Multiple targets for flecainide action: implications for cardiac arrhythmogenesis

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Multiple targets for flecainide action: implications for cardiac arrhythmogenesis

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Short title: Cardiac actions of flecainide

Abstract

Flecainide suppresses cardiac tachyarrhythmias including paroxysmal atrial fibrillation, supraventricular tachycardia, arrhythmic long QT syndromes (LQTS), as well as the Ca^{2+}-mediated, catecholaminergic polymorphic ventricular, tachycardia (CPVT). However, flecainide can also exert pro-arrhythmic effects most notably following myocardial infarction and when used to diagnose Brugada Syndrome (BrS). These divergent actions result from its physiological and pharmacological actions at multiple, interacting, levels of cellular organisation. These were studied in murine genetic models with modified Nav channel or intracellular ryanodine receptor (RyR2)-Ca^{2+} channel function. Flecainide accesses its transmembrane Nav1.5 binding site during activated, open, states producing a use-dependent antagonism. Closing either activation or inactivation gates traps flecainide within the pore. An early peak I_{Na} related to Nav channel ***activation*** followed by rapid de-activation drives action potential (AP) upstrokes and their ***propagation***. This is diminished in pro-arrhythmic conditions reflecting ***loss*** of Nav1.5 function such as BrS, accordingly ***exacerbated*** by flecainide challenge. Contrastingly, pro-arrhythmic effects attributed to prolonged AP ***recovery*** by abnormal late I_{NaL} following ***gain-of-function*** Nav1.5 modifications in LQTS3 are ***reduced*** by flecainide. Anti-arrhythmic effects of flecainide that reduce ***triggering*** in CPVT models mediated by sarcoplasmic reticular Ca^{2+} release could arise from its primary Nav channel actions ***indirectly*** decreasing $[Ca^{2+}]_i$ through a reduced $[Na^+]_i$ and/or ***direct*** open-state RyR2-Ca^{2+} channel antagonism. The consequent $[Ca^{2+}]_i$ alterations could also modify AP propagation velocity and therefore arrhythmic ***substrate*** through its actions on Nav1.5 function. This is consistent with the paradoxical differences between flecainide actions upon Na^+ currents, AP conduction and arrhythmogenesis under circumstances of normal and increased RyR2 function.

Abbreviations

AP- Action potential

APD- Action potential duration

AV- Atrioventricular

BrS- Brugada syndrome

CaM- Calmodulin

CaMKII- Calmodulin Kinase II

CASQ- Calsequestrin

CAST- Cardiac Arrhythmia Suppression Trial

CPVT- Catecholaminergic polymorphic ventricular tachycardia

DAD- Delayed afterdepolarisation

ECG- Electrocardiographic

I_{CaL}- L-type calcium current

I_{Kr}- Rapidly activating delayed rectifier current

I_{Ks}- Slowly activating delayed rectifier current

I_{Na}- Inward sodium current

I_{NaL}- Late inward sodium current

I_{to}- Transient outward current

LQTS- Long QT syndrome

NCX- Sodium-Calcium exchanger

PR- standard P to R interval on ECG recording

QRS- standard QRS interval on ECG recording

QT- standard QT interval on ECG recording

RyR- Ryanodine receptor

SR- Sarcoplasmic reticular

VT- Ventricular tachycardia

Introduction

Cardiac arrhythmias constitute an important clinical and public health problem. Pharmacological modes of action, effectiveness, and specific indications of anti-arrhythmic agents are therefore of particular interest (Huang, 2017). This is particularly so when they exert contrasting, beneficial, ineffective, or even harmful, actions dependent upon the particular physiological or clinical circumstances under which they are applied. The class Ic anti-arrrhythmic agent flecainide ((*RS*)-*N*-(piperidin-2-ylmethyl)-2,5-bis(2,2,2-trifluoroethoxy)benzamide); $C_{17}H_{20}F_6N_2O_3$) (Fig. 1A) originated from explorations of 2,5-bis(2,2,2-trifluoroethoxy)benzamide compounds as pharmaceutical candidates. Early studies in intact canine hearts demonstrated that flecainide markedly increased ventricular fibrillation thresholds following supraventricular beats and ventricular premature beats and slowed ectopic atrial and ventricular pacemakers. It also prolonged atrioventricular (AV) conduction (at plasma concentrations of 0.4 to 0.7 μg ml^{-1}) and overall excitation delay (at >6.5 μg ml^{-1}) (Hodess et al., 1979). Standard microelectrode techniques attributed these findings to reductions in maximal rates of rise of the action potential (AP), $(dV/dt)_{max}$, in the absence of stimulation. These became accentuated during stimulus trains with stimulus intervals <4800 ms over 20 to 50 beats in guinea-pig ventricle even at normal resting potentials. Flecainide also produced negative steady-state shifts in the relationship between $(dV/dt)_{max}$ and membrane potential of possible clinical relevance in ischaemic states (Campbell and Vaughan Williams, 1983).

In common with other anti-arrhythmic agents, flecainide also affected cardiac contractile activation processes, dose-dependently decreasing peak left ventricular isovolumic pressure and peak isovolumic rate of pressure generation, $(dP/dt)_{max}$, in intact rat hearts (Hoffmeister et al., 1987; Fernandes et al., 2014). These findings correlated with decreased aequorin luminescence and isometric tension signals in isolated canine ventricular trabeculae and reduced Ca^{2+} current, I_{CaL}, in isolated myocytes from the same ventricle (Kihara et al., 1996). These findings translated to effects on peak isometric contractile force and maximal rates of force development and decline in human ventricular muscle (Lynch et al., 2013). These results suggest multiple, potentially interacting, actions requiring analysis at the systems level, whose mechanisms and pharmacological implications are reviewed in this present article.

Anti-arrhythmic effects of flecainide

Initial clinical studies reported encouraging effects of flecainide on occurrences of premature ventricular or atrial contractions in arrhythmic patients whilst minimally altering their electrocardiographic (ECG) PR, QRS, or QT intervals, or producing other side effects (Somani, 1980). The compound has a high bioavailability. Its amide group has a pKa of ~9.3 (Liu et al., 2003) and so it is 99% protonated as a water soluble monovalent cation at physiological pH. Peak blood levels are reached 1 to 6 hours after oral ingestion (Smith, 1985). Its plasma half-life is 12 to 27 hours (Padrini et al., 1993). Subsequent reports similarly confirmed that even low (100 mg twice daily) flecainide doses reduced both triggering events represented by premature ventricular contractions (Abitbol et al., 1983), and substrate reflected in the appearance of ventricular tachycardia (VT) following programmed electrical stimulation, during Holter monitoring and electrophysiological testing (Somberg and Tepper, 1986). Its pharmacokinetics permitted oral administration (Anderson et al., 1981; Pottage, 1983; Holmes and Heel, 1985). Orally administered flecainide also suppressed premature ventricular complexes, and ventricular tachycardia (Anderson et al., 1981) and proved acceptable for long term use (Meinertz et al., 1984).

These findings led to use of flecainide in preventing and treating ventricular ectopic events and tachycardias, paroxysmal atrial fibrillation (Anderson et al., 1989) and supraventricular tachycardia, including AV nodal re-entrant tachycardia and Wolff-Parkinson-White syndrome (Henthorn et al., 1991; Pritchett et al., 1991). It was also beneficial for long QT syndromes particularly long QT syndrome type 3 (LQTS3), associated with gain-of-function Nav1.5 mutations (Shimizu and Antzelevitch, 1999). Low-dose, oral flecainide consistently shortened corrected QT (QTc) intervals and normalised repolarisation T-wave patterns in LQTS3 patients with *SCN5A*-ΔKPQ mutations (Windle et al., 2001; Moss et al., 2005), consistent with its application as a mutation-specific therapy for LQTS3 (Benhorin et al., 2000). Flecainide was relatively free of adverse, particularly neurological and gastrointestinal, side effects at effective dose levels (Anderson et al., 1981; Pottage, 1983; Holmes and Heel, 1985).

Pro-arrhythmic effects of flecainide: the CAST trial

Through its long history of clinical therapeutic benefit, use of flecainide has been shadowed by pro-arrhythmic consequences under some clinical circumstances, particularly in the presence of ischaemic or morphological change. Ventricular tachyarrhythmias and severe bradycardia occur when its narrow therapeutic index is exceeded by frank overdose or with chronic cardiac disease. Such cases show increased PR and QRS intervals suggesting depressed conduction and signs and symptoms attributable to overt heart failure likely reflecting acutely decreased myocardial contractility (Winkelmann and Leinberger, 1987). An early study reported that 7 of 152 patients showed pro-arrhythmic effects including ventricular tachycardia or ventricular fibrillation over a ~22 month period, similarly associated with increased PR intervals and widened QRS complexes rather than QTc prolongation (Nathan et al., 1984). Additionally, in the Cardiac Arrhythmia Suppression Trial (CAST) anti-arrhythmic therapy with encainide, flecainide, or moricizine initially suppressed arrhythmia in 1727 of 2309 post-myocardial infarction patients with asymptomatic or mildly symptomatic ventricular arrhythmia during Holter recording. However, encainide or flecainide-treated patients showed a higher incidence (8.9%) of arrhythmic death than patients assigned to placebo (1.2%) over a 10 month follow-up (CAST Investigators, 1989; Echt et al., 1991; Greenberg et al., 1995). Finally, flecainide has proved pro-arrhythmic in individuals suspected of having BrS where it can unmask its characteristic

ECG findings, a fact used in its clinical diagnosis in equivocal cases (Gasparini et al., 2003; Wolpert et al., 2005; Meregalli et al., 2006).

Nav channel activation and inactivation processes

These multifarious actions of flecainide under different clinical circumstances may reflect arrhythmias being multicellular phenomena. They involve ***triggering*** mechanisms formed by spontaneous electrophysiological events occurring independently of the normal cardiac pacing process. In addition, the presence of ***arrhythmic substrate*** in the form of further electrophysiological abnormalities could perpetuate this arrhythmic event. These depend upon the electrophysiological stability of cellular excitation involving the interacting properties of numerous channel types, alterations in AP conduction between myocytes, and the effects of myocardial anatomy. Understanding the effects of flecainide therefore not only concern its actions at the molecular level, but also their systems-level consequences. It would then be necessary to consider interacting functional changes at the cellular, tissue and organ levels, and to correlate these with the targeted clinical outcome. The following sections explore the extent to which these diverse actions of flecainide under various disease paradigms are accounted for by its actions upon multiple interacting cellular targets, of which the most prominent are Nav channels.

The Nav channel function itself poses intrinsic complexities. First, it entails distinct activation and inactivation processes. Channel *activation* depends on movements of S4 α-helices predominantly in domains DI-III whose positive charges underly their voltage-sensing function (Catterall, 2012). This process drives the rapid initial, phase 0 depolarisation that activates the cardiac AP as well as its *propagation* to neighbouring and previously quiescent regions. Channel *inactivation* results from similar voltage-sensitive movement of the S4 α-helix in domain DIV, which drives pore occlusion by the cytoplasmic III-IV linker (Kühn and Greeff, 1999). A further slow inactivation may involve further conformational changes in the α-subunit pore region (Ulbricht, 2005). Secondly, the resulting Na^+ current, I_{Na}, may include one or more current components each with different kinetics These might reflect either modulations in the function of individual cardiac Nav1.5 channels or distinct channel subpopulations (Saint et al., 1992; Saint, 2009). An early peak I_{Na} related to Nav activation drives the rapid early AP upstroke, thereby generating local circuit currents underlying AP propagation, rapidly inactivating within a few milliseconds. The resulting membrane depolarisation activates a variety of further ion channels.

In addition to I_{Na} inactivation, *AP recovery* initially involves activation of transient outward (I_{to}) currents. The initial Nav1.5-mediated depolarisation also activates plateau Ca^{2+} currents (I_{CaL}) that locally elevate cytosolic [Ca^{2+}] triggering RyR2-mediated sarcoplasmic reticular (SR) Ca^{2+} release and therefore mechanical activation, with consequences for Ca^{2+} homeostasis and possible reciprocal interactions with surface channel excitability. Finally a variety of voltage-gated K^+ channels including the delayed rectifiers I_{Kr} and I_{Ks}, and the inwardly rectifying I_{K1}, drives the final repolarisation restoring the resting potential. This recovery is opposed by late inward Na^+ current, I_{NaL}, of magnitude ~1-2% of the peak $I_{Na,}$ (Noble and Noble, 2006; Makielski, 2016). Although I_{NaL} shows a more negative (by -20 mV) voltage dependence in its activation properties (Saint et al., 1992), its channel conductance, mean open times and selectivity properties are otherwise identical to the remaining I_{Na} (Ju et al., 1992). Increased I_{NaL} influences AP duration and the refractory period. Finally, with repolarisation to the resting potential, the Nav1.5 channels recover

their capacity for re-excitation, resulting in absolute and relative refractory periods. These respectively correspond to the time intervals over which the channels either cannot be re-excited whatever the stimulus intensity, or require increased stimulus amplitudes for such re-excitation.

Molecular pharmacology of flecainide

Studies of clinically occurring Nav1.5 variants implicated mutations in the IV-S6 helix as most commonly associated with altered responses to flecainide and overlapping interactions with other, similarly cationic and hydrophobic, local anaesthetics. They thus suggested that the flecainide binding site on Nav1.5 is close to this region (Fig. 2) (Viswanathan et al., 2001; Liu et al., 2002, 2003; Viswanathan and Balser, 2004; Fozzard et al., 2011). Other amino acid substitution studies revealed that only two IV-S6 residues affected Nav1.5 interactions with anaesthetics (Ragsdale et al., 1994; Yarov-Yarovoy et al., 2002; Hanck et al., 2009). In particular, unnatural amino acid mutagenesis showed that high-affinity binding of lignocaine highly depended upon cation-π interactions with phenylalanine-1759; it is possible that the positive charge of flecainide could similarly interact (Fig. 2A) (Ahern et al., 2008). Fig. 2B illustrates this region by docking flecainide into a proteobacterial homologue of $Na_v1.5$ (NavRh) (Zhang et al., 2012). In this docked pose, flecainide occupies a hydrophobic cavity at the interface of adjacent subunits and makes contact with the important phenylalanine in IV-S6.

Flecainide may have similar K^+ channel, including Kv11.1, binding sites that also overlap with binding sites for other ligands, such as the structurally related propafenone (Fig. 1B). Binding is again heavily influenced by interactions with a phenylalanine residue in the S6 helix (Madeja et al., 2010; Melgari et al., 2015). These effects appeared to be mediated by charged rather than uncharged flecainide accessing the channel from the cell interior. Studies of its effects in I_{KR} from expressed Kv11.1 containing a range of single site mutations suggested that flecainide binds low in the inner channel cavity (Melgari et al., 2015). These similarities suggest that flecainide binding is constrained even between receptor subtypes. Such shared sites of action are perhaps not surprising: elsewhere different members of the Cys-loop family of ligand-gated ion channels also share a common transmembrane binding site for anaesthetics (Forman et al., 1995; Nury et al., 2011).

