

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% CO₂ and 80-90% humidity at a density of 2×10^5 per 100 mmol/L plate (60 cm²) 24 hours prior to transfection with pcDNA3/eGFP-hRyR2 (6 µg per 1×10^5 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 $\sim 50 \times 10^6$ 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 CaCl₂, 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 described¹ 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). Ca²⁺ activation was measured using [³H]-ryanodine binding to ER membranes (100 µg) from transfected HEK293 cells and was carried out as reported previously.² The binding of [³H]-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 Ca²⁺ can be measured directly by varying the buffered free Ca²⁺ 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 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ free⁴) 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 $\mu\text{mol/L}$ 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.

(Bruyton) 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) algorithm¹² based on Hidden Markov Models (HMM) and a dead time of 75-120 μ s 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) $\leftrightarrow$ open (O) $\leftrightarrow$ 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 $\leftrightarrow$ 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

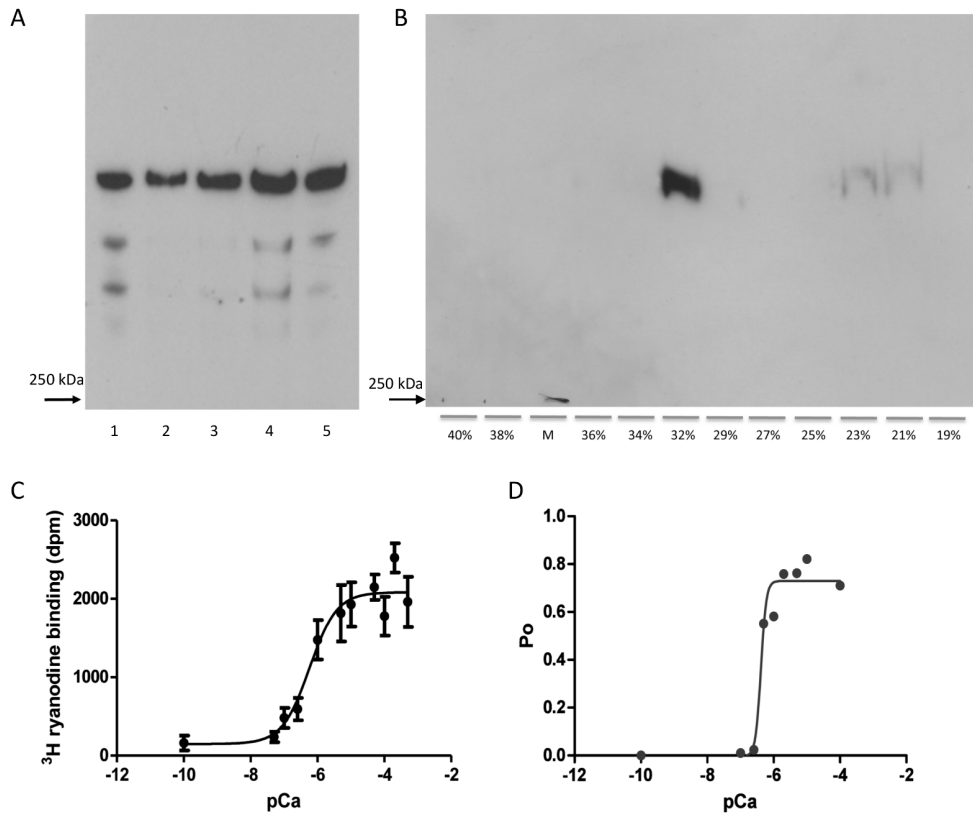
All animal surgical procedures and peri-operative management were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) under assurance number A5634-01. Imperial College Ethical Review 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_2 ATP, 10 mmol/L creatine phosphate, 5.5 mmol/L (total) $MgCl_2$, 0.05 mmol/L K_2 EGTA, 0.02 mmol/L $CaCl_2$. The fluorophore used was 5 μ mol/L fluo5F pentapotassium salt (LifeTech) for waves and fluo4 pentapotassium salt for sparks. Cells were permeabilised in this solution containing 0.1 mg/ml eschin (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 μ mol/L). The same cell was imaged in the absence of drug (0), or following the addition of 5- and 25 μ mol/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

