Effect of flecainide derivatives on sarcoplasmic reticulum calcium release suggests a lack of direct action on the cardiac ryanodine receptor

Running title: Flecainide derivatives and ryanodine receptors

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Abstract

Background and purpose

Flecainide is a use-dependent blocker of cardiac Na^+ channels. The mechanisms involved in block were elucidated using fully charged (QX-FL) and neutral (NU-FL) derivatives of flecainide, establishing that Na^+ channel block requires the cationic form of the molecule to enter the cytosolic vestibule of the open channel. Flecainide is also effective in the treatment of catecholaminergic polymorphic ventricular tachycardia (CPVT), however its mechanism of action is contentious. We investigated how flecainide derivatives influence RyR2-mediated Ca^{2+}-release from the sarcoplasmic reticulum and whether this correlates with their effectiveness as blockers of Na^+ channels and/or RyR2.

Experimental approach

We compared the ability of QX-FL and NU-FL to block individual recombinant human RyR2 channels incorporated into planar phospholipid bilayers, and their effects on the properties of Ca^{2+} sparks in intact adult rat cardiac myocytes.

Key results

Both QX-FL and NU-FL were partial blockers of the non-physiological cytosolic to luminal flux of cations through RyR2 but were significantly less effective than flecainide. None of the compounds influenced the physiologically relevant luminal to cytosol cation flux through RyR2. Intracellular flecainide or QX-FL reduced Ca^{2+} spark frequency whereas NU-FL didn't.

Conclusions and implications

Given its inability to block physiologically-relevant cation flux through RyR2, and its lack of efficacy in blocking the cytosolic-to-luminal current, the effect of QX-FL on Ca^{2+} sparks is likely, by analogy with flecainide, to result from Na^+ channel block. Our data reveal important differences in the nature of flecainide interaction with sites in the cytosolic

vestibules of Na^+ and RyR2 channels.

Abbreviations

CPVT catecholaminergic polymorphic ventricular tachycardia

RyR2 cardiac ryanodine receptor

SR sarcoplasmic reticulum

TPeA tetrapentyl ammonium

Table of links

TARGETS
Voltage-gated ion channels[a]
$Na_v1.5$
Ligand-gated ion channels[b]
RyR2

LIGANDS
Flecainide

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al*., 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al*., 2015a,b).

Introduction

Flecainide is a well-characterized blocker of sarcolemmal Na^+ channels (Liu et al. 2002; Liu et al. 2003) and K^+ channels (Follmer and Colatsky 1990; Paul et al. 2002). In addition, flecainide has been shown to be effective in the treatment of CPVT and its action has been attributed to an ability to reduce inappropriate release of Ca^{2+} from the SR by directly blocking open RyR2 channels (Watanabe et al. 2009; Hilliard et al. 2010). However, in previous studies we have established that while flecainide can act as a partial blocker of cytosolic to luminal flux of cations in RyR2, it is unable to influence the physiologically relevant flux of cations from the SR lumen to the cytosol that occurs during Ca^{2+} release. As a consequence we have concluded that the mechanism of action of flecainide in CPVT depends on its ability to block Na^+ channels and does not involve a direct action on RyR2 (Bannister et al. 2015). This issue is important to resolve because disruption of intracellular calcium homeostasis is also linked to dysfunction in skeletal muscle (muscular dystrophy, malignant hyperthermia), smooth muscle (asthma), brain (stroke, Alzheimer's disease) and the endocrine system (type 2 diabetes mellitus). Defective RyR-mediated Ca^{2+} release thus impacts on diseases of global prevalence and importance, and RyR is therefore an important therapeutic target (see e.g. Mackrill 2010; Santulli and Marks 2015).

Block of Na^+ flux into the cell by flecainide results from entry of the ligand from the cytosol and interaction with a site in the cytosolic vestibule of the Na^+ channel (Liu et al. 2003). Similarly, flecainide interacts with an equivalent site within the cytosolic vestibule of RyR2 and gains access to this site from the cytosol (Bannister et al. 2015; Mehra et al. 2014). Flecainide bound in RyR2 influences cation flux from the cytosolic side of the channel to the SR lumen; a current that may contribute to charge compensation during Ca^{2+} release

(Gillespie and Fill 2008), but is rapidly displaced by cations moving in the physiologically relevant direction (from the SR lumen to the cytosol).

Given the very different abilities of flecainide to block the equivalent, physiologically relevant, flux of cations in the Na^+ and RyR2 channels, it is important to establish the respective mechanisms governing interaction. Important information on the molecular characteristics underlying Na^+ channel block were obtained using derivatives of flecainide with similar structural features but differing net charge (Liu et al. 2003). As highlighted in Figure 1, at physiological pH, 100% of QX-FL will be cationic while 90% of NU-FL will be neutral. Investigations of Na^+ channel block by flecainide, QX-FL and NU-FL established that block results from the interaction of the cationic form of the molecule with the Na^+ channel (Liu et al. 2003). Flecainide has also been shown to block hERG channels at physiologically relevant concentrations (Paul et al. 2002) and QX-FL and NU-FL have been used to demonstrate that, as is the case in the Na^+ channel, hERG block requires entry of a cationic blocking molecule from the cell interior (Melagri et al. 2015). In the present study we have used QX-FL and NU-FL to establish the mechanisms underlying the interaction of flecainide with RyR2.

Our investigations demonstrate that the structural and chemical features governing flecainide interaction with Na^+ channels and RyR2 are different. While ligand charge is the primary determinant of blocking ability in Na^+ channels, small structural changes severely reduce the ability of flecainide to block the potential charge compensating, (cytosolic to SR lumen), flux of cations through RyR2 during Ca^{2+} release from the SR. Consequently, the flecainide derivatives give us new tools with which to test the mechanism of action of flecainide in the regulation of Ca^{2+} release from the SR in CPVT. Both flecainide and QX-FL (applied inside

the cell) are effective blockers of sarcolemmal Na^+ channels. Data presented here demonstrate that while intracellular flecainide might produce some reduction in a potential RyR2-mediated cation counter current during Ca^{2+} release from the SR, QX-FL will not. Therefore, intracellular QX-FL is a Na^+ channel-specific ligand and we have compared its action with those of flecainide and NU-FL on the properties of Ca^{2+} sparks in intact adult rat cardiac myocytes.

Methods

Cell culture and transfection HEK293 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% (v/v) foetal bovine serum, 2 mM glutamine and 100 $\mu g.mL^{-1}$ penicillin/streptomycin (LifeTech). Cells were incubated at 37°C, 5% CO_2 and 80-90% humidity at a density of 2 x 10^6 per 100 mm plate (60 cm^2) 24 h prior to transfection with pcDNA3/eGFP-hRyR2 (6 µg per 1 x 10^6 cells). After overnight incubation, transfected cells were treated with sodium butyrate (2 mM) for a further 24 h before assessment of expression (by eGFP visualisation), harvesting by centrifugation (500g 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 mM Tris-HCl, 5 mM EDTA; pH 7.4) containing protease inhibitor cocktail (Roche), by passing them twenty times through a 23G needle. Unbroken cells and nuclei were removed by low speed centrifugation (1500 g, 4°C, AllegraR, Beckman) and the resulting lysate was subjected to a high-speed spin (100,000 g, 90 mins, 4°C, Optima L-90K, Beckman) to collect the microsomal membranes. Solubilization of these membranes was carried out (at a concentration of 2.5 $mg.mL^{-1}$) in a

solution containing 1 M NaCl, 0.15 mM $CaCl_2$, 0.1 mM EGTA, 25 mM PIPES, 0.6% (w/v) CHAPS and 0.3% (w/v) phospatidylcholine, with protease inhibitor cocktail (Sigma) at 4°C. Insoluble material was removed by centrifugation (15,000 g, 1 h 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 h) centrifugation at 4°C and stored at -80°C until use.

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 $mg.mL^{-1}$). Bilayers were formed in a solution containing 610 mM KCl, 20 mM 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 M 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 (Sitsapesan and Williams 1994; Bannister et al. 2015). After channel incorporation, symmetrical ionic conditions were re-instated by perfusion of the *cis* chamber with a 610 mM KCl, 20 mM HEPES (pH 7.4) solution. All experiments were carried out at room temperature (20-22°C). The effects of flecainide, QX-FL and NU-FL 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 mM K^+) in the presence of 20 μM EMD 41000, a RyR2 agonist shown previously to act via the caffeine-binding site (McGarry and Williams 1994).

