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Invited Minireview

Moving in the right direction – elucidating mechanisms of interaction between flecainide and the cardiac ryanodine receptor

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Abstract

Flecainide is used to treat catecholaminergic polymorphic ventricular tachycardia (CPVT), an arrhythmia caused by disrupted cellular Ca^{2+} handling following ß-adrenergic stimulation. The clinical efficacy of flecainide in this context involves complex effects on multiple ion channels that may be influenced by the disease state. A compelling narrative has been constructed around flecainide's non-selective block of sarcoplasmic reticulum (SR) lumen-to-cytoplasm Ca^{2+} release through intracellular calcium release channels (RyR2). However, ion fluxes across the SR membrane during heart contraction are *bidirectional* and here we review experimental evidence that flecainide's principal action on RyR2 involves the partial block of ion flow in the cytoplasm-to-lumen direction (*i.e*. flecainide inhibits RyR2-mediated SR 'countercurrent'). Experimental approaches that could advance new knowledge on the mechanism of RyR2 block by flecainide are proposed. Some impediments to progress in this area, that must be overcome to enable the development of superior drugs to treat CPVT, are also considered.

Introduction

Cardiac ryanodine receptors (RyR2) regulate the release of Ca^{2+} from the sarcoplasmic reticulum (SR) that triggers heart muscle contraction. In the genetic arrhythmogenic disorder catecholaminergic polymorphic ventricular tachycardia type 1 (CPVT1), mutations in RyR2 promote structural instability in the channel leading to abnormal diastolic Ca^{2+} leak during beta-adrenergic stimulation. Given the centrality of RyR2 in this arrhythmogenic mechanism, the presumed ideal pharmacologic therapy for CPVT1 would be achieved by highly specific modulation of diastolic SR lumen-to-cytoplasmic Ca^{2+} flux with inhibitory effects biased towards structurally-defective (mutant) RyR2 channels.

Allosteric modulation of CPVT-linked diastolic Ca^{2+} leak through RyR2 using a series of channel stabilizers ('RyCals' *e.g*. S107) has been reviewed (Marks, 2013). A wide range of structurally diverse ligands have been demonstrated to interact directly with the RyR pore. However, the only known ligands with selectivity for RyR are the diterpenoid, ryanodine, and structurally related 'ryanoids'. These compounds, which are used commercially as insecticides, exhibit highly selective binding to RyR but their multi-modal action on channel function (activation, sustained activation by substate locking, and inactivation/'shut down') and resultant toxicity profiles make them also unsuitable for clinical use.

In the absence of the 'ideal' CPVT drug, efforts have focussed on establishing the therapeutic potential of non-RyR-selective drugs with known pharmacologic and safety profiles. Direct interactions of RyR2 with propafenone (Hwang *et al*., 2011) and carvedilol (Zhou *et al*., 2011; Smith *et al*., 2013), and also with derivatives of the local anaesthetic tetracaine (Li *et al*., 2017; Klipp *et al*., 2018), have been investigated. In all of these studies, while block of RyR2 has been demonstrated, the primary effect of these drugs is likely mediated by actions on other cellular targets (Table 1).

The concept of drug pleiotropy also applies to flecainide, a class 1c antiarrhythmic with well-described effects on Na^+ channels, around which a compelling narrative of CPVT therapeutic effect has been constructed. Flecainide is of evident clinical benefit (van der Werf *et al*., 2011) and it conforms to the profile of a 'repurposed' drug that binds with comparatively high affinity to multiple voltage-gated Na^+ and K^+ channels (Table 1) (Salvage *et al*., 2017).

The multiplicity of actions of flecainide have enabled several different, non-exclusive interpretations of its effects on reducing pro-arrhythmic behaviour to be developed (Watanabe *et al*., 2009; Liu *et al.,* 2011; Sikkel *et al.,* 2013a, b; Steele *et al.,* 2013; Mehra *et al*., 2014; Bannister *et al.,* 2015; Williams *et al.,* 2016). These are subject to the interdependence of intracellular Na^+ and Ca^{2+} homeostasis in the heart. The evidence supporting the conclusion that flecainide-mediated reduction in Na^+ influx decreases the frequency of Ca^{2+} sparks and Ca^{2+} waves is credible. The underlying hypothesis is that the decrease in cytosolic (or local "fuzzy space") Na^+ reduces the level of Ca^{2+} around RyR2 clusters as the result of NCX–mediated Ca^{2+} efflux so reducing the probability of RyR2 opening.

