consistent with those used to inhibit SR $Ca^{2+}$ release in permeabilized cardiac myocytes.[41] As demonstrated by Hilliard et al[10], rather than blocking the open channel, tetracaine reduces RyR2 Po by prolonging the duration of closed events (Online Figure V). These investigations demonstrate that the mechanisms of action of, on the one hand, flecainide and propafenone, and on the other, tetracaine, differ and can explain the differing abilities of these 2 classes of drug to regulate the physiological release of $Ca^{2+}$ from the cardiac SR. It should be noted that a recent publication[42] identifies

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a tetracaine-like slow block effect of flecainide, which was not reported in earlier publications from this group.[8,10,38] This new observation, which is not seen in our experiments, is still reported to occur during cytosolic-to-luminal cation flux through RyR2 and as a consequence, is not relevant to the physiological situation.

The experiments on which our conclusions are based were performed on wild-type human RyR2 and some may question their relevance to the potential action of flecainide in the treatment of CPVT. Two lines of evidence suggest strongly that they are. Flecainide has proven to be effective in humans with, and animal models of, both CPVT1 and 2.[7,8,11] CPVT2 is caused by disrupted control of luminal $Ca^{2+}$ as a consequence of mutations in the $Ca^{2+}$-binding protein cardiac calsequestrin. Individuals with CPVT2 have wild-type RyR2 channels and these channels will respond to flecainide in the ways described in this communication.

CPVT1 is caused by mutations in RyR2 and to date >170 different mutations have been identified,[43] however, the phenotypes of only a relatively small number of these have been examined in detail.[44] For flecainide to block the physiologically relevant, luminal-to-cytosolic, flux of $Ca^{2+}$ in these channels, the CPVT1 mutation, in addition to altering RyR2 function, would also have to result in a greatly increased affinity for flecainide in the PFR and hence prevent the destabilization of bound flecainide by luminal-to-cytosolic cation flux. We have investigated the actions of cytosolic flecainide on recombinant human RyR2 channels, in which we introduced the N4104K CPVT1 mutation. Data presented in Online Figure VI demonstrate that the mechanisms of action of flecainide on hRyR2 are unaffected by this mutation. As in the wild-type hRyR2, cytosolic flecainide is a concentration-dependent, partial blocker of cytosolic-to-luminal cation flux, which has no effect on the luminal-to-cytosolic flux of cations.

We also note that the original experiments demonstrating flecainide block of RyR2 were performed using channels in sheep cardiac SR, however, we do not anticipate that species differences will affect the functional consequence of flecainide interaction with RyR2 as the amino acid sequences for the channel PFR (4731–4967) are identical in human (NP_001026.2) and sheep (XP_004021663.1).

### Why Does Flecainide Inhibit Movement of $Na^+$ into the Cell While Failing to Block the Equivalent Flux of Cations Through RyR2?

The movement of extracellular $Na^+$ into the cytosol during the action potential is facilitated by voltage sensitive, $Na^+$ selective, channels in the sarcolemma and flecainide is a well characterized, potent, blocker of these channels.[45,46] RyR2-mediated release of $Ca^{2+}$ from the SR into the cytosol is an equivalent flux and the channels involved in both processes have equivalent orientations within their respective membrane systems. As highlighted above, both channels have large, water-filled cytosolic vestibules, and selectivity filter regions located toward, respectively, the extracellular and luminal end of the PFR. As is the case with RyR2, studies have established that block of sarcolemmal $Na^+$ channels by flecainide results from interactions of these molecules, present in the cytosol, with sites in an open conformation of the channel.[46] This raises an obvious question, if cytosolic flecainide inhibits the physiologically relevant flux of $Na^+$ into the cell, why does it not inhibit the equivalent cation flux through RyR2? The answer can be found by an inspection of the mechanisms underlying the actions of flecainide on the 2 species of channel. As is clear from the work presented in this communication, flecainide is a simple open state, partial blocker of RyR2 that exerts no influence on channel gating. Block of cytosolic-to-luminal flux results from the relatively low affinity interaction of cytosolic flecainide with a site in the cytosolic vestibule of the channel. Flecainide at this site is rapidly displaced by luminal-to-cytosolic cation flux.

Flecainide has an equivalent mode of action in the open sarcolemmal $Na^+$ channel but exerts an additional influence on function. The $Ca^{2+}$-dependent gating of RyR2 has been defined[32] and is mechanistically different from the voltage-dependent gating of the $Na^+$ channel, where the open channel must pass through structurally distinct inactivated, deactivated, and closed, nonconducting, states before it can reopen. Cytosolic flecainide, bound in the open $Na^+$ channel, significantly slows recovery from the inactivated and deactivated states[47] and, as a result, prevents $Na^+$ influx for an extended period.

## Conclusions

Our data demonstrate that flecainide is unable to regulate RyR2-mediated $Ca^{2+}$ release from the SR either by directly blocking $Ca^{2+}$ release through this channel or by inhibiting the charge-compensating monovalent cation current carried by the SR $K^+$ channel and RyR2. Flecainide is a proven use-dependent blocker of $Na^+$ entry and in the absence of any evidence for an action of flecainide on the release of $Ca^{2+}$ from the SR membrane network, it is logical to propose that the $Na^+$ channel is its primary therapeutic target in CPVT. Use-dependent block of $I_{Na}$ could, in turn, increase the threshold for triggered activity[11] or reduce the probability of RyR2 opening by reducing the level of cytosolic $Ca^{2+}$ as the result of sodium–calcium exchanger–mediated $Ca^{2+}$ efflux driven by reduced cytosolic $Na^+$.[12]

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## Disclosures

None.