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). Single channel traces of 2-3 min (containing >3000 events) were idealized using the Segmental K-means (SKM) algorithm (Qin and Li 2004) 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 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 described (Mukherjee et al. 2012). Po values are shown as mean ± SEM and fitted using non-linear regression. Rates of association (K_{on}) and disassociation (K_{off}) were calculated as the reciprocal of To and Tb, respectively for each drug concentration and holding potential and fitted using linear regression (through the origin when calculating K_{on} for drug concentration only). Due to the large difference in voltage-dependence of block by flecainide, QX-FL and NU-FL, K_{on} and K_{off} are plotted in a dose-normalised manner. Although different example traces are used in Figures 2 and 4, the mean data for flecainide in Figures 3 and 5 are the same as those published in Bannister et al., 2015. These data serve as controls against which the actions of QX-FL and NU-FL are compared.

Cardiomyocyte isolation Ventricular myocytes from healthy adult male Sprague Dawley rats were enzymatically isolated as previously described (Sato et al. 2005). Briefly, hearts were rapidly dissected and underwent retrograde perfusion using the Langendorff apparatus. Hearts were perfused sequentially with low calcium and an enzyme (collagenase, Worthington) solution. Ventricles were cut into small pieces and gently minced with a Pasteur pipette. The cell suspension was filtered and cardiomyocytes were allowed to settle by gravity.

Electrophysiological recordings combined with calcium imaging To assess the effects of intracellularly applied drug molecules on the spatial and temporal dynamics of Ca^{2+} sparks, confocal microscopy was combined with the whole-cell voltage clamp recordings. Dual experiments were performed on adult rat ventricular myocytes at room temperature (20-22 ^{o}C). Cells were superfused with an external physiological solution of the following composition (in mM): 137 NaCl, 10 HEPES, 10 glucose, 6 KCl, 2 $MgCl_2$, 1 $CaCl_2$ (pH adjusted to 7.4 with NaOH). Borosilicate microelectrodes were pulled to give a resistance between 2.5 and 3.5 MΩ. Vehicle, flecainide (Sigma) or its analogues QX-FL and NU-FL (all at 5 μM) were introduced into the cell cytosol via the patch pipette. Pipettes were filled with an intracellular solution containing either drug or vehicle in addition to membrane-impermeant dye Fluo-3 pentapotassium salt (Invitrogen) of the following composition (in mM): 100 K-Aspartate, 15 KCl, 10 HEPES, 5 KH_2PO_4, 5 Mg-ATP, 5 EGTA, 0.75 $MgCl_2$, 0.12 $CaCl_2$, 0.04 Fluo-3-$5K^+$ salt (pH adjusted to 7.2 with 2 M KOH).

After establishing a whole-cell configuration, vehicle- or drug-containing pipette solution was allowed to equilibrate with the cytosol for 10-15 min. To facilitate steady-state SR calcium-loading, cells underwent a series of depolarising steps of 200 ms duration to +30 mV applied from a holding potential of -80 mV at a frequency of 3 Hz for 30 s. Ca^{2+} sparks were captured following cessation of voltage-clamp stimulation. Electrophysiological recordings were carried out using an Axopatch-1D amplifier (Axon Instruments) and a Digidata1322A acquisition system (Axon Instruments). Series resistance and whole cell capacitance were electronically compensated. Live cell calcium imaging was performed using a laser scanning confocal system (BioRad Microscience Ltd, UK) and an inverted microscope (Nikon Eclipse TE300, Nikon UK Ltd). Ca^{2+} imaging data were acquired and processed using LaserSharp2000 and ImageJ (National Institutes of Health, USA) software, respectively.

Materials

QX-FL was synthesised as described (Liu et al. 2003) while NU-FL was synthesised using the novel procedure described in Supplemental Material. The characterisation of use-dependent block of voltage gated Na^{+} channels by the synthesised QX-FL and NU-FL used in this study is also described in Supplemental Material. The drug/molecular target nomenclature conforms to British Journal of Pharmacology's Concise Guide to Pharmacology (Alexander *et al*., 2015c).

Animal welfare ethical statement

All procedures were carried out in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act 1986, which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH

publication No. 85-23, revised 1996). Ethical approvals were granted by the United Kingdom Home Office. Rats were sacrificed by cervical dislocation following exposure to 5% isoflurane until the righting reflex was lost. All procedures involving animals are reported according to the ARRIVE guidelines (Kilkenny et al., 2010).

Design and statistical analysis

Data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al*., 2015). All cardiac myocytes were isolated following the sacrifice of healthy adult male Sprague Dawley rats and there was no requirement for randomization and blinding. For experiments using cardiac myocytes under voltage-clamp conditions, after an initial assessment of Ca^{2+} handling in control (drug-naïve) cells, the inclusion of flecainide, QX-FL or NU-FL in the patch pipette was randomly assigned. Data analysis was performed by operators blinded to the drugs under test.

In all experiments, data subjected to statistical analysis are from at least 5 independent values and are reported as mean ± SEM. The exact number of n in each dataset is given in each instance in the corresponding figure legends. Data were tested for normality using the D'Agostino and Pearson omnibus test and normally and non-normally distributed data were tested using unpaired Student's t-test or Mann-Whitney test, respectively. Data were considered significant if $P < 0.05$. All statistical analysis was performed using Prism 6.0 software (GraphPad, CA, USA).

Results

The structures of flecainide, QX-FL and NU-FL, together with the proportion of each molecule present as cationic or neutral species at pH 7.4, are shown in Figure 1.

Previous work (Bannister et al. 2015) has established that flecainide, present at the cytosolic face of the RyR2 channel, can enter the cytosolic vestibule of the channel and interact with residues in the helices lining this portion of the pore-forming region (PFR). When bound, flecainide introduces a physical and/or electrostatic barrier to the movement of cations from the cytosolic to the luminal side of the channel. The affinity of interaction of flecainide at this site is relatively weak and bound flecainide is destabilised by movement of cations in the physiologically relevant, luminal to cytosolic direction. In this report, we have examined the influence of QX-FL and NU-FL on cation translocation in individual recombinant hRyR2 channels reconstituted into planar phospholipid bilayers and compared these effects with those of flecainide.

The single channel traces in Figure 2 (A) show that like flecainide, QX-FL and NU-FL, present in the solution at the cytosolic face of the channel, are open channel blockers of hRyR2 when net cation current is driven cytosolic to luminal by applying a holding potential of +40 mV across the bilayer. The increase in occurrence of blocking events with increasing concentration is evident in the traces themselves and is depicted in the accompanying frequency amplitude histograms. As is the case with flecainide, QX-FL and NU-FL do not fully occlude the hRyR2 pore and residual current continues to flow with the blocking molecule bound. The expanded frequency amplitude histograms (Figure 2 (B)) highlight a small but significant difference ($p < 0.05$) in the residual current of the blocked states

produced by flecainide (19.16 ± 0.61%) and QX-FL (17.27 ± 0.51%). For NU-FL the residual current in the blocked state shows considerable variation.

In all cases block is manifest as a decrease in Po (Figure 3 (A)). The K_d for flecainide is 13.14 ± 1.89 μM (n=6 channels). The fully charged and neutral analogues block considerably less effectively, and their K_ds cannot be calculated from this plot. More quantitative information can be obtained from the rates of association (K_{on}) and dissociation (K_{off}) for flecainide, QX-FL and NU-FL (n=6 channels) shown in Figures 3 (B) and (C). Whilst no significant differences exist between the dissociation rates of the three compounds, rates of flecainide association are significantly higher than those of either QX-FL or NU-FL (all in nM^{-1} s^{-1}: flecainide: 9.54 ± 2.20, QX-FL: 0.91 ± 0.03, NU-FL: 0.62 ± 0.05; $p<0.05$).

We next examined whether cytosolic QX-FL or NU-FL was able to influence the physiologically relevant flux of cations through hRyR2. This was done by reversing the holding potential across the bilayer to -40 mV and so driving net K^+ current in the luminal to the cytosolic direction (Figure 4). These experiments demonstrate that, as previously shown for flecainide (Bannister et al. 2015), neither cytosolic QX-FL nor NU-FL is able to influence the physiologically relevant, luminal to cytosolic flux of cations through the hRyR2 channel.

We have extended this investigation by monitoring the effects of varying holding potential on the ability of all three forms of flecainide to block K^+ flux through the open hRyR2 channel. Figure 5 (A) shows the variation in block caused by 50 μM cytosolic flecainide, QX-FL and NU-FL at holding potentials between ± 70 mV. Under these conditions the probability of block for both flecainide and QX-FL is dependent on potential; being more pronounced as the potential is made increasingly positive. Voltage dependence of block by the predominantly

neutral NU-FL at 50 μM is very weak, but is more pronounced when its association rate is increased on raising the concentration to 200 μM (Figure 5 (A)). These data confirm that for all three compounds block is observed only when net K^+ flux is in the cytosolic to luminal direction. No block is seen at negative holding potentials when net flux is luminal to cytosolic. In all cases both rates of blocker association (Figure 5 (B)) and dissociation (Figure 5 (C)) are influenced by trans-membrane potential.