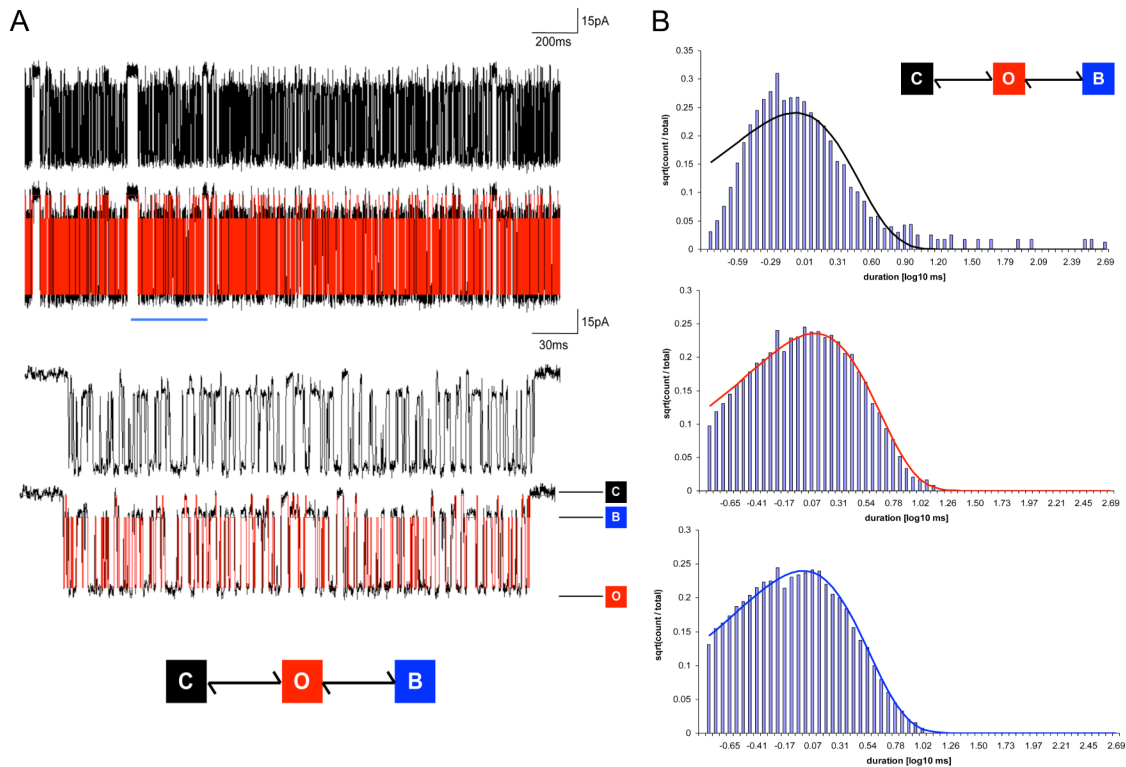


Online Figure I

Assessment of hRyR2 channel expression and function

A. Full-length recombinant hRyR2 is detected as a discrete band with few breakdown products in microsomes (50 μg) 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 [^3H]-ryanodine binding (as disintegrations per minute (dpm), see Methods) with an EC_{50} of $0.58 \pm 0.27 \mu\text{mol/L}$ ($n=5$). **D.** Ca^{2+} activation curve for a representative individual purified hRyR2 channel which activated similarly ($\text{EC}_{50} = 0.41 \mu\text{mol/L}$). 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.