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## Novelty and Significance

### What Is Known?

- Flecainide, a class 1c antiarrhythmic and potent $Na^+$-channel blocker, is a clinically effective antiarrhythmic in individuals with catecholaminergic polymorphic ventricular tachycardia (CPVT), refractory to β-adrenergic receptor blockade.
- In the context of CPVT, flecainide has been suggested to block intracellular $Ca^{2+}$ release through open cardiac ryanodine receptor (RyR2) channels.
- Studies testing the effects of flecainide on RyR2 channels have focused on ion flow in the nonphysiological direction (cytosol to sarcoplasmic reticulum lumen).

### What New Information Does This Article Contribute?

- Flecainide does not inhibit RyR2-mediated cation flow in the physiologically relevant direction.
- The primary mode of flecainide action in CPVT is $Na^+$ channel–mediated rebalancing of intracellular $Ca^{2+}$.
- Using flecainide, and other class 1c antiarrhythmics, as prototypical compounds is unlikely to yield new compounds with improved specificity for RyR2.

CPVT is a malignant arrhythmia characterized by dysfunctional sarcoplasmic reticulum $Ca^{2+}$ release and triggered by increased adrenergic drive. β-Adrenergic receptor blockers attenuate aberrant, $Ca^{2+}$-linked, arrhythmia and are the cornerstone of CPVT therapy. Flecainide, a class 1c antiarrhythmic and a potent $Na^+$-channel blocker is effective in patients with CPVT refractory to β-blockade and it has been reported that the mechanism of action is due, at least in part, to its direct blocking action on RyR2. We investigated the effects of flecainide on human RyR2 in planar lipid bilayers under defined experimental conditions. Consistent with earlier reports, we show that flecainide blocked cation movement through the channel in the nonphysiological direction. Crucially, flecainide, even at supraphysiological concentrations, had no effect on the physiologically relevant sarcoplasmic reticulum-to-cytosol cation flux through RyR2, nor did it affect other mechanisms that impinge on sarcoplasmic reticulum $Ca^{2+}$ release (eg, $K^+$ counter current). These findings suggest that the mechanism of flecainide does not involve a direct action on RyR2. Our data do not negate the clinical use of flecainide but serve to highlight that class 1c compounds should not be considered as prototypical RyR2 blockers.

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# The Mechanism of Flecainide Action in CPVT Does Not Involve a Direct Effect on RyR2

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# Correction

In the *Circulation Research* article by Bannister et al (The Mechanism of Flecainide Action in CPVT Does Not Involve a Direct Effect on RyR2. *Circ Res*. 2015:116:1324–1335. DOI: 10.1161/CIRCRESAHA.116.305347.), a correction was needed.

The copyright footnote has been amended to indicate the CC-BY-NC Open Access license.

This has been corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/116/8/1324.full.

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**Supplemental Material**

# The mechanism of flecainide action in CPVT does not involve a direct effect on RyR2

Short title: Bannister/Thomas Flecainide and RyR2

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**Detailed Methods**

Cell culture and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% (v/v) foetal bovine serum, 2 mmol/L glutamine and 100 µg/ml penicillin/streptomycin (LifeTech). Cells were incubated at 37°C, 5% $CO_2$ and 80-90% humidity at a density of 2 x $10^5$ per 100 mmol/L plate (60 $cm^2$) 24 hours prior to transfection with pcDNA3/eGFP-hRyR2 (6 µg per 1 x $10^5$ cells). After overnight incubation, transfected cells were treated with 2 mmol/L sodium butyrate for a further 24 hours before assessment of expression (by eGFP visualisation), harvesting by centrifugation (1500 rpm, AllegraR, Beckman) and storage at -80°C.

Purification of recombinant hRyR2 channels

Frozen cell pellets (typically ~50 x $10^6$ cells) were lysed on ice in a hypo-osmotic buffer (20 mmol/L Tris-HCl, 5 mmol/L EDTA; pH 7.4) containing protease inhibitor cocktail (Roche), by passing them approximately 20 times through a 23G needle. Unbroken cells and nuclei were removed by low speed centrifugation (1500 rpm, 4°C, AllegraR, Beckman) and the resulting lysate was subjected to a high-speed centrifugation (100,000 g, 90 minutes, 4°C, Optima L-90K, Beckman) to collect the microsomal membranes. Solubilization of these membranes was carried out (at a concentration of 2.5 mg/ml) in a solution containing 1 mol/L NaCl, 0.15 mmol/L $CaCl_2$, 0.1 mmol/L EGTA, 25 mmol/L PIPES, 0.6% (w/v) CHAPS and 0.3% (w/v) phosphatidylcholine, with protease inhibitor cocktail (Sigma) at 4°C. Insoluble material was removed by centrifugation (15,000 g, 1 hour at 4°C) and the supernatant loaded onto a 5-30% (w/v) continuous sucrose gradient. Fractions containing channel proteins were collected after overnight (18 hours) centrifugation at 4°C and stored at -80°C until use.