These characterizations establish that both QX-FL and NU-FL, present in the solution at the cytosolic face of hRyR2 are open channel blockers of the non-physiological flux of cations but are significantly less effective than flecainide. None of the compounds block the physiologically relevant flux of cations when present at the cytosolic face of the channel.

Figure 6 shows the effect of adding each of the blockers to the luminal face of the channel. Under these conditions no blocking events are seen either at a holding potential of -40 mV, when net cation flux is in the physiologically relevant luminal to cytosolic direction, or initially at +40 mV when net flux is cytosolic to luminal (top panel). These experiments demonstrate that no sites of interaction for flecainide blocking molecules are present at the luminal side of the hRyR2 channel. If a net cytosolic to luminal cation flux is maintained at +40 mV in the presence of luminal flecainide and NU-FL, brief blocking events are apparent after several minutes and open channel block is well established after 10-20 min (lower panel). The neutral forms of these compounds are able to cross the membrane, passing into the solution in the cytosolic chamber and re-equilibrate here with the cationic form (see Figure 1). Only then are the cationic species able to access the cytosolic vestibule of the channel and partially block cytosolic to luminal cation flux. Luminal fully charged QX-FL

can not cross the bilayer or pass through hRyR2 and, as a consequence, has no access to blocking sites in the cytosolic vestibule.

The data presented to this point indicate that neither QX-FL nor NU-FL are as effective as blockers of cytosolic to luminal K^+ flux through the RyR2 channel as flecainide and, consequently, would be unable to inhibit any contribution that this current might make to RyR2-mediated charge compensation during Ca^{2+} release from the SR. Previous investigations have established that, when present in the cytosol, both flecainide and QX-FL are potent use-dependent blockers of sarcolemmal Na^+ channels (Liu et al. 2003). Their very different ability to block a potential charge compensating current through RyR2 means that intracellular QX-FL is a powerful tool for establishing the relative contribution of Na^+ channel block, and a potential action on RyR2, to flecainide's inhibition of inappropriate RyR2-mediated Ca^{2+} release in CPVT.

Towards this end we examined the effects of intracellular flecainide and its derivatives on Ca^{2+} regulation in rat ventricular myocytes. To measure Ca^{2+} sparks intracellular buffering was adjusted with EGTA to prevent the occurrence of Ca^{2+} waves. Lowering the Ca^{2+}-buffering capacity of the internal solution to 0.4 mM EGTA gave optimal conditions for selectively targeting SR-mediated spark activity. Before Ca^{2+} spark measurement, myocytes from the control and drug treated group were voltage-clamp stimulated for 30 sec at 3Hz from a resting membrane potential to +30 mV in order to bringthe SR [Ca^{2+}] to a steady-state level. Intracellular diastolic [Ca^{2+}] was calculated to be 77 nM. The effects of intracellular application of flecainide, QX-FL and NU-FL on Ca^{2+} spark parameters are shown in Figure 7. A 3D topological view of the spatial and temporal properties of Ca^{2+} sparks under voltage-clamp stimulation is presented in Figure 7 (A). Flecainide and QX-FL were equally effective

at reducing the frequency of Ca^{2+} sparks (Figure 7 (B)), while NU-FL had no effect on this parameter. None of the compounds had a significant effect on spark amplitude (Figure 7 (C)) or mass (Figure 7 (D)). A detailed analysis of the influence of flecainide, QX-FL and NU-FL on Ca^{2+} spark parameters is presented in a table in the Supplemental Material.

Discussion

Flecainide is a well-characterized use-dependent blocker (UDB) of Na^{+} channels (Liu et al. 2002; Liu et al. 2003). More recently flecainide has emerged as an effective therapeutic agent for the treatment of CPVT both in combination with conventional β blockade (van der Werf et al. 2011; van der Werf et al. 2012; Watanabe et al. 2013) and as a mono-therapy (Padfield et al. 2016; Napolitano 2016).

Given its effectiveness as a Na^{+} channel blocker, it is difficult to envisage a therapeutic action for flecainide that does not involve this target, however its primary action in CPVT is considered (or has been suggested) to be on RyR2, with the proposition that flecainide blocks the open channel and hence reduces, or prevents, inappropriate RyR2-mediated release of Ca^{2+} from the SR (Hilliard et al. 2010).

However, recent work from our group (Bannister et al. 2015) has established that while flecainide does interact, with relatively low affinity, with a site within the cytosolic cavity of RyR2, and when bound can reduce the flux of monovalent cations in the cytosolic to SR luminal direction through the channel; bound flecainide is displaced by cation flux in the luminal to cytosol direction (i.e. the flux corresponding to the physiologically relevant movement of Ca^{2+} from the SR to the cytosol, to initiate contraction during excitation-

contraction coupling). Given this, the only feasible mechanism by which direct interaction of flecainide with RyR2 could influence SR Ca^{2+} release is by reducing a monovalent cation, charge compensating, counter-current through the channel (Bannister et al. 2015).

While casting doubt on the therapeutic significance of a direct action of flecainide on RyR2, this observation does raise an important mechanistic issue. In the cardiac myocyte Na^{+} channels and RyR2 are located in different membrane systems, however their orientation, the direction of physiologically relevant cation flux through the channels, the access route of flecainide to its binding site and the location of the binding site, are all equivalent (Figure 8A). Given this equivalence, why then is flecainide a therapeutically relevant blocker of the Na^{+} channel but not of RyR2?

The factors governing flecainide's blocking interaction in the Na^{+} channel cytosolic cavity were revealed by studying the blocking efficiency of flecainide derivatives with similar three-dimensional structure but differing net charge (Figure 1)(Liu et al. 2003). We have used these derivatives in the current investigation to gain new insights into the contribution of structural and charge characteristics to the ability of flecainide to interact with RyR2 and to block a potential charge compensating, cytosolic to luminal, monovalent cation flux.

Our data demonstrate that (although with dramatically lower affinity), as is the case for flecainide, both QX-FL and NU-FL are concentration- and voltage-dependent blockers of a potential charge compensating, monovalent cation flux through RyR2. All three ligands interact within the cytosolic vestibule of the channel pore and gain access to this site from the cytosolic side of the channel. When bound, the ligands do not fully-occlude the RyR2 pore, rather, they create a steric and/or electrostatic barrier that limits cytosolic to luminal cation

flux. The different residual currents seen with the blocking molecules bound indicate that the magnitude of the barrier is dependent upon characteristics of the blocking molecule.

Detailed investigation of the effects of flecainide, QX-FL and NU-FL on Na^+ channels established that the efficacy of these ligands as blockers was determined, primarily, by net charge rather than the structural features of the molecule (Liu et al. 2003). The data presented here establish that this is not the mechanism that governs the blocking interaction with RyR2. Under the experimental conditions used in our experiments, the proportion of flecainide and QX-FL molecules that will be positively charged is essentially the same (99% for flecainide and 100% for QX-FL). However QX-FL is a dramatically less effective blocker of the potential charge compensating, cytosolic to luminal, flux of K^+ through RyR2. This observation demonstrates that factors other than net charge contribute to the ability of flecainide to block cytosolic to luminal cation flux in RyR2. This conclusion is strengthened by the observation that NU-FL (~10% cationic) is only slightly less efficient as a blocker of RyR2 than QX-FL. No significant difference exists in the rate of dissociation of the three blocking molecules from RyR2 and the variation in blocking efficiency of flecainide and its derivatives arises from differences in their rates of association with the channel, with this rate, in comparison with flecainide, approximately 10 fold and 15 fold lower for QX-FL and NU-FL respectively. What characteristics of the molecules underlie this variation?

Previous work from our group has established that a wide range of large mono- and polyvalent cations are concentration- and voltage-dependent blockers of cytosolic to luminal cation flux through the open RyR2 channel (Tinker, Lindsay and Williams 1992b; Tinker, Lindsay and Williams 1992a; Tinker and Williams 1993; Mead and Williams 2004; Mason et al. 2012). Given this, it is logical to conclude that the open channel block reported here arises

from the interaction of the cationic component of flecainide, QX-FL and NU-FL, with a site within the cytosolic vestibule of RyR2. Therefore the difference in the absolute concentration of cationic species in flecainide and NU-FL could be a contributing factor to the differing abilities of these molecules to block RyR2. However, as the absolute concentrations of cationic species in flecainide and QX-FL are essentially the same, other molecular characteristics must contribute to the difference in the efficacy of these blockers.

