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Multiple targets for flecainide action: implications for cardiac arrhythmogenesis

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Flecainide suppresses cardiac tachyarrhythmias including paroxysmal atrial fibrillation, supraventricular tachycardia and 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 organization. These were studied in murine genetic models with modified Na_v channel or intracellular ryanodine receptor (RyR2)-Ca^{2+} channel function. Flecainide accesses its transmembrane $Na_v1.5$ channel 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 activation of Na_v channels followed by rapid de-activation, drives action potential (AP) upstrokes and their propagation. This is diminished in pro-arrhythmic conditions reflecting loss of function of $Na_v1.5$ channels, 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 modifications of $Na_v1.5$ channels 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 actions on Na_v channels 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 $Na_v1.5$ channel 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 afterdepolarization; 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

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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); $C_{17}H_{20}F_6N_2O_3$) (Figure 1A) was derived 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 $\mu g \cdot mL^{-1}$) and overall excitation delay (at >6.5 $\mu g \cdot mL^{-1}$) (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 L-type 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 while 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 h after oral ingestion (Smith, 1985). Its plasma half-life is 12 to 27 h (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 VT (Anderson *et al.*, 1981) and proved acceptable for long-term use (Meinertz *et al.*, 1984).

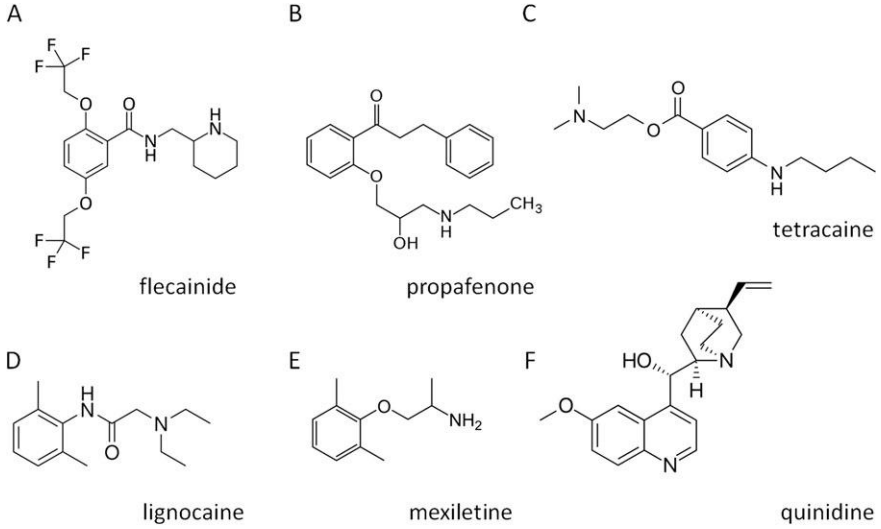

Figure 1

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.

These findings led to the 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 the long QT syndrome type 3 (LQTS3), associated with gain-of-function mutations in **$Na_v1.5$** channels (Shimizu and Antzelevitch, 1999). Low-dose, oral flecainide shortened corrected QT (QTc) intervals and normalized repolarization 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 benefit, the use of flecainide has been shadowed by pro-arrhythmic consequences under some clinical circumstances, particularly in the presence of ischaemic or morphological changes. 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 to reflect acutely decreased myocardial contractility (Winkelmann and Leinberger, 1987). An early study reported that 7 of 152 patients showed pro-arrhythmic effects including VT or ventricular fibrillation over an ~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 Brugada syndrome (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).

Na_v 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 concerns 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 the voltage-gated Na_v ion channels.

The Na_v 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 depolarization 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 inward 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 $Na_v1.5$ channels or distinct channel subpopulations (Saint *et al.*, 1992; Saint, 2009). An early peak I_{Na} related to Na_v channel activation drives the rapid early AP upstroke, thereby generating local circuit currents underlying AP propagation, rapidly inactivating within a few milliseconds. The resulting membrane depolarization 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 $Na_v1.5$ channel-mediated depolarization also activates plateau Ca^{2+} currents (**I_{CaL}**) that locally elevate cytosolic [Ca^{2+}] triggering Ca^{2+} release from the sarcoplasmic reticulum (SR), mediated by the **ryanodine receptor type 2 (RyR2)**. This leads to mechanical activation, with consequences for Ca^{2+} homeostasis and possible reciprocal interactions with surface channel excitability. Finally, a variety of voltage-gated K^+ channels, carrying the delayed rectifiers **I_{Kr}** and **I_{Ks}**, and the inwardly rectifying **I_{K1}**, drives the final repolarization 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

