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

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ABSTRACT

***Rationale*:** Flecainide, a class I_c anti-arrhythmic, has emerged as an effective therapy in preventing arrhythmias in catecholaminergic polymorphic ventricular tachycardia (CPVT) patients refractory to β-adrenergic receptor blockade. It has been proposed that the clinical efficacy of flecainide in CPVT is due to 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 supra-physiological 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 (SR) charge-compensating counter current. Using permeabilised 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 SR Ca^{2+} release.

***Conclusion*:** 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.

Keywords:
Ryanodine receptor, flecainide, arrhythmia, catecholaminergic polymorphic ventricular tachycardia, antiarrhythmic drug.

Nonstandard Abbreviations and Acronyms:

β-AR	β-adrenergic receptor
CPVT	catecholaminergic polymorphic ventricular tachycardia
CSQ2	cardiac calsequestrin
DAD	delayed afterdepolarizations
NCX	sodium-calcium exchanger
PFR	pore-forming region
RyR2	cardiac ryanodine receptor
SR	sarcoplasmic reticulum
TPeA	tetrapentyl ammonium

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INTRODUCTION

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a genetic condition characterised by an increased propensity to adrenergic-induced polymorphic or bidirectional VT in structurally normal hearts. The autosomal dominant form (CPVT1) is caused by mutations in the cardiac ryanodine receptor (RyR2) whilst 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 (e.g. exercise or emotional stress) that triggers arrhythmogenic delayed afterdepolarizations (DADs) via the sodium-calcium exchanger (NCX).[4]

β-adrenergic receptor (β-AR) blockers are the first line pharmacotherapy in the clinical management of CPVT but the approach of limiting cardiac excitability is sub-optimal and some patients exhibit persistent tachyarrhythmias.[5,6] Flecainide, a class I_c anti-arrhythmic and potent Na^+ channel blocker, has emerged as an effective alternative in CPVT patients refractory to β-AR 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 DAD-mediated triggered activity.[8,10] Although plausible based on the data reported, the biophysical characterization of blockade of sheep RyR2 by flecainide favoured the net flow of ions through the RyR2 channel in the cytosolic-to-luminal direction i.e. the non-physiological 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 reported that the efficacy of flecainide in the R4496C$^{+/-}$ RyR2 mouse model of CPVT1 depended on its well-characterised action on Na^+ channels by increasing the threshold for triggered activity.[11] Sikkel et al proposed that the flecainide-induced attenuation of Ca^{2+} wave generation was due predominantly to reduced cytosolic Ca^{2+} ensuing from modulation of the NCX/I_{Na} axis.[1-3,12] The proposed 'triple mode' model of flecainide effect, which attempts to reconcile the observed actions of flecainide on RyR2, I_{Na} and NCX, 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 (i.e. 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 RyR2. Consistent with these findings, flecainide did not inhibit RyR2-mediated Ca^{2+} release from the SR in permeabilised adult cardiac ventricular myocytes. Our data refute the proposal that the clinical effectiveness of flecainide in CPVT patients is dependent upon 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 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.

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. 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.

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RESULTS

Concentration-dependent block of cytosolic-to-luminal cation flux by flecainide.

We have investigated the interactions of flecainide with individual purified, recombinant, human RyR2 (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 non-physiological 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. As flecainide is reported to be a blocker of the open RyR2 channel,[8,10] in these experiments we maximized channel open probability 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) prior to, and following, 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, 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-D. The probability of block is dependent upon 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 three state scheme (Online Figure II) in which block occurs as the result of a bimolecular interaction between the open channel and flecainide:

$$\text{Closed} \leftrightarrows \text{Open} \underset{[\text{Flec}]}{\leftrightarrows} \text{Blocked} \qquad (1)$$

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 Scheme 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 trans-membrane 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.

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, trans-membrane 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

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effectiveness of blocking molecules, with similar modes of action to flecainide in RyR2, is increased at positive trans-membrane 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 trans-membrane 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 trans-membrane 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 (i.e. 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 TPeA: 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 trans-membrane 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 two 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 likely further equilibrate with the SR lumen (see Materials & Methods). 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 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 carried out using K^+ as the permeant cation in hRyR2. The use of K^+ is valuable because a) it maximizes the resolution of hRyR2 gating and block and b) it allows manipulation of the direction of net current flow through the open channel by varying either trans-membrane 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 trans-membrane potential is clamped at 0 mV. Channel Po is regulated by the inclusion of cytosolic Ca^{2+}, Mg^{2+} and ATP (see Materials and Methods 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 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 TRIC,[25] RyR2 can carry a K^+ counter current during Ca^{2+} release.[23,26] The relative contribution of these two SR charge-compensating mechanisms during RyR2-mediated Ca^{2+} release and 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). As it is not possible to unequivocally determine the orientation of the SR K^+ channel following 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 very high 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

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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 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 approximately 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 & D). 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 Ca^{2+} release from the cardiac SR.

Flecainide does not block SR Ca^{2+} release in permeabilised 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, permeabilised 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 & B) and waves (Figure 8C & D) determined under these experimental conditions.