Nav channel antagonism by flecainide

Flecainide acts upon the activated, open, state of Nav1.5 (Anno and Hondeghem, 1990; Nitta et al., 1992; Nagatomo et al., 2000) (Fig. 3A), gaining access to a transmembrane binding site where it blocks the pore, and inhibits I_{Na} (Liu et al., 2002, 2003) (Fig. 3B). I_{Na} inhibition takes place with a low-affinity (IC_{50} = 345 μM) during brief depolarising steps. However the affinity dramatically increases (IC_{50} = 7.4 μM) with increasing stimulation frequency as expected for use-dependent binding. This use-dependent antagonism occurs at concentrations as low as 0.5 μM and saturates at ≥50 μM flecainide (Nitta et al., 1992). It is reflected in an increasing inhibition of I_{Na} (Fig. 4A, left panel), and a consequent shift in the dependence of I_{Na} inhibition towards lower flecainide concentrations under conditions of increasing pulsing frequency (Fig. 4A, right panel)(Penniman et al., 2010). This accounts for progressive increases in AP refractory periods, decreases in $(dV/dt)_{max}$ and increases in APD with increasing stimulus frequencies in hearts of a range of species (Fig. 4B)(Wang et al., 1990). Consistent with this, in a non-inactivating Nav1.5 mutant, flecainide produced decays in I_{Na} with a timecourse suggesting a simple pore blocking mechanism (K_D = 11 μM). Once bound,

flecainide reduces Nav channel open times (Grant et al., 2000). Flecainide binding to channels then inactivated by sustained depolarisation does not contribute to Nav channel inhibition (Nitta et al., 1992; Nagatomo et al., 2000; Liu et al., 2002; Wang et al., 2003). Flecainide action was not enhanced with sustained depolarisation producing channel inactivation (Ramos and O'Leary, 2004)

Flecainide does not directly bind to closed or inactivated Nav channels but closing either the activation or the inactivation gate traps flecainide within the pore (Fig. 3C), slowing recovery of drug-bound channels at hyperpolarised voltages. Thus, flecainide slowed recovery of both rapidly inactivating (τ~81 s) and non-inactivating (τ~42 s) channels with hyperpolarisation. The mutation of a conserved isoleucine, SCN5A-I1756C, within the pore forming region (DIV- S6), accelerated recovery of both rapidly inactivating (τ ~ 12.6 s) and non-inactivating (τ ~ 7.4 s) channels. These observations suggest that flecainide is trapped rather than tightly bound within the pore when channels are closed or inactivated (Ramos and O'Leary, 2004).

Contrasting actions of flecainide in ion channel models for arrhythmia

Experimental studies suggest that some of the contrasting effects of flecainide reflect the differing mechanisms underlying arrhythmia in the particular models under study (Fig. 5). They suggest that flecainide exerts ***pro-arrhythmic effects*** upon arrhythmic substrate attributable to compromised AP activation and propagation resulting from a reduced peak I_{Na} correspondingly compromising AP upstroke velocities, $(dV/dt)_{max}$. This situation is likely in the Brugada syndrome (BrS), whose commonest genetic accompaniment is an inherited loss-of-function Nav1.5 deficiency associated with increased risks of potentially fatal ventricular arrhythmias particularly in middle aged (40–45 y) males (Brugada et al., 2002). It has been modelled in isolated murine heterozygotic Nav1.5 haplo-insufficient *Scn5a+/-* cardiac preparations (Papadatos et al., 2002). These preparations replicated clinically observed arrhythmic tendencies and attributed these to compromised AP conduction particularly following extrasystolic stimuli, findings that correlate with biophysical observations of a reduced peak I_{Na} (Martin et al., 2011b, 2012). Flecainide challenge also recapitulated clinical observations as it increased these ventricular arrhythmic tendencies (Stokoe et al., 2007a; Martin et al., 2010; Matthews et al., 2013). The altered balance between inward I_{Na} and outward I_{to} mediating early AP repolarisation would also be expected to increase the likelihood of pro-arrhythmic phase II re-entry phenomena (Lukas and Antzelevitch, 1996), although these would be made less likely as flecainide also increases effective refractory periods (Martin et al., 2011a).

In contrast, flecainide exerts ***anti-arrhythmic effects*** under conditions associated with abnormal AP recovery, particularly when arising from increased I_{NaL}. The open channel antagonist nature of flecainide action on Nav1.5 could make it particularly effective on Nav channels showing prolonged dwell times, as with the increased I_{NaL} in LQTS3. Patch-clamp studies on the HEK293 expression system demonstrated that flecainide exerted a more marked tonic and use-dependent I_{Na} antagonism in *Scn5a*+/ΔKPQ than wild-type (WT). *Scn5a*+/ΔKPQ channels showed a greater use-dependent antagonism of both peak I_{Na} and I_{NaL} than WT channels. In both cases, flecainide preferentially inhibited I_{NaL} (IC_{50} ~ 19 vs 44 μM) over peak I_{Na} (IC_{50} ~ 80 vs 127 μM) (Nagatomo et al., 2000).

In LQTS3, both AP prolongation and increased arrhythmic tendency is attributed to increased late Na^+ current I_{NaL} and a consequent persistent Nav channel opening. This thus provides a pro-arrhythmic exemplar distinct from arrhythmia arising from deficient peak I_{Na} in BrS. Murine *Scn5a*+/ΔKPQ hearts modeled this clinical arrhythmic phenotype. For example, isolated, Langendorff-perfused, *Scn5a*+/ΔKPQ hearts showed increased arrhythmogenicity on programmed electrical stimulation. Their monophasic APs were prolonged, accounting for the observed increases in electrocardiographic QT intervals. Biophysical studies attributed these changes to increased I_{NaL} (Bennett et al., 1995; Nuyens et al., 2001; Head et al., 2005). This was accompanied by increased frequencies of early afterdepolarisation events that could potentially act as arrhythmic triggers (Damiano and Rosen, 1984; Wang et al., 1995a; Thomas et al., 2008; Belardinelli et al., 2015). The latter have been attributed to elevations of $[Na^+]_i$ promoting reverse mode Na^+-Ca^{2+} exchanger (NCX) activity. This results in the pro-arrhythmic alterations in cellular Ca^{2+} homeostasis further discussed below (Shryock et al., 2013). The ventricles also showed altered transmural action potential duration (APD) gradients across the ventricular wall potentially providing arrhythmic substrate (January and Riddle, 1989; Sabir et al., 2008; Horvath et al., 2013). Both these abnormalities and their associated arrhythmic tendencies were abolished by flecainide (Stokoe et al., 2007b; Sabir et al., 2008). This feature replicates clinically established anti-arrhythmic effects of flecainide in LQTS3 (Windle et al., 2001; Moss et al., 2005) thus further demonstrating that murine hearts provide useful models for the human condition.

Occurrences of flecainide exerting pro- rather than anti-arrhythmic effects in LQTS3 have also been reported. However, these were observed when LQTS3 phenotypic features were combined with abnormalities normally associated with a $Na_v1.5$ haplo-insufficient BrS, resulting in an *overlap syndrome* (Bezzina et al., 1999). The latter has been modelled by murine *Scn5a+/1795insD* hearts. In addition to the increased QTc intervals, bradycardia and bradycardic pauses expected from a LQTS3 phenotype, these showed increased PQ intervals, and QRS durations suggesting slowed ventricular conduction. Patch-clamped ventricular myocytes correspondingly showed increased AP durations and increased I_{NaL}. The voltage-dependences of activation, of steady-state rapid or slow inactivation, and of recovery from inactivation, were of normal $Na_v1.5$. In addition, reduced peak I_{Na} and $(dV/dt)_{max}$, correlated with multi-electrode recordings in Langendorff-perfused hearts revealing slowed conduction of excitation (Remme et al., 2006). Overlap features were also shown by ageing *Scn5a*+/ΔKPQ (Guzadhur et al., 2010; Wu et al., 2012). These findings could account for reports that flecainide produced ST-segment elevation characteristic of BrS in some LQT3 patients, suggesting that this Nav channel antagonist could paradoxically be pro-arrhythmic in LQTS3 in the presence of accompanying conduction abnormalities (Priori et al., 2000). Such dual phenotypes have also been attributed to myocardial heterogeneities (Clancy and Rudy, 2002) or simultaneous shifts in Nav1.5 inactivation characteristics (Grant et al., 2002). They could also arise from differences in the effects of flecainide upon inactivation gating. Both *Scn5a*+/1795insD and *Scn5a*+/ΔKPQ channels expressed in tsA-201 cells exhibited modified inactivation gating from the closed channel state. However, flecainide antagonised I_{NaL} to different extents in the sequence WT < *Scn5a*+/ΔKPQ < *Scn5a*+/1795insD. *Scn5a*-+/1795insD channels further showed delayed recoveries from inactivation further exacerbated by flecainide (Viswanathan et al., 2001).

Further complexities arise because Nav channels do not occur as isolated molecules in the plasma membrane, but instead are anchored within larger, extended multi-component complexes. Examples of such associated proteins

include auxiliary Nav channel β subunits (Cusdin et al., 2010), cytoskeletal proteins (Jeevaratnam et al., 2016; Huang, 2017) and other ion channels such as the inward rectifier Kir2.1 (Willis et al., 2015). These proteins can influence Nav channel gating behaviour both directly through protein-protein contacts and indirectly by affecting surface expression and trafficking (Abriel and Kass, 2005; Cusdin et al., 2008; Abriel et al., 2015).

Relatively little attention has been paid to how this supra-molecular channel clustering could influence flecainide pharmacology. To our knowledge, the only example where such effects on flecainide behaviour were studied is the case of the auxiliary Nav β3 subunit, the product of the *Scn3b* gene (Hakim et al., 2010). The β3 subunit is expressed in heart and modulates Nav1.5 gating (Yu et al., 2005). Patch-clamped *Scn3b*$^{-/-}$ murine cardiomyocytes showed reduced I_{Na} likely reflecting reduced Nav1.5 trafficking into the surface membrane. This was combined with negative shifts in Nav1.5 inactivation characteristics that would be expected to reduce I_{NaL} but shorten refractory periods. The genetic variant accordingly shows arrhythmic phenotypes resembling that of the *Scn5a*+/- murine model (Hakim et al., 2008). Indeed, several mutations in *SCN3B* are associated with inherited cardiac arrhythmias in humans (Namadurai et al., 2015).

Curiously however, in *Scn3b*-/- hearts, flecainide produced reduced arrhythmic incidences combined with prolonged refractory periods and shortened APDs (Hakim et al., 2010). This is in direct contrast to its effects in *Scn5a*+/- mice (see above). The reasons for this difference are unclear, but they further confirm suggestions that flecainide exerts dual pro- and anti-arrhythmic actions through effects on both conduction and refractoriness. Thus, in the case of *Scn5a*+/- hearts the negative conduction velocity effects predominate in producing arrhythmia *exacerbated* by flecainide. In the case of LQTS3, refractoriness and recovery effects predominate in producing arrhythmia *reduced* by flecainide. The presence or absence of β3 subunits may differentially modify these two competing effects so that an anti-arrhythmic effect predominates.

How this might work is currently unknown and will probably require detailed structural insights into how the β3 subunit interacts and modulates the Nav1.5 α subunit. The β3 subunit contains a single extracellular immunoglobulin domain, a single-pass transmembrane domain and an intracellular domain and interacts with Nav1.5 through both its extracellular and intracellular domains (Namadurai et al., 2015). It is striking however, that neither of these two interaction sites are close to the flecainide binding site on Nav1.5 (Fig. 2B). This suggests that the β3 subunit most likely modulates the effects of flecainide on Nav1.5 indirectly, either by affecting channel opening probability or by its known effects on Nav1.5 oligomerisation (Namadurai et al., 2014, 2015).

K^+ channel antagonism by flecainide

Flecainide also acts on K^+ channels (Figure 6). At <10 μM it inhibits rapid K^+ current, I_{KR}, tails that followed voltage clamp pulses to +30 mV in the HEK293 expression system. The effect was most noticeable in the steepest part of the I_{KR} (Kv11.1, hERG) activation curve reflecting a voltage-dependent inhibition consistent with a rapid open channel state I_{KR} antagonism similar to that described for I_{Na} (Paul et al., 2002). Flecainide (>10 μM) also inhibits rapid transient outward (Kv4.2) currents, I_{tof}, in both native cells (Slawsky and Castle, 1994) and heterologous expression systems (Rolf et al., 2000), to extents increasing with channel inactivation and consistent with its higher affinity for

the inactivated state of Kv4.2 (Wang et al., 1995b). Finally, flecainide (~100 μM) inhibits ultrarapid delayed rectifier (Kv1.5) current, I_{Kur} (Tamargo et al., 2004; Herrera et al., 2005)

Anti-arrhythmic effects of flecainide in catecholaminergic polymorphic ventricular tachycardia (CPVT)

More recently, flecainide proved to exhibit potential therapeutic efficacy in the Ca^{2+}-mediated catecholaminergic polymorphic ventricular tachycardia (CPVT). CPVT is predominantly associated with genetic abnormalities involving the cardiac ryanodine receptor type 2 sarcoplasmic reticular (SR) Ca^{2+} release channel (RYR2*)* and the SR binding protein calsequestrin type 2 (CASQ2) respectively. CPVT results in aberrant RYR2-mediated SR Ca^{2+} release precipitated by adrenergic stress. The leaky RyR2-Ca^{2+} release initiates delayed afterdepolarisations (DADs) that might trigger polymorphic VT.

Initial findings that flecainide prevented ventricular arrhythmia in two patients with respective CASQ2 and RYR2 mutations in exercise stress tests suggested a mechanism involving reduced triggering activity (Watanabe et al., 2009). These clinical effects were corroborated by further case reports in which flecainide was added to prior conventional β-adrenergic antagonist therapy (Biernacka and Hoffman, 2011; Pott et al., 2011; Jacquemart et al., 2012; Mantziari et al., 2013; Wangüemert-Pérez et al., 2014).

Combination therapy using a β-adrenergic antagonist and flecainide partially or completely suppressed ventricular arrhythmias in 76 % of one *CASQ2* and 32 *RYR2* mutation carriers with intractable CPVT (Van Der Werf et al., 2011). It also completely suppressed exercise-induced ventricular arrhythmia in all of 10 *CASQ2-D307H* patients who were experiencing exercise-induced events on β-blocker therapy alone or in combination with a Ca^{2+} channel antagonist. This remission was maintained in 8 of the 10 patients over a ~15 month follow-up period (Khoury et al., 2013). Furthermore, addition of flecainide completely prevented ventricular arrhythmias during exercise testing and over long-term follow-up in 7 of 12 patients with *RYR2*, *CASQ2* or *KCNJ2* genotype-negative CPVT resistant to conventional β-blocker therapy (Watanabe et al., 2013).

Flecainide monotherapy was pursued in patients carrying *RyR2* mutations in which one patient did not tolerate β-blockers, and 7 other patients were switched to flecainide monotherapy from combined therapy. Monotherapy with flecainide proved more effective or equal to β-blocker monotherapy, while combination therapy only proved more successful in 2 of the 8 patients over a ~37 months follow up period (Padfield et al., 2016).

The paediatric CPVT phenotype is often more severe than the adult presentation (Hayashi et al., 2009). Flecainide was used in 24% of patients in a retrospective paediatric (<19 yr age) cohort study of 226 CPVT patients. Treatment failure never occurred in any adherent patient receiving optimal doses of both flecainide and β-blocker. Flecainide monotherapy was used in a limited number of 5 patients. Results then compared well with results from β-blockers, implantable cardioverter defibrillators and left cardiac sympathetic denervation. All these cases showed suppression of exercised induced events; 78% remained asymptomatic, and there was no mortality on follow-up (Roston et al., 2015). Proarrhythmic effects of flecainide of the kind observed in BrS have not been observed in the context of CPVT.