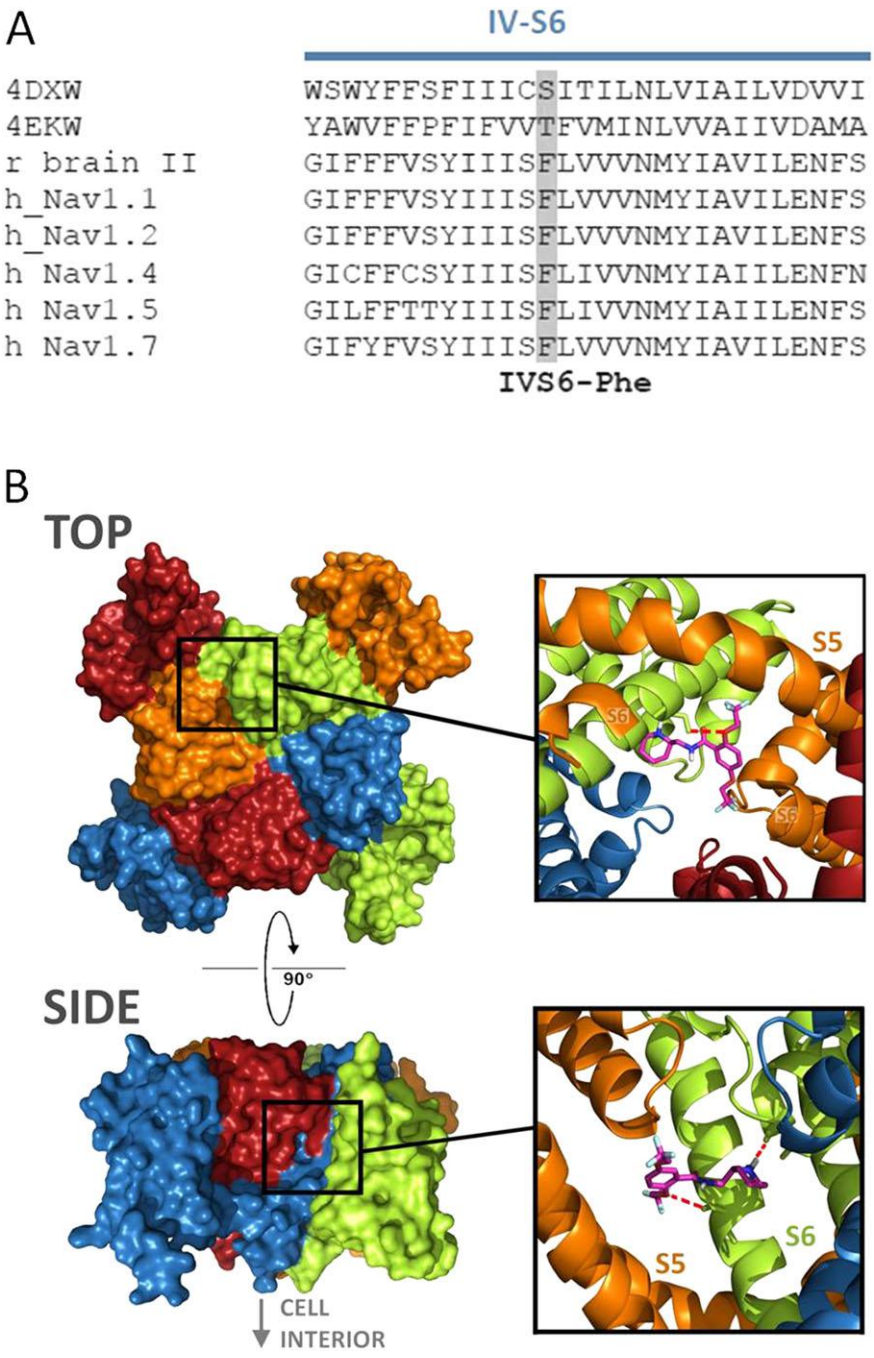

Figure 2

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 visualized 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 *et al.*, 2008). (R)-flecainide was generated *ab initio* using Chem3D Prov14.0 (CambridgeSoft, Cambridge, UK), energy minimized 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.

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 repolarization to the resting potential, the $Na_v1.5$ channels recover their capacity for re-excitation, resulting in absolute and relative refractory periods. These 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 $Na_v1.5$ channel variants showed that mutations in the IV-S6 helix were 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 $Na_v1.5$ channels is close to this region (Figure 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 interactions of $Na_v1.5$ channels 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 (Figure 2A) (Ahern *et al.*, 2008). Figure 2B illustrates this region by docking flecainide into a proteobacterial homologue of the $Na_v1.5$ channel (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.

In K^+ channels, including the **$K_v11.1$** channels, flecainide may have similar binding sites that also overlap with binding sites for other ligands, such as the structurally related **propafenone** (Figure 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 on I_{KR} from expressed **$K_v11.1$** channels, 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).

