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LETTER

Reply from Pei-Chi Yang, Jonathan D. Moreno, Mao-Tsuen Jeng, Xander H. T. Wehrens, Sergei Noskov and Colleen E. Clancy

We appreciate Williams *et al.* (2016) taking the time to comment on our recently published study (Yang *et al.* 2016). In their letter, the authors question the 'usefulness' of the computational modelling and simulation approaches that we used in part because as they state, 'The blocking parameters used in Yang *et al.* (2016) are based on values reported in Hilliard *et al.* (2010) and subsequent publications from the same group.'

This statement does not reflect the careful process that we actually used in building our modelling approaches, where we rather considered the full range of experimentally measured IC_{50} values for flecainide interaction that have been reported in multiple studies. In addition to the assumption of $IC_{50} = 0$ μM (i.e. no interaction with RyR) as reported by the Williams group (Bannister *et al.* 2015), we reported the following in our paper (Yang *et al.* 2016): 'Isoproterenol-stimulated Ca^{2+} waves in CASQ2 knockout (KO) CASQ2(−/−) mice were inhibited by flecainide with an IC_{50} of 2.0 ± 0.2 μM (Hwang *et al.* 2011), while other experimental preparations measured an IC_{50} range from 2 to 17 μM (Brunton *et al.* 2010; Hilliard *et al.* 2010; Hwang *et al.* 2011; Mehra *et al.* 2014) ... We also predicted cases for variable flecainide $IC_{50} = 3$, 4, and 5 μM shown in Fig. 1.'

The model simulations led to the predictions that IC_{50} values above 5 μM are too low to show therapeutic benefit to normalize the catecholaminergic polymorphic ventricular tachycardia (CPVT) phenotype. An alternative interpretation is that the concentration of flecainide near the receptor is considerably higher than in the bulk water compartments, a possibility supported by our physics-based approach (Fig. 5 in Yang *et al.* 2016) that shows accumulation of flecainide on the membrane surface and very favourable conditions for neutral flecainide in the hydrophobic core of the membrane. Detailed investigations into membrane partitioning of drugs are ongoing in our group.

The point of the simulations in our study was to make predictions about the necessary and sufficient targets of flecainide and the range of IC_{50} that would allow for normalization of the CPVT phenotype since the experimental literature has shown such variety in reported values. When we started the investigation reported in Yang *et al.* (2016), we had no preconceived intent or notion about the results. The predictions are the resulting outputs of the model, and suggest that Na^+ channel block alone is not sufficient to prevent the CPVT phenotype. The critical point here is that the disparity in sensitivity of the dose–response for flecainide interaction with the RyR depends on the experimental approach being used. This issue has been the subject of discussion by others (Steele *et al.* 2013; Sikkel *et al.* 2013*b*; Smith & MacQuaide, 2015).

Williams *et al.* describe their recent work in their letter. It is important to mention, however, the numerous other studies that report alternative data and explanations. Some in native myocytes show very clear effects of flecainide on spontaneous Ca^{2+} release (i.e. Ca^{2+} waves) under experimental conditions where cytosolic $[Ca^{2+}]$ and $[Na^+]$ are clamped, demonstrating a direct action of flecainide on RyR2-mediated sarcoplasmic reticulum (SR) Ca^{2+} release (Savio-Galimberti & Knollmann, 2015; Hilliard *et al.*, 2010; Galimberti & Knollmann, 2011). Moreover, in native myocytes, flecainide does not inhibit physiological Ca^{2+} current-induced SR Ca^{2+} release but only inhibits spontaneous SR Ca^{2+} release, which occurs in the setting of diastolic $[Ca^{2+}]$ (i.e. 100 nM) (Hilliard *et al.* 2010). Such conditions are difficult to model using RyR2 channels incorporated into artificial bilayers and hence were never tested by the group of Williams *et al.* Other studies demonstrate a clear benefit of flecainide in the clinical CPVT setting, but not in experiments with other Na^+ channel blockers (Watanabe *et al.* 2009; Hwang *et al.* 2011; van der Werf *et al.* 2011).

Williams *et al.* performed single-channel experiments in an experimental model comprising phosphatidylethanolamine (PE) bilayers to show that flecainide does not block ion current by binding to a site within the cytosolic domain of the pore-forming domain of RyR2. However, other data and the physics-based computational approaches in our paper suggest that lipophilic drug access may be critical and is a vital component of drug interactions with membrane protein targets such as RyR2. The potential of mean force calculations we performed in our study suggest that flecainide concentration in the lipid phase could be substantially greater than what would be expected in the bilayer studies. Carvedilol is another example of a very hydrophobic/lipophilic drug that interacts with RyR2 without blocking unitary conductance in single-channel experiments. Liposome partitioning experiments suggest that up to 90% of carvedilol molecules are lipid-phase localized (Cheng *et al.* 1996). The lipophilic access mechanism would imply different dose–response ratios and use-dependent features of drug interaction with the RyR2 target in contrast to a single-site drug block mechanism endorsed by Williams *et al.* It is important to point out that lipophilic access mechanisms have been shown recently for various membrane targets found in the heart (Lees-Miller *et al.* 2015; Boiteux *et al.* 2014) and are likely to exist for RyR2 given the lipophilicity of many drugs interacting with this channel.