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DISCUSSION

Flecainide, a class I_c antiarrhythmic local anaesthetic, is a well characterised 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 due to 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 focussed upon 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 CPVT patients involves suppression of Ca^{2+} waves due a triple mode of action comprising a) a direct action of flecainide on RyR2,[8,10] b) a I_{Na}-dependent reduced probability of DAD triggered action potentials as observed by Liu et al[11] and c) a reduction in cytosolic Ca^{2+} concentration resulting from the change in Na^+/Ca^{2+} homeostasis due to 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 CPVT patients is dependent upon its ability to directly modulate RyR2-mediated release of Ca^{2+} from the cardiac SR. Our data demonstrate that, while very high concentrations of cytosolic flecainide can, to some extent, block cytosolic-to-luminal flux of monovalent cations through RyR2, this local anaesthetic 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 permeabilised adult cardiac ventricular myocytes.

These conclusions are based principally on observations of single hRyR2 function following reconstitution into planar bilayers and are therefore dependent upon 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, trans-membrane 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 asymmetric 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 TPeA (Online Figure III), both of which define the cytosolic side of the channel.[16,32]

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Why doesn't flecainide block the physiologically relevant movement of cations through RyR2?

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Our data establish that interactions between flecainide and RyR2 only occur when the local anaesthetic is added to the solution at the cytosolic side of the channel. When bound, the flecainide molecule adds a steric and/or electrostatic barrier to the RyR2 conduction pathway that reduces the rate of cation translocation from the cytosolic side of the channel to the lumen i.e. in the non-physiological direction. The rate at which flecainide associates with its binding site is dependent upon its concentration and, consistent with a simple bimolecular interaction, cytosolic flecainide concentration does not influence its rate of dissociation.

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Both rates of association and dissociation of cytosolic flecainide are influenced by trans-membrane potential. The voltage drop across membrane channels is concentrated in a short, narrow, region of the PFR within which discrimination between ions occurs, i.e. the selectivity filter. Focusing of the voltage drop is made possible by intrusion of the cytosolic solution into the trans-membrane 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 trans-membrane 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. Modelling 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 over which 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 occluding the pore. Significant residual current in the blocked state (Figure 1C) and direct competition between flecainide and TPeA (Online Figure III) indicates 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 trans-membrane potential must be due to interactions between flecainide and permeant cations within the PFR. This conclusion is consistent with the observation that, in the absence of an applied trans-membrane potential, cytosolic flecainide cannot block the luminal-to-cytosolic flux of either K^+ (Figure 3C and D) 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 I_C 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 permeablilzed cardiac myocytes.[41] As demonstrated by Hilliard et al, 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 two 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 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 CSQ2. Individuals with CPVT2 have wild type RyR2 channels and these channels will respond to flecainide in the ways described in this communication.

CPTV1 is caused by mutations in RyR2 and to date more than 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 carried out 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 characterised, 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 towards, 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 two 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 very different from the voltage-dependent gating of the Na^+ channel, where the open channel must pass thorough structurally distinct inactivated, deactivated and closed, non-conducting, states before it can re-open. 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.

Conclusion.

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, reduce the probability of DADs[11] and/or reduce the probability of RyR2 opening by reducing the level of cytosolic Ca^{2+} as the result of NCX-mediated Ca^{2+} efflux driven by reduced cytosolic Na^+.[12]

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DISCLOSURES
None

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FIGURE LEGENDS

Figure 1: **Flecainide partially blocks a cytosolic-to-luminal current through hRyR2.**
A. Representative single channel traces recorded at + 40 mV. Openings are downwards 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 emphasise 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-D are from n=6 channels.

Figure 2: Flecainide does not block voltage driven luminal-to-cytosolic cation flux through hRyR2.
Representative single channel traces, recorded at – 40 mV (luminal-to-cytosolic current) in the presence of cytosolic flecainide. Openings are upwards from the closed level (black line), no blocking events or significant changes in Po were observed (n=6).

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 hRyR2 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}) and dissociation (k_{off}) 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 upwards from the closed level (black line). **D.** Po was not significantly altered by 50 µmol/L cytosolic flecainide under these conditions (n=3).

Figure 4: **Luminal flecainide does not block a luminal-to-cytosolic cation flux through hRyR2**
A. Representative single channel traces recorded at - 40 mV. Openings are upwards 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.

Figure 5: **Flecainide does not affect hRyR2 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).

Figure 6: **Flecainide does not block the physiologically relevant Ca^{2+} flux through hRyR2**
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 upwards 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).

Figure 7: **Flecainide does not markedly inhibit the charge-compensating counter current.**
A. Representative single 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 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 downwards from the closed level (solid line) **D.** Reduction in Po and C-C resulting from the partial block of hRyR2 (n=3-6).

Figure 8: Flecainide does not affect SR Ca^{2+} release in permeabilised rat cardiac myocytes.
Representative confocal line-scans of: **A.** Ca^{2+} sparks and **C.** Ca^{2+} waves in escin-permeabilised rat cardiac myocytes following 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 means as determined by ANOVA (n=19 cells).

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Novelty and Significance

What Is Known?

- Flecainide, a class I_c anti-arrhythmic and potent Na^+ channel blocker, is a clinically effective anti-arrhythmic in individuals with catecholaminergic polymorphic ventricular tachycardia (CPVT), refractory to β-adrenoceptor (β-AR)-blockade.

- In the context of CPVT, flecainide has been suggested to block of 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 non-physiological direction (cytosol to sarcoplasmic reticulum (SR) 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 I_c anti-arrhythmics, as prototypical compounds is unlikely to yield new compounds with improved specificity for RyR2.