Na_v channel antagonism by flecainide

Flecainide acts upon the activated, open, state of $Na_v1.5$ channels (Anno and Hondeghem, 1990; Nitta *et al.*, 1992; Nagatomo *et al.*, 2000) (Figure 3A), gaining access to a transmembrane binding site where it blocks the pore, and inhibits I_{Na} (Liu *et al.*, 2002, 2003) (Figure 3B). I_{Na} inhibition takes place with a low affinity (IC_{50} = 345 μM) during brief depolarizing 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} (Figure 4A, left panel) and a consequent shift in the dependence of I_{Na} inhibition towards lower flecainide concentrations under conditions of increasing pulsing frequency (Figure 4A, right panel) (Penniman *et al.*, 2010). This accounts for progressive increases in AP refractory periods, decreases in $(dV/dt)_{max}$ and increases in action potential duration (APD) with increasing stimulus frequencies in hearts of a range of species (Figure 4B) (Wang *et al.*, 1990). Consistent with this, in a non-inactivating $Na_v1.5$ channel mutant, flecainide produced decays in I_{Na} with a time course suggesting a simple pore blocking mechanism (K_D = 11 μM). Once bound, flecainide reduces Na_v channel open times (Grant *et al.*, 2000). Flecainide binding to channels then inactivated by sustained depolarization does not contribute to Na_v 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 depolarization producing channel inactivation (Ramos and O'Leary, 2004).

Flecainide does not directly bind to closed or inactivated Na_v channels, but closing either the activation or the inactivation gate traps flecainide within the pore (Figure 3 C), slowing recovery of drug-bound channels at hyperpolarized voltages. Thus, flecainide slowed recovery of both rapidly inactivating (τ ~ 81 s) and non-inactivating (τ ~ 42 s) channels with hyperpolarization. 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 (Figure 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 BrS, whose commonest genetic accompaniment is an inherited loss-of-function in $Na_v1.5$ channels, associated with increased risks of potentially fatal ventricular arrhythmias particularly in middle aged (40–45 years) males (Brugada *et al.*, 2002). It has been modelled in isolated murine heterozygotic $Na_v1.5$ channel haplo-insufficient *Scn5a*$^{+/-}$ cardiac preparations (Papadatos *et al.*, 2002). These preparations replicated the clinically observed arrhythmic

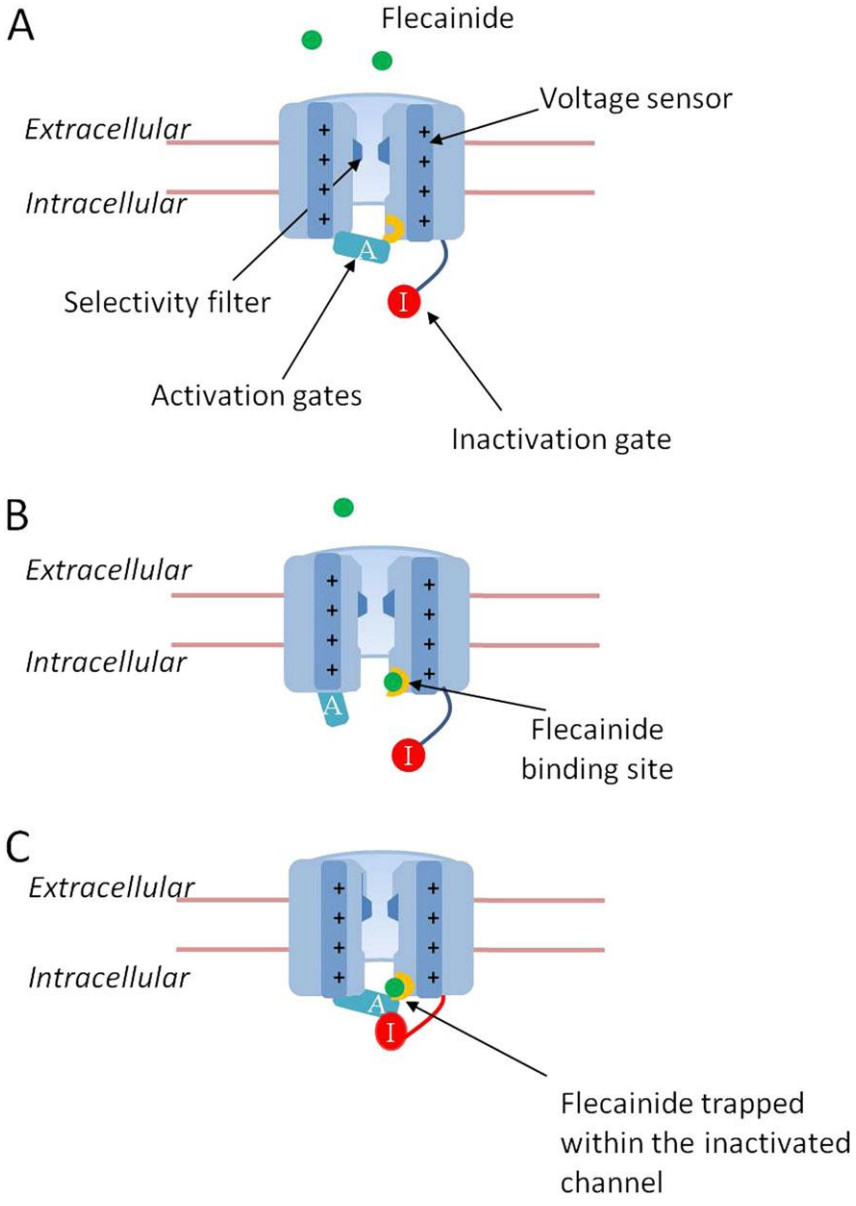

Figure 3

Open state antagonism of the voltage-gated sodium channel by flecainide. (A) Voltage-gated Na_v channel is represented in its closed, resting state. Surface membrane depolarization 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 depolarizations that allow for the refractory period of the inactivated state result in higher potency.