Assessment of hRyR2 tetramer expression and function.

Expression of hRyR2 from ER membranes (50µg) and solubilised sucrose fractions was assessed using immunoblotting as described[1] using an anti-eGFP primary antibody (B-2, Santa Cruz). Sucrose fractions were concentrated (from 500 to 100 µl) using Amicon Ultra 100K centrifugal filters, before loading (15 µl). $Ca^{2+}$ activation was measured using [$^3$H]-ryanodine binding to ER membranes (100 µg) from transfected HEK293 cells and was carried out as reported previously.[2] The binding of [$^3$H]-ryanodine to RyR2 is an indirect measure of channel open probability (Po) in populations of channels as the site of interaction is only accessible in the open state of the channel.[3] The dependence of single channel Po on cytosolic $Ca^{2+}$ can be measured directly by varying the buffered free $Ca^{2+}$ concentration of the *cis* chamber, this was carried out as reported previously.[4]

Isolation of SR $K^+$ channels

Light and heavy SR membrane vesicle fractions were prepared by differential centrifugation of cardiac muscle homogenates derived from adult male Sprague-Dawley rats using protocols described previously.[5] All procedures were carried out according to Institutional and UK ethical guidelines.

Effective concentration range of flecainide

Flecainide has a pKa of 9.3 so that at pH 7.4 99% of flecainide molecules will be cationic with the remaining 1% uncharged. It has been suggested that the intracellular effects of flecainide are best observed after incubation with the drug[6,7] and that flecainide can also accumulate in the heart.[8,9] However the cited studies give no indication of where in the heart, or how, accumulation might occur and provide no information on achievable intracellular concentrations. Over a period of time the flecainide concentrations in the extracellular space, the cytosol and the SR lumen should equilibrate, however cytosolic and luminal concentrations will never exceed the extracellular concentration unless the molecule is bound with high affinity to sites within these compartments. Flecainide, bound to

hypothetical high affinity intracellular sites, would not be free to interact with sarcolemmal or SR membrane channels.

In the studies reported in this communication we have used intracellular flecainide concentrations several fold higher than the extracellular concentrations necessary to inhibit $Ca^{2+}$ waves in isolated cardiac myocytes, to enable us to establish the mechanisms of action of flecainide on intracellular membrane proteins responsible for the regulation of SR $Ca^{2+}$ release.

Conditions for recording single hRyR2 channels

Single hRyR2 channels were incorporated into bilayers formed using a suspension of phosphatidylethanolamine (Avanti Polar Lipids) in n-decane (35 mg/ml). Bilayers were formed in a solution containing 610 mmol/L KCl, 20 mmol/L HEPES (pH 7.4) in both *cis* (0.5 ml) and *trans* (1 ml) chambers. Channel incorporation from the *cis* chamber was facilitated by the introduction of an osmotic gradient (using 200 µl 3 mol/L KCl). On stirring, hRyR2 incorporates in a fixed orientation such that the *cis* chamber corresponds to the cytosolic side of the channel and the *trans* chamber to the luminal side. After channel incorporation, symmetrical ionic conditions were re-instated by perfusion of the *cis* chamber with a 610 mmol/L KCl, 20 mmol/L HEPES (pH 7.4) solution. All experiments were carried out at room temperature (20-22°C).

The effects of flecainide, propafenone and tetracaine (Sigma) were determined after addition of the drug to either *cis* or *trans* chambers at concentrations indicated in the text. We optimized the quantification of block by using conditions that maximize the open duration of the channel i.e. high permeant ion concentration (610 mmol/L $K^+$) in the presence of 20 µmol/L EMD 41000, a RyR2 agonist shown previously to act via the caffeine-binding site.[10] In competition assays, tetrapentylammonium (TPeA) (Sigma) was added to the *cis* chamber at the concentrations stated.

The ability of cytosolic flecainide to block RyR2 was also determined at 0 mV in the presence of an ionic gradient (210 mmol/L KCl *cis*, 850 mmol/L *trans*) with cytosolic $Ca^{2+}$ (approx. 1 µmol/L free[4]) as the only activating ligand.

The effect of flecainide on hRyR2 gating was determined with cytosolic $Ca^{2+}$ as the sole activating ligand at -40 mV (current flowing in the physiologically relevant, luminal-to-cytosolic direction) in the presence of various concentrations of cytosolic flecainide.

The potential effect of flecainide on hRyR2 channel function was also assessed in cell-like salt solutions (with $Ca^{2+}$ as the permeant ion) as follows: Bilayers were formed in symmetrical 120 mmol/L KCl and channels were incorporated using an osmotic gradient as before. After re-instating symmetrical ionic conditions (120 mmol/L KCl), 1 mmol/L $MgCl_2$ and 5 mmol/L $CaCl_2$ were added to the *trans* chamber, while the solution in the *cis* chamber was adjusted to contain 10 µmol/L free $Ca^{2+}$ (using $CaCl_2$), 1 mmol/L free $Mg^{2+}$ and 5 mmol/L total ATP (using $MgCl_2$ and MgATP). Free ion concentrations were calculated using MaxChelator (http://web.stanford.edu/~cpatton/webmaxcS.htm). Recordings were at 0 mV holding potential and the current recorded was due to the net movement of $Ca^{2+}$ ions from the luminal to the cytosolic side of the channel.