Flecainide also increases the current required to produce an action potential (AP) in cardiac myocytes carrying a RyR2 mutation associated with CPVT (R4496C) (Liu *et al*., 2011). The hypothesis arising as a consequence is that delayed afterdepolarisations (DADs) resulting from spontaneous releases of Ca^{2+} would be required to be much larger to depolarise the cells to the threshold for sufficient Na^+ channel activation and AP production. In other words, the reduction in availability of Na^+ channels in the presence of flecainide appears to shift the threshold for premature AP triggering. Flecainide also exhibits pro-arhythmic liability since it can inhibit ventricular voltage-gated K^+ channels involved in repolarisation (I_{Kr} (Melgari *et al*., 2015) and $I_{to,f}$ (Slawsky and Castle, 1994)) potentially resulting in AP prolongation.

The net effect of flecainide's action is therefore complex, remains difficult to predict, and is likely determined by its influences on Na^+, Ca^{2+} and K^+ homeostasis which may also be dependent on disease state. However, the defining feature of flecainide's MOA is claimed to be the direct block of RyR2 (Watanabe *et al*., 2009; Hwang *et al*., 2011; Hilliard *et al.,* 2010). The assertion that the clinical efficacy of flecainide in CPVT is due principally to its inhibition of Ca^{2+} flux through RyR2 is contentious given the evidence that flecainide has much higher affinity for surface membrane Na^+ and K^+ channels (Table 1).

Insights from the latest data

Recently, Kryshtal and colleagues (Kryshtal *et al*., 2021) sought to provide clarity on this issue using differentially charged flecainide analogues, an approach that had been previously taken to probe mechanisms of ion channel block in Na^+- K^+ and

RyR2 channels (Liu *et al*., 2003; Melgari *et al*., 2015; Bannister *et al*., 2016). Central to their conclusion that "RyR2 channel inhibition is the principal mechanism of flecainide action in CPVT" were data acquired using the 'single channel technique' in which single RyR2 channels are studied following their incorporation into artificial lipid bilayers. It is considered to be the gold-standard for investigating mechanism and for resolving gating behaviour of single ion channels under defined conditions (*e.g*. following addition of drug). It benefits from unrivalled control of experimental variables, including the directionality of current flow through RyR channels via manipulation of voltage or ionic gradients. Using this technique, and consistent with previous data (Hilliard *et al.,* 2010; Hwang *et al*., 2011; Bannister et al., 2015 and 2016), Kryshtal and colleagues showed that flecainide blocked ion flow through RyR2 in the direction equivalent to cytoplasm-to-SR lumen (Figure 1A). The permanently charged, membrane impermeant QX-FL derivative and the novel NMFL analogue, which is predicted to have similar charge properties to flecainide but lacks the donor N-H group, were less effective. Kryshtal and colleagues also reported that flecainide analogues that do not block RyR2, but do block Na^+ channels are ineffective against CPVT. Moreover, flecainide remains effective even when Na^+ channels were blocked by tetrodotoxin (TTX) (Kryshtal *et al*., 2021). However, Kryshtal and co-workers did not report any data showing the ability of flecainide or its derivatives to directly block RyR2-mediated ion flow in the physiologically relevant SR lumen-to-cytoplasm direction.

There is a need therefore to reconcile the widely portrayed status of flecainide as an effective blocker of RyR2-mediated SR Ca^{2+} efflux with available experimental evidence demonstrating only a partial block of ions moving through RyR2 in the 'opposite' direction (cytoplasm-to-SR lumen) (Figure 1A). We offer some thoughts on how this paradox may be resolved below. This minireview is focussed specifically on the mechanisms of flecainide inhibition of RyR2 and we do not consider other modulatory elements that may regulate RyR2-mediated ion handling *in vivo* (e.g. accessory co-proteins, PKA- and CaMKII-mediated phosphorylation, spatial clustering of RyR2 channels, redox status).

Building on consensus - the charge-compensating countercurrent

The study of Ca^{2+} release from the SR has dominated investigations on the mechanisms of cardiac muscle force generation and rhythmicity. However, ion fluxes

across the SR membrane during the initiation of contraction are bidirectional; a charge compensating cytoplasm-to-SR luminal "countercurrent" ensures that trans-SR membrane potential remains unchanged during the SR luminal-to-cytoplasmic Ca^{2+} release (Sanchez *et al*., 2018) (Figure 1A). RyR2 has been shown to carry its own countercurrent (Gillespie and Fill, 2008), and it is plausible that retarding the charge compensation via partial/substate block of a cytoplasmic-to-luminal countercurrent through RyR2, may indeed result in inhibition of Ca^{2+} release (Figure 1B) (Bannister *et al*., 2015, 2016; Benitah and Gomez, 2021). This should not be confused with direct block of Ca^{2+} efflux from the SR. Mehra *et al*. (2014) have published an effect of flecainide acting directly on luminal-to-cytoplasmic current but at concentrations of the drug that could not be achieved via clinical dosing regimens (IC_{50} ~ 3000 µM; Table 1).