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 reproduced 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 repolarization 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 $Na_v1.5$ channels could make it particularly effective on Na_v channels showing prolonged dwell times, as with the increased I_{NaL} in LQTS3. Patch-clamp studies on the HEK293 expression

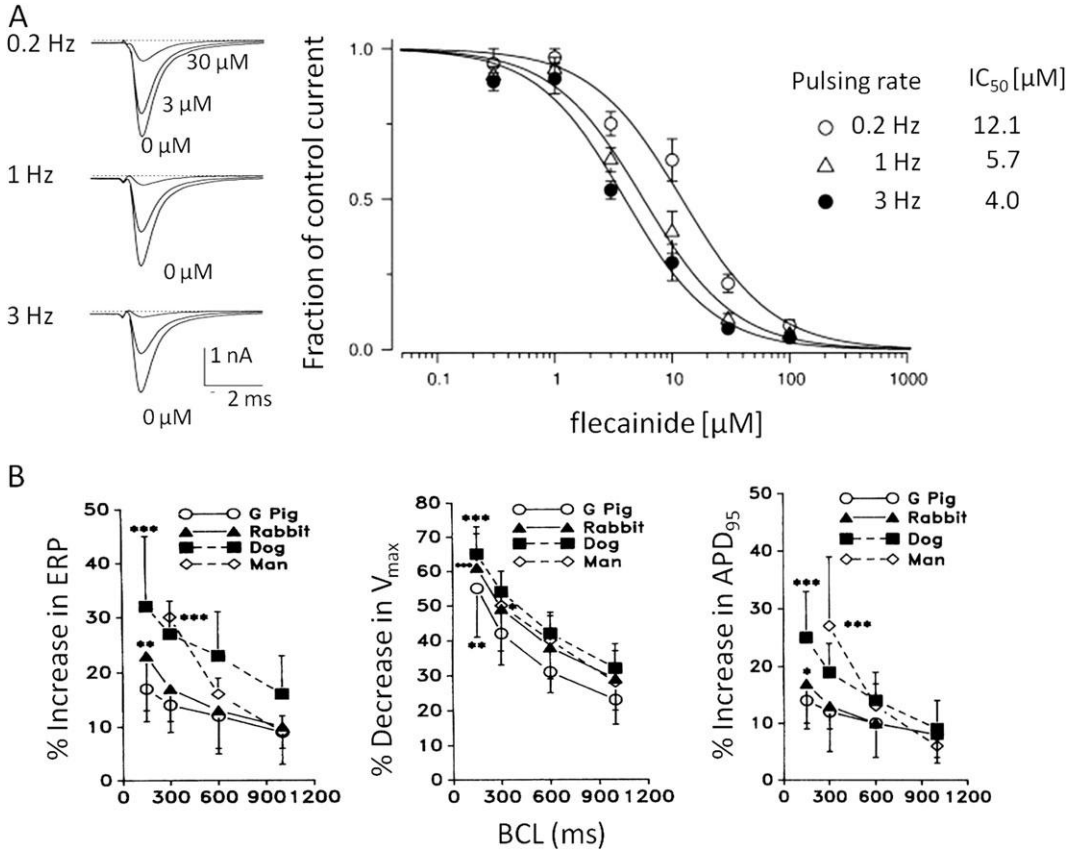

Figure 4

Rate-dependent effects of flecainide on 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 $hNa_v1.5$ channels before and after application of 3 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 Figure 6A and B of Penniman *et al.*, (2010)). (B) The effects of flecainide on (from left to right) ERP, maximum rate of AP depolarization (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 Figures 2–4 of Wang *et al.*, (1990)).

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 Na_v channel opening. This thus provides a pro-arrhythmic exemplar distinct from arrhythmia arising from deficient peak I_{Na} in BrS. Murine *Scn5a*$^+$/ΔKPQ hearts modelled 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 afterdepolarization 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 activity of the 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 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 reproduces the clinically established, anti-arrhythmic effects of flecainide in LQTS3 (Windle *et al.*, 2001; Moss *et al.*, 2005), further demonstrating that murine hearts can provide useful models for the human condition.

Occurrences of flecainide exerting pro- rather than anti-arrhythmic effects in LQTS3 have also been reported.