CPVT is a malignant arrhythmia characterized by dysfunctional SR Ca^{2+} release and triggered by increased adrenergic drive. β-AR blockers attenuate aberrant Ca^{2+}-linked electrical activity and are the cornerstone of CPVT therapy. Flecainide, a class I_c anti-arrhythmic and a potent Na^+-channel blocker is effective in CPVT patients 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 non-physiological direction. Crucially though, flecainide, even at supra-physiological concentrations, had no effect on the physiologically-relevant SR-to-cytosol cation flux through RyR2 nor did it affect other mechanisms that impinge on SR Ca^{2+} release (e.g. 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 I_c compounds should not be considered as prototypical RyR2 blockers.

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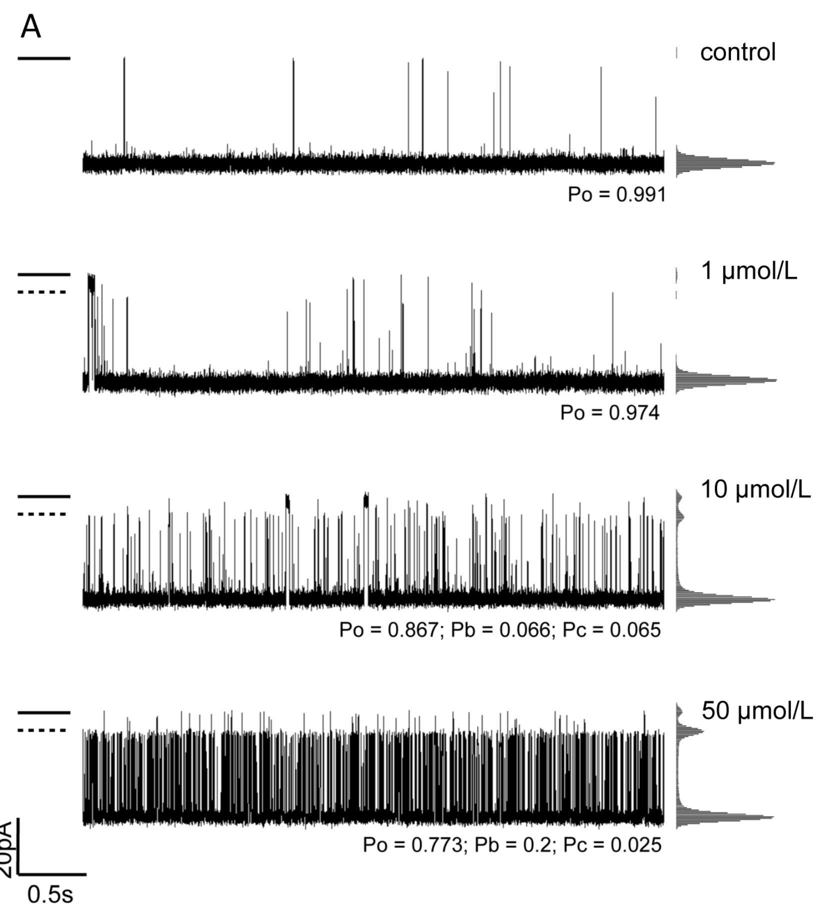
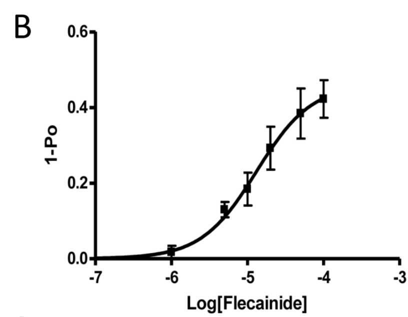
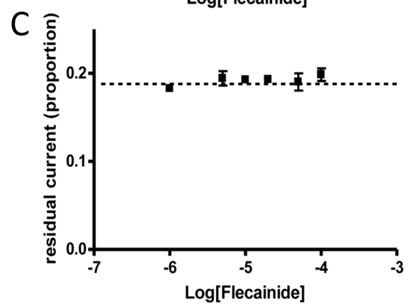
Figure 1