Conditions for recording single SR $K^+$ channels

LSR vesicles were fused to PE bilayers in 75 mmol/L $K_2SO_4$ (10 mmol/L HEPES, pH 7.4) using osmotic gradients. The hyperosmotic *cis* chamber was perfused out following successful channel incorporation. Recordings were made in the absence and presence of symmetrical flecainide at a *cis* holding potential of 40 mV with polarity alternating every 30 seconds. Block by succinyl choline was used at the end of each experiment to confirm channel identity. Data were filtered at 1 kHz and open probability and mean dwell times of the full conductance state were determined using TACx4.1.5 (Bruxton).

Analysis of single channel recordings

Single channel currents were low-pass filtered at 5 kHz with an 8-pole Bessel filter then digitized at 20 kHz with a PCI-6036E AD board (National Instruments). Acquire 5.0.1.

(Bruxton) was used for viewing and acquisition of the single channel traces. Data analysis was carried out using QuB v2.0.0.13. www.qub.buffalo.edu).[11] Single channel traces of 2-3 minutes (containing >3000 events) were idealized using the Segmental K-means (SKM) algorithm[12] based on Hidden Markov Models (HMM) and a dead time of 75-120 μs was imposed. In traces where substate block was detected (by evaluation of the amplitude histogram), idealization was carried out using a three state (closed (C) ↔ open (O) ↔ blocked (B)) scheme. QuB can accurately distinguish between blocked and closed levels (Online Figure I) and idealization using this scheme resulted in the calculation of mean amplitudes, open (Po), blocked (Pb) and closed (Pc) probabilities and mean open (To), blocked (Tb) and closed (Tc) times. In all other instances where block was not observed a 2-state (C↔O) scheme was used for idealization, which yielded amplitude, Po, To and Tc as previously.[4] Rates of flecainide association ($K_{on}$) and disassociation ($K_{off}$) were calculated by plotting the reciprocal of To and Tb, respectively against drug concentration or voltage.

Maximal counter current through hRyR2 in the absence of flecainide was estimated by the product of open state amplitude and To over 30 seconds of channel recording in the presence of EMD 41000 (designated 100%). The counter current in the presence of flecainide was estimated by the sum of (open state amplitude x To) and (blocked state amplitude x Tb) over 30 seconds of channel recording, and expressed as a proportion of counter current in the absence of flecainide.

Ventricular myocyte isolation, permeabilisation and imaging of $Ca^{2+}$ sparks

All animal surgical procedures and peri-operative management were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) under assurance number A5634-01. Imperial College Ethical Review Committee authorized the project licence. Rats were sacrificed by cervical dislocation following exposure to 5% isoflurane until righting reflex was lost. Cardiac myocytes were enzymatically isolated from the left ventricle of healthy adult male Sprague-Dawley rats by Langendorff perfusion.[13] A gravity driven superfusion system with a low volume chamber (Warner RC-24N) was used to allow rapid solution exchanges at 37°C. Cells were attached to coverslips using mouse laminin (Sigma-Aldrich) and superfused with a solution containing 90 mmol/L KCl, 10 mmol/L NaCl, 5 mmol/L (total) $K_2ATP$, 10 mmol/L creatine phosphate, 5.5 mmol/L (total) $MgCl_2$, 0.05 mmol/L $K_2EGTA$, 0.02 mmol/L $CaCl_2$. The fluorophore used was 5 μmol/L fluo5F pentapotassium salt (LifeTech) for waves and fluo4 pentapotassium salt for sparks. Cells were permeabilised in this solution containing 0.1 mg/ml escin (Sigma) for ~2 minutes, until $Ca^{2+}$ waves were observed (denoting permeabilisation), whereupon cells were superfused with the original imaging solution containing varying concentrations of flecainide (0, 5 or 25 μmol/L). The same cell was imaged in the absence of drug (0), or following the addition of 5- and 25 μmol/L flecainide using a cross-over protocol as described.[14] Sparks were assessed using line-scan images collected at a rate of 500 lines per second and detected using SparkMaster as previously described.[14-16]