In Na^+ channels, local anesthetic antiarrhythmics, including flecainide, are stabilized within the cytosolic vestibule by interactions with two aromatic residues separated by two turns on the S6 pore-lining helix of repeat IV (Ragsdale et al. 1994; Ragsdale et al. 1996), and high affinity binding is believed to involve cation-pi interactions between these aromatic residues and amine groups of the blockers (Ahern et al. 2008). In RyR2 cation-pi interactions are unlikely to underpin flecainide block since there are no aromatic residues in the proposed binding region (the lower cytosolic vestibule of the channel) (Bannister et al. 2015). The variation in blocking efficiency of flecainide and its derivatives suggests that the capacity of the molecules to act as hydrogen bond acceptors or donors may be important in determining their ability to bind in the RyR2 cytosolic cavity. Considering functional group differences, the secondary amine flecainide can form two hydrogen bonds with the piperidine ring as its conjugate acid, whereas NU-FL, a tertiary amine, can form only one. QX-FL, as a quaternary compound, has no hydrogen bonding capacity in that region. Small structural differences between flecainide, QX-FL and NU-FL (Figure 1) may also contribute to the likelihood of interaction with RyR2. The bonding around the ring nitrogen in QX-FL and the cationic forms of flecainide and NU-FL is not identical, and steric encumbrance around this atom may result in reduced efficacy.

Crucially, the consequence of flecainide interaction with the Na^+ channel differs from that with RyR2 in one very important way: although access to the flecainide binding site in Na^+ channels requires the channel to be open, once bound, flecainide interacts preferentially with the inactivated closed conformation of the channel, prolonging this state, thereby limiting the channel's availability for further activation (Liu et al. 2002). This limited egress of flecainide from the Na^+ channel facilitates the accumulation of block (or use-dependence) with repetitive depolarizations of the cell (Liu et al. 2003). Therefore, in Na^+ channels flecainide does not merely act as a blocking substrate, but also effectively alters the gating of the channel. RyR2 gating involves transitions between open or closed states; unlike the Na^+ channel, the channel does not need to pass through an inactivated state, so in RyR2 flecainide acts simply as an open channel partial blocker of cytosolic to luminal cation flux and use-dependent block does not occur. The absence of an effect on gating, and the low affinity with which flecainide binds within the cytosolic vestibule of RyR2, means that bound flecainide is readily displaced by the physiologically relevant, luminal to cytosolic, flux of Ca^{2+} from the SR during E-C coupling (Bannister et al. 2015). The mechanisms governing flecainide interaction in Na^+ and RyR2 channels are summarised in Figure 8 (B) and (C) respectively.

The data presented here and in Bannister et al (2015) establish that the only way in which flecainide could act directly on RyR2 to inhibit Ca^{2+} release would be via a very small reduction of an RyR2-mediated counter current. The demonstration that QX-FL is essentially without effect on this current identifies this ligand as a tool with which to examine if counter current inhibition contributes, in any way, to flecainide's therapeutic action in CPVT. Intracellular QX-FL and flecainide are both highly effective blockers of the sarcolemmal Na^+ channel (Liu et al. 2003, Supplementary Figure 2), while even the small, potential, effect of flecainide on RyR2-mediated counter current will be absent with intracellular QX-FL.

Consistent with the conclusion that the action of flecainide on Ca^{2+} handling in cardiac myocytes results solely from inhibition of the Na^+ channel, intracellular flecainide and QX-FL have equivalent effects on Ca^{2+} sparks. NU-FL, a considerably less efficient Na^+ channel blocker, has no effect on Ca^{2+} handling.

In intact cardiomyocytes whole-cell patch clamp-mediated intracellular application of 5 μM flecainide and QX-FL, but not NU-FL, decreased spark frequency with no effect on spark amplitude and mass (Figure 7 and Supplementary Table). These data suggest that it is the charged form of flecainide that mediates this effect and corroborate and extend previous findings that flecainide suppresses Ca^{2+} spark frequency (Sikkel et al 2013a). Taken together with the present investigations that confirmed a lack of effect of these concentrations of flecainide and QX-FL on the physiologically relevant luminal-to-cytoplasmic ion flux through RyR2 and, in the case of QX-FL, any inhibition of a potential charge compensating cytosolic-to-luminal K^+ flux (Figures 4-6), these data support the conclusion that the only mode of action underpinning flecainide's efficacy in CPVT patients is the inhibition of I_{Na} (Sikkel et al 2013a; Bannister et al. 2015; Liu et al. 2011). In addition, our demonstration that QX-FL is a Na^+ channel specific ligand, yet has identical effects to flecainide on Ca^{2+} spark parameters (Supplementary Table) reinforces this conclusion.

Sikkel et al. (2013a) showed that the flecainide-mediated reduction in Na^+ influx into the cardiomyocyte can, via the enhancement of Ca^{2+} efflux through the Na^+/ Ca^{2+} exchanger (NCX), decrease $[Ca^{2+}]_i$ in the vicinity of the RyR2 and thus reduce the frequency of spontaneous SR Ca^{2+} release events. These authors also showed that this was a class effect of I_{Na} blockers and moreover, flecainide caused no reduction in SR Ca^{2+} leak when I_{Na} was eliminated by altering the holding potential via voltage clamp. By amalgamating these findings with their existent hypothesis that flecainide's mechanism of action was dependent

on direct effects on I_{Na} and through direct interaction with RyR2, Steele and co-workers proposed a 'triple mode' of flecainide action namely via its direct effects on I_{Na} and RyR2 and an indirect effect on NCX (Steele et al. 2013). The present investigations, which corroborates previous work by Bannister and colleagues (Bannister et al. 2015), present the unequivocal demonstration that flecainide does not have a *direct* effect on luminal-to-cytosolic Ca^{2+} flux through RyR2 and fundamentally challenges reports that flecainide efficacy in RyR2- and CSQ-mutation linked CPVT patients is due to an additive effect of I_{Na} inhibition and a direct block of RyR2 (Watanabe et al. 2009; Hilliard et al. 2010; Lee et al. 2012; Hwang et al. 2011; Galimberti and Knollmann 2011). Further corroborating the lack of direct effect of flecainide on RyR2-mediated Ca^{2+} release, Supplementary Figure 3 shows the persistence of RyR2-dependent spontaneous Ca^{2+} release events in $Na_v1.5$-null human embryonic kidney (HEK) in the presence of flecainide (5 μM).

The contention that flecainide is less effective in inhibiting RyR2 under circumstances that promote higher spark frequency (i.e. conditions of Ca^{2+} overload) (Steele et al. 2013; Sikkel et al 2013b) is not applicable here since the present studies in intact control cells are characterised by comparable Ca^{2+} spark frequencies to those reported by Hilliard et al (≈ 1.5 versus 0.8, respectively) suggesting similar SR Ca^{2+} loading. Adding to the confusion, the same group have recently described the *increased* potency of flecainide in suppressing intracellular Ca^{2+} waves in permeabilized cardiomyocytes following manoeuvres that increase Ca^{2+} spark frequencies (Savio-Galimberti and Knollmann 2015).

We have established that 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. These findings are crucial as they will contribute to the rational design of improved therapies, and prevent the clinical misuse of flecainide in the treatment of phenotypically similar but mechanistically distinct arrhythmias.

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Author contributions

A.J.W., M.L.B., N.L.T., A.A.L. and C.H.G. designed the study; M.L.B. and N.L.T. performed single channel experiments; A.A.L. performed myocyte Ca^{2+} imaging experiments; S.C. and C.H.G. created the H-Flp/$Na_v1.5$ cells; S.A.M. characterised Na^+ channel function; C.d.P., A.T.M., D. N-H., H.O. and M.C.B. synthesised NU-FL and QX-FL; M.L.B., N.L.T., A.A.L., S.A.M, K.T.M. and C.H.G. analysed the data; M.L.B., N.L.T., A.J.W., C.H.G., A.A.L., K.T.M., S.A.M. and M.C.B. wrote the paper.

Conflicts of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1 Chemical structures of flecainide, QX-FL and NU-FL. At pH 7.4, flecainide (pKa 9.3) is >99% charged; NU-FL (pKa 6.4) is >90% neutral. QX-FL is permanently charged.

Figure 2 Open channel block of voltage driven cytosolic to luminal cation flux through hRyR2. (A) Representative single channel traces recorded at + 40 mV in symmetrical 610 mM KCl. Openings are downwards from the closed level (black line). Channels are fully activated with 20 μM EMD 41000. The increase in blocking events (marked with a dotted line) with increasing concentration of flecainide, QX-FL or NU-FL is depicted in the frequency amplitude histograms and manifests as a decrease in Po and increase in Pb (B) Expanded frequency amplitude histograms demonstrating the nature of the blocked states, tables above show the mean residual current (R.C.), Pb and Tb achieved with 50 μM of each drug. n is given in each instance.