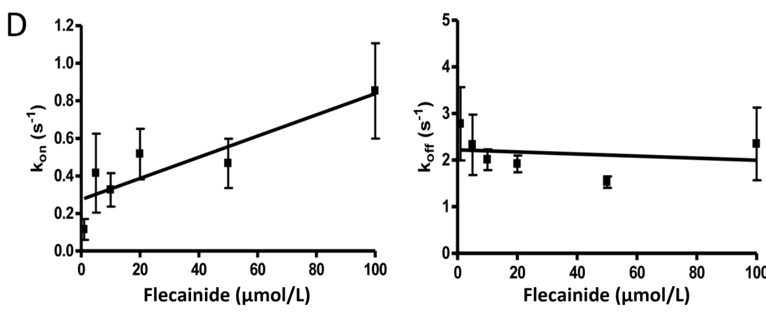


Figure 2

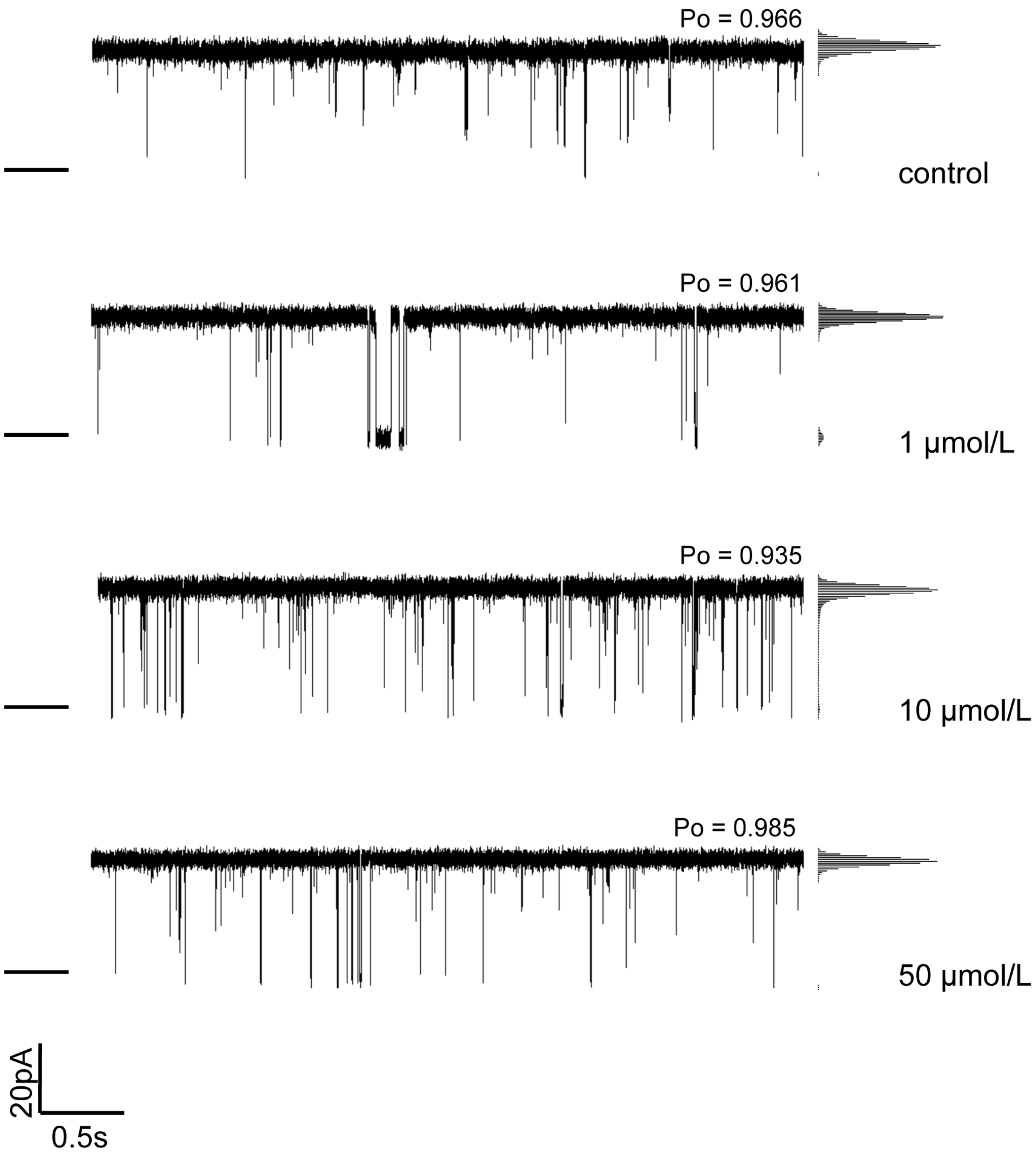
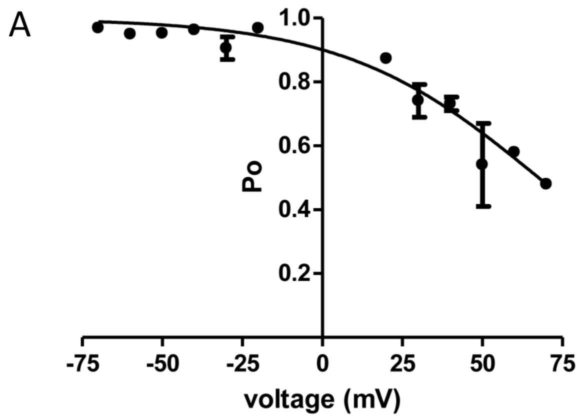
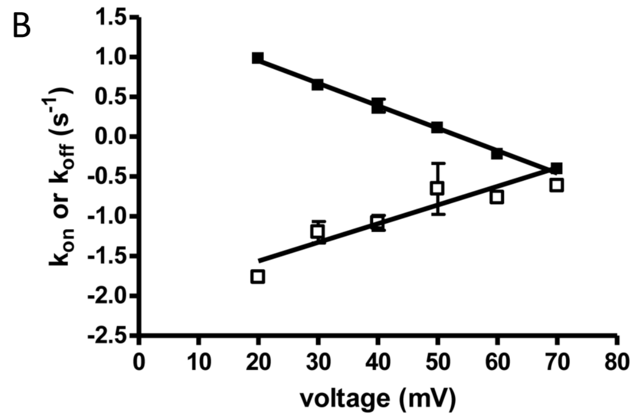
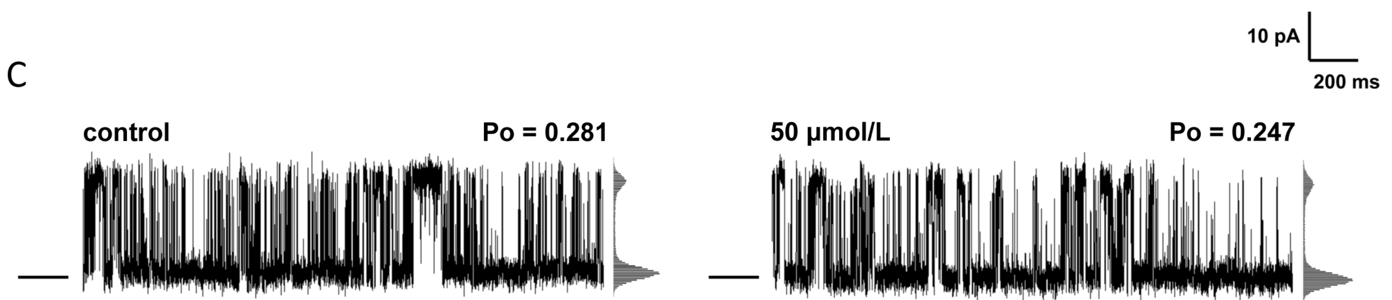