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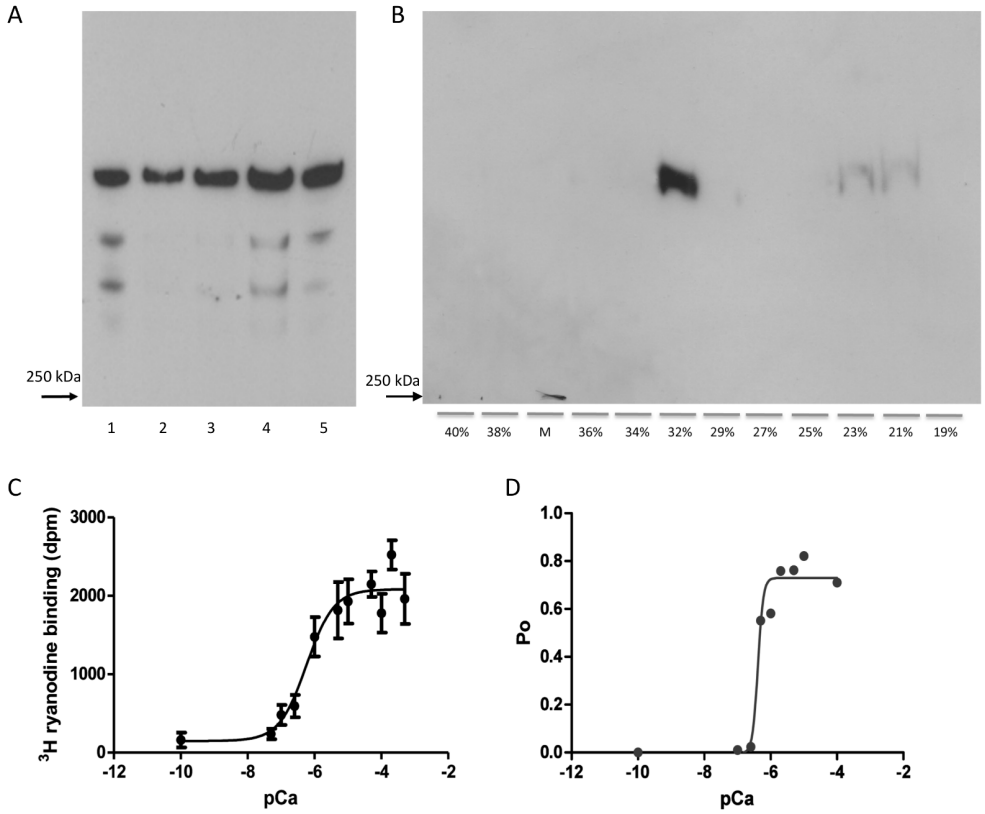
**Supplemental Figures**

**Online Figure I**

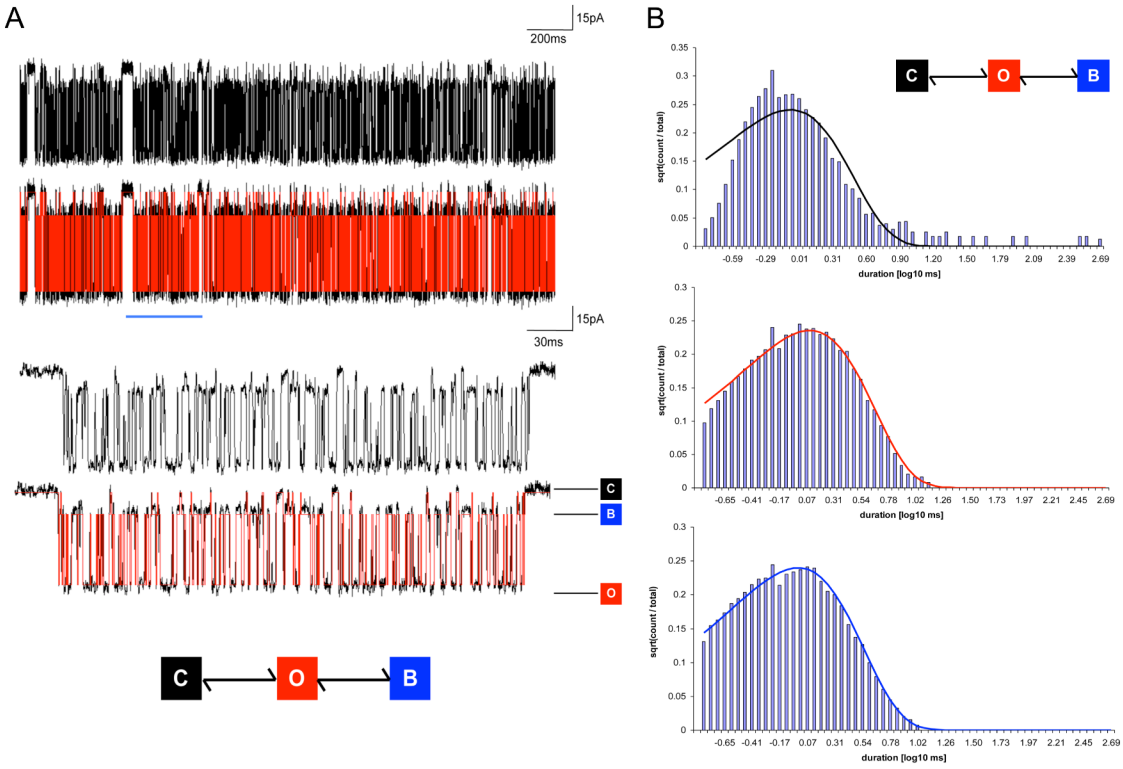



Online Figure I

**Assessment of hRyR2 channel expression and function**

**A.** Full-length recombinant hRyR2 is detected as a discrete band with few breakdown products in microsomes (50µg) from 5 different transfections (labelled 1-5). **B.** Isolation of tetrameric hRyR2 channels (~2.2 MDa) with no breakdown products in a fraction containing approximately 30% sucrose, following sucrose density gradient separation (40-19% fractions shown here, M = molecular weight marker). Recombinant channels are functional both before and after purification: **C.** hRyR2s from microsomes demonstrated typical $Ca^{2+}$ activation, measured using [$^3$H]-ryanodine binding (as disintegrations per minute (dpm), see Methods) with an $EC_{50}$ of 0.58 ± 0.27µmol/L (n=5). **D.** $Ca^{2+}$ activation curve for a representative individual purified hRyR2 channel which activated similarly ($EC_{50}$ = 0.41 µmol/L). It should be noted that both methods demonstrate activation over the range of $Ca^{2+}$ concentrations compatible with the variation in cytosolic $Ca^{2+}$ expected during cardiac excitation-contraction coupling.