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Figure 7 Effects of intracellular application of flecainide and its analogues on Ca^{2+} spark parameters. (A) 3D topological view of the spatial and temporal properties of Ca^{2+} sparks under voltage-clamp stimulation. Cells were dialyzed via patch pipette (10-12 min) with either vehicle control solution containing Fluo-3-5K^{+} salt, or with flecainide, QX-FL or NU-FL (5 μM). Myocytes were stimulated via depolarization from -80 to +30 mV (2Hz, 30s) and spontaneous sparks recorded during a subsequent 20s quiescent period. (B-D): The effect of intracellular dialysis of flecainide, QX-FL or NU-FL on mean Ca^{2+} spark frequency (B),

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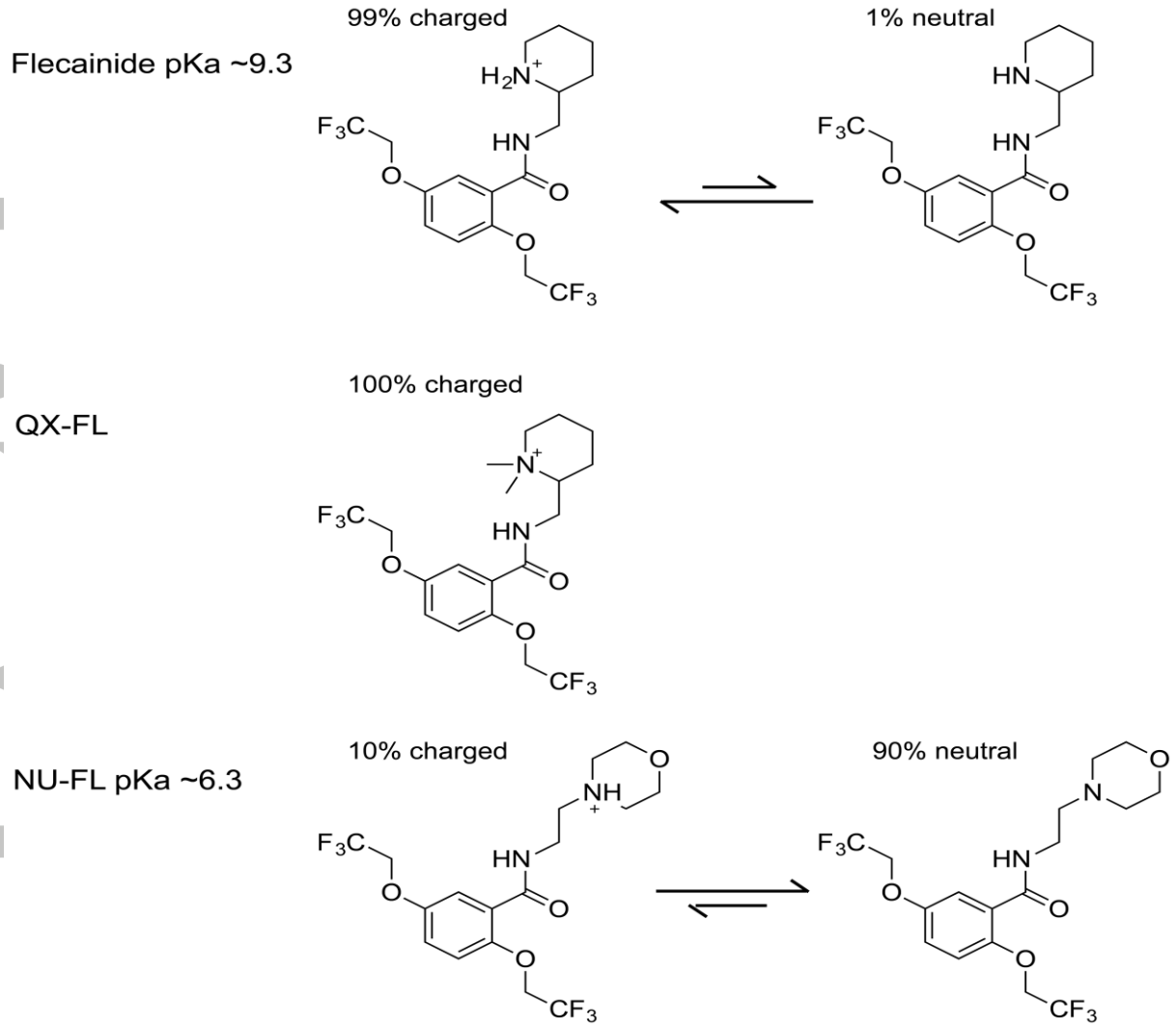
Figure 8 Flecainide inhibits the physiologically relevant cation flux through Na^+ channels but does not block Ca^{2+} release through RyR2. Cartoons showing the orientation of Na^+ and RyR2 channels in the cardiac myocyte and the consequences of flecainide interaction.. (A) The topology of sarcolemmal Na^+ and sarcoplasmic reticulum RyR2 channels is equivalent. Cation fluxes are indicated by arrows (Na^+ influx in blue, Ca^{2+} release in red and a charge-compensating K^+ flux into the SR in black). (B) i) Simplified scheme for Na^+ channel gating in the absence of flecainide. 1. – closed; 2. – open; 3. – inactivated; 4. - closed (inactivated). ii) Simplified scheme for Na^+ channel gating in the presence of flecainide. 1. – flecainide enters the open channel from the cytosol; 2. and 3. – flecainide prolongs the inactivated and closed (inactivated) conformations; 4. – closed; 5. – flecainide leaves the open channel. (C) i) Simplified scheme for RyR2 gating in the absence of flecainide. 1. – closed; 2. – open allowing Ca^{2+} release from the SR (red arrow) and a potential, charge compensating, cytosolic to luminal K^+ counter current (black arrow). ii) Simplified scheme for RyR2 gating in the presence of flecainide. 1. – closed; 2. – flecainide enters the cytosolic vestibule from the cytosol; 3. – bound flecainide partially blocks the potential, charge compensating, cytosolic to luminal K^+ counter current (dotted black arrow); 4. – bound flecainide is displaced by the physiologically relevant flux of Ca^{2+} during release from the sarcoplasmic reticulum.



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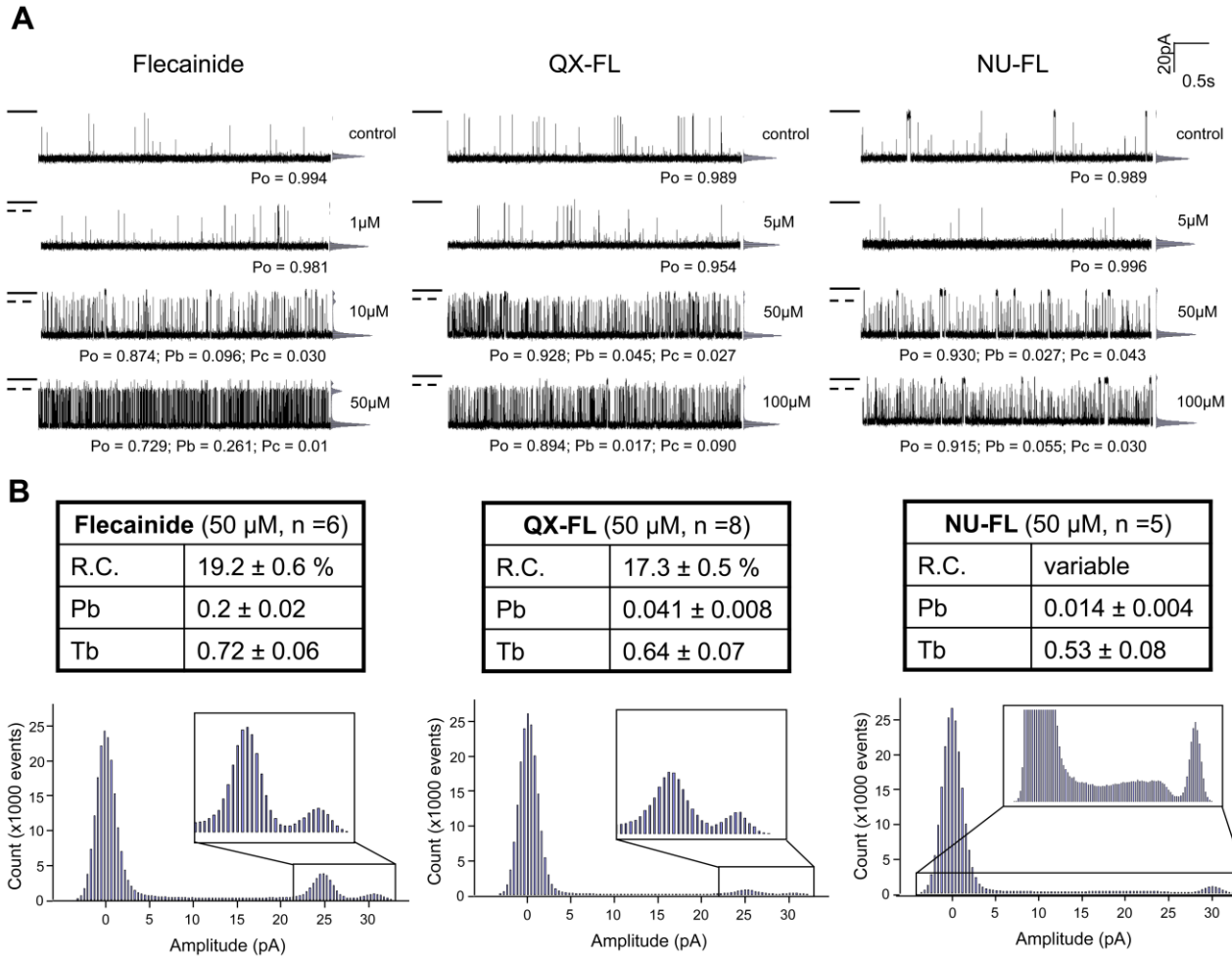