Nevertheless, given the underlying catecholaminergic trigger for CPVT, their efficacy and wide therapeutic window the first line of current therapy continues to utilize β-blocker monotherapy. However, β-blockers are not well tolerated or do not have an adequate therapeutic efficacy in as many as 30% of cases. These are often the younger, healthier patients. In these situations the addition of flecainide as a combined therapy may prove more effective. Thus, flecainide is an appealing therapeutic addition to traditional β-blocker monotherapy, particularly in patients resistant to such therapy or requiring high dose β-blockers. Adverse side effects might then be reduced through the use of smaller doses of two as opposed to a larger dose of a single pharmacological agent. Recent reports have progressed to introduce flecainide monotherapy in particular cases, with encouraging preliminary results. Flecainide monotherapy emerges as an available and effective next step, where β-blockers are not tolerated or ineffective. However, the current data relies on limited studies. Further investigation is required to conclusively assess flecainide monotherapy as an earlier line of treatment, given its narrow therapeutic window (Priori et al., 2013; Lieve et al., 2016).

Indirect actions of flecainide on Ca^{2+}-mediated triggering of arrhythmia

Flecainide also acts upon Ca^{2+} mediated arrhythmia, as exemplified by its use in the management of CPVT outlined above. This action may involve cellular-level interactions following its effects upon its primary molecular targets. Thus, two contrasting groups of observations both suggest ***indirect*,** feed-forward effects arising from its Nav channel antagonism, and additionally implicate such actions in increased thresholds for triggered pro-arrhythmic activity.

In the first of these, flecainide pre-treatment reduced incidences of sustained VT in *RyR2*-R4496C+/- mice studied by ECG telemetry, following epinephrine and caffeine challenge, from 70% to 8%. In isolated intact regularly paced (1 Hz) *RyR2*-R4496C+/- ventricular myocytes, isoproterenol (1 μM) increased the amplitudes and accelerated the decays of spontaneous Ca^{2+} transients and increased SR Ca^{2+} load. Permeabilised *RyR2*-R4496C+/- ventricular myocytes similarly demonstrated greater spontaneous Ca^{2+} spark and wave activity than WT, particularly following isoproterenol challenge. Both these groups of Ca^{2+} release phenomena persisted with flecainide (6 μM) challenge but were abolished by tetracaine (Fig. 1C). Patch-clamped *RyR2*-R4496C+/- myocytes showed increased incidences of DADs and triggered activity with isoproterenol challenge. Flecainide reduced the occurrences of the triggered but not the DAD activity. These findings suggest flecainide actions attributable to its primary effects on Nav channel availability (Liu et al., 2011). Secondly, flecainide (5 μM) reduced Ca^{2+} spark and wave frequency, but not amplitude, waveform or associated levels of SR Ca^{2+} loading in superfused, regularly-paced healthy adult rat ventricular myocytes. However, tetrodotoxin, propafenone (Fig. 1B) and lignocaine (Fig. 1D) exerted similar actions (Sikkel et al., 2013). These agents all known to decrease I_{Na}, and correspondingly reducing $[Na^+]_i$, could thereby decreasing $[Ca^{2+}]_i$, through an enhanced reverse mode NCX (Bers and Ellis, 1982; Eisner et al., 1984). This would decrease SR luminal $[Ca^{2+}]$ (Bers, 2002), reducing spontaneous SR Ca^{2+} release (Diaz et al., 1997; Györke et al., 2004; Lindegger and Niggli, 2005; Sikkel et al., 2013).

Direct actions of flecainide on Ca^{2+}-mediated triggering of arrhythmia

Flecainide may also act ***directly*** on SR RyR2-Ca^{2+} release channels likely through open state block (Hilliard et al. 2011), with efficacies and potencies varying with channel activity (Savio-Galimberti and Knollmann, 2015). Open state RyR2 antagonism may be specific to flecainide in contrast to the prolonged RyR2 channel closure produced by

tetracaine (Huang, 1997; Hilliard et al., 2010; Huang et al., 2011). Flecainide would then produce optimal antagonist actions in association with the increased activity of 'leaky' CPVT as opposed to WT RyR2s. Lipid bilayer studies reported that flecainide antagonised WT-RyR2 opening with a half maximal inhibitory concentration (IC_{50}) of ~15 μM with the high luminal [Ca^{2+}] expected to produce spontaneous SR Ca^{2+} release (Watanabe et al., 2009), reducing RyR2 open probabilities particularly when channels were in the open state (Hilliard et al., 2010). The IC_{50} values for flecainide action became progressive lower as bilayer voltage became more positive in a direction that would increase cation current flow from the cytoplasmic to the luminal side of the bilayer. The latter would correspond to a direction opposite to that expected with spontaneous Ca^{2+} release (Watanabe et al., 2009; Hilliard et al., 2010; Mehra et al., 2014). Conversely, IC_{50} values increased 1000-fold to mM levels at negative bilayer potentials that would result in a current flow from the lumen to the cytoplasm. This would correspond to a direction aligned with that expected for spontaneous Ca^{2+} release (Mehra et al., 2014). Similarly in WT RyR2 exposed to EMD41000, consequently with high open probabilities, flecainide (10 μM) reduced cytoplasmic-to-luminal currents. but not luminal-to-cytosolic current even at higher (50 μM) concentrations (Bannister et al., 2015). The fully charged (QX-FL) and neutral (NU-FL) flecainide derivatives were less effective antagonists of cytoplasmic-to-luminal currents and similarly did not affect luminal-to-cytosolic current (Bannister et al., 2016).

Nevertheless, flecainide may show multiple modes of RyR2 inhibition (Hwang et al., 2011; Mehra et al., 2014). Both cytoplasmic and luminal flecainide induced two modes of inhibition respectively associated with millisecond and second timescale channel closures under conditions of near-maximal RyR2 activation. The latter was achieved by the presence of 100 μM cytoplasmic Ca^{2+} and 2 mM cytoplasmic ATP. Reducing cytoplasmic free [Ca^{2+}] to 100 nM, adding 1 mM free [Mg^{2+}], and increasing (cytoplasmic – luminal) membrane potential decreased the flecainide IC_{50}. Some of the differing observations may also reflect use of differing, native sheep or recombinant human, RyR2, preparations, levels of associated proteins, ionic conditions and directions of charge flow in the different reports. Finally flecainide could potentially bind calmodulin or other intermediary proteins with differing effects from those resulting from its direct RyR2 binding (Smith and MacQuaide, 2015).

In *Casq2*-/- mice, flecainide pre-administration reduced incidences of ventricular arrhythmic patterns such as bigeminy and biventricular tachycardia (Watanabe et al., 2009). Flecainide treatment also reduced occurrences of SR Ca^{2+} release events and triggered activity in isoproterenol-treated *Casq2*-/- ventricular myoctes (Watanabe et al., 2009). Permeabilised *Casq2*-/- ventricular myocytes demonstrated greater Ca^{2+} spark and wave activity than WT. This was inhibited by flecainide and R-propafenone with greater inhibitory potencies and efficacies in *Casq2*-/- compared to WT myocytes. Tetracaine contrastingly exerted similar effects in both groups. Furthermore, increasing Ca^{2+} spark and wave activity in WT myocytes by caffeine increased the potencies of both flecainide and propafenone but not of tetracaine. Other class I antiarrhythmic drugs, such as lignocaine, mexiletine and quinidine (Fig. 1D, E and F) did not exhibit such anti-arrhythmic efficacy in CPVT models (Savio-Galimberti and Knollmann, 2015). This difference was attributed to the different extents to which these test agents antagonised RyR2-mediated SR Ca^{2+} release (Hwang et al., 2011). Additionally, in both WT and *RyR2*-R4496C+/- murine Purkinje cells, flecainide suppressed spontaneous Ca^{2+} release events as effectively as did tetracaine (Kang et al., 2010).

The hypothesis in Fig. 5 summarises the above feed-forward effects of flecainide ultimately arising from its actions on Nav1.5. Its action in reducing peak, I_{Na}, would result in a reduction of action potential (AP) conduction velocity. This would exacerbate arrhythmia in BrS as the phenotype in this variant is attributable to a loss of Nav1.5 function. In contrast, its actions in reducing late, I_{NaL}, would reduce arrhythmia in LQTS3 as this phenotype results from a gain of Nav1.5 function which prolongs AP duration. Nav1.5 inhibition also increases triggering threshold. Finally a reduced Na^+ entry resulting from reductions in I_{Na} reduces $[Na^+]_i$. This then indirectly reduces $[Ca^{2+}]_i$ through modifying NCX activity, in turn leading to a reduction of RyR2-mediated SR Ca^{2+} release and the incidence of DADs.

Paradoxical effects of flecainide on arrhythmic substrate produced by RyR2-mediated Ca^{2+} release

A final group of experiments suggested that these flecainide actions on RyR2-Ca^{2+} release channels, particularly those with genetic modifications related to CPVT might further reciprocally modify Nav channel function, and the associated AP conduction velocity, with potential implications for arrhythmic substrate. Increased $[Ca^{2+}]_i$ within the physiological range produced concentration-dependent decreases in I_{Na} in rat ventricular cardiomyocytes (Casini et al., 2009). This could reflect direct Ca^{2+} actions at an EF hand motif in the Nav1.5 C-terminal region (Wingo et al., 2004). In addition, indirect actions of Ca^{2+} binding may involve an IQ domain binding site for Ca^{2+}-calmodulin (Ca^{2+}/CaM). Nav1.5 also contains phosphorylatable serine, 516 and 571, and threonine, 594, sites within its DI-II linker. These are targeted by CaM kinase II (CaMKII) following Ca^{2+} binding to the EF hand motifs of calmodulin (CaM) or CaM kinase II (CaMKII). All these mechanisms positively shift the voltage dependence of Nav current inactivation (Wingo et al., 2004; Ashpole et al., 2012), and may also enhance slow Na^+ current inactivation (Tan et al., 2002).

RyR2-P2328S mice demonstrated isoproterenol-induced arrhythmic episodes resembling CPVT in ECG studies (Zhang et al., 2013). Their intact isolated Langendorff-perfused hearts showed pro-arrhythmic atrial and ventricular triggering and arrhythmic events associated with altered Ca^{2+} homeostasis during monophasic action potential recordings (Goddard et al., 2008; King et al., 2013b; Zhang et al., 2013). In addition, they showed arrhythmic substrate resulting from delayed AP conduction. Atrial multi-electrode array, and ventricular micro-electrode recordings following isoproterenol challenge, showed pro-arrhythmic reductions in conduction velocity compared to WT. Intracellular microelectrode AP recordings showed correspondingly reduced maximum rates of depolarisation $(dV/dt)_{max}$ (King et al., 2013b; Zhang et al., 2013). These changes could be attributed to (a) chronic downregulation of Nav1.5 expression, demonstrated in *RyR2*-P2328S ventricles (Ning et al., 2016) and (b) acute actions of increased $[Ca^{2+}]_i$ upon Nav1.5 function. Loose-patch clamp recordings demonstrated reduced peak I_{Na} in whole isolated *RyR2*-P2328S compared to WT atria to extents comparable to those reported in Nav1.5-haploinsufficent *Scn5a*+/- hearts (King et al., 2013a; Salvage et al., 2015) (Fig. 6A, left traces). These conduction abnormalities could not be attributed to either fibrotic change or altered connexin expression. The I_{Na} reductions were acutely replicated in WT atria with increased $[Ca^{2+}]_i$ produced by elevated extracellular $[Ca^{2+}]$, or challenge by caffeine or cyclopiazonic acid (King et al., 2013a).

Flecainide (1 μM) modified arrhythmic tendency and conduction velocity in *RyR2*-P2328S hearts, in directions that paradoxically contrasted with its corresponding effects upon either WT and *Scn5a*+/- hearts. It exerted pro-arrhythmic atrial and ventricular effects in *Scn5a*+/- and some WT hearts. Yet it produced consistently anti-arrhythmic effects in

RyR2-P2328S atria (Salvage et al., 2015). Multi-electrode recording array studies demonstrated marked conduction slowing in *RyR2*-P2328S compared to WT atria. Flecainide reduced conduction velocity and indicators of AP upstroke velocity in WT hearts but did not do so in *RyR2*-P2328S hearts (Fig. 7B left panel). *RyR2*-P2328S atria similarly showed a reduced peak I_{Na} compared to WT (Fig. 7A, left panel). However, whereas 1 μM flecainide reduced peak I_{Na} in WT atria, it rescued the previously reduced peak I_{Na} in *RyR2*-P2328S atria to magnitudes indistinguishable from untreated WT (Fig. 7A, centre panels) while further increases to 5 μM flecainide inhibited I_{Na} in common with effects on WT (Fig. 7A, right panels). Effective refractory periods were similar in untreated *RyR2*-P2328S and WT atria but were increased in flecainide-treated *RyR2*-P2328S (Fig. 7B centre panel). As a result, flecainide shortened AP wavelength as computed from the product of conduction velocity and refractory period in WT in a direction towards increased arrhythmic substrate. In contrast, flecainide increased AP wavelength in *RyR2*-P2328S hearts consistent with its observed anti-arrhythmic effects (Fig. 7B, right panel) (Salvage et al., 2015).

Figure 8 summarises these effects of flecainide upon arrhythmic substrate in terms of a hypothesis invoking *RyR2*-P2328S as a primary pharmacological target in addition to Nav1.5. It represents an increased RyR2-mediated SR Ca^{2+} leak associated with the *RyR2*-P2328S variant as exerting downregulatory effects upon Nav channel expression or function, thereby compromising AP conduction and potentially producing arrhythmic substrate. Flecainide is suggested to reduce the RyR2-mediated SR-Ca^{2+} leak. This would drive a feedback rescue of the compromised Nav1.5 function, restoring I_{Na} and thereby AP conduction. This would account for a net reduction in the arrhythmic substrate associated with the *RyR2*-P2328S mutation. These findings would be consistent with dual Nav1.5 and RyR2-Ca^{2+} channel blocking effects of flecainide and propafenone (Fig. 1A, B), in contrast to selective effects of tetracaine (Fig. 1C), and lignocaine and mexiletine (Fig. 1D,C) on RyR2 and Nav1.5 respectively, in turn consistent with patterns represented by their comparative chemical structures.

Summary and conclusions

The Class Ic anti-arrhythmic agent flecainide shows both pro- and anti-arrhythmic actions depending on clinical and experimental circumstances. Flecainide therapy had initially been introduced to suppress cardiac tachyarrhythmias including paroxysmal atrial fibrillation, supraventricular tachycardia and arrhythmic LQTS. It subsequently proved useful in the management of Ca^{2+}-mediated arrhythmias exemplified by CPVT. However, the CAST trial reported its pro-arrhythmic effects following myocardial infarction. In addition, pro-arrhythmic effects of flecainide have been used in diagnostic tests for BrS.