Figure 3







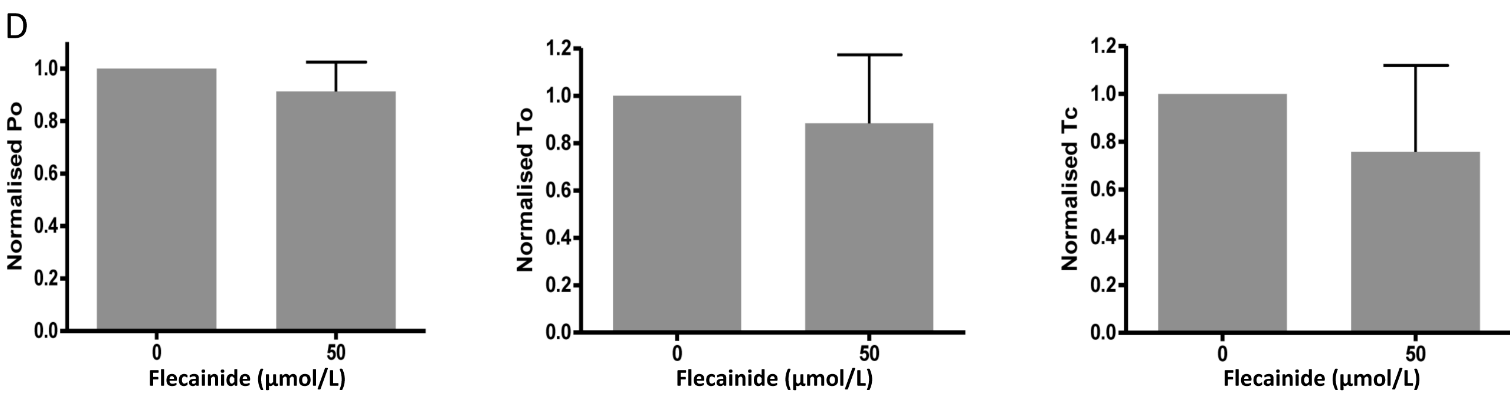
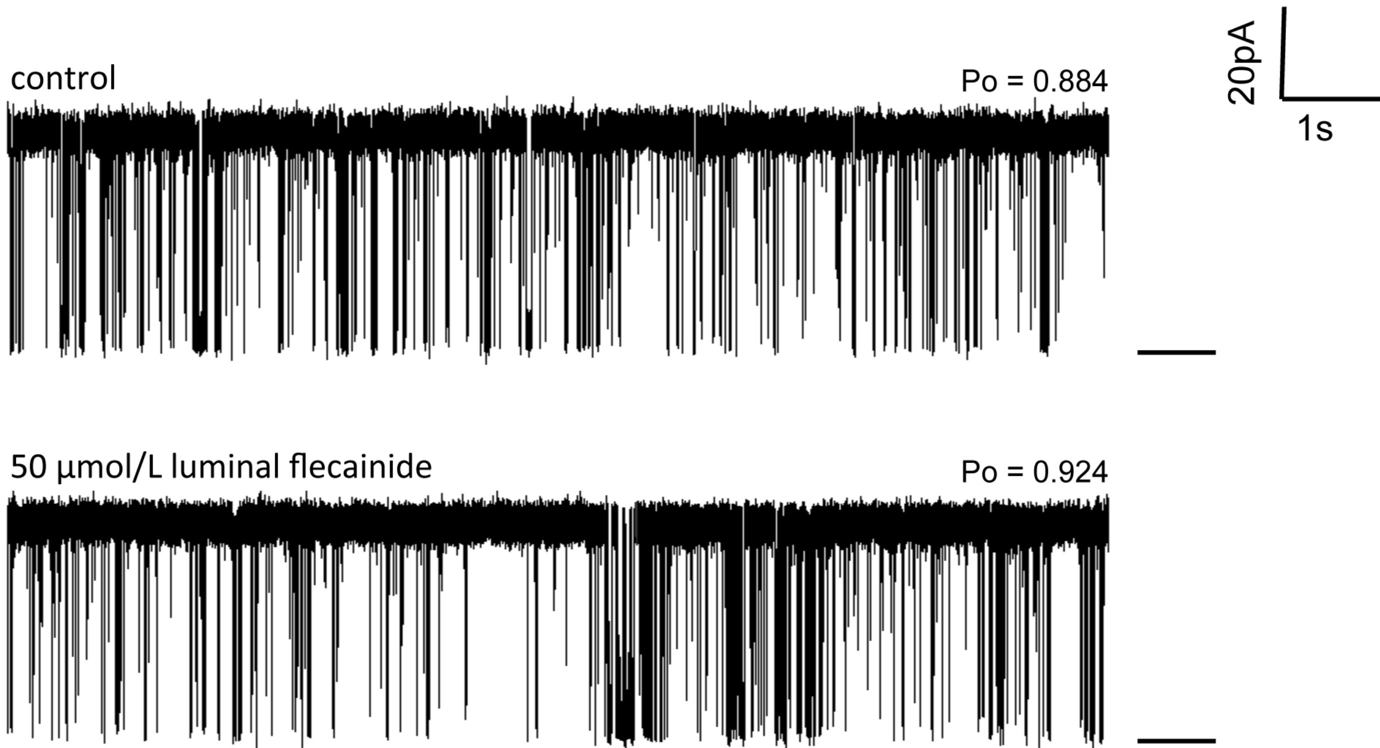