Williams *et al.* have undertaken valuable biophysical studies using purified recombinant channels in artificial lipid bilayers. We argue, however, that such a system is far removed from the physiological reality and cannot unequivocally prove the absence of a flecainide interaction with RYR2 channels in a native cellular environment. For example, Cannon *et al.* (2003) reconstituted RyR2 into a bilayer composed by 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) showing that channel activity depends critically on the bilayer composition. Another study showed that the polyunsaturated fatty acid eicosapentanoic acid (EPA) exerts its antiarrhythmic effect by reducing the opening probability of RyR2 (Swan *et al.* 2003). This is important, because the artificial bilayer used by Williams *et al.* was composed of 100% (PE), but the actual SR lipid content from dog hearts showed the presence of triglycerides, cholesterol and other phospholipids like phosphatidylinositol (PI), phosphatidylcholine (PC), sphingomyelin (SM) and phosphatidylserine (PS). Most of these

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lipids have been found to regulate the gating (and hence the activity) of other channels as well (Suh & Hille, 2008).

Williams and coauthors also mention the potential for I_{Na} block to result in reduced junctional Ca^{2+} concentration through modulated I_{NCX} activity. As has been discussed previously (Steele *et al.* 2013), the experimental conditions used in Sikkel *et al.* (2013*a*) employed fast pacing that is well known to cause Na^+ loading and resultant Ca^{2+} loading that can trigger sparks and waves. Early experimental and computational studies support this mechanism and showed that Na^+ channel blockers are effective to suppress these events (Leblanc & Hume, 1990; Faber & Rudy, 2000). In our models, fast pacing rates also caused Na^+ accumulation (Morotti *et al.* 2014, Shannon *et al.* 2004), but I_{Na} block only led to modest reduction in junctional $[Na^+]$ and thus $[Ca^{2+}]$.

In conclusion, the study by Williams *et al.* has shown that flecainide does not inhibit recombinant RyR2 channels in artificial bilayers by the pore channel block that they observed. Given the contrasting plethora of evidence from other experimental work and our modelling studies predicting that flecainide inhibition of Na^+ currents alone is insufficient to explains its efficacy in CPVT, we contend that further studies are warranted to reveal the mechanism of flecainide action on RyR2, which thus far remains elusive and may not be discoverable using a reductionist approach alone.

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References

Bannister ML, Thomas NL, Sikkel MB, Mukherjee S, Maxwell CE, MacLeod KT, George CH & Williams AJ (2015). The mechanism of flecainide action in CPVT does not involve a direct effect on RyR2. *Circ Res* **116**, 1324–1335.

Boiteux C, Vorobyov I, French RJ, French C, Yarov-Yarovoy V & Allen TW (2014). Local anesthetic and antiepileptic drug access and binding to a bacterial voltage-gated sodium channel. *Proc Natl Acad Sci USA* **111**, 13057–13062.

Brunton LL, Chabner BA & Knollmann BC (2010). *Goodman and Gilman's The Pharmacological Basis of Therapeutics.* McGraw-Hill Professional, New York.

Cannon B, Hermansson M, Gyorke S, Somerharju P, Virtanen JA & Cheng KH (2003). Regulation of calcium channel activity by lipid domain formation in planar lipid bilayers. *Biophys J* **85**, 933–942.

Cheng HY, Randall CS, Holl WW, Constantinides PP, Yue TL & Feuerstein GZ (1996). Carvedilol-liposome interaction: evidence for strong association with the hydrophobic region of the lipid bilayers. *Biochim Biophys Acta* **1284**, 20–28.

Faber GM & Rudy Y (2000). Action potential and contractility changes in $[Na^+]_i$ overloaded cardiac myocytes: a simulation study. *Biophys J* **78**, 2392–2404.

Galimberti ES & Knollmann BC (2011). Efficacy and potency of class I antiarrhythmic drugs for suppression of Ca^{2+} waves in permeabilized myocytes lacking calsequestrin. *J Mol Cell Cardiol* **51**, 760–768.

Hilliard FA, Steele DS, Laver D, Yang Z, Le Marchand SJ, Chopra N, Piston DW, Huke S & Knollmann BC (2010). Flecainide inhibits arrhythmogenic Ca^{2+} waves by open state block of ryanodine receptor Ca^{2+} release channels and reduction of Ca^{2+} spark mass. *J Mol Cell Cardiol* **48**, 293–301.