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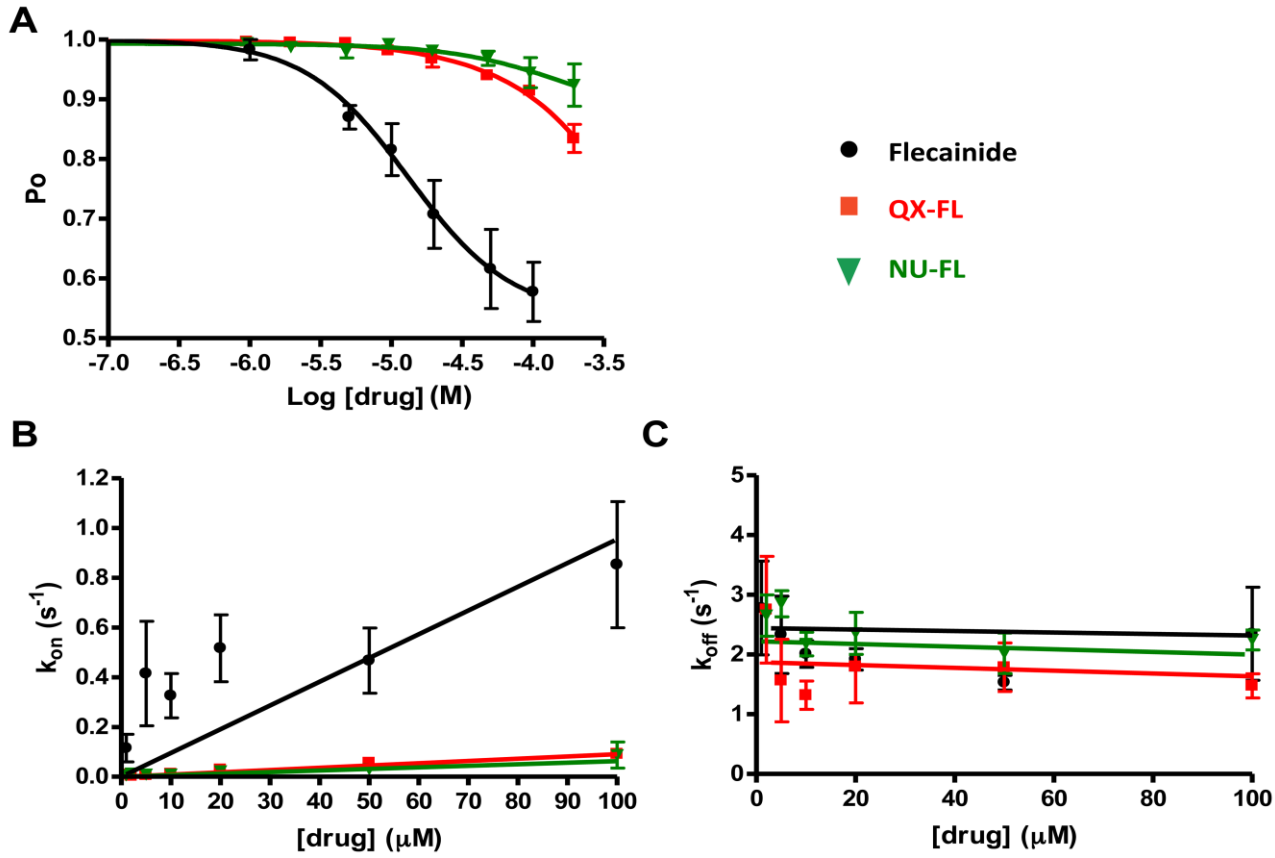

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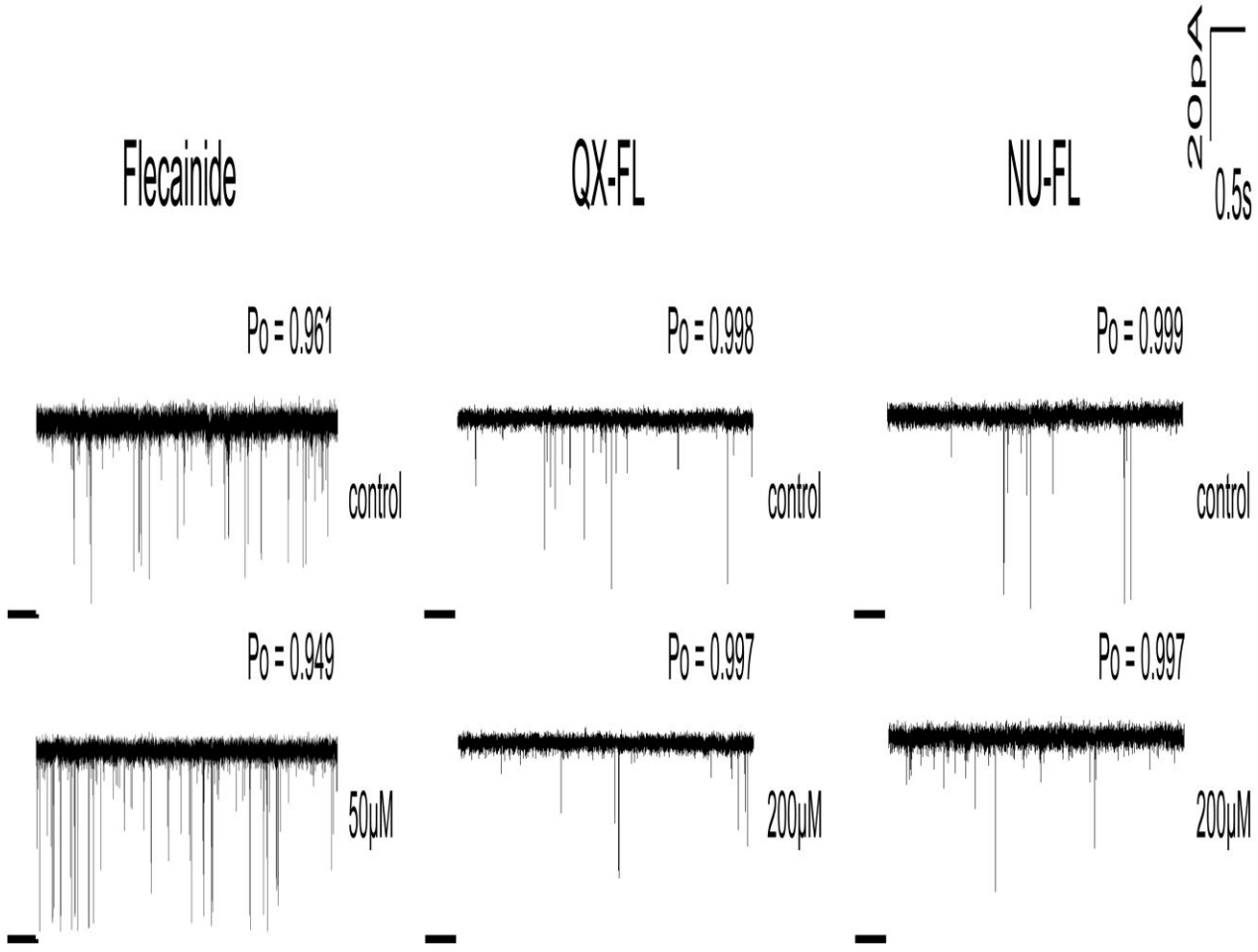