However, while a therapeutic effect involving flecainide inhibition of RyR2 countercurrent is consistent with available data, this remains to be demonstrated. TRIC-A and Cl^- channels also contribute to maintaining the trans-SR potential near 0mV (Zsolnay *et al*., 2018) but are not blocked by flecainide (Bannister *et al*., 2015). We would have a better idea of the precise contribution to charge compensation made by RyR2, and could verify the utility of this mechanism experimentally, if there was a selective inhibitor of the RyR2 countercurrent. To our knowledge, such a blocker does not exist. Conceivably, the chemical development of low affinity and reversible ryanoids that elicited small (residual) fractional conductance might fulfil this purpose for mechanism-based *in vitro* experiments.

If it is indeed the case that partial block of the RyR2 countercurrent is the primary mechanism of action for flecainide *in vivo* then this justifies the reconsideration of other antiarrhythmics and local anaesthetic-type drugs that could potentially have an effect on RyR2 (in addition to their action on surface-membrane voltage-gated channels).

Mechanisms of block and membrane-drug equilibration.

Similar to its mechanism of action in blocking I_{Kr} (Follmer *et al*., 1992) and $K_v2.1$ channels (Madeja *et al*., 2010), single channel bilayer experiments have revealed that flecainide produces open channel block of the monovalent RyR2 countercurrent (Hilliard *et al*., 2010; Hwang *et al*., 2011; Bannister *et al*., 2015). This involves H-bonding between the RyR2 channel and the amine group of the drug (Bannister *et*

al., 2016; Kryshtal *et al.,* 2021) and the asymmetry (sidedness) is a feature of other drug-like molecules that inhibit RyR2 via open channel block. It has also been shown that flecainide inhibits RyR2 via a mechanism involving closed channel block with increased durations in the closed state reported (Mehra *et al*., 2014). Conceptually, closed channel block is consistent with Marks' argument that the optimal therapeutic approach for CPVT would target diastolic Ca^{2+} leak occurring when the RyR2 channel is supposed to be tightly shut, not open (Marks 2013). However, the kinetics of open channel block are not dependent on Ca^{2+} concentration (*i.e*. block would be consistent at diastolic and systolic concentrations (Mehra *et al*., 2014)). Crucially though, it is the open channel block of the cytoplasm-to-SR luminal countercurrent that is proposed to distinguish the cellular action of flecainide from that of tetracaine (Watanabe *et al*., 2009; Hilliard *et al*., 2010).

It has been previously reasoned that the degree of blockade that flecainide could exert on RyR2 would be restricted by its aqueous solubility in the cytoplasm (Bannister *et al*., 2015). Flecainide has been suggested to "accumulate in the heart" (Piovan *et al*., 1986; Zhou *et al*., 1987) and intracellular partitioning and free drug-bound drug equilibria *in vivo*, even of charged hydrophilic drugs, are determined by multiple factors including the actions of organic anion transporter proteins (OATP) and cytoplasmic 'buffering' (George *et al*., 2016). Relating to open- and closed-channel block though, it is extremely unlikely that testing higher concentrations of the drug above those already used experimentally would yield additional insights into these mechanisms. Specifically, in single-channel experiments, at a concentration of 50μM a drug would be in approximately $1x10^{16}$ (ten million billion) excess relative to the (single) channel. Also, the relatively low affinity for flecainide for RyR2 (~13μM (cytoplasm-to-SR lumen block) (Bannister *et al*., 2015) and ~3000μM (SR lumen-to-cytoplasm block) (Mehra *et al*., 2014); Table 1), combined with the torrent of Ca^{2+} ions moving through the channel, means that, even at $1x10^{16}$ excess, the occupancy of the drug in the channel pore during SR lumen-to-cytoplasm ion flow is negligible (Figure 1B).