These divergent actions may reflect physiological and pharmacological actions of flecainide at multiple, interacting, levels of cellular organisation. There are also complexities in the interactions of flecainide with its primary Nav1.5 target as well as other possible cellular targets, in particular RyR2-Ca^{2+} release channels. Nevertheless flecainide appears to act specifically through accessing a cytoplasmic binding site on Nav1.5 in its activated, open state. This results in a use-dependent antagonism. It also acts on other, K^+ and RyR2-Ca^{2+} release channels, but the resulting antagonism appears similarly to involve open channel block. Closing either the activation or the inactivation gates in Nav1.5 traps flecainide within its pore. Nav1.5 function itself involves an activation which triggers the action potential upstroke, and inactivation that influences recovery from excitation and the refractory period. An early peak I_{Na} related

to Nav channel ***activation*** followed by rapid de-activation drives AP upstrokes and ***propagation***. Peak I_{Na} is diminished in pro-arrhythmic conditions reflecting ***loss*** of Nav1.5 function in experimental genetic exemplars for BrS. Experimental data confirms predictions that these conditions would be ***exacerbated*** by the Nav1.5 inhibition following flecainide challenge. In contrast, the experimental data demonstrate that pro-arrhythmic phenotype effects attributed to abnormalities in AP ***recovery*** owing to increased I_{NaL} following the ***gain-of-function*** Nav1.5 modifications in LQTS3 are ***reduced*** by flecainide.

Anti-arrhythmic effects of flecainide on Ca^{2+} mediated arrhythmia in experimental CPVT models could arise from its primary Nav channel antagonism. Through NCX activity, the resulting reduced $[Na^+]_i$ would ***indirectly*** decrease $[Ca^{2+}]_i$. Alternatively a ***direct*** open-state RyR2-Ca^{2+} channel antagonism would also reduce SR Ca^{2+} release. In both cases, the consequently reduced $[Ca^{2+}]_i$ would decrease the likelihood of NCX-mediated DADs that could trigger arrhythmia. Such alterations in $[Ca^{2+}]_i$ could also reduce the inhibitory effects of $[Ca^{2+}]_i$ on Nav channel function and their associated effects on AP propagation velocity and arrhythmic ***substrate.*** Thus, experimental studies confirm predictions of paradoxical differences between flecainide actions upon Nav channel function, AP conduction and arrhythmia in the *RyR2*-P2328S model that contrast with its effects under circumstances of normal WT RyR2 function.

The apparently complex actions of flecainide upon cardiac arrhythmias are thus clarified by a systems analysis of actions upon different membrane proteins and their interaction with cellular Na^+ and Ca^{2+} homeostasis, using experimental models for particular arrhythmic disease states. They also lead to expectations that flecainide action would be particularly effective in conditions associated with increased channel activity. At all events, clinical use of flecainide would require physiological assessment of the underlying cause of the arrhythmia.

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Conflicts of Interest.

None declared.

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Figure legends

Figure 1. Chemical structures of flecainide and related pharmacological agents.

(A) Flecainide and (B) propafenone are class Ic cardiotropic agents. (C) tetracaine is a ryanodine receptor antagonist, and (D) lignocaine and (E) mexiletene are class Ib cardiotropic agents. (F) quinidine is a class Ia cardiotropic agent.

Figure 2. Flecainide docking into the voltage-gated sodium channel crystal structure NavRh (pdbid: 4DXW).

(A). Alignment of the IV-S6 region of different voltage-gated Na+ channels, highlighting the phenylalanine residue (IV-S6-phe) that is strongly implicated in flecainide binding. (B). In silico docking of flecainide into NavRh locates the ligand in a hydrophobic pocket. The upper panels show NavRh as viewed from the top, and the lower panels as viewed from the side. The four colours represent the four domains that constitute the functional protein. The boxes to the right show the flecainide (pink) binding site represented as a cartoon. Note that as flecainide binds within the pore of the channel, the site has been visualised as a slice through the protein; this excludes some of the overlying helices. Hydrogen bond interactions (dashed red line) are predicted with IV-S6-phe. At Nav1.4 a cation-π interaction is seen at the same location (Ahern, Eastwood, Dougherty & Horn, 2008). (R)-Flecainide was generated ab initio using Chem3D Prov14.0 (CambridgeSoft, Cambridge, UK), energy minimised using the implemented MM2 force field and docked using GOLD Suite v5.3 (The Cambridge Crystallographic Data Centre, Cambridge, UK) with the GoldScore function and default settings. Amino acid sequences used in the ClustalW alignment are: 4DXW and 4EKW taken directly from structures of bacterial sodium channels; r_brain II = P04775; h_Nav1.1 = NP_001189364; h_Nav1.2 = NP_001035232; h_Nav1.4 = NP_000325; h_Nav1.5 = NP_932173; hNav1.7 = ABI51981.

Figure 3. Open state antagonism of the voltage-gated sodium channel by flecainide.

(A) Voltage-gated Nav channel represented in its closed, resting state. Surface membrane depolarisation detected by the S4 segment-voltage sensor drives opening of the activation gates. This switches the channel to the (B) open state for a finite ~1 ms interval permitting selective Na^+ entry. Flecainide gains access to its binding site on the cytoplasmic side of the channel pore thereby preventing or reducing Na^+ entry into the intracellular compartment. Subsequent inactivation involving the cytoplasmic III-IV linker results in occlusion of the pore, and can result in (C) trapping of flecainide in the channel. The use-dependent action of flecainide reflects its gaining access to its binding site only when the channel is in the open state. Thus repetitive depolarisations that allow for the refractory period of the inactivated state result in higher potency.

Figure 4. Rate dependent effects of flecainide on Na^+ current (I_{Na}), effective refractory period (ERP), V_{max} and APD_{95} in different species.

(A) Left hand panel: superimposed Na^+ currents, I_{Na}, recorded from HEK293 cells expressing hNav1.5 channels before and after application of 3 μM and 30 μM flecainide at different pulsing rates, illustrating use-dependent antagonism. Right hand panel: average steady-state I_{Na} inhibition by flecainide at each pulsing rate expressed as a

fraction of control current obtained in the absence of flecainide at that pulsing rate. IC_{50} of flecainide at each pacing rate was determined from the Hill coefficient (Adapted with permission from Fig. 6A and B of (Penniman et al., 2010)). (B). The effects of flecainide on (from left to right): effective refractory period (ERP), maximum rate of AP depolarisation (V_{max}) and action potential duration at 95% recovery (APD_{95}) with changing basic cycle lengths (BCL) in guinea-pig, rabbit, dog and human cardiac action potentials. With decreasing BCL there was an increasing effect of flecainide on prolongation of both ERP and APD_{95}, and a decreasing V_{max}. (Figure adapted with permission from left hand panels of Figure 2, 3, and 4 of (Wang et al., 1990)).

Figure 5. Feed-forward effects of flecainide attributable to its actions on Nav1.5.

In this hypothesis, flecainide reduces peak, I_{Na}, thereby reducing action potential (AP) conduction velocity, exacerbating pro-arrhythmic conditions arising from *loss* of Nav1.5 function occurring in conditions such as BrS. In contrast, its reduction of late, I_{NaL}, would be anti-arrhythmic in conditions associated with gain of Nav1.5 function increasing I_{NaL} and prolonging AP duration such as LQTS. The inhibitory effect of flecainide on Nav1.5 also increases triggering threshold. Finally reduced Na^+ entry resulting from reductions in I_{Na} reduces $[Na^+]_i$. This then indirectly reduces $[Ca^{2+}]_i$ through NCX action, thereby reducing incidences of RyR2-mediated SR Ca^{2+} release and its resulting DADs.

Figure 6. Flecainide actions on potassium channel subtypes

Figure 7. Paradoxical actions of flecainide on Na^+ current (I_{Na}), conduction velocity, refractory period and action potential (AP) wavelength in homozygotic RyR2-P2328S ($RyR2^{s/s}$) hearts.

(A). Loose patch clamp measurements of Na^+ current, I_{Na}, in isolated atrial preparations from WT (top row) and $RyR2^{S/S}$ murine hearts (bottom row) respectively demonstrate contrasting decreases and increases in peak I_{Na} with 1 μM flecainide treatment. Increasing flecainide concentration to 5 μM resulted in a reduced I_{Na} in both $RyR2^{S/S}$ and WT. (B). Left panel: flecainide reduced conduction velocity in the WT whilst conserving conduction velocity in $RyR2^{S/S}$ atria. Centre panel: flecainide increased atrial effective refractory periods in both WT and $RyR2^{S/S}$, but did so more markedly in the $RyR2^{S/S}$. Right panel: The product of conduction velocity and refractory period, wavelength (λ), was shorter in $RyR2^{s/s}$ atria than WT. However, flecainide shortened λ in WT but increased λ in $RyR2^{s/s}$ atria (Figure adapted with permission from Fig 3(a) and (b) and Fig. 7 (a)-(c) of (Salvage et al., 2015)).

Figure 8. Feed-backward effects of flecainide on arrhythmic substrate attributable to its possible actions on RyR2-Ca^{2+} release channels.

A model invoking *RyR2*-P2328S as a primary pharmacological target for flecainide in addition to Nav1.5 may account for its effect in diminishing arrhythmic substrate. Increased RyR2-mediated SR Ca^{2+} leak associated with *RyR2*-P2328S downregulates Na^+ channel expression or function, compromising AP conduction and potentially producing arrhythmic substrate. Reduction of the RyR2-mediated SR-Ca^{2+} leak by flecainide rescues the compromised Nav1.5 function, restoring I_{Na} and thereby AP conduction and reduces arrhythmic substrate.

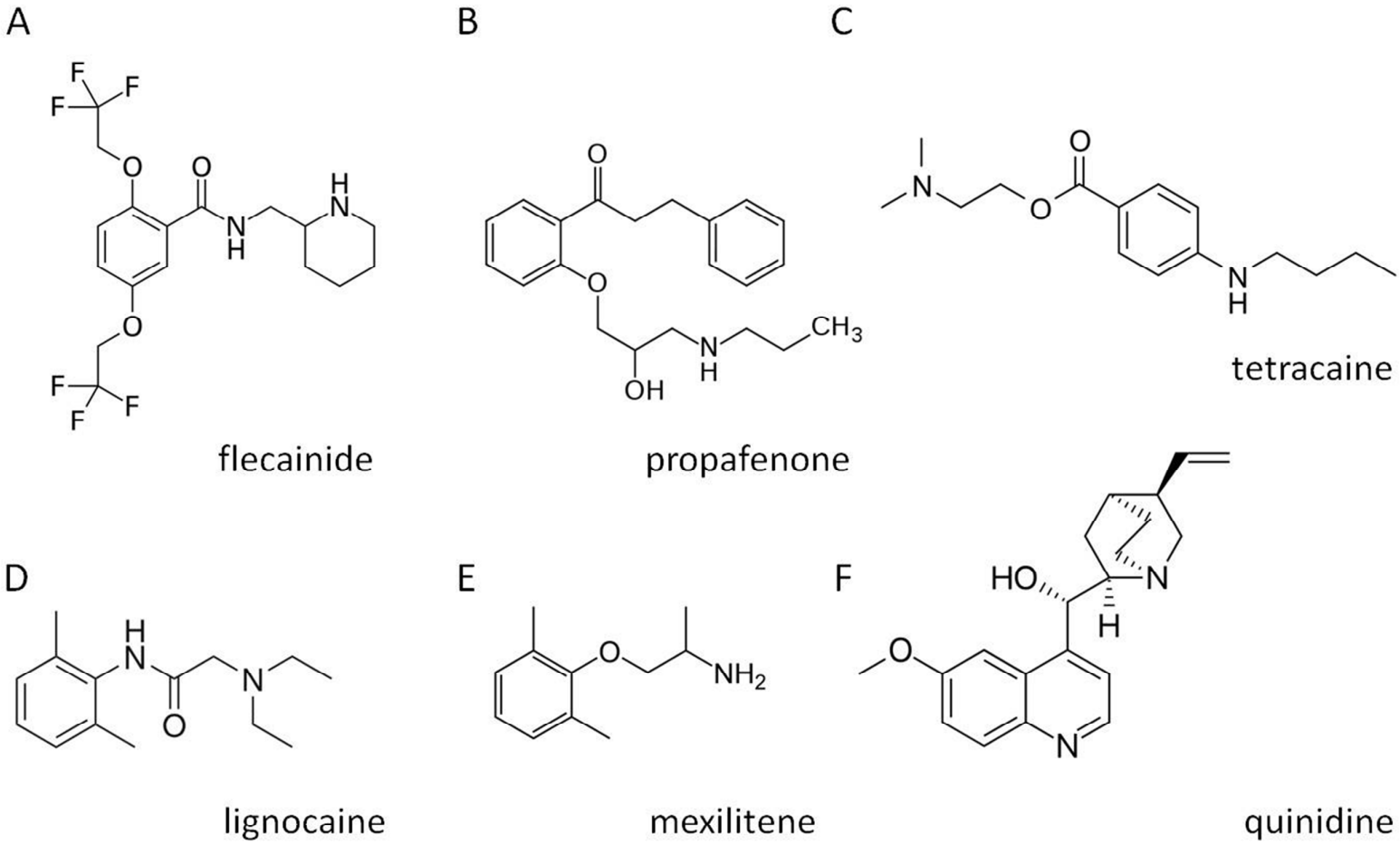
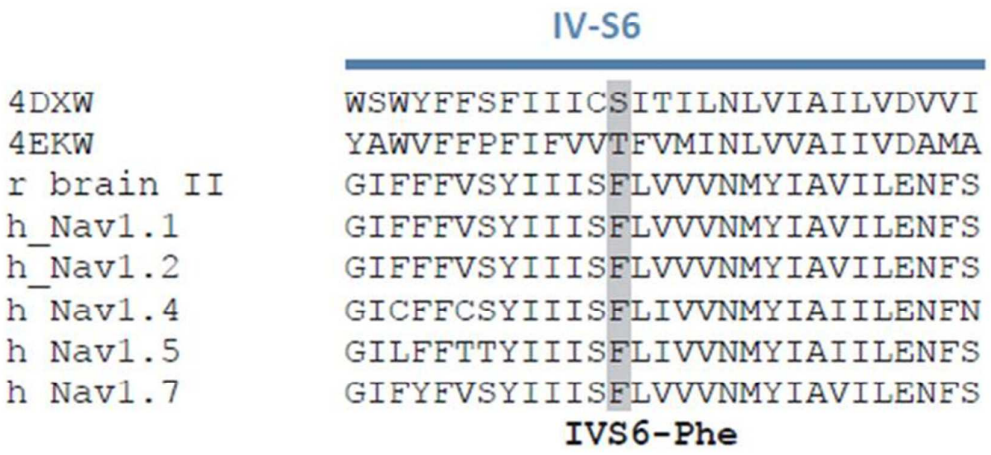

A
B
C
flecainide
propafenone
tetracaine
D
E
F
lignocaine
mexilitene
quinidine

Figure 1

A


IV-S6
4DXW WSWYFFSFIIICSITILNLVIAILVDVVI
4EKW YAWVFFPFIFVVTFVMINLVVAIIVDAMA
r brain II GIFFFVSYIIISFLVVVNMYIAVILENFS
h_Nav1.1 GIFFFVSYIIISFLVVVNMYIAVILENFS
h_Nav1.2 GIFFFVSYIIISFLVVVNMYIAVILENFS
h Nav1.4 GICFFCSYIIISFLIVVNMYIAIILENFN
h Nav1.5 GILFFTTYIIISFLIVVNMYIAIILENFS
h Nav1.7 GIFYFVSYIIISFLVVVNMYIAVILENFS
IVS6-Phe