Figure 4



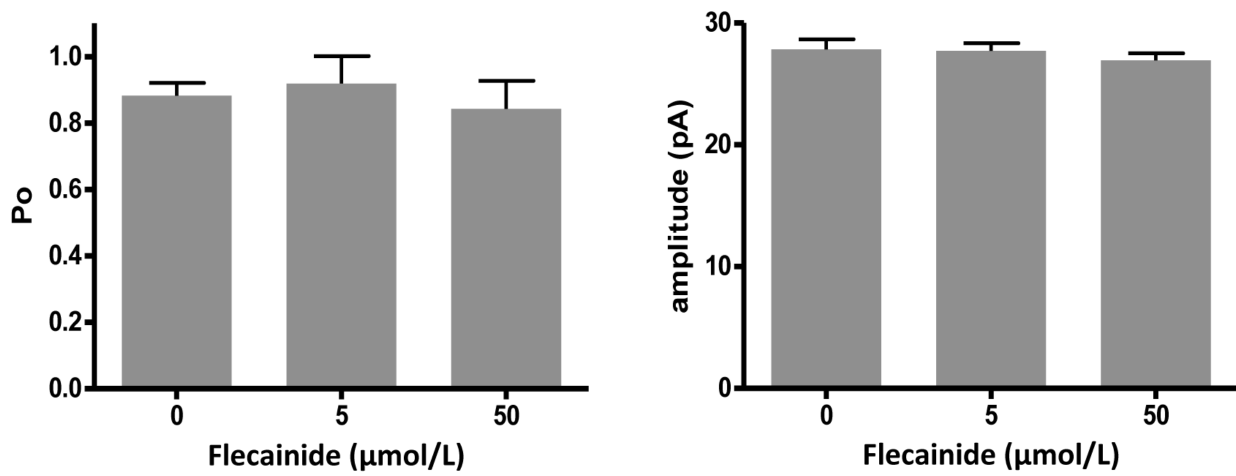
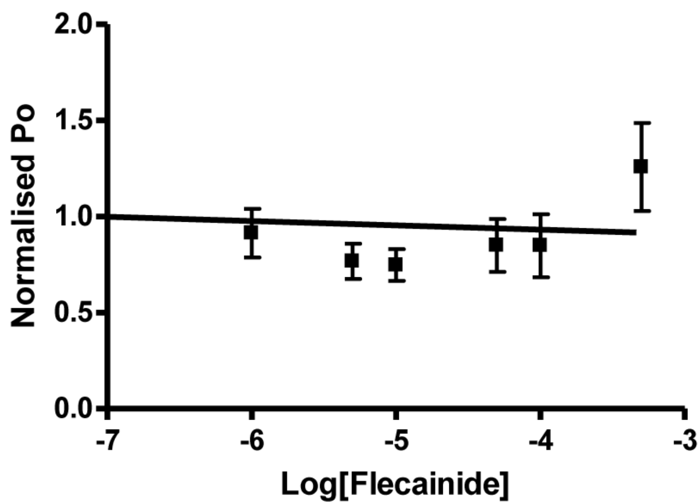
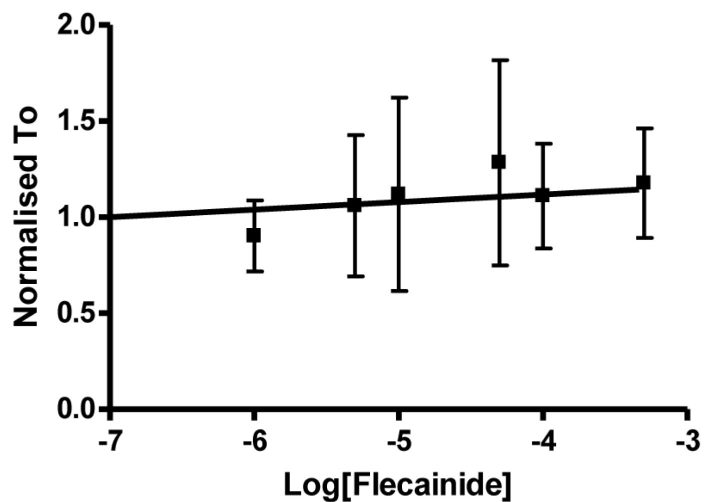
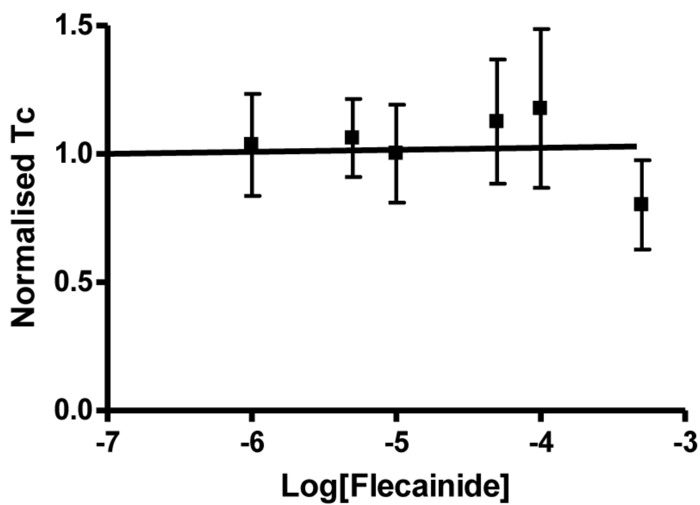