Countercurrent block of RyR2 reconciles much of the data focused on resolving the mechanism of flecainide inhibition of RyR2, but it is just one potential mechanism. It remains possible, and needs to be tested, that flecainide inhibition of RyR2 involves other mechanisms of channel block (*e.g*., allosteric modulation or 'non-pore' block). Indeed, Mehra *et al.* have previously speculated on an additional

fast inhibition mechanism to explain the observed changes in spark morphology induced by flecainide (Mehra *et al*., 2014). Regarding MOA, it is also essential to consider the possibility that the drug acts from the membrane phase which negates arguments centred on aqueous solubility and cytoplasmic concentrations. Membrane accumulation of flecainide and subsequent inhibition of RyR2 via other 'non-classical' channel block might therefore play a crucial role in restricting ion flow through the channel (Figure 2B). To address this conclusively, time-dependent effects of flecainide on lipid membrane-incorporated RyR2 needs to be investigated.

This is currently impeded by the remarkable fragility of the lipid bilayer system. Typically, data can only be acquired for a few tens of minutes prior to bilayer rupture and this precludes prolonged study of RyR2 channel-drug interactions. It is relevant to note that to overcome this issue, in their characterisation of interactions between RyR2 and carvedilol (and analogues), Zhou and colleagues pre-incubated the channel with the drug prior to incorporation into lipid membranes (Zhou *et al*., 2011). Quantifying the effects of prolonged exposure to flecainide on single RyR2 channels already incorporated into lipid bilayer systems needs new technical solutions to enable longer periods of data acquisition.

Conclusions

The evidence is that the pleiotropic actions of flecainide on several cellular targets contribute to its therapeutic efficacy in CPVT. Specifically relating to its ability to block RyR2- which is considered an essential factor in its antiarrhythmic action (Kryshtal *et al*., 2021) - interpretation of the existing data indicates that the major action of flecainide on the channel occurs under non-physiological voltages (e.g. Mehra *et al*., 2014; Bannister *et al*., 2015; Bannister *et al*., 2016; Williams *et al*., 2016). Thus, current knowledge of flecainide MOA is insufficient to explain the block of pathologic RyR2-mediated SR Ca^{2+} release in CPVT. In this minireview, we posit an alternative hypothesis, supported by published data but not yet resolved experimentally, that a component of flecainide's therapeutic action involves the partial block of the cytoplasm-to-SR lumen RyR2 countercurrent. Some experimental approaches that might help address conclusively the possibility that inhibition of RyR2 countercurrent results in the attenuation of SR-to-cytoplasm Ca^{2+} release are considered. It will be important to determine whether the lack of change in SR

voltage during Ca^{2+} release previously reported in skeletal muscle (Sanchez *et al*., 2018) is also a defining feature of SR Ca^{2+} release in cardiac muscle. Understanding the mechanism of flecainide block of RyR2 is critical in advancing knowledge and developing safe next-generation drugs for the treatment of CPVT.

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Table 1. Comparison of IC_{50} values for block of ion channels by flecainide

(studies utilising human channels are highlighted in bold)

Channel	***Current***	***IC_{50} / µM***	***Species***	***Reference***
K_v11.1	**I_{Kr}**	**1.49**	**human**	**Melgari *et al*., 2015 *J Mol Cell Cardiol* 86:42–53**
K_v1.5	I_{Kur}	2.9	dog	Yue *et al*., 2000 *Cardiovasc Res* 46:151-161
K_v4.2/3	$I_{to,f}$	3.7	rat	Slawsky and Castle, 1994 *J Pharmacol Exp Ther* 269:66-74
Na_v1.5	**I_{Na}**	**7.4**	**human**	**Ramos and O'Leary 2004 *J Physiol* 560:37–49**
K_v4.3	I_{to1}	**10**	**human**	**Radicke *et al*., 2008 *Br J Pharmacol* 136:717–729**
RyR2	**Cytoplasm-to-SR 'counter-current'**	**13.1**	**human**	**Bannister *et al*., 2015 *Circ Res* 116:1324–1335**
		16	sheep	Hilliard *et al*., 2010 *J Mol Cell Cardiol* 48:293–301
Ca_v1.2	I_{Ca}	20	frog	Scamps *et al*., 1989 *Am J Physiol* 256:C549–559
K_v3.1	I_{Kur}	29	dog	Herrera *et al*., 2005 *Mol Pharmacol* 68:305–316
K_v1.5	I_{Kur}	**211**	**human**	**Herrera *et al*., 2005 *Mol Pharmacol* 68:305–316**
HCN4	I_f	1700	rabbit	Tamura *et al.*, 2009 *J Pharmacol Sci* 110:150 – 159
RyR2	SR-to-cytoplasm	3000	sheep	Mehra *et al*., 2014 *Mol Pharmacol* 86:696–706