B

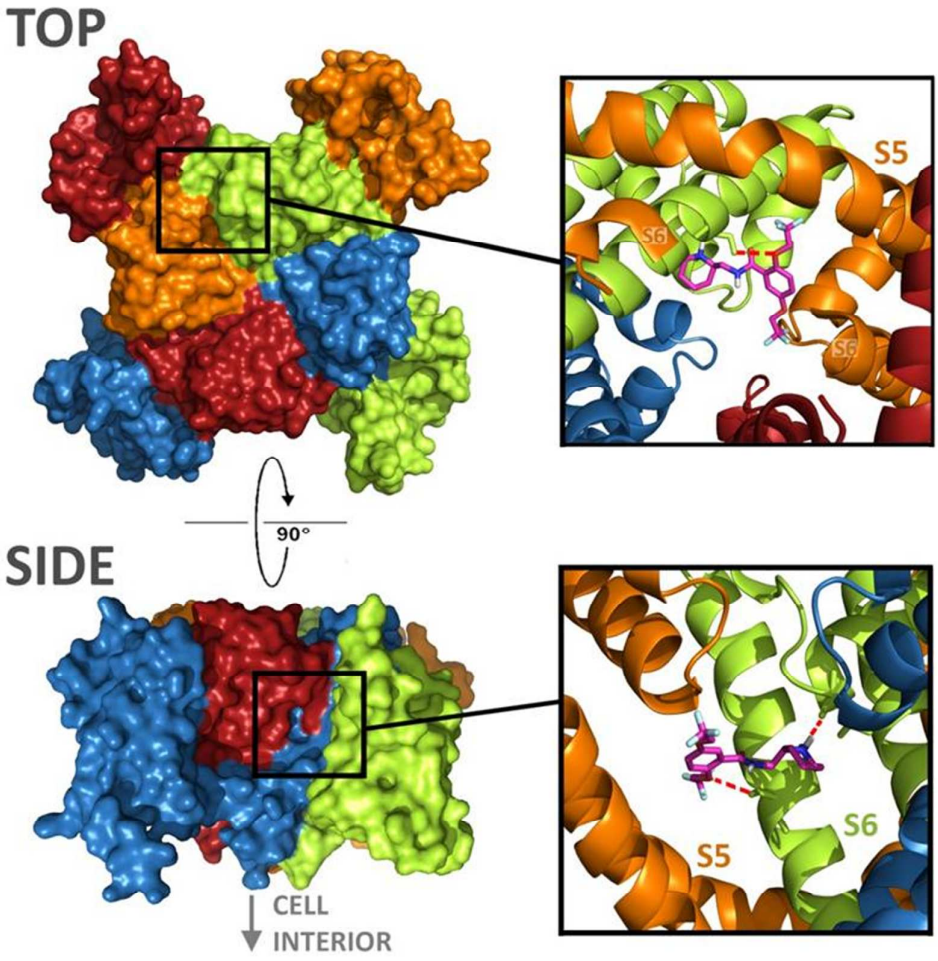

TOP
S5
S6
S6
90°
SIDE
S5
S6
CELL
INTERIOR

Figure 2

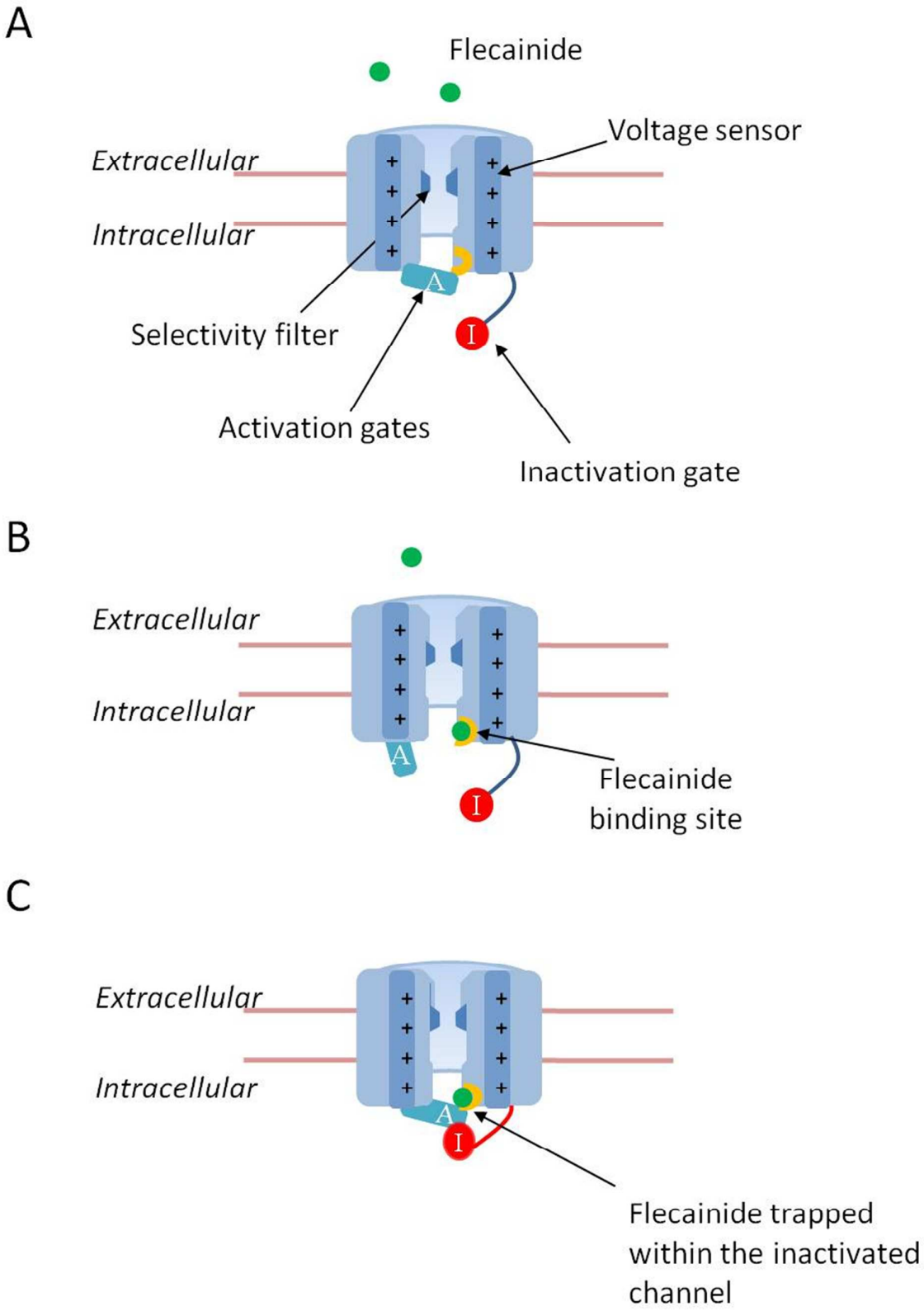

A
Flecainide
Voltage sensor
Extracellular
Intracellular
Selectivity filter
Activation gates
Inactivation gate
B
Extracellular
Intracellular
Flecainide
binding site
C
Extracellular
Intracellular
Flecainide trapped
within the inactivated
channel

Figure 3

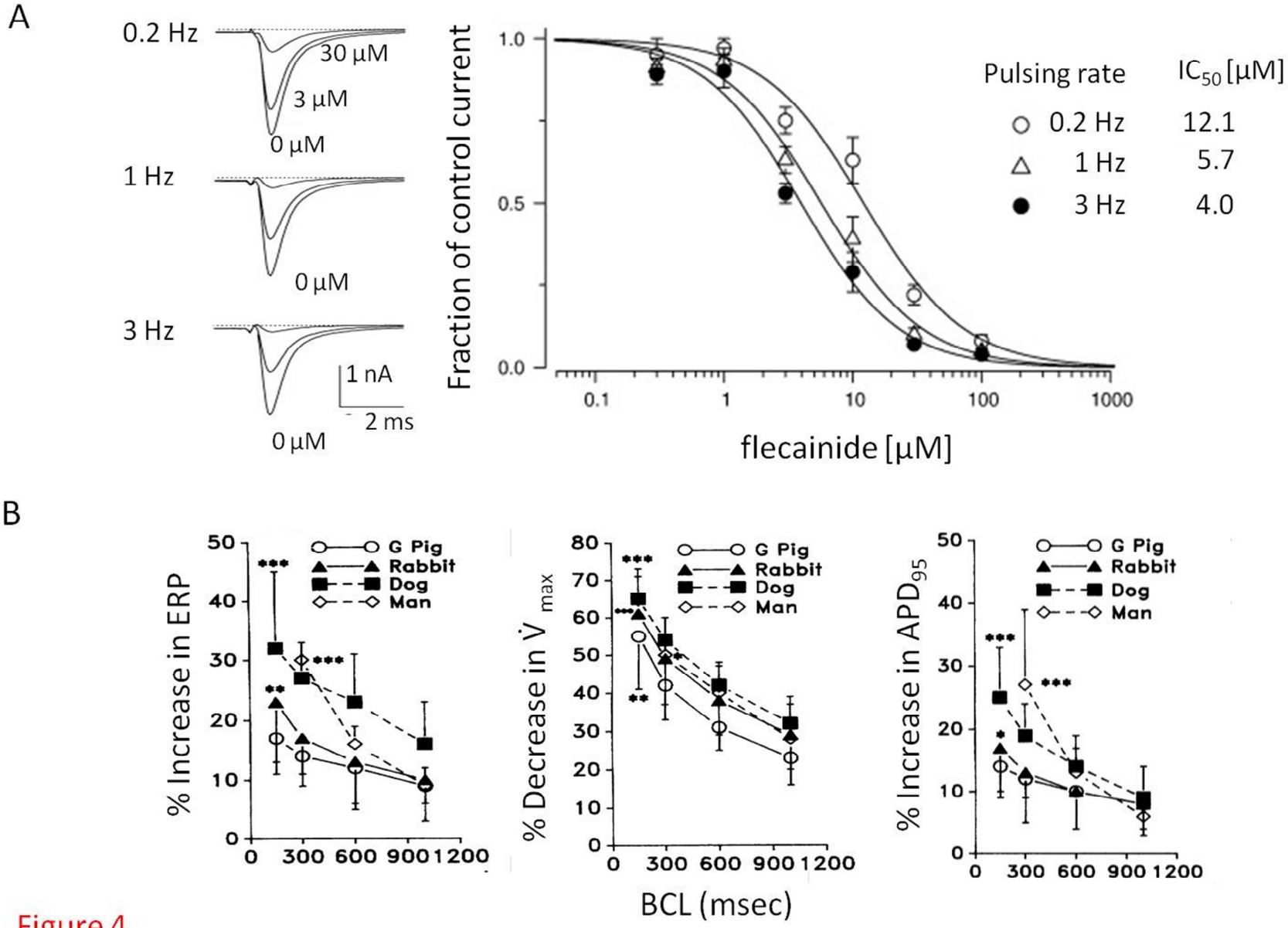
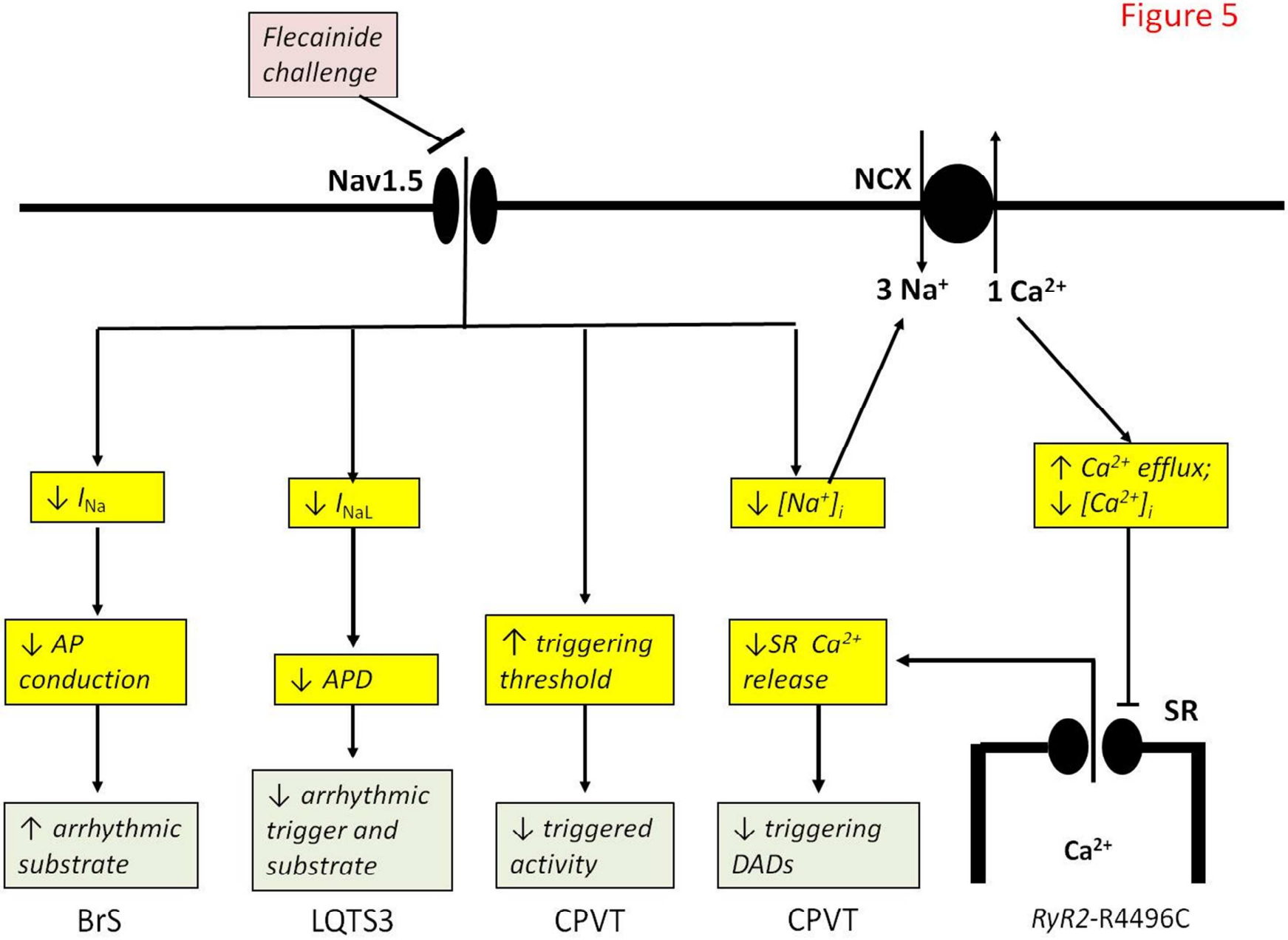

A
0.2 Hz
30 µM
3 µM
0 µM
1 Hz
0 µM
3 Hz
1 nA
2 ms
0 µM
Fraction of control current
1.0
0.5
0.0
0.1
1
10
100
1000
flecainide [µM]
Pulsing rate
IC50 [µM]
0.2 Hz 12.1
1 Hz 5.7
3 Hz 4.0
B
% Increase in ERP
% Decrease in V̇max
% Increase in APD95
G Pig
Rabbit
Dog
Man
0 300 600 900 1200
BCL (msec)