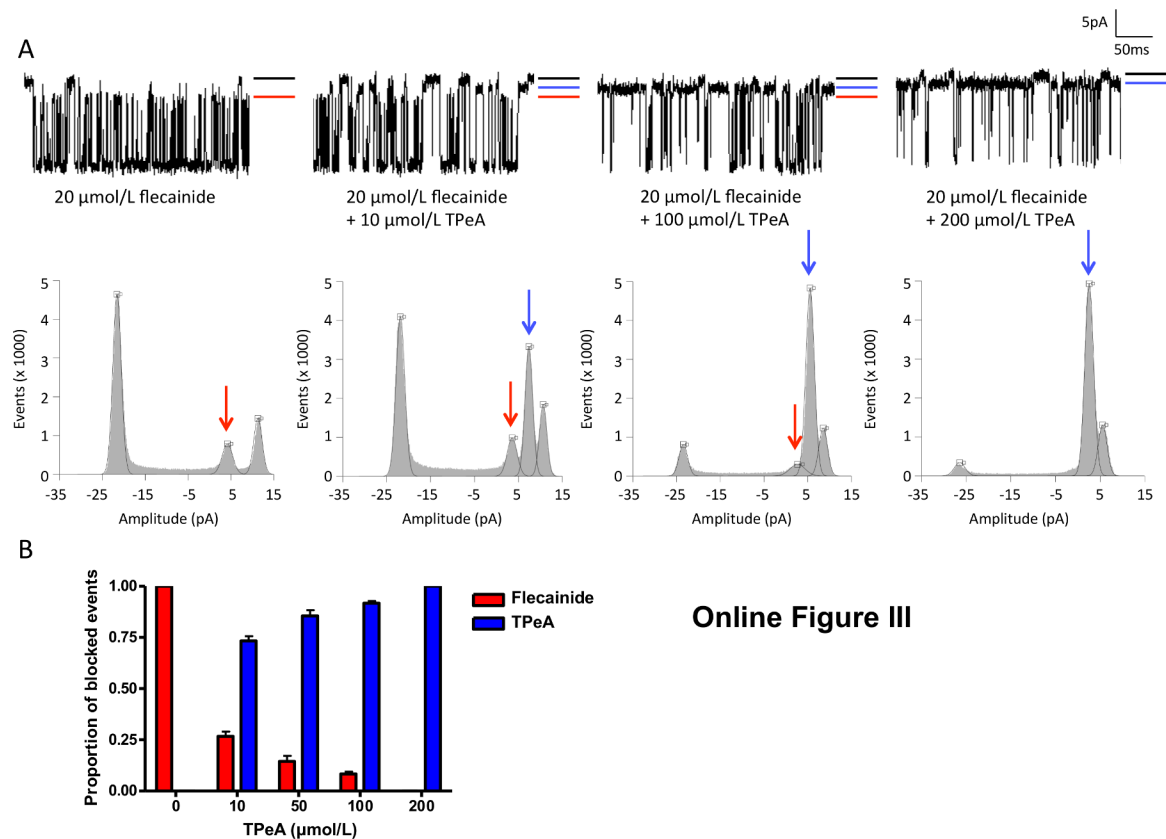
Online Figure II



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 $\mu\text{mol/L}$) 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.