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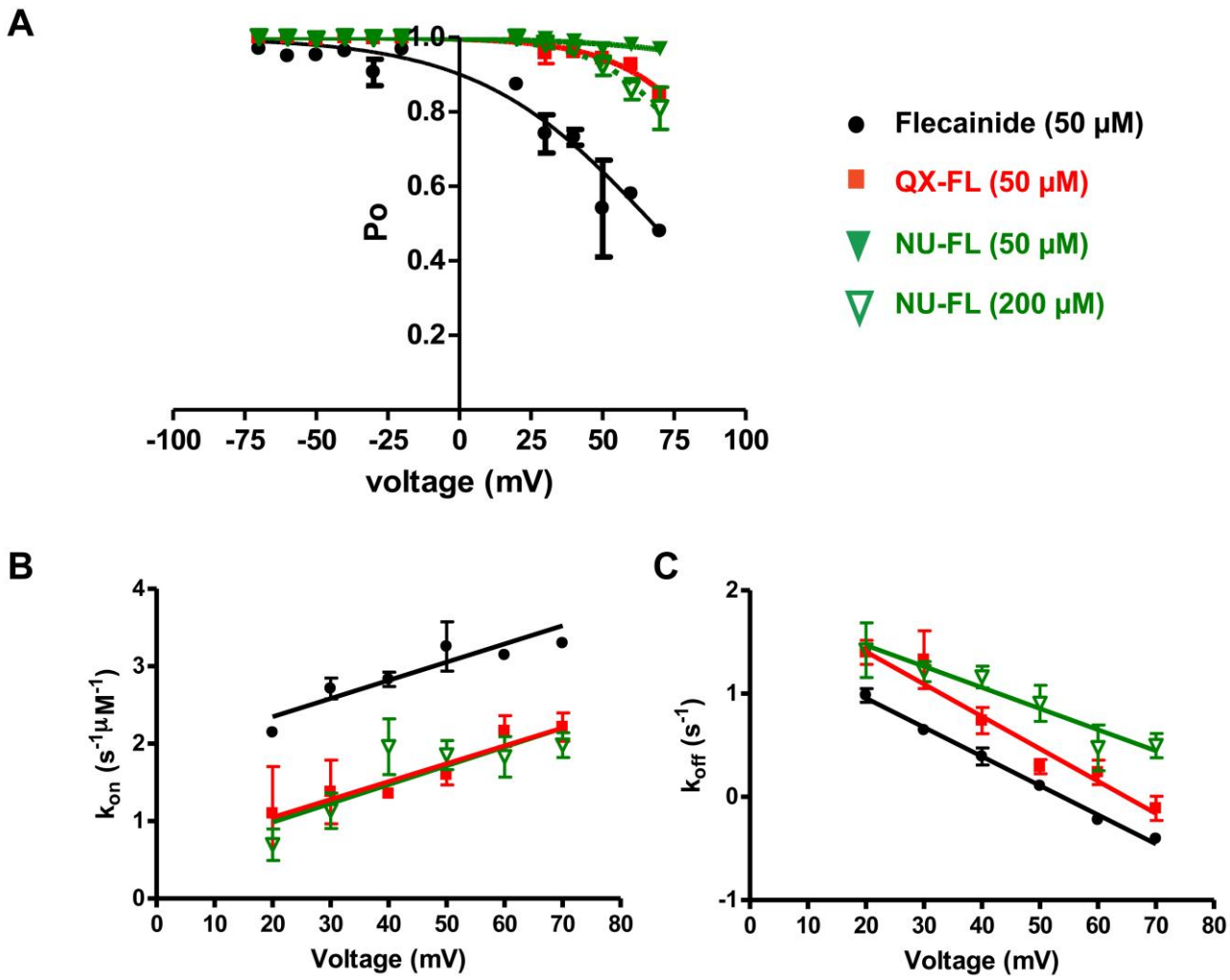

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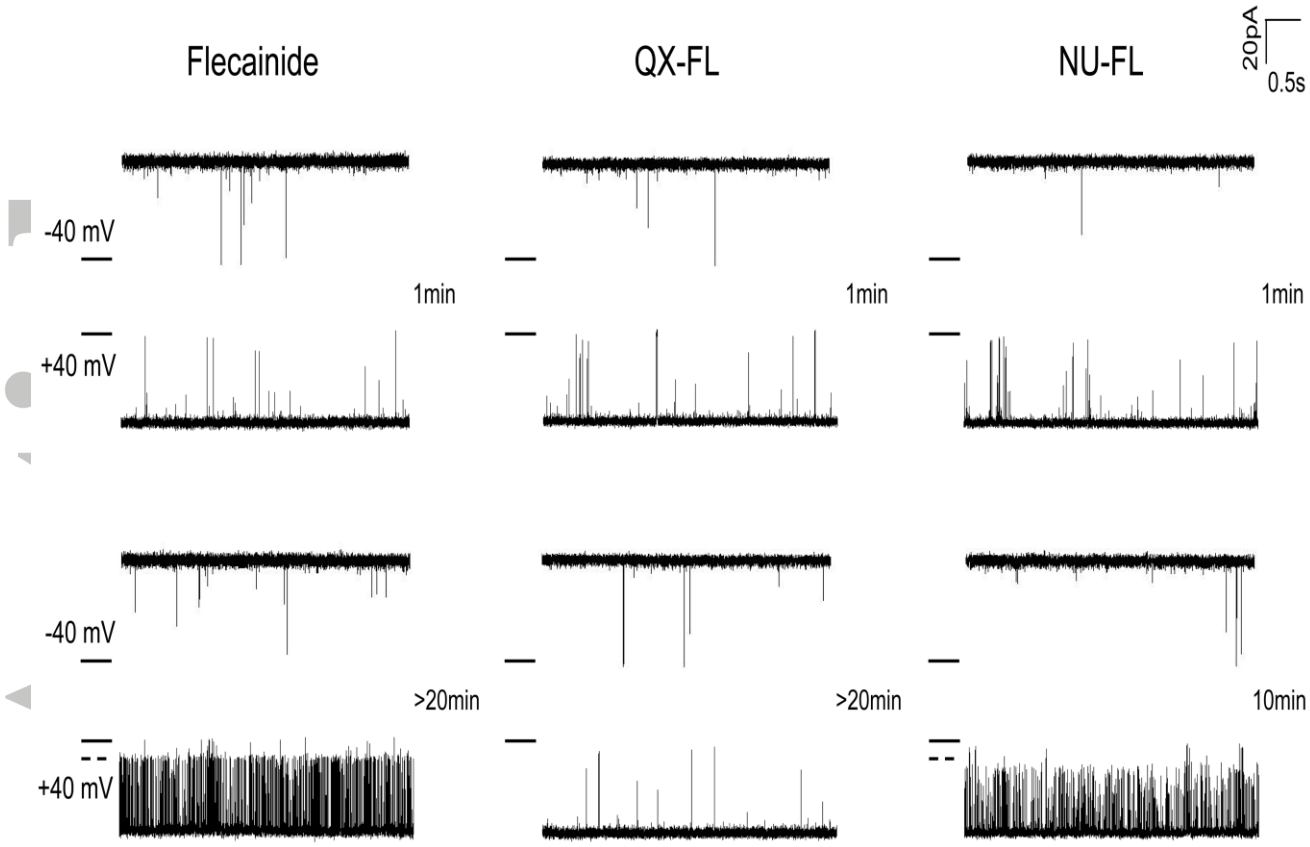