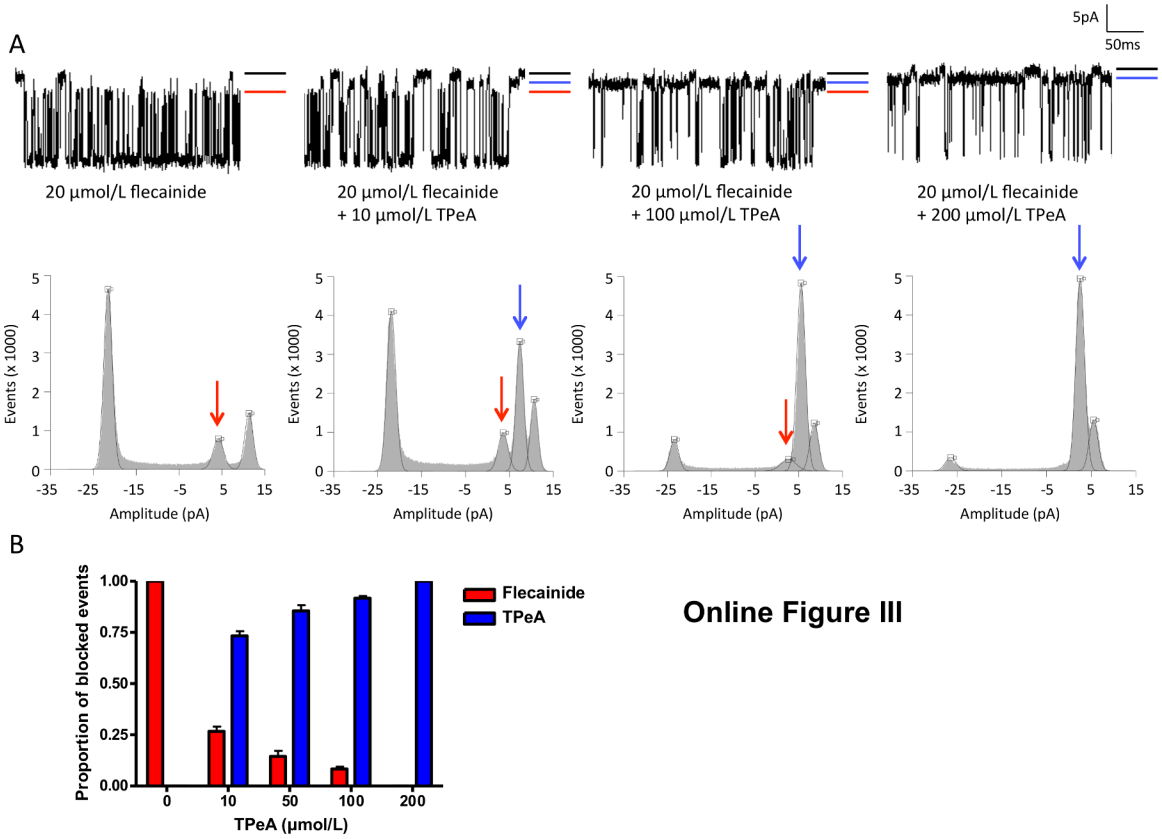
**Online Figure II**




Online Figure II

**Block of the cytosolic-to-luminal ion flux through hRyR2 by flecainide can be described by a three-state scheme.**

**A.** Representative single channel traces recorded at + 40 mV showing flecainide (50 μmol/L) block idealized using the SKM function of QuB (the portion of trace underlined in blue is expanded below). These events were best described by the closed (C) to open (O) to blocked (B) transitions represented by the model shown (and labelled on the trace). **B.** Dwell time histograms (plotted as $\log_{10}$ duration vs square root of counts/total) for closed, open and blocked states (represented by the black, red and blue curves respectively) can each be described by a single exponential.