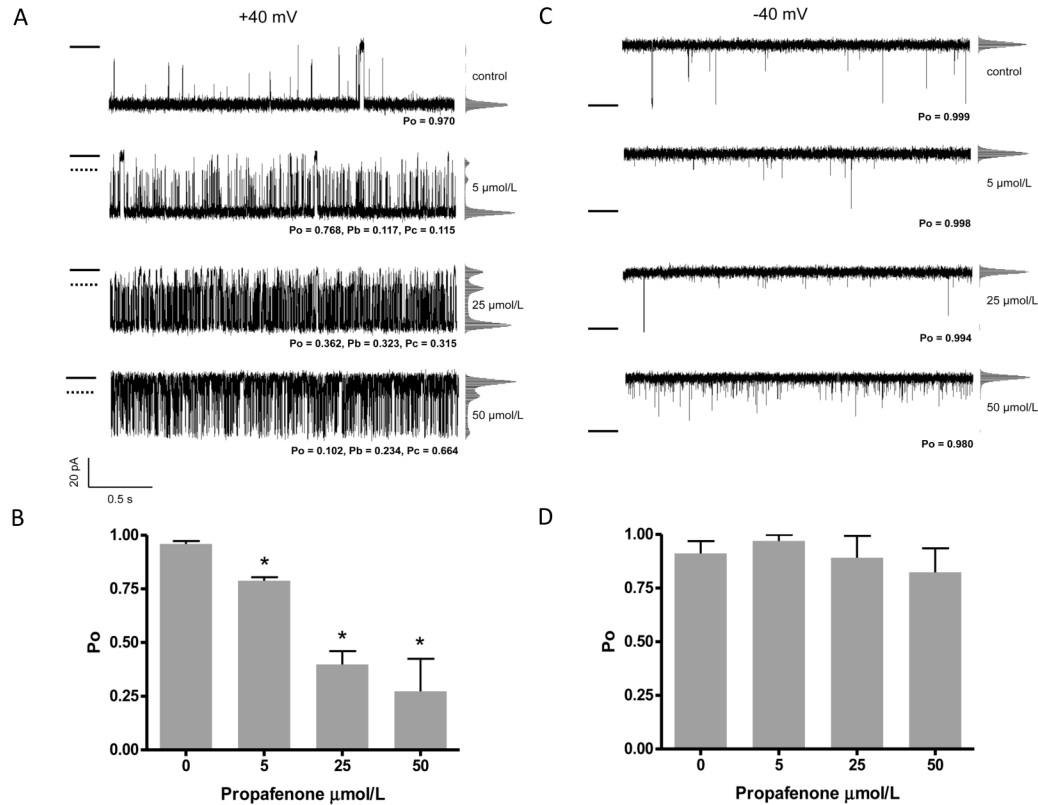
Online Figure III

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 $\mu\text{mol/L}$ 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