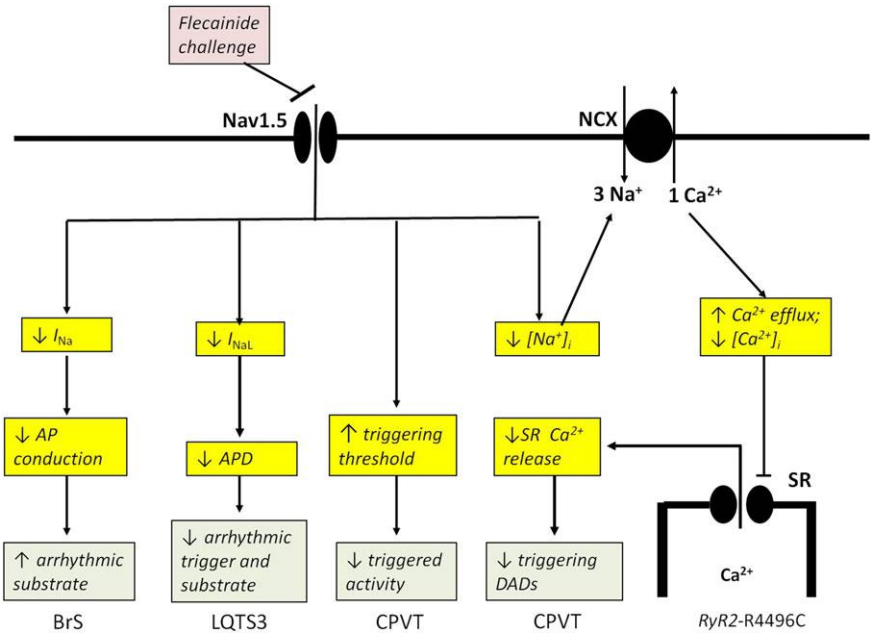

Figure 5

Feed-forward effects of flecainide attributable to its actions on $Na_v1.5$ channels. In this hypothesis, flecainide reduces peak, I_{Na}, thereby reducing AP conduction velocity, exacerbating pro-arrhythmic conditions arising from *loss* of function in $Na_v1.5$ channels, occurring in conditions such as BrS. In contrast, its reduction of late, I_{NaL}, would be anti-arrhythmic in conditions associated with *gain* of function in $Na_v1.5$ channels, increasing I_{NaL} and prolonging AP duration such as LQTS. The inhibitory effect of flecainide on $Na_v1.5$ channels 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.

However, these were observed when LQTS3 phenotypic features were combined with abnormalities normally associated with a $Na_v1.5$ channel 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 $Na_v1.5$ channels. 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 Na_v 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 the inactivation characteristics of $Na_v1.5$ channels (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 antagonized I_{NaL} to different extents in the sequence WT < *Scn5a*$^+$/ΔKPQ < *Scn5a*$^+$/1795insD. *Scn5a*$^+$/1795insD channels also showed delayed recoveries from inactivation further exacerbated by flecainide (Viswanathan *et al.*, 2001).

Further complexities arise because Na_v 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 **Na_v channel β subunits** (Cusdin *et al.*, 2010), cytoskeletal proteins (Jeevaratnam *et al.*, 2016; Huang, 2017) and other ion channels such as the inwardly rectifying **$K_{ir}2.1$** channels (Willis *et al.*, 2015). These proteins can influence Na_v 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 Na_v channel β3 subunit, the product of the *Scn3b* gene (Hakim *et al.*, 2010). The β3 subunit is expressed in heart and modulates gating of $Na_v1.5$ channels (Yu *et al.*, 2005). Patch-clamped *Scn3b*$^{-/-}$ murine cardiomyocytes showed reduced I_{Na} , most likely reflecting reduced trafficking of $Na_v1.5$ channels to the surface membrane. This was combined with negative shifts in $Na_v1.5$ channel 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 $Na_v1.5$ channel α subunit. The β3 subunit contains a single extracellular immunoglobulin domain, a single-pass transmembrane domain and an intracellular domain and interacts with $Na_v1.5$ channels 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 $Na_v1.5$ channels (Figure 2B). This suggests that the β3 subunit most likely modulates the effects of flecainide on $Na_v1.5$ channels indirectly, either by affecting channel opening probability or by its known effects on oligomerization of $Na_v1.5$ channels (Namadurai *et al.*, 2014, 2015).

K^+ channel antagonism by flecainide

Flecainide also acts on voltage-gated K^+ channels (Figure 6). At <10 μM, it inhibited the 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} (carried by $K_v11.1$ channels, 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 (**$K_v4.2$ channels**) 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 $K_v4.2$ channels (Wang *et al.*, 1995b). Finally, flecainide (~100 μM) inhibits the ultrarapid delayed rectifier (**$K_v1.5$** channels) current, I_{Kur} (Tamargo *et al.*, 2004; Herrera *et al.*, 2005).