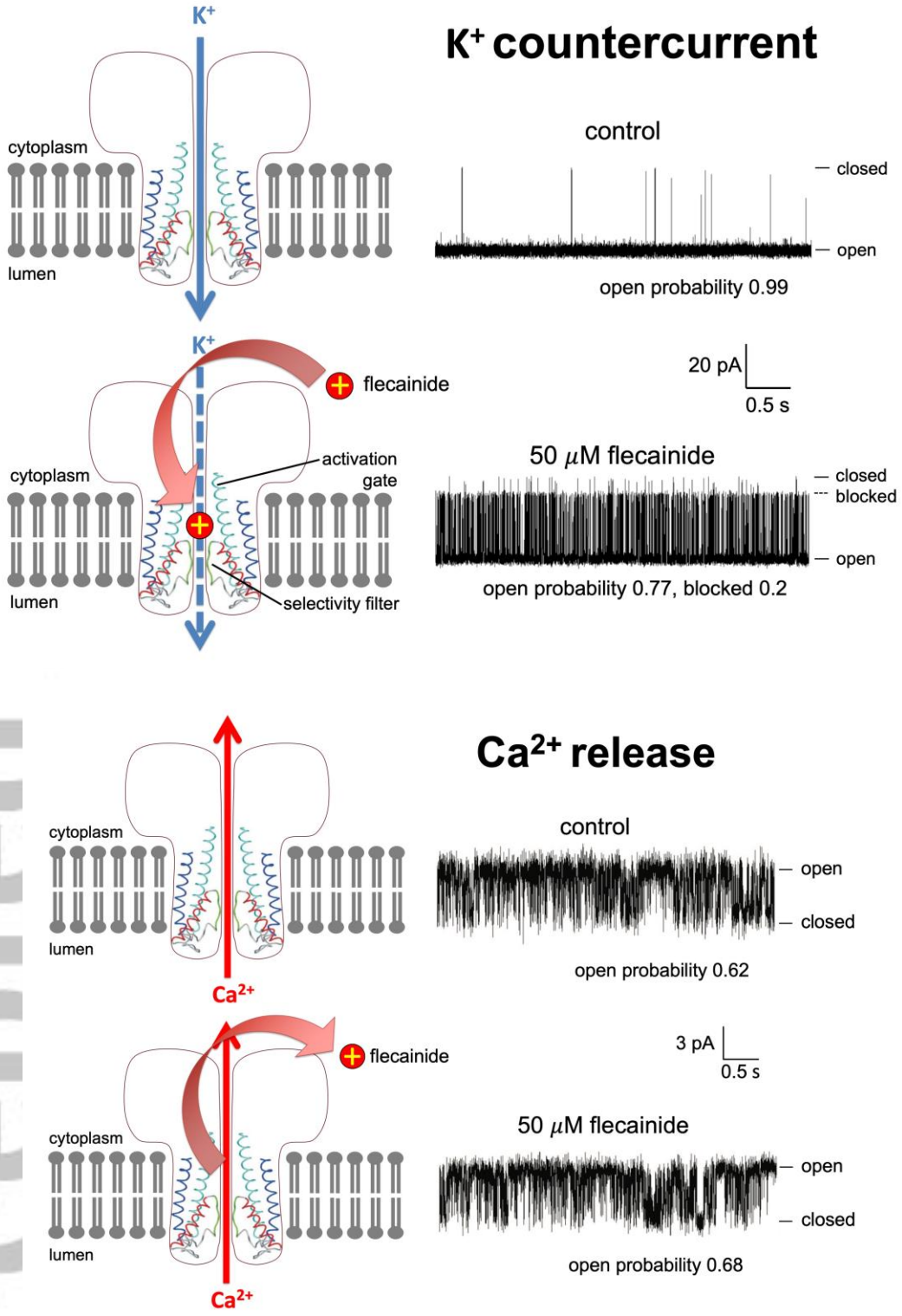

Figure 1 Mechanism of open channel block (A) RyR-mediated influx of K^+ into the SR contributes to the charge compensating countercurrent during Ca^{2+} release. This current flows from cytoplasm to lumen in the cell. The single channel trace shows a predominantly fully open channel (open probability of '1' describes a channel that is

exclusively ‘open’). (upper panel). Flecainide in the cytoplasm moves via the channel activation gate into the pore lumen where it remains since it is too large to pass through the selectivity filter (lower panel). The resultant partial occlusion of cytoplasm-to-SR lumen current is manifest as block to a conductance substate and a reduction in channel open probability **(B)** In experimental single-channel investigations, Ca^{2+} efflux from the SR lumen to cytoplasm expels flecainide from the pore lumen and there is no substate block. It is plausible however that, *in vivo,* the directionally opposing ion currents comprised of SR-to-cytoplasm Ca^{2+} release and cytoplasm-to-SR ‘countercurrent’ stabilise flecainide in the RyR2 pore. This possibility remains to be tested. (Single channel traces are from Bannister *et al.,* 2015 used under CC-BY license.)