Hwang HS, Hasdemir C, Laver D, Mehra D, Turhan K, Faggioni M, Yin H & Knollmann BC (2011). Inhibition of cardiac Ca^{2+} release channels (RyR2) determines efficacy of class I antiarrhythmic drugs in catecholaminergic polymorphic ventricular tachycardia. *Circ Arrhythm Electrophysiol* **4**, 128–135.

Leblanc N & Hume JR (1990). Sodium current-induced release of calcium from cardiac sarcoplasmic reticulum. *Science* **248**, 372–376.

Lees-Miller JP, Guo JQ, Wang YB, Perissinotti LL, Noskov SY & Duff HJ (2015). Ivabradine prolongs phase 3 of cardiac repolarization and blocks the hERG1 (KCNH2) current over a concentration-range overlapping with that required to block HCN4. *J Mol Cell Cardiol* **85**, 71–78.

Mehra D, Imtiaz MS, van Helden DF, Knollmann BC & Laver DR (2014). Multiple modes of ryanodine receptor 2 inhibition by flecainide. *Mol Pharmacol* **86**, 696–706.

Morotti S, Edwards AG, McCulloch AD, Bers DM & Grandi E (2014). A novel computational model of mouse myocyte electrophysiology to assess the synergy between Na+ loading and CaMKII. *J Physiol* **592**, 1181–1197.

Savio-Galimberti E & Knollmann BC (2015). Channel activity of cardiac ryanodine receptors (RyR2) determines potency and efficacy of flecainide and R-propafenone against arrhythmogenic calcium waves in ventricular cardiomyocytes. *PLoS One* **10**, e0131179.

Shannon TR, Wang F, Puglisi J, Weber C & Bers DM (2004). A mathematical treatment of integrated Ca dynamics within the ventricular myocyte. *Biophys J* **87**, 3351–3371.

Sikkel MB, Collins TP, Rowlands C, Shah M, O'Gara P, Williams AJ, Harding SE, Lyon AR & MacLeod KT (2013*a*). Flecainide reduces Ca^{2+} spark and wave frequency via inhibition of the sarcolemmal sodium current. *Cardiovasc Res* **98**, 286–296.

Sikkel MB, Collins TP, Rowlands C, Shah M, O'Gara P, Williams AJ, Harding SE, Lyon AR & MacLeod KT (2013*b*). Triple mode of action of flecainide in catecholaminergic polymorphic ventricular tachycardia: reply. *Cardiovasc Res* **98**, 327–328.

Smith GL & MacQuaide N (2015). The direct actions of flecainide on the human cardiac ryanodine receptor: keeping open the debate on the mechanism of action of local anesthetics in CPVT. *Circ Res* **116**, 1284–1286.

Steele DS, Hwang HS & Knollmann BC (2013). Triple mode of action of flecainide in catecholaminergic polymorphic ventricular tachycardia. *Cardiovasc Res* **98**, 326–327.

Suh BC & Hille B (2008). PIP2 is a necessary cofactor for ion channel function: how and why? *Annu Rev Biophys* **37**, 175–195.

Swan JS, Dibb K, Negretti N, O'Neill SC & Sitsapesan R (2003). Effects of eicosapentaenoic acid on cardiac SR Ca^{2+}-release and ryanodine receptor function. *Cardiovasc Res* **60**, 337–346.

van der Werf C, Kannankeril PJ, Sacher F, Krahn AD, Viskin S, Leenhardt A, Shimizu W, Sumitomo N, Fish FA, Bhuiyan ZA, Willems AR, van der Veen MJ, Watanabe H, Laborderie J, Haissaguerre M, Knollmann BC & Wilde AA (2011). Flecainide therapy reduces exercise-induced ventricular arrhythmias in patients with catecholaminergic polymorphic ventricular tachycardia. *J Am Coll Cardiol* **57**, 2244–2254.

Watanabe H, Chopra N, Laver D, Hwang HS, Davies SS, Roach DE, Duff HJ, Roden DM, Wilde AA & Knollmann BC (2009). Flecainide prevents catecholaminergic polymorphic ventricular tachycardia in mice and humans. *Nat Med* **15**, 380–383.

Williams AJ, Bannister ML, Lowri Thomas N, Sikkel MB, Mukherjee S, Maxwell C, MacLeod KT & George CH (2016). Questioning flecainide's mechanism of action in the treatment of catecholaminergic polymorphic ventricular tachycardia. *J Physiol* **594**, 6431–6432.

Yang PC, Moreno JD, Miyake CY, Vaughn-Behrens SB, Jeng MT, Grandi E, Wehrens XH, Noskov SY & Clancy CE (2016). In silico prediction of drug therapy in catecholaminergic polymorphic ventricular tachycardia. *J Physiol* **594**, 567–593.

Additional information

Competing interests

None declared.

Author contributions

All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.