Figure 4

Figure 5


Flecainide challenge
Nav1.5
NCX
3 Na+
1 Ca2+
↓ INa
↓ INaL
↓ [Na+]i
↑ Ca2+ efflux; ↓ [Ca2+]i
↓ AP conduction
↓ APD
↑ triggering threshold
↓SR Ca2+ release
SR
↑ arrhythmic substrate
↓ arrhythmic trigger and substrate
↓ triggered activity
↓ triggering DADs
Ca2+
BrS
LQTS3
CPVT
CPVT
RyR2-R4496C

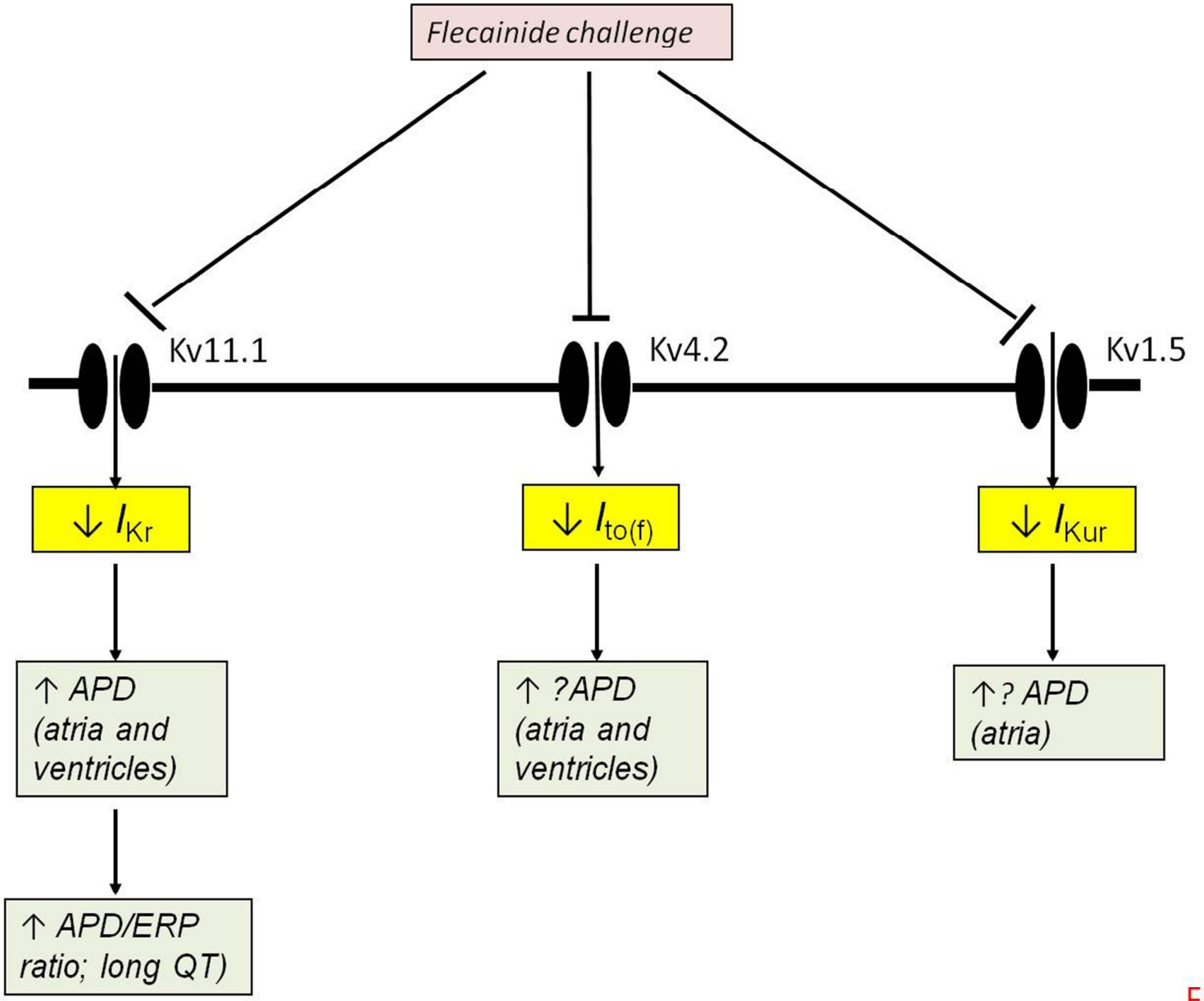

Flecainide challenge
Kv11.1
Kv4.2
Kv1.5
↓ I_{Kr}
↓ $I_{to(f)}$
↓ I_{Kur}
↑ APD (atria and ventricles)
↑ ?APD (atria and ventricles)
↑ ? APD (atria)
↑ APD/ERP ratio; long QT)

Figure 6

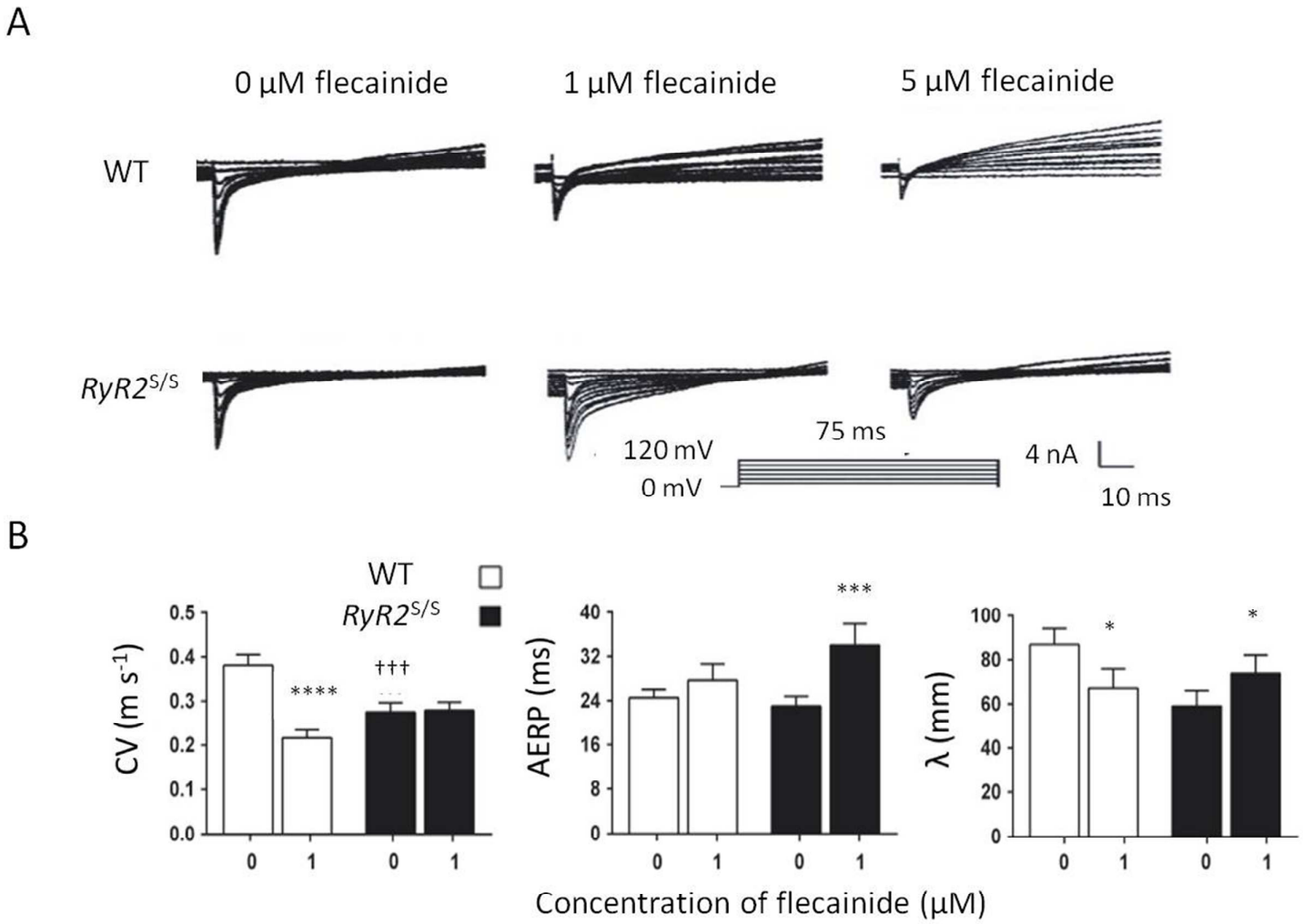

A
0 µM flecainide
1 µM flecainide
5 µM flecainide
WT
RyR2S/S
75 ms
120 mV
0 mV
4 nA
10 ms
B
WT
RyR2S/S
CV (m s-1)
****
†††
AERP (ms)
***
λ (mm)
*
*
Concentration of flecainide (µM)

Figure 7

Figure 8

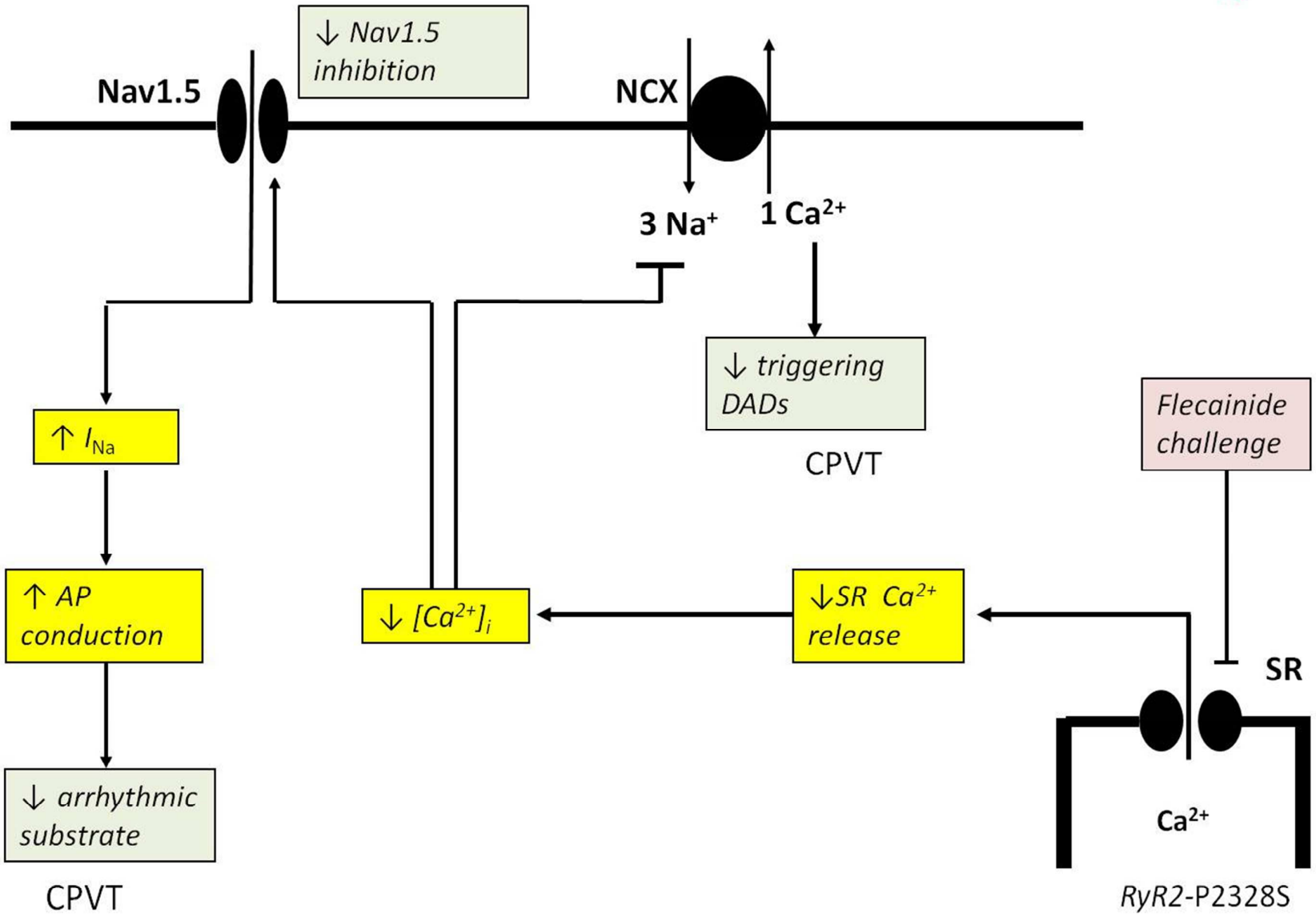

↓ Nav1.5 inhibition
Nav1.5
NCX
3 Na+
1 Ca2+
↓ triggering DADs
CPVT
↑ INa
↑ AP conduction
↓ arrhythmic substrate
CPVT
↓ [Ca2+]i
↓SR Ca2+ release
Flecainide challenge
SR
Ca2+
RyR2-P2328S

Fig. 4A reproduced by permission from Fig. 6A and B of (Penniman et al., 2010)

Figure 4B reproduced by permission from left hand panels of Figure 2, 3,4 of (Wang et al., 1990)

Fig 6 obtained from Fig 3(a) and (b) and Fig. 7 (a)-(c) of (Salvage et al., 2015)

January 18, 2017

Christopher Huang
Physiology Department
University of Cambridge
Downing Street
Cambridge CB2 3EG
United Kingdom

Email: clh11@cam.ac.uk

Dear Dr. Huang:

This is to grant you permission to reproduce the following figures in your journal article tentatively titled "A systems analysis of flecainide action" to appear in *British Journal of Pharmacology* (John Wiley):

> Figures 2, 3, and 4 from Z Wang, B Fermini, and S Nattel, Mechanism of flecainide's rate-dependent actions on action potential duration in canine atrial tissue, *J Pharmacol Exp Ther* 1993, 267(2):575-581