Figure 5







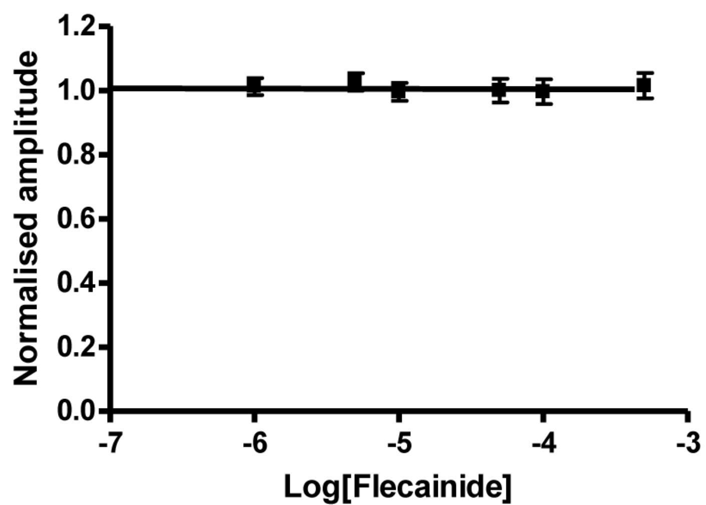
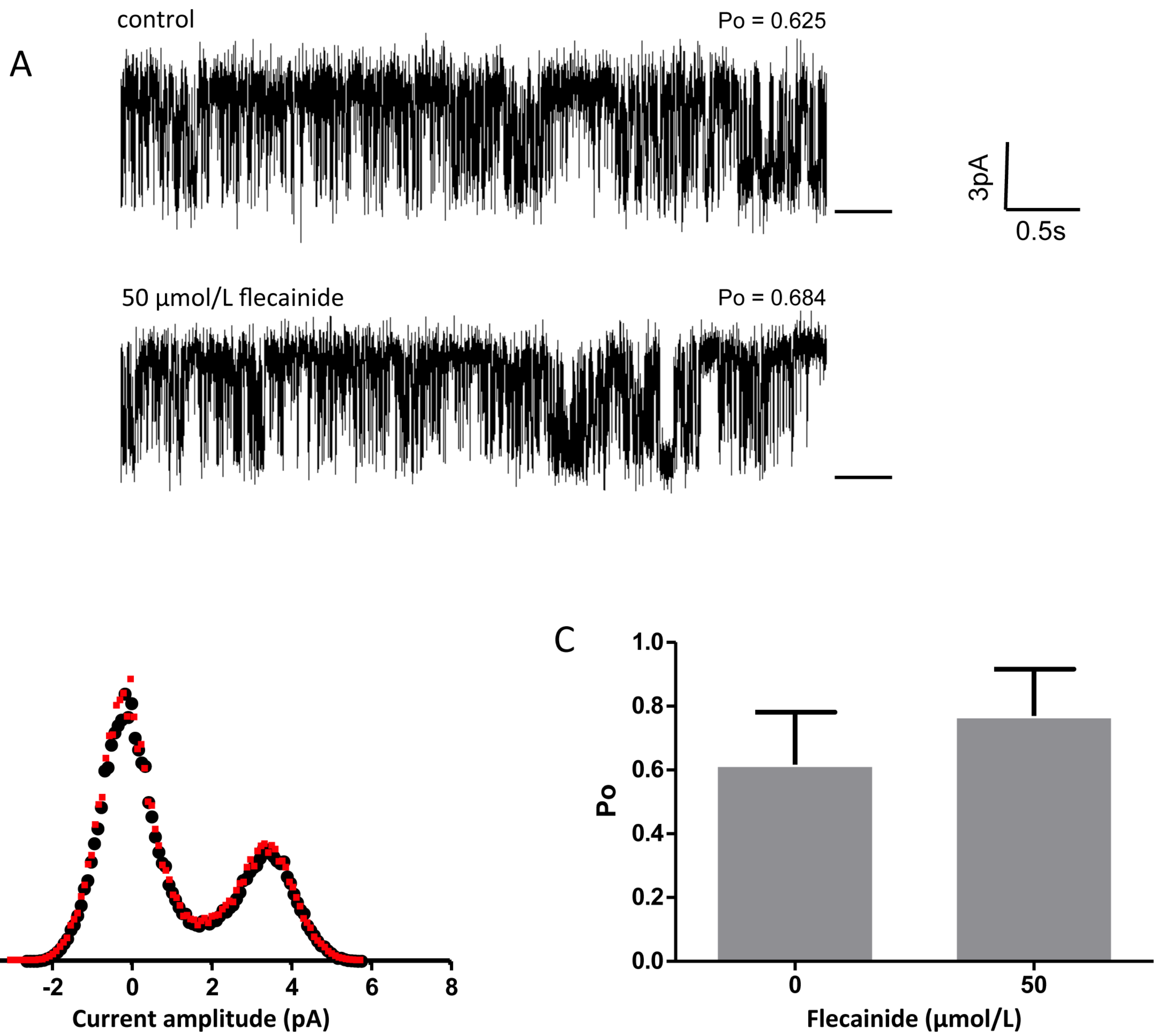
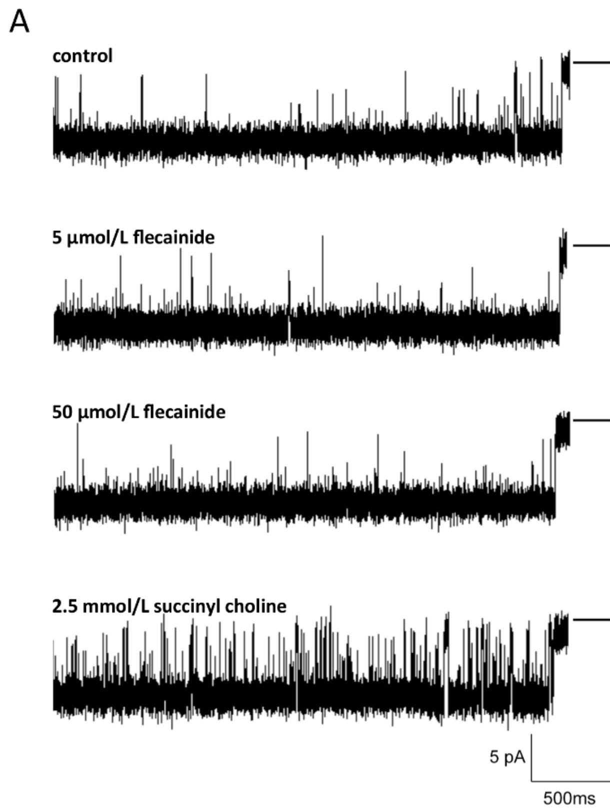
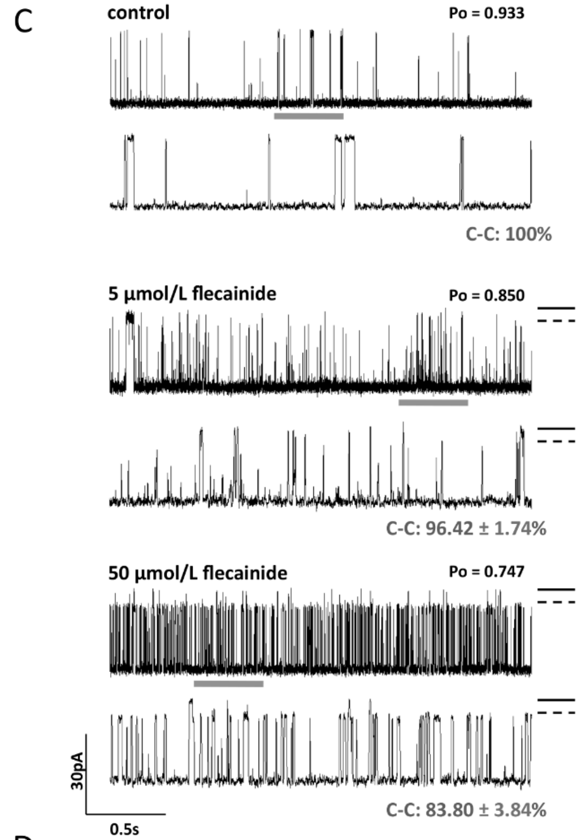