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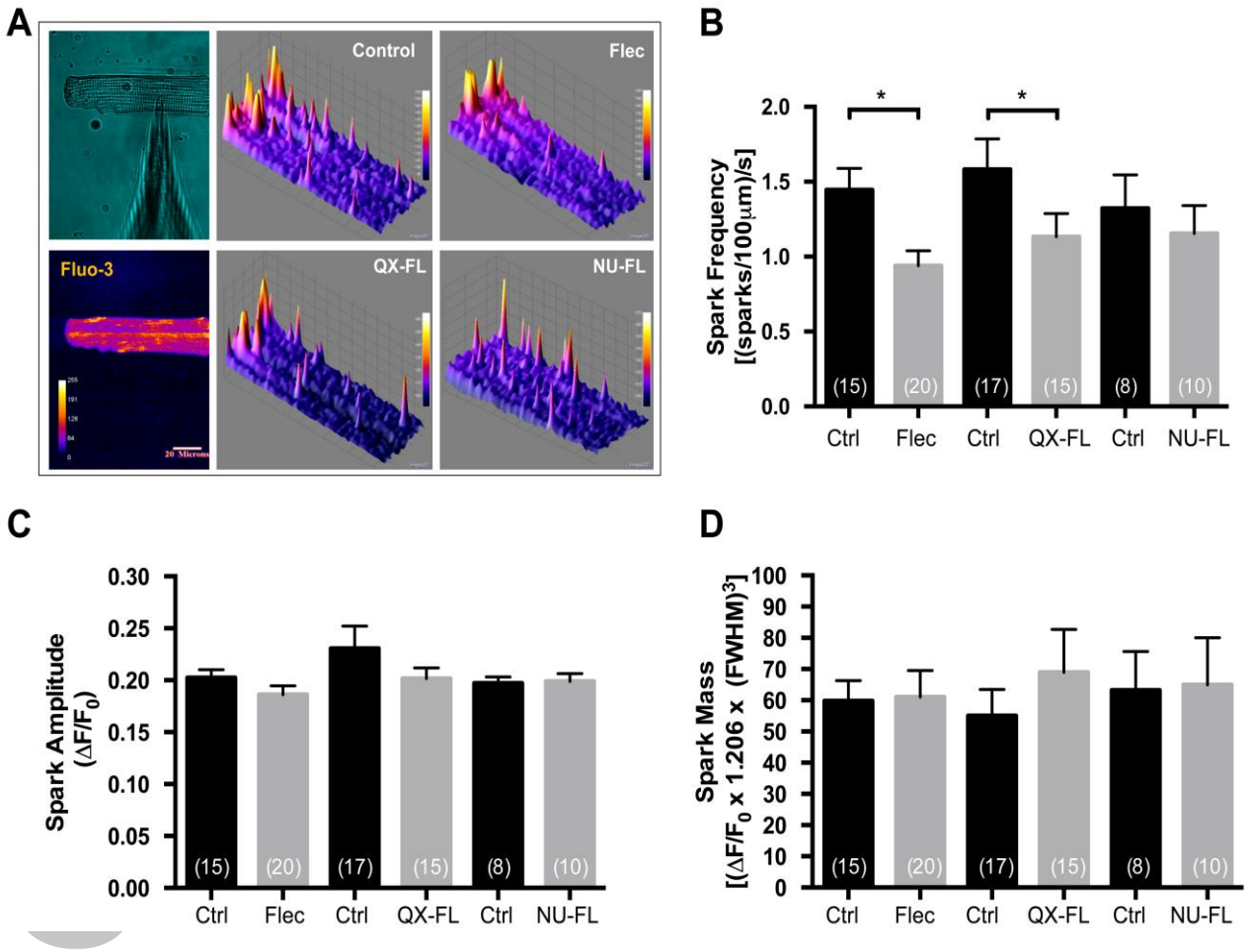

Figure 7 Effects of intracellular application of flecainide and its analogues on Ca^{2+} spark parameters. (A) 3D topological view of the spatial and temporal properties of Ca^{2+} sparks under voltage-clamp stimulation. Cells were dialyzed via patch pipette (10-12 min) with either vehicle control solution containing Fluo-3-$5K^{+}$ salt, or with flecainide, QX-FL or NU-FL (5 μM). Myocytes were stimulated via depolarization from -80 to +30 mV (2Hz, 30s) and spontaneous sparks recorded during a subsequent 20s quiescent period. (B-D): The effect of intracellular dialysis of flecainide, QX-FL or NU-FL on mean Ca^{2+} spark frequency (B), amplitude (C) and mass (D). The number of cardiac myocytes used in each instance is given in parentheses and they were isolated from multiple hearts (flecainide n=10/11, QX-FL n=10, NU-FL n=5; see Supplmentary Table). *, $p<0.05$ compared with the corresponding control (untreated) cells in each group.

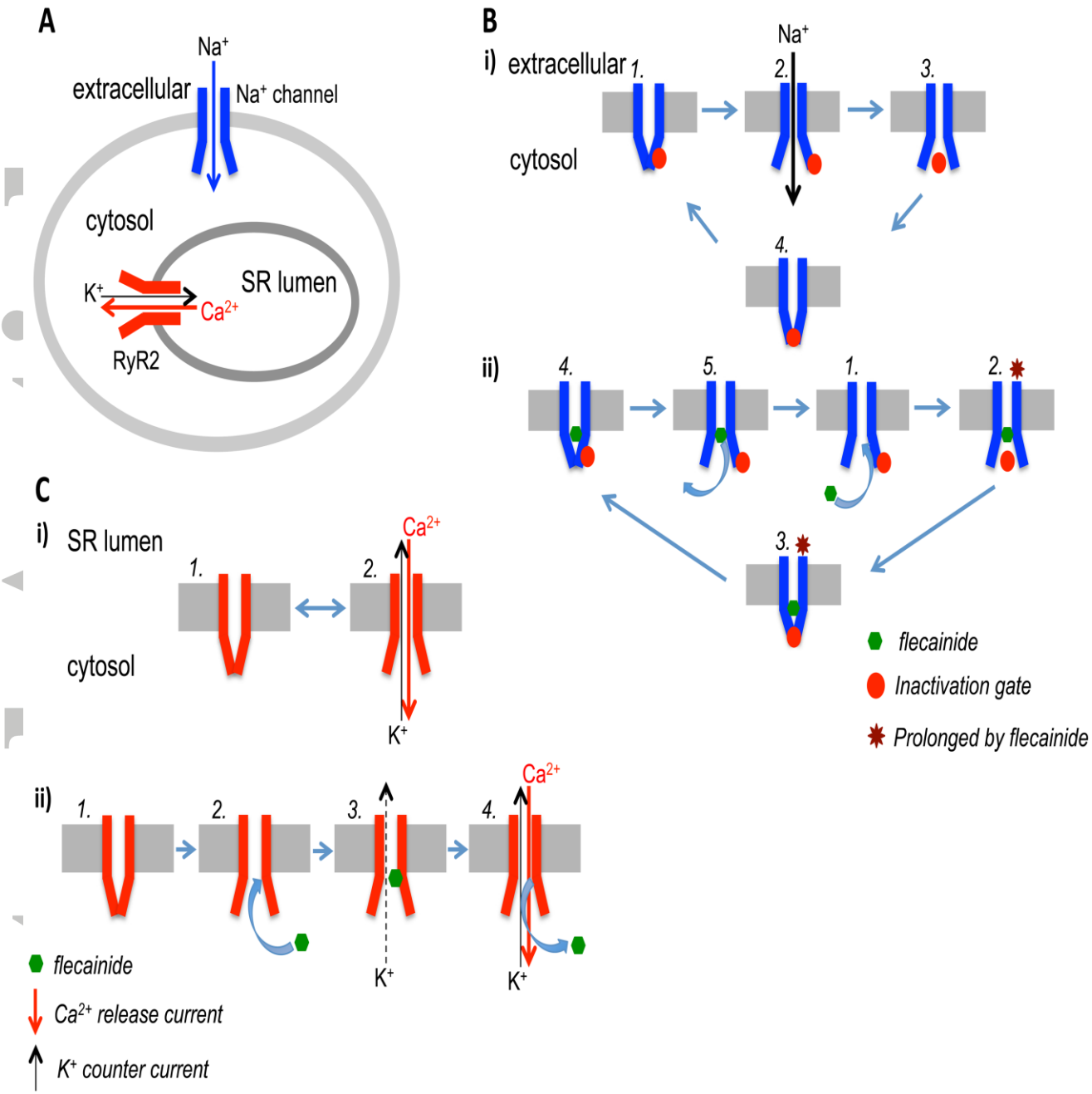

Figure 8 Flecainide inhibits the physiologically relevant cation flux through Na^+ channels but does not block Ca^{2+} release through RyR2. Cartoons showing the orientation of Na^+ and RyR2 channels in the cardiac myocyte and the consequences of flecainide interaction.. (A) The topology of sarcolemmal Na^+ and sarcoplasmic reticulum RyR2 channels is equivalent. Cation fluxes are indicated by arrows (Na^+ influx in blue, Ca^{2+} release in red and a charge-compensating K^+ flux into the SR in black). (B) i) Simplified scheme for Na^+ channel gating in the absence of flecainide. 1. – closed; 2. – open; 3. – inactivated; 4. - closed (inactivated). ii) Simplified scheme for Na^+ channel gating in the presence of flecainide. 1. – flecainide enters the open channel from the cytosol; 2. and 3. – flecainide prolongs the inactivated and

closed (inactivated) conformations; 4. – closed; 5. – flecainide leaves the open channel. (C) i) Simplified scheme for RyR2 gating in the absence of flecainide. 1. – closed; 2. – open allowing Ca^{2+} release from the SR (red arrow) and a potential, charge compensating, cytosolic to luminal K^+ counter current (black arrow). ii) Simplified scheme for RyR2 gating in the presence of flecainide. 1. – closed; 2. – flecainide enters the cytosolic vestibule from the cytosol; 3. – bound flecainide partially blocks the potential, charge compensating, cytosolic to luminal K^+ counter current (dotted black arrow); 4. – bound flecainide is displaced by the physiologically relevant flux of Ca^{2+} during release from the sarcoplasmic reticulum.