Anti-arrhythmic effects of flecainide in CPVT

More recently, flecainide proved to exhibit potential therapeutic efficacy in the Ca^{2+}-mediated catecholaminergic

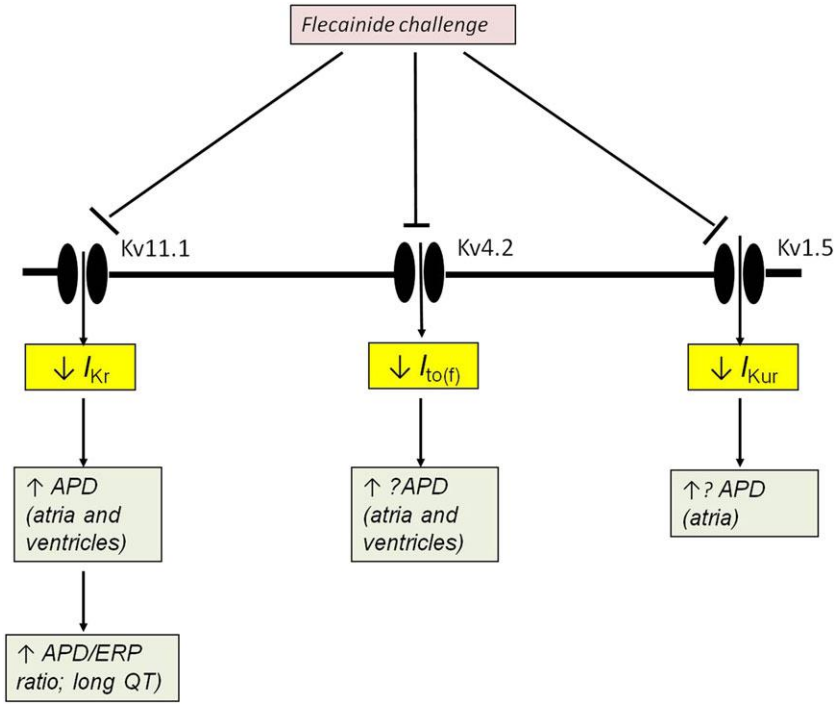

Figure 6
Flecainide actions on voltage-gated K_v channel subtypes.

polymorphic ventricular tachycardia (CPVT). CPVT is predominantly associated with genetic abnormalities involving the cardiac RyR2 SR Ca^{2+} release channel 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 afterdepolarizations (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-adrenoceptor** 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 an ~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 seven 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 two of the eight patients over an ~37 month 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 years of 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 five patients. Results then compared well with results from β-blockers, implantable cardioverter defibrillators and left cardiac sympathetic denervation. All these cases showed suppression of exercise induced events; 78% remained asymptomatic, and there was no mortality on follow-up (Roston *et al.*, 2015). Pro-arrhythmic 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 Na_v channel antagonism and additionally implicate such actions in increased thresholds for triggered pro-arrhythmic activity.