Figure 6







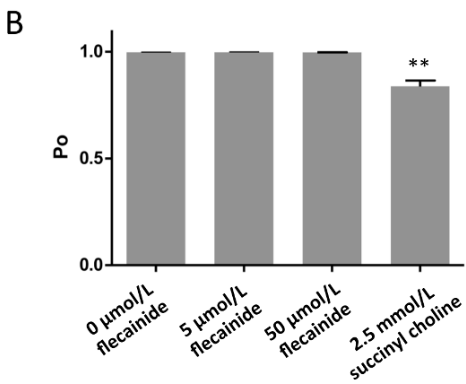


Figure 7

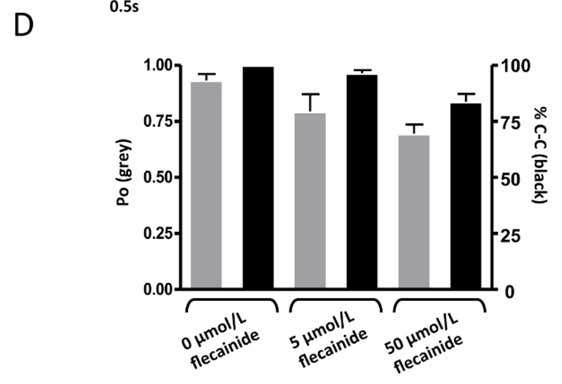


Figure 8