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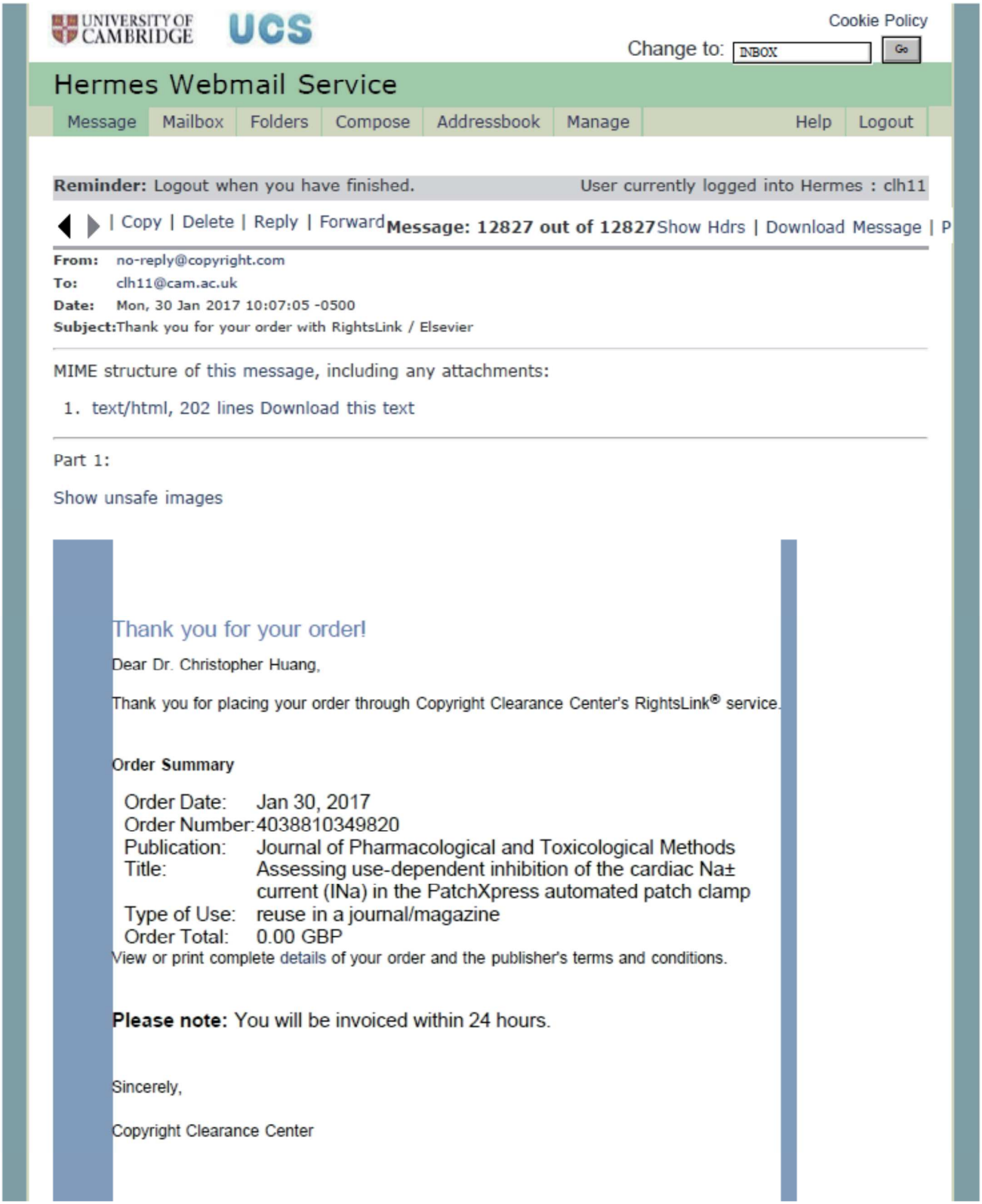

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Multiple targets for flecainide action: implications for cardiac arrhythmogenesis

Responses to Reviewers Comments.

Reviewer: 1

In the current review article, Salvage et al. elucidated that flecainide not only suppresses cardiac tachyarrhythmias, but also exert pro-arrhythmic effects by its involvement of modifying Na and Ca channels etc. This is a comprehensive review article apart from some flaws.

Thank you for the positive reaction to our paper and drawing our attention to aspects requiring attention. We have revised the paper responding to all the reviewers' reports, and the following changes are relevant to the comments made by the present reviewer.

Major points:

1, the authors should provide more information about combination usage of flecainide and beta-blockers or other ion channel blockers on human or animal models. At least, this should be discussed.

We have revised the relevant paragraph, concerning the use of flecainide either alone or in combination with beta blockers. Note the paragraph has been placed later in the review in response to comment 3 below. Thus for the paragraph:

"More recently, flecainide was also found to exhibit potential therapeutic efficacy in the Ca^{2+}-mediated catecholaminergic polymorphic ventricular tachycardia (CPVT) (Watanabe et al). CPVT is predominantly associated with genetic abnormalities involving the cardiac ryanodine receptor type 2 sarcoplasmic reticular (SR) Ca^{2+} release channel (RYR2*)* and the SR binding protein calsequestrin type 2 (CASQ2) respectively. CPVT results in aberrant RYR2-mediated SR Ca^{2+} release precipitated by adrenergic stress. The leaky RyR2-Ca^{2+} release initiates delayed afterdepolarisations (DADs) that might trigger polymorphic VT. Initial findings that flecainide prevented ventricular arrhythmia in two patients with respective CASQ2 and RYR2 mutations in exercise stress tests suggested a mechanism involving reduced triggering activity (Watanabe *et al.*, 2009) and were corroborated by further case reports (Biernacka & Hoffman, 2011; Pott *et al.*, 2011; Jacquemart *et al.*, 2012; Mantziari *et al.*, 2013; Wangüemert-Pérez *et al.*, 2014). Flecainide mainly used in combination with a β-adrenergic antagonist partially or completely suppressed ventricular arrhythmias in 76 % of one *CASQ2* and 32 *RYR2* mutation carriers with intractable CPVT (Van Der Werf *et al.*, 2011). It suppressed exercise-induced ventricular arrhythmia in all of 10 and relieved symptoms in 8 of 10 *CASQ2* -D307H patients with CPVT resistant to β-adrenergic antagonist therapy over a ~15 month follow-up period (Khoury *et al.*, 2013). Flecainide treatment compared well with β-adrenergic antagonists, implantable cardioverter defibrillators and left cardiac sympathetic denervation in a retrospective paediatric (<19y age) cohort study of 226 CPVT patients (Roston *et al.*, 2015). Additionally, flecainide completely prevented ventricular arrhythmias during exercise testing and over long-term follow-up in 7 of 12 patients with *RYR2*, *CASQ2* or *KCNJ2* genotype-negative CPVT resistant to conventional therapy (Watanabe *et al.*, 2013). A number of the above cases employed flecainide monotherapy particularly where there were significant side-effects of β-adrenergic antagonists. Furthermore, none of a cohort of 9 patients carrying *RYR2* mutations intolerant of β-adrenergic antagonists experienced treatment failure with flecainide monotherapy over a ~37 months follow up period (Padfield *et al.*, 2016). Therefore, flecainide is now included in treatment guidelines in patients with recurrent syncope or polymorphic ventricular tachycardia on β- adrenergic antagonists (Priori *et al.*, 2013; Lieve *et al.*, 2016)."

This material covering the clinical observations that flecainide acts on Ca^{2+} mediated arrhythmias has been revised to cover the background bearing on the initial use of adrenergic blockers for such conditions as CPVT, working through the use of combined adrenergic blocker and flecainide, to the more recent adoption of flecainide therapy alone. We also add a summarising paragraph unifying the available information. Note that in response to a further request below, we have repositioned this section to occur with the material on Ca2+ mediated arrhythmias:

"***Anti-arrhythmic effects of flecainide in catecholaminergic polymorphic ventricular tachycardia (CPVT)***

More recently, flecainide proved to exhibit potential therapeutic efficacy in the Ca^{2+}-mediated catecholaminergic polymorphic ventricular tachycardia (CPVT). CPVT is predominantly associated with genetic abnormalities involving the cardiac ryanodine receptor type 2 sarcoplasmic reticular (SR) Ca^{2+} release channel (RYR2*)* and the SR binding protein calsequestrin type 2 (CASQ2) respectively. CPVT results in aberrant RYR2-mediated SR Ca^{2+} release precipitated by adrenergic stress. The leaky RyR2-Ca^{2+} release initiates delayed afterdepolarisations (DADs) that might trigger polymorphic VT.

Initial findings that flecainide prevented ventricular arrhythmia in two patients with respective CASQ2 and RYR2 mutations in exercise stress tests suggested a mechanism involving reduced triggering activity (Watanabe *et al.*, 2009). These clinical effects were corroborated by further case reports in which flecainide was added to prior conventional β-adrenergic antagonist therapy (Biernacka & Hoffman, 2011; Pott *et al.*, 2011; Jacquemart *et al.*, 2012; Mantziari *et al.*, 2013; Wangüemert-Pérez *et al.*, 2014).

Combination therapy using a β-adrenergic antagonist and flecainide partially or completely suppressed ventricular arrhythmias in 76 % of one *CASQ2* and 32 *RYR2* mutation carriers with intractable CPVT (Van Der Werf *et al.*, 2011). It also completely suppressed exercise-induced ventricular arrhythmia in all of 10 *CASQ2-D307H* patients who were experiencing exercise-induced events on β-blocker therapy alone or in combination with a Ca^{2+} channel antagonist. This remission was maintained in 8 of the 10 patients over a ~15 month follow-up period (Khoury *et al.*, 2013). Furthermore, addition of flecainide completely prevented ventricular arrhythmias during exercise testing and over long-term follow-up in 7 of 12 patients with *RYR2*, *CASQ2* or *KCNJ2* genotype-negative CPVT resistant to conventional β-blocker therapy (Watanabe *et al.*, 2013).

Flecainide monotherapy was pursued in patients carrying *RyR2* mutations in which one patient did not tolerate β-blockers, and 7 other patients were switched to flecainide monotherapy from combined therapy. Monotherapy with flecainide proved more effective or equal to β-blocker monotherapy, while combination therapy only proved more successful in 2 of the 8 patients over a ~37 months follow up period (Padfield *et al.*, 2016).

The paediatric CPVT phenotype is often more severe than the adult presentation (Hayashi *et al.*, 2009). Flecainide was used in 24% of patients in a retrospective paediatric (<19 yr age) cohort study of 226 CPVT patients. Treatment failure never occurred in any adherent patient receiving optimal doses of both flecainide and β-blocker. Flecainide monotherapy was used in a limited number of 5 patients. Results then compared well with results from β-blockers, implantable cardioverter defibrillators and left cardiac sympathetic denervation. All these cases showed suppression of exercised induced events; 78% remained asymptomatic, and there was no mortality on follow-up (Roston *et al.*, 2015). Proarrhythmic effects of flecainide of the kind observed in BrS have not been observed in the context of CPVT.

Nevertheless, given the underlying catecholaminergic trigger for CPVT, their efficacy and wide therapeutic window the first line of current therapy continues to utilize β-blocker

monotherapy. However, β-blockers are not well tolerated or do not have an adequate therapeutic efficacy in as many as 30% of cases. These are often the younger, healthier patients. In these situations the addition of flecainide as a combined therapy may prove more effective. Thus, flecainide is an appealing therapeutic addition to traditional β-blocker monotherapy, particularly in patients resistant to such therapy or requiring high dose β-blockers. Adverse side effects might then be reduced through the use of smaller doses of two as opposed to a larger dose of a single pharmacological agent. Recent reports have progressed to introduce flecainide monotherapy in particular cases, with encouraging preliminary results. Flecainide monotherapy emerges as an available and effective next step, where β-blockers are not tolerated or ineffective. However, the current data relies on limited studies. Further investigation is required to conclusively assess flecainide monotherapy as an earlier line of treatment, given its narrow therapeutic window (Priori *et al.*, 2013; Lieve *et al.*, 2016)."

Note: the following points emerging from the initial case reports make clear the grounds upon which we have summarized the situation concerning flecainide/beta-block combination therapy versus flecainide monotherapy: these early studies all involved combined therapy with monotherapy occurring later in a more limited group of reported cases.

a) (Biernacka & Hoffman, 2011): one patient unable to tolerate propranolol so the patient was given a combined flecainide and bisoprolol regime. Effective suppression of exertion induced arrhythmia after 3 days.
b) (Pott *et al.*, 2011): Flecainide added to beta blocker/verapamil treatment as the initial treatment was ineffective at arrhythmia suppression. BB dose was subsequently reduced but not stopped.
c) (Jacquemart *et al.*, 2012) Flecainide added to beta blocker therapy as the BB was not effective alone. The combination was a success.
d) (Mantziari *et al.*, 2013) combination therapy- metoprolol and flecainide with implantable defibrillator.
e) (Padfield *et al.*, 2016): One patient who did not tolerate BB therapy whatsoever and seven patients who were removed from BB therapy (total 8 patients). Transition from beta blocker to flecainide monotherapy occurred over a 2-8 week period.

2, Schematic figure should also be provided for information of K channel antagonism by flecainide.

We have provided New figure 6, to cover flecainide actions on K+ channels, with a re-arrangement of the text so the section on flecainide actions of K+ channels occurs in a more logical place.

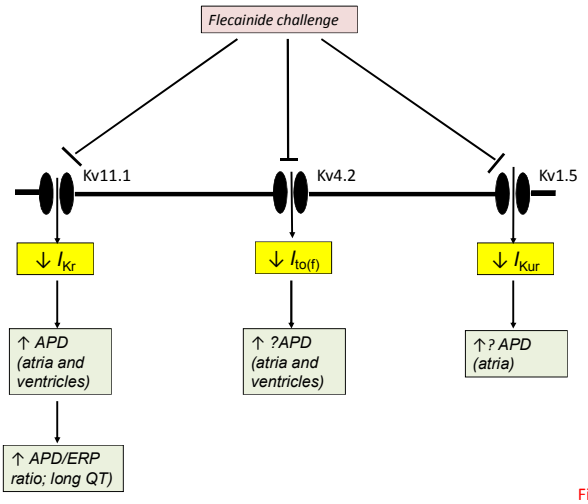

Figure 6

New Figure 6. Flecainide actions on potassium channel subtypes.

We have also made good a number of omissions in our nomenclature so the passage:

"***K^+ channel antagonism by flecainide***
"Flecainide also acts on K^+ channels, where it inhibits rapid K^+ current, I_{KR}, tails that followed voltage clamp pulses to +30 mV in the HEK293 expression system. The effect was most noticeable in the steepest part of the I_{KR} activation curve reflecting a voltage-dependent inhibition consistent with a rapid open channel state I_{KR} antagonism similar to that described for I_{Na} (Paul *et al.*, 2002). Flecainide also inhibits rapid transient outward currents, I_{tof}, in both native cells (Slawsky & Castle, 1994) and heterologous expression systems (Rolf *et al.*, 2000), to extents increasing with channel inactivation and consistent with its higher affinity for the inactivated state of Kv4.2 (Wang *et al.*, 1995). Finally, flecainide inhibits ultrarapid delayed rectifier, I_{Kur} with preference for current carried by Kv3.1 over human Kv1.5 (Herrera *et al.*, 2005)"

Is modified to (with clarification of K+ channel molecular types and flecainide concentrations and an additional reference(Tamargo *et al.*, 2004)):

"***K^+ channel antagonism by flecainide***
Flecainide also acts on K^+ channels (Figure 6). At <10 μM it inhibits rapid K^+ current, I_{KR}, tails that followed voltage clamp pulses to +30 mV in the HEK293 expression system. The effect was most noticeable in the steepest part of the I_{KR} (Kv11.1, hERG) activation curve reflecting a voltage-dependent inhibition consistent with a rapid open channel state I_{KR} antagonism similar to that described for I_{Na} (Paul *et al.*, 2002). Flecainide (>10 μM) also inhibits rapid transient outward (Kv4.2) currents, I_{tof}, in both native cells (Slawsky & Castle, 1994) and heterologous expression systems (Rolf *et al.*, 2000), to extents increasing with channel inactivation and consistent with its higher affinity for the inactivated state of Kv4.2 (Wang *et al.*, 1995). Finally, flecainide (~100 μM) inhibits ultrarapid delayed rectifier (Kv1.5) current, I_{Kur} (Tamargo *et al.*, 2004; Herrera *et al.*, 2005)"

"

3, Logically, restructure of article writing is highly suggested. The authors should demonstrate anti-arrhythmic effects and pro-arrhythmic effects of flecainide by clinical levels followed by molecular (channels' alterations) for each part.