In the first of these, flecainide pretreatment reduced incidences of sustained VT in *RyR2*-R4496C$^{+/-}$ mice studied by ECG telemetry, following **adrenaline** and **caffeine** challenge, from 70 to 8%. In isolated intact regularly paced (1 Hz) *RyR2*-R4496C$^{+/-}$ ventricular myocytes, **isoprenaline** (1 μM) increased the amplitudes and accelerated the decays of spontaneous Ca^{2+} transients and increased SR Ca^{2+} load. Permeabilized *RyR2*-R4496C$^{+/-}$ ventricular myocytes similarly demonstrated greater spontaneous Ca^{2+} spark and wave activity than WT, particularly following isoprenaline challenge. Both these groups of Ca^{2+} release phenomena persisted with flecainide (6 μM) challenge but were abolished by tetracaine (Figure 1C). Patch-clamped *RyR2*-R4496C$^{+/-}$ myocytes showed increased incidences of DADs and triggered activity with isoprenaline challenge. Flecainide reduced the occurrences of the triggered but not the DAD activity. These findings suggest flecainide actions attributable to its primary effects on Na_v 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 (Figure 1B) and lignocaine (Figure 1D) exerted similar actions (Sikkel *et al.*, 2013). These agents, all known to decrease I_{Na}, and correspondingly reducing $[Na^+]_i$, could thereby decrease $[Ca^{2+}]_i$, through an enhanced reverse mode action of the 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, probably through open-state block (Hilliard *et al.*, 2010), with efficacies and potencies varying with channel activity (Savio-Galimberti and Knollmann, 2015). Antagonism of open-state RyR2 channels 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 RyR2 channels. Lipid bilayer studies reported that flecainide antagonized WT-RyR2 channel 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 the channels were in the open state (Hilliard *et al.*, 2010). The IC_{50} values for flecainide action became progressively 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 channels exposed to EMD41000, consequently with high open probabilities, flecainide (10 μM) reduced cytoplasmic-to-luminal currents, but not the 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 inhibition of the RyR2 channels (Hwang *et al.*, 2011; Mehra *et al.*, 2014). Both cytoplasmic and luminal flecainide induced two modes of inhibition respectively associated with millisecond and second time-scale channel closures under conditions of near-maximal RyR2 channel 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 binding to RyR2 channels (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 isoprenaline-treated *Casq2*$^{-/-}$ ventricular myocytes (Watanabe *et al.*, 2009). Permeabilized *Casq2*$^{-/-}$ ventricular myocytes demonstrated greater Ca^{2+} spark and wave activity than those from WT mice. This was inhibited by flecainide and R-propafenone with greater inhibitory potencies and efficacies in *Casq2*$^{-/-}$ compared with 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** (Figure 1D–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 antagonized 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 scheme in Figure 5 summarizes the above feed-forward effects of flecainide ultimately arising from its actions on $Na_v1.5$ channels. 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 function in $Na_v1.5$ channels. In contrast, its actions in reducing late, I_{NaL}, would reduce arrhythmia in LQTS3 as this phenotype results from a gain of function in $Na_v1.5$ channels which prolongs AP duration. Inhibition of $Na_v1.5$ channels 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 Na_v 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 C-terminal region of $Na_v1.5$ channels (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)**. The Nav1.5 channels also contain phosphorylatable sites ($Ser^{516,\ 571}$, and Thr^{594}) 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 CaMKII. All these mechanisms positively shift the voltage dependence of Na_v 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 isoprenaline-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 isoprenaline challenge, showed pro-arrhythmic reductions in conduction velocity compared with WT. Intracellular microelectrode AP recordings showed correspondingly reduced maximum rates of depolarization $(dV/dt)_{max}$ (King *et al.*, 2013b; Zhang *et al.*, 2013). These changes could be attributed to (a) chronically down-regulated expression of $Na_v1.5$ channels, demonstrated in *RyR2*-P2328S ventricles (Ning *et al.*, 2016), and (b) acute actions of increased $[Ca^{2+}]_i$ upon $Na_v1.5$ channel function. Loose-patch clamp recordings demonstrated reduced peak I_{Na} in whole isolated *RyR2*-P2328S compared with WT atria to extents comparable with those reported in Nav1.5 channel-haploinsufficent *Scn5a*$^{+/-}$ hearts (King *et al.*, 2013a; Salvage *et al.*, 2015) (Figure 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, although 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 with WT atria. Flecainide reduced conduction velocity and indicators of AP upstroke velocity in WT hearts but did not do so in *RyR2*-P2328S hearts (Figure 7B, left panel). *RyR2*-P2328S atria similarly showed a reduced peak I_{Na} compared with WT (Figure 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 (Figure 7A, centre panels) while further increases to 5 μM flecainide inhibited I_{Na} in common with effects on WT (Figure 7A, right panels). Effective refractory periods were similar in untreated *RyR2*-P2328S and WT atria but were increased in flecainide-treated *RyR2*-P2328S (Figure 7B, centre panel). As a result, flecainide shortened AP wavelength as computed from the product of conduction

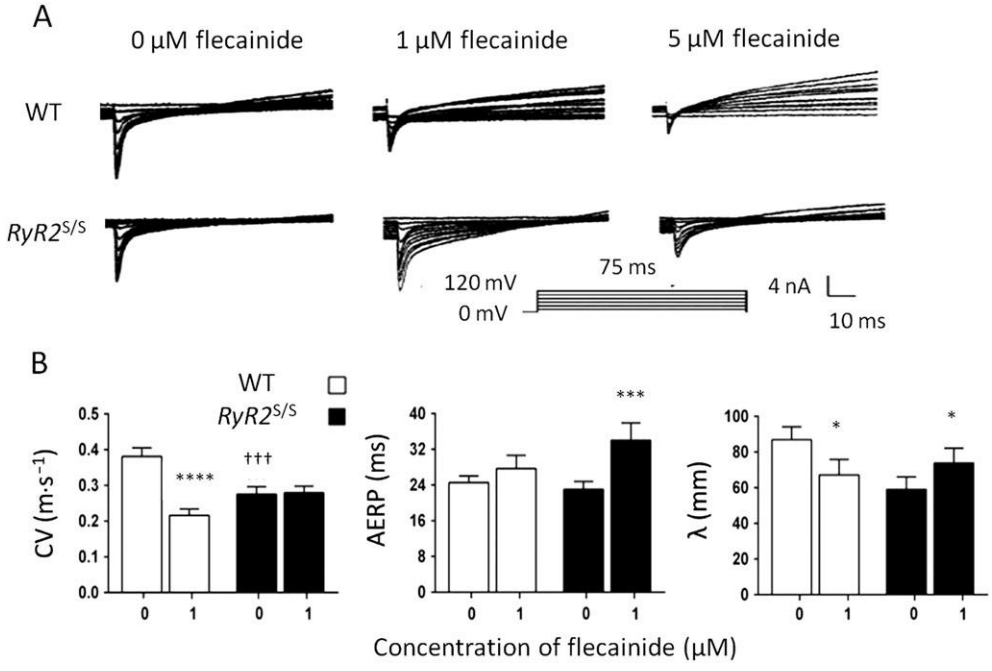

Figure 7

Paradoxical actions of flecainide on Na^+ current (I_{Na}), conduction velocity, refractory period and 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 while 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 in those from WT mice. However, flecainide shortened λ in WT but increased λ in $RyR2^{s/s}$ atria (figure adapted with permission from Figure 3(a) and (b) and Figure 7 (a)–(c) of Salvage *et al.*, (2015)).