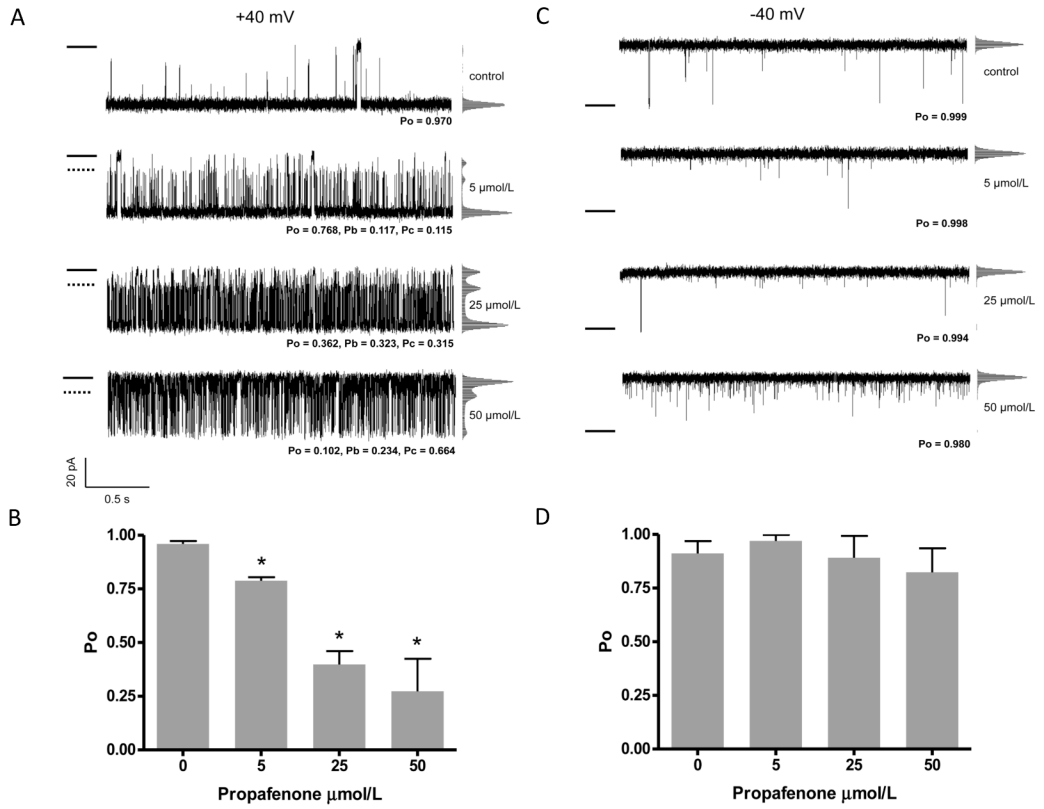

**Online Figure III**

Online Figure III

**Flecainide competes with TPeA to bind at a site in the cytosolic vestibule of hRyR2**

**A.** Representative single channel traces recorded at +40 mV in the presence of 20 µmol/L flecainide and increasing concentrations of TPeA. Openings are downwards from the closed level (black line) with flecainide and TPeA blocked states shown in red and blue respectively. Current amplitude histograms below show that with increasing concentration of TPeA the proportion of blocking events due to flecainide (red arrow) decreases and those due to TPeA (blue arrow) increases. These data (n=3) are represented graphically in **B.** In earlier investigations it has been established that block of RyR2 by cytosolic TPeA involves interactions between the cation and residues of the cavity-lining helices of the channel PFR. Direct competition in the ability of flecainide and TPeA to block hRyR2 identifies the cytosolic vestibule of the hRyR2 PFR as the binding site for flecainide during block.

**Online Figure IV**

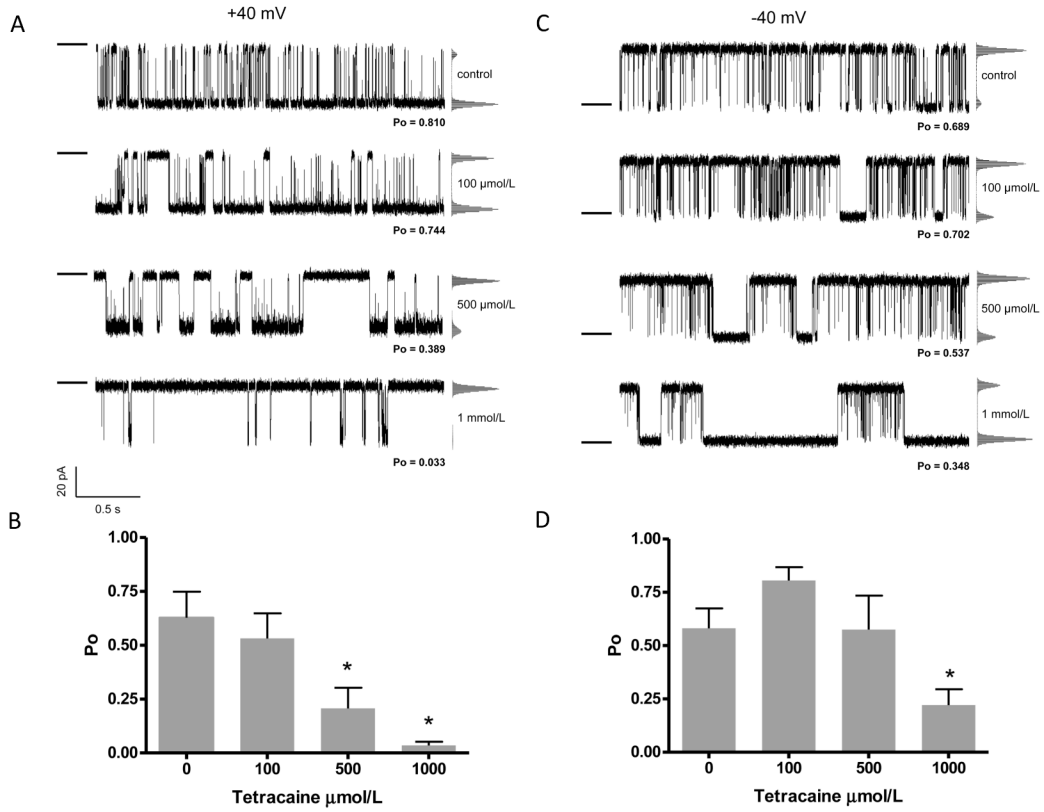



Online Figure IV

**Cytosolic propafenone blocks cytosolic-to-luminal but not luminal-to-cytosolic flux of cations through hRyR2 channels**