As requested, there has been a rearrangement of topics as suggested with each major section preceded by clinical findings, and this is then followed by possible molecular and physiological mechanisms. This involves a re-arrangement of material covering clinical observations that flecainide acts on Ca^{2+} mediated arrhythmias so that it precedes the experimental data exploring its mechanisms. The relevant paragraph (which was one that was revised as described above) is:

""More recently, flecainide proved to exhibit potential therapeutic efficacy in the Ca^{2+}-mediated catecholaminergic polymorphic ventricular tachycardia (CPVT). CPVT is predominantly associated with….. However, the current data relies on limited studies. Further investigation is required to conclusively assess flecainide monotherapy as an earlier line of treatment, given its narrow therapeutic window (Priori *et al.*, 2013; Lieve *et al.*, 2016)."

Reviewer: 2

The review entitled 'multiple targets for flecainide action: implications for cardiac arrhythmogenesis' summarises and synthesises the complex actions of the Class Ic anti-arrhythmic drug flecainide on the mammalian heart. The review takes a mechanistic approach and discusses what is known about the

modes of interaction between the drug and its primary targets – Nav1.5 and RyR2. The review summaries the relevant data and has chosen appropriate 'case studies' for inclusion in figures. The paper also makes a strong attempt to pull apart the multiple and often conflicting actions of flecainide on arrhythmias. The authors propose a feed-forward and feed-backward schema to integrate the diverse physiological, biophysical and pharmacological data with clinical findings. My suggestions for improvement are minor and are listed below.

> Thank you for the positive reaction to our paper, encapsulating our objectives so effectively and advising us about aspects requiring attention. We have revised the paper responding to all the reviewers' reports. The following changes are relevant to the comments made by the present reviewer.

P5 paragraph 2 second last sentence – remove one of the 'over a 10 month period' statements.

> Thank you: the passage:
>
> "However, over a 10 month follow-up, encainide or flecainide-treated patients showed a higher incidence (8.9%) of arrhythmic death than patients assigned to placebo (1.2%) over a 10 month follow-up (CAST Investigators, 1989; Echt *et al.*, 1991; Greenberg *et al.*, 1995)."
>
> Is now corrected to:
>
> "However, encainide or flecainide-treated patients showed a higher incidence (8.9%) of arrhythmic death than patients assigned to placebo (1.2%) over a 10 month follow-up (CAST Investigators, 1989; Echt *et al.*, 1991; Greenberg *et al.*, 1995)."

P5 top of page – does this need to start with 'However'? Perhaps just begin the paragraph with The Nav channel….

> Thank you: The passage:
> "However, even Nav channel function itself poses intrinsic complexities."
> Is corrected to:
> "The Nav channel function itself poses intrinsic complexities."

Same paragraph – can you explain how INa 'may compromise on of more currents components each with different kinetics'

> Thanks. The word should have been 'comprise' rather than 'compromise' and the passage reads:
> "Secondly, the resulting Na^+ current, I_{Na}, may comprise one or more current components each with different kinetics."
>
> However, the wording has been improved to:
> "Secondly, the resulting Na^+ current, I_{Na}, may include one or more current components each with different kinetics."

P5 Bottom of second paragraph – may be useful to explain more what is meant by absolute and relative refractory periods.

> Thank you. We have incorporated definitions of absolute and relative refractory periods into the revised text as suggested:
>
> The passage:
>
> "Finally, with repolarisation to the resting potential, the Nav1.5 channels recover their capacity for re-excitation over absolute and relative refractory periods."

Is now clarified to:

"Finally, with repolarisation to the resting potential, the Nav1.5 channels recover their capacity for re-excitation, resulting in absolute and relative refractory periods. These respectively correspond to the time intervals over which the channels either cannot be re-excited whatever the stimulus intensity, or require increased stimulus amplitudes for such re-excitation."

P5 3rd paragraph – the idea of protein complexes influencing channel function could be expanded upon here to lead better into the later discussion of flecainide interactions with B3-subunits.

Thank you. We identify the point at issue as existing at the paragraphs:

(A) "Further complexities arise from Nav channel anchoring into localised plasma membrane clusters within larger, extended protein complexes. These include not only auxiliary Nav channel β subunits (Cusdin *et al.*, 2010), but also cytoskeletal proteins (Jeevaratnam *et al.*, 2016; Huang, 2017) and even other ion channels such as the inward rectifier Kir2.1 (Willis *et al.*, 2015). These associated proteins can influence Nav channel gating behaviour both directly through protein-protein contacts and indirectly by affecting surface expression and trafficking (Abriel & Kass, 2005; Cusdin *et al.*, 2008)."

And:

(B) "As noted above, Nav channels *in vivo* are associated with a range of auxiliary and interacting proteins, many of which can modulate channel gating. However, relatively little attention has been paid to this particular aspect of flecainide pharmacology. To our knowledge, the only example where effects of auxiliary subunits on flecainide behaviour were studied is the case of the β3 subunit, the product of the *Scn3b* gene (Hakim *et al.*, 2010). The β3 subunit is expressed in heart and modulates Nav1.5 gating (Yu *et al.*, 2005). The β3 subunit contains a single extracellular immunoglobulin domain, a single-pass transmembrane domain and an intracellular domain. It interacts with Nav1.5 through both its extracellular and intracellular domains (Namadurai *et al.*, 2015). However, neither of these two interaction sites are close to the flecainide binding site on Nav1.5 (Fig. 2B). Nevertheless, β3 could potentially modulate the effects of flecainide on Nav1.5 indirectly, either affecting channel opening probability or by its known effects on Nav1.5 oligomerisation (Namadurai *et al.*, 2014, 2015)."

And:

(C) "Patch-clamped *Scn3b*$^{-/-}$ murine cardiomyocytes showed reduced I_{Na} likely reflecting reduced Nav1.5 trafficking into the surface membrane. This was combined with negative shifts in Nav1.5 inactivation characteristics that would be expected to reduce I_{NaL} but shorten refractory periods. The genetic variant accordingly shows arrhythmic phenotypes resembling that of the *Scn5a*+/- murine model (Hakim et al., 2008). Indeed, several mutations in *SCN3B* are associated with inherited cardiac arrhythmias in humans (Namadurai et al., 2015). However, in contrast to its effects in *Scn5a*+/-, in *Scn3b*-/- hearts, flecainide produced reduced arrhythmic incidences combined with prolonged refractory periods and shortened APDs (Hakim et al., 2010). The reasons for this difference are unclear, but they further confirm suggestions that flecainide exerts dual pro- and anti-arrhythmic actions through both conduction and refractoriness effects. Thus, in the case of *Scn5a*+/- hearts the negative conduction velocity effects predominate in producing arrhythmia *exacerbated* by flecainide. In the case of LQTS3, refractoriness and recovery effects predominate in producing

arrhythmia *reduced* by flecainide. The presence or absence of β3 subunits may differentially modify these two competing effects so that an anti-arrhythmic effect predominates."

We have rewritten and relocated these paragraphs to (1) expand the idea of protein complexes influencing channel function to (2) lead better into the later revised discussion of flecainide interactions with β3-subunits to:

"Further complexities arise because Nav channels do not occur as isolated molecules in the plasma membrane, but instead are anchored within larger, extended multi-component complexes. Examples of such associated proteins include auxiliary Nav channel β subunits (Cusdin *et al.*, 2010), cytoskeletal proteins (Jeevaratnam *et al.*, 2016; Huang, 2017) and other ion channels such as the inward rectifier Kir2.1 (Willis *et al.*, 2015). These proteins can influence Nav channel gating behaviour both directly through protein-protein contacts and indirectly by affecting surface expression and trafficking (Abriel & Kass, 2005; Cusdin *et al.*, 2008; Abriel *et al.*, 2015).

Relatively little attention has been paid to how this supra-molecular channel clustering could influence flecainide pharmacology. To our knowledge, the only example where such effects on flecainide behaviour were studied is the case of the auxiliary Nav β3 subunit, the product of the *Scn3b* gene (Hakim *et al.*, 2010). The β3 subunit is expressed in heart and modulates Nav1.5 gating (Yu *et al.*, 2005). Patch-clamped *Scn3b*$^{-/-}$ murine cardiomyocytes showed reduced I_{Na} likely reflecting reduced Nav1.5 trafficking into the surface membrane. This was combined with negative shifts in Nav1.5 inactivation characteristics that would be expected to reduce I_{NaL} but shorten refractory periods. The genetic variant accordingly shows arrhythmic phenotypes resembling that of the *Scn5a*+/- murine model (Hakim *et al.*, 2008). Indeed, several mutations in *SCN3B* are associated with inherited cardiac arrhythmias in humans (Namadurai *et al.*, 2015).

Curiously however, in *Scn3b*-/- hearts, flecainide produced reduced arrhythmic incidences combined with prolonged refractory periods and shortened APDs (Hakim *et al.*, 2010). This is in direct contrast to its effects in *Scn5a*+/- mice (see above). The reasons for this difference are unclear, but they further confirm suggestions that flecainide exerts dual pro- and anti-arrhythmic actions through effects on both conduction and refractoriness. Thus, in the case of *Scn5a*+/- hearts the negative conduction velocity effects predominate in producing arrhythmia *exacerbated* by flecainide. In the case of LQTS3, refractoriness and recovery effects predominate in producing arrhythmia *reduced* by flecainide. The presence or absence of β3 subunits may differentially modify these two competing effects so that an anti-arrhythmic effect predominates.

How this might work is currently unknown and will probably require detailed structural insights into how the β3 subunit interacts and modulates the Nav1.5 α subunit. The β3 subunit contains a single extracellular immunoglobulin domain, a single-pass transmembrane domain and an intracellular domain and interacts with Nav1.5 through both its extracellular and intracellular domains (Namadurai *et al.*, 2015). It is striking however, that neither of these two interaction sites are close to the flecainide binding site on Nav1.5 (Fig. 2B). This suggests that the β3 subunit most likely modulates the effects of flecainide on Nav1.5 indirectly, either by affecting channel opening probability or by its known effects on Nav1.5 oligomerisation (Namadurai *et al.*, 2014, 2015)."

P7 3rd paragraph – which 'confounding complexities' are you referring to here?

Thank you. This sentence:

"Confounding these complexities further are the elaborate actions of flecainide upon the Nav channel, each with implications for channel activation and recovery."

Is indeed unclear, and in fact is completely unnecessary. It has been omitted and the paragraph now begins with the next sentence:

"Flecainide acts upon the activated, open, state of Nav1.5 (Anno & Hondeghem, 1990; Nitta *et al.*, 1992; Nagatomo *et al.*, 2000)…"

P10 top of page – i am not clear from this paragraph what the action of flecainide on the B3 subunit is? Or how it affects Ina function. May be worth trying to rephrase this paragraph.

We have dealt with this issue under a query above ["P5 3rd paragraph – the idea of protein complexes influencing channel function could be expanded upon here to lead better into the later discussion of flecainide interactions with B3-subunits."]. Briefly we point out that little is known about the effect of flecainide on beta subunits.

"How this might work is currently unknown and will probably require detailed structural insights into how the β3 subunit interacts and modulates the Nav1.5 α subunit. The β3 subunit contains a single extracellular immunoglobulin domain, a single-pass transmembrane domain and an intracellular domain and interacts with Nav1.5 through both its extracellular and intracellular domains (Namadurai *et al.*, 2015). It is striking however, that neither of these two interaction sites are close to the flecainide binding site on Nav1.5 (Fig. 2B). This suggests that the β3 subunit most likely modulates the effects of flecainide on Nav1.5 indirectly, either by affecting channel opening probability or by its known effects on Nav1.5 oligomerisation (Namadurai *et al.*, 2014, 2015)."

Figure legend 2 – I don't see hydrogen bonds in the figure?

We have changed the figure in response to the comment that no hydrogen bonds were visible. We have also taken the opportunity to slightly simplify. The new figure and its legend are as below; we deleted a few words to make the legend compatible with the new fig:

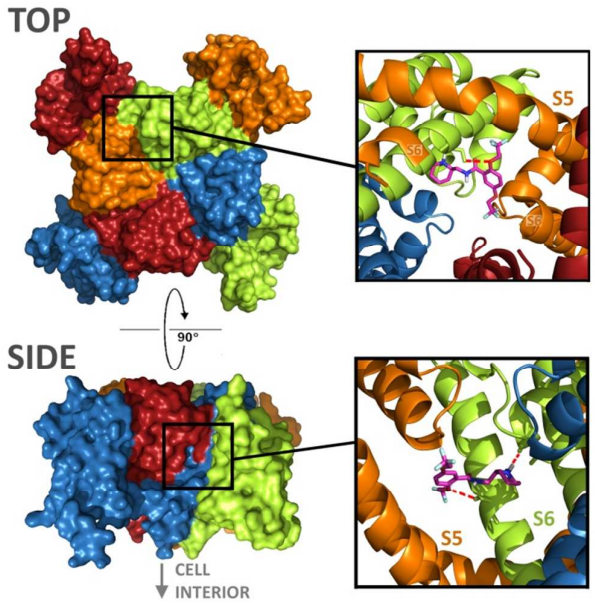

Fig 2. Flecainide docking into the voltage-gated sodium channel crystal structure NavRh (pdbid: 4DXW). (A). Alignment of the IV-S6 region of different voltage-gated Na+ channels, highlighting the phenylalanine residue (IV-S6-phe) that is strongly implicated in flecainide binding. (B). In silico docking of flecainide into NavRh locates the ligand in a hydrophobic pocket. The upper panels show NavRh as viewed from the top, and the lower panels as viewed from the side. The four colours represent the four domains that constitute the functional protein. The boxes to the right show the flecainide (pink) binding site represented as a cartoon. Note that as flecainide binds within the pore of the channel, the site has been visualised as a slice through the protein; this excludes some of the overlying helices. Hydrogen bond interactions (dashed red line) are predicted with IV-S6-phe. At Nav1.4 a cation-π interaction is seen at the same location (Ahern, Eastwood, Dougherty & Horn, 2008). (R)-Flecainide was generated ab initio using Chem3D Prov14.0 (CambridgeSoft, Cambridge, UK), energy minimised using the implemented MM2 force field and docked using GOLD Suite v5.3 (The Cambridge Crystallographic Data Centre, Cambridge, UK) with the GoldScore function and default settings. Amino acid sequences used in the ClustalW alignment are: 4DXW and 4EKW taken directly from structures of bacterial sodium channels; r_brain II = P04775; h_Nav1.1 = NP_001189364; h_Nav1.2 = NP_001035232; h_Nav1.4 = NP_000325; h_Nav1.5 = NP_932173; hNav1.7 = ABI51981.

Figure legend 4 – add brackets around (ERP) in title for consistency and around (B) later in the paragraph.

(1) The title of figure legend 4 is corrected from" *Rate dependent effects of flecainide on Na^+ current (I_{Na}), effective refractory period ERP, V_{max} and APD_{95} in different species.*" to "*Rate dependent effects of flecainide on Na^+ current (I_{Na}), effective refractory period (ERP), V_{max} and APD_{95} in different species.*"

(2) Brackets placed around "B" in "B. The effects of flecainide on (from left to right):" to give "(B). The effects of flecainide on (from left to right):"

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