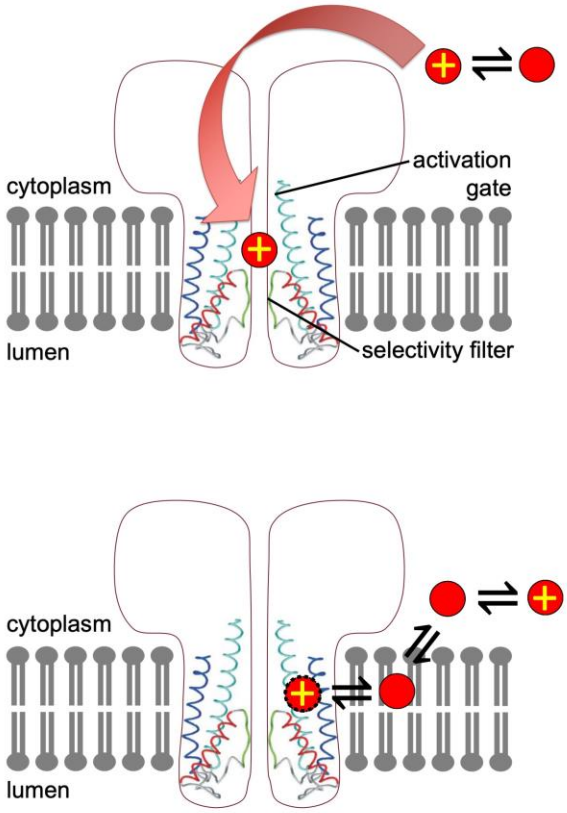

Figure 2 Access routes to inner helix binding site (A) Block of the RyR pore requires the cationic form of flecainide (+). The established route of entry to the pore vestibule is from the cytoplasmic solution via the activation gate (upper panel). **(B)** An alternative route would be for flecainide from the membrane phase to interact with the channel. This might be at membrane-facing binding sites, inter-helical locations or via access to the pore. This mechanism would not apply to permanently-charged cationic drugs such as QX-FL.

Table 1. Comparison of IC_{50} values for block of ion channels by flecainide

(studies utilising human channels are highlighted in bold)

Channel	*Current*	*IC_{50} / μM*	*Species*	*Reference*
$K_v11.1$	**I_{Kr}**	**1.49**	**human**	**Melgari *et al*., 2015 *J Mol Cell Cardiol* 86:42–53**
$K_v1.5$	I_{Kur}	2.9	dog	Yue *et al*., 2000 *Cardiovasc Res* 46:151-161
$K_v4.2/3$	$I_{to,f}$	3.7	rat	Slawsky and Castle, 1994 *J Pharmacol Exp Ther* 269:66
$Na_v1.5$	**I_{Na}**	**7.4**	**human**	**Ramos and O'Leary 2004 *J Physiol* 560:37–49**
$K_v4.3$	I_{to1}	**10**	**human**	**Radicke *et al*., 2008 *Br J Pharmacol* 136:717–729**
RyR2	**Cytoplasm-to-SR 'counter-current'**	**13.1**	**human**	**Bannister *et al*., 2015 *Circ Res* 116:1324–1335**
		16	sheep	Hilliard *et al*., 2010 *J Mol Cell Cardiol* 48:293–301
$Ca_v1.2$	I_{Ca}	20	frog	Scamps *et al*., 1989 *Am J Physiol* 256:C549–559
$K_v3.1$	I_{Kur}	29	dog	Herrera *et al*., 2005 *Mol Pharmacol* 68:305–316
$K_v1.5$	I_{Kur}	**211**	**human**	**Herrera *et al*., 2005 *Mol Pharmacol* 68:305–316**
HCN4	I_f	1700	rabbit	Tamura *et al*., 2009 *J Pharmacol Sci* 110:150 – 159
RyR2	SR-to-cytoplasm	3000	sheep	Mehra *et al*., 2014 *Mol Pharmacol* 86:696–706