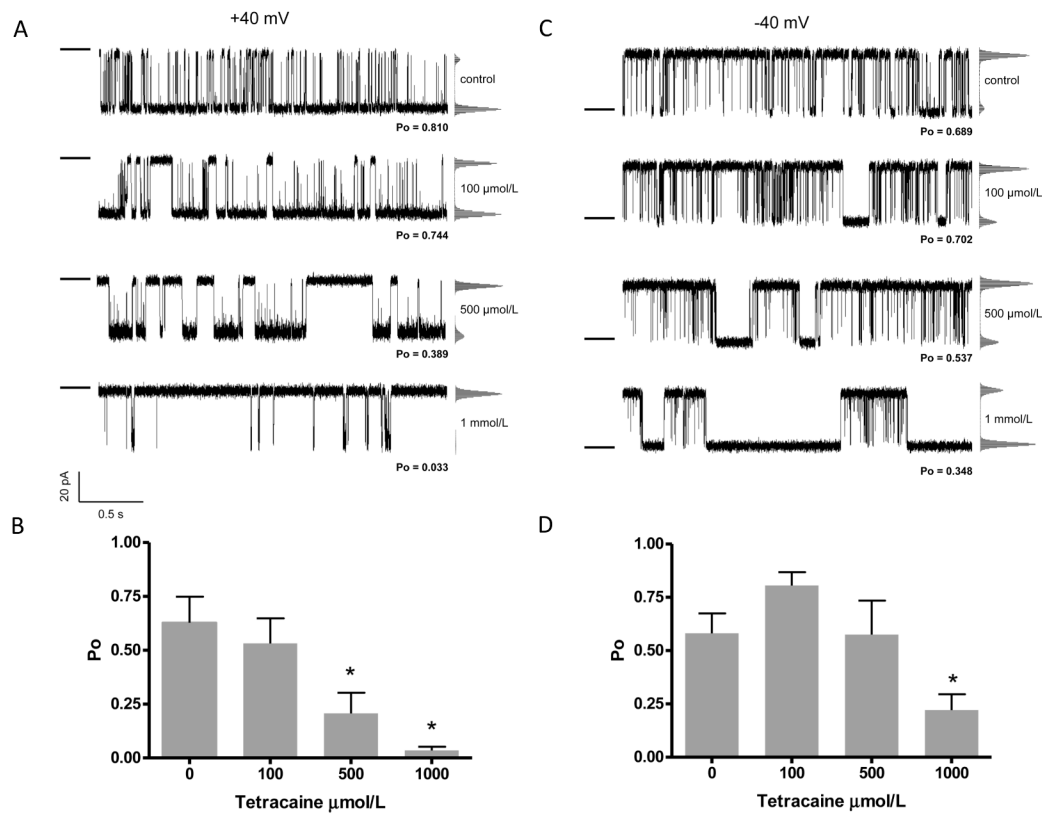


Online Figure IV

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 ($31.23 \pm 0.27\%$ of full conductance) and does not vary significantly with blocker concentration. **B.** Quantification of the decrease in P_o 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 $\mu\text{mol/L}$, produces no significant reduction in P_o , quantified in **D**.

Online Figure V

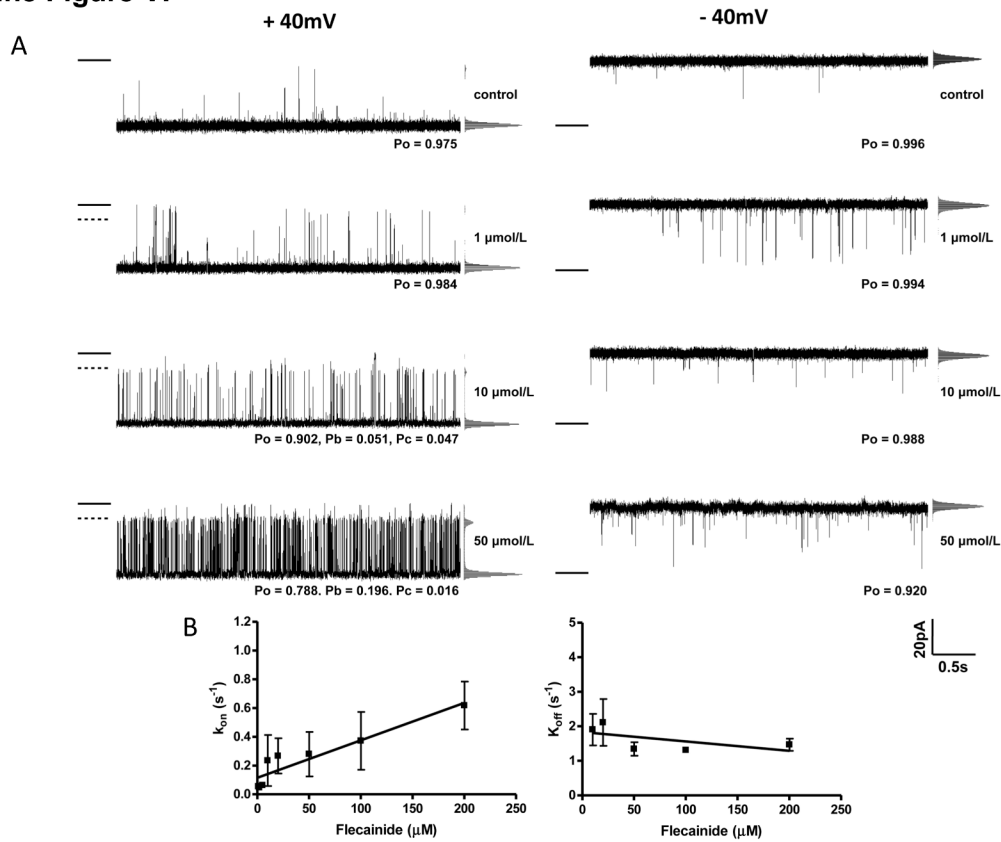


Online Figure V

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 (-40 mV), 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.