**A.** Current fluctuations through a representative hRyR2 channel at a holding potential of +40 mV prior to, and following the addition of, increasing concentrations of propafenone to the cytosolic side of the channel (closed level indicated by the solid line at the left of each trace). Under these conditions the net cation flux through the channel is cytosolic-to-luminal. As is the case with flecainide (see Figure 1), propafenone induces short-lived, but clearly resolved, transitions from the open state to a reduced conductance state (marked with a dotted line). The residual current that continues to flow during propafenone block is larger than that seen with flecainide (31.23 ± 0.27% of full conductance) and does not vary significantly with blocker concentration. **B.** Quantification of the decrease in Po with increasing propafenone concentration (n=5). **C.** Consistent with our observations with flecainide, when net cation flux through RyR2 is reversed to the physiologically relevant direction by imposing a holding potential of -40 mV, propafenone, at concentrations up to 50 µmol/L, produces no significant reduction in Po, quantified in **D**.

**Online Figure V**

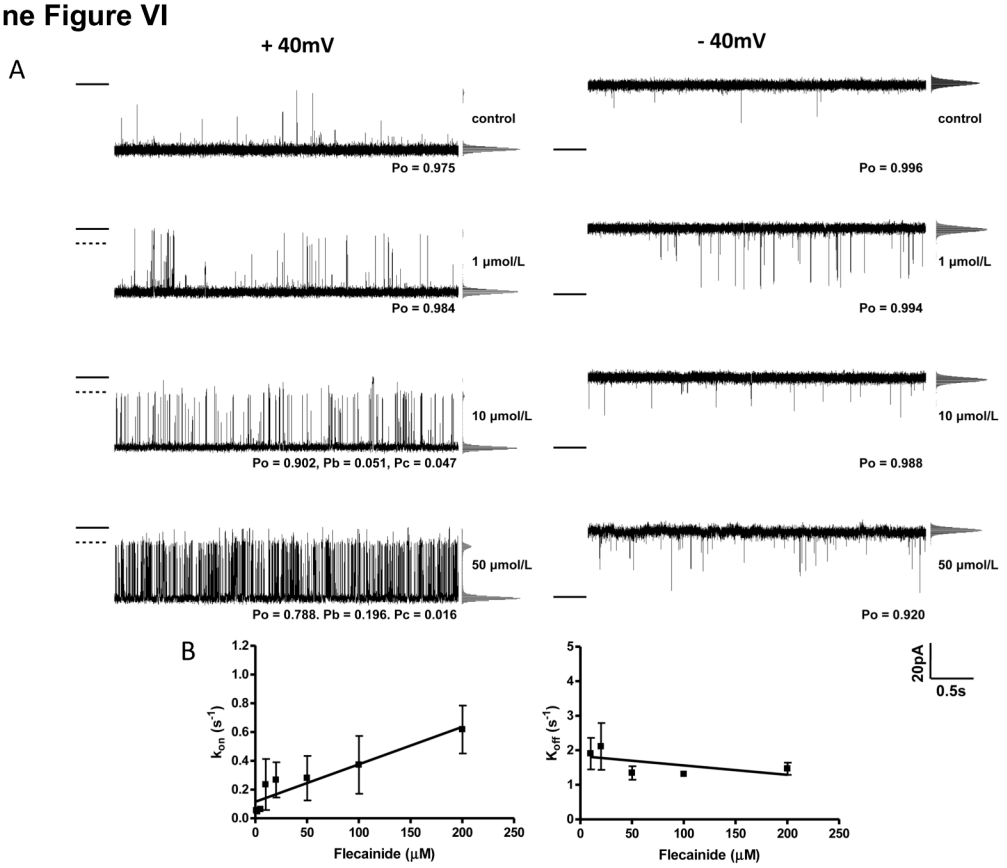



Online Figure V

**Cytosolic tetracaine inhibits both cytosolic-to-luminal and luminal-to-cytosolic flux of cations through hRyR2 channels**

**A.** Current fluctuations through a representative hRyR2 channel at a holding potential of +40 mV prior to, and following the addition of, increasing concentrations of tetracaine to the cytosolic side of the channel (closed level indicated by the solid line at the left of each trace). Under these conditions, tetracaine reduces RyR2 Po in a concentration-dependent manner, as quantified in **B.** In contrast to flecainide and propafenone, the reduction in RyR2 Po induced by tetracaine results from increases in the duration of closed events. **C.** Unlike flecainide and propafenone, high concentrations of cytosolic tetracaine significantly reduce RyR2 Po when cation flux through the channel is in the physiologically relevant direction (-40mV), quantified in **D.**

**Online Figure VI**




Online Figure VI

**Mechanism of interaction of flecainide with hRyR2 is unaltered in the N4104K CPVT mutant channel**

**A.** Representative single channel traces recorded at -/+40 mV, where openings are upwards or downwards from the close level (black line), respectively. As for the WT channel, block by flecainide only occurs at positive holding potentials (when ionic flux is in the cytosolic-to-luminal direction). **B.** Association ($k_{on}$) and dissociation ($k_{off}$) rates of flecainide interaction with N4104K channels.