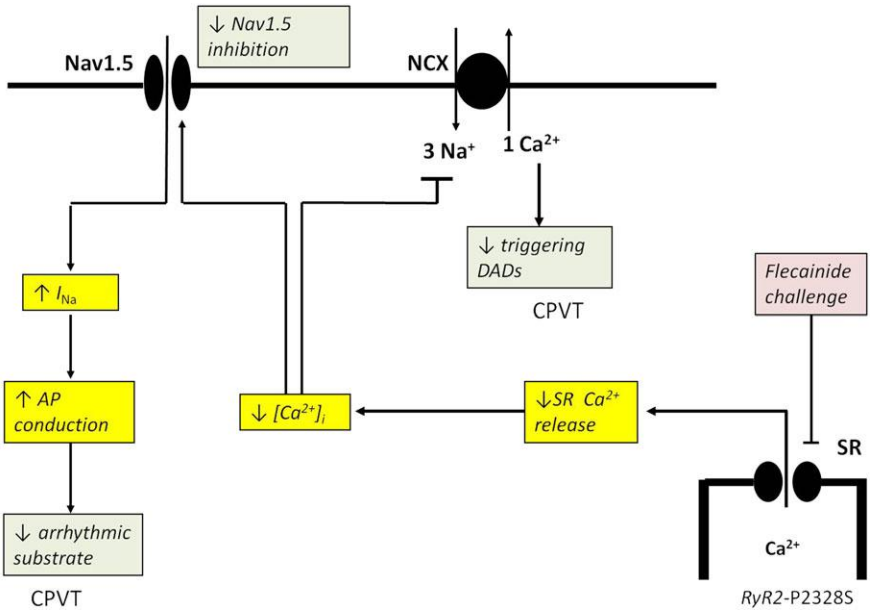

Figure 8

Feed-backward effects of flecainide on arrhythmic substrate attributable to its possible actions on RyR2-Ca^{2+} release channels. A model invoking *RyR2*-P2328S channels as a primary pharmacological target for flecainide, in addition to $Na_v1.5$ channels, may account for its effect in diminishing arrhythmic substrate. Increased RyR2-mediated SR Ca^{2+} leak, associated with *RyR2*-P2328S, down-regulates Na_v 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 function of $Na_v1.5$ channels, restoring I_{Na} and thereby AP conduction and reduces arrhythmic substrate.

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 (Figure 7B, right panel) (Salvage *et al.*, 2015).

Figure 8 summarizes these effects of flecainide upon arrhythmic substrate in terms of a hypothesis invoking *RyR2*-P2328S as a primary pharmacological target in addition to $Na_v1.5$ channels. It represents an increased RyR2-mediated SR Ca^{2+} leak associated with the *RyR2*-P2328S variant as exerting down-regulatory effects upon Na_v 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 function of $Na_v1.5$ channels, restoring I_{Na} and thereby rescuing 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 $Na_v1.5$ and RyR2-Ca^{2+} channel blocking effects of flecainide and propafenone (Figure 1A, B), in contrast to selective effects of tetracaine (Figure 1C), and lignocaine and mexiletine (Figure 1D,C) on RyR2 and $Na_v1.5$ channels 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 organization. There are also complexities in the interactions of flecainide with its primary target, the $Na_v1.5$ channels, as well as other possible cellular targets, in particular the RyR2-Ca^{2+} release channels. Nevertheless, flecainide appears to act specifically through accessing a cytoplasmic binding site on $Na_v1.5$ channels in their activated, open state. This results in a use-dependent antagonism. It also acts on other, K_v 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 the $Na_v1.5$ channel traps flecainide within its pore. Na_v channel function itself involves an activation, which triggers the action potential upstroke and inactivation that influences both recovery from excitation and the refractory period. An early peak I_{Na} related to Na_v channel *activation* followed by rapid de-activation drives AP upstrokes and *propagation*. Peak I_{Na} is diminished in pro-arrhythmic conditions reflecting *loss* of function in $Na_v1.5$ channels in experimental genetic exemplars for BrS. Experimental data

confirms predictions that these conditions would be *exacerbated* by the inhibition of $Na_v1.5$ channels, following flecainide challenge. In contrast, the experimental data demonstrate that pro-arrhythmic phenotype effects attributed to abnormalities in AP *recovery,* due to increased I_{NaL} following the *gain-of-function* modifications in $Na_v1.5$ channels, 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 Na_v 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 Na_v 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 Na_v 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.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b,c,d).

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Conflict of interest

The authors declare no conflicts of interest.

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