Mechanisms of Cardiac Ryanodine Receptor Inhibition by Anti Arrhythmic Drugs

By
Divya Rajendra Mehra
Bachelor of Pharmacy

Thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy (PhD) in Human Physiology
January 2014

School of Biomedical Sciences and Pharmacy
University of Newcastle
Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

Divya Rajendra Mehra

University of Newcastle
Statement of Authorship

I hereby certify that the work embodied in this thesis contains a published paper/s/scholarly work of which I am a joint author. I have included as part of my thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publications/scholarly work.

Divya Rajendra Mehra

University of Newcastle
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List of Publications

Peer Reviewed Publications

- Hyun Seok Hwang, Can Hasdemir, Derek Laver, Divya Mehra, Kutsal Turhan, Michela Faggioni, Huiyong Yin, Bjorn C. Knollmann: Inhibition of Cardiac Ca$^{2+}$ Release Channels (RyR2) Determines Efficacy of Class I Antiarrhythmic Drugs in Catecholaminergic Polymorphic Ventricular Tachycardia. *Circ Arrhythm Electrophysiol* 2011;4;128-135

- Divya Mehra, Mohammad Imtiaz, Dirk van Helden, and Derek Laver. Flecainide and magnesium block of the ryanodine receptor of the heart. Manuscript for submission to *British Journal of Pharmacology*. (Manuscript in preparation)

- Divya Mehra, Mohammad Imtiaz, Dirk van Helden and Derek Laver. Class I anti arrhythmic drug blocking kinetics of the ryanodine receptor of the heart. Manuscript for submission to *Molecular Pharmacology*. (Manuscript in preparation)

- Divya Mehra, Mohammad Imtiaz, Dirk van Helden and Derek Laver. Effect of K201, carvedilol and verapamil blocking kinetics on the ryanodine receptor of the heart. (Manuscript in preparation)

Conference Oral Presentations

2012 Divya Mehra, Dirk van Helden, Bjorn Knollmann and Derek Laver; talk on “Role of cardiac Na$^+$ channel blockers and Mg$^{2+}$ in inhibiting the cardiac calcium release channel” abstract accepted for presentation at *Australian Physiological Society* on 2nd Dec at Sydney.

2011 Divya Mehra, Dirk van Helden, Bjorn Knollmann and Derek Laver; talk on “Role of cardiac Na$^+$ channel blockers in inhibiting the cardiac calcium release channel” at *Australian Physiological Society* at Perth.

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abstract selected for Poster presentation on the 6th March at the Biophysical Society Meeting at Baltimore. Finalist for the SRAA event.

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2010 Divya Mehra, Dirk van Helden, Bjorn Knollmann, Hyun S. Hwang and Derek Laver; “Inhibition of cardiac ca\(^{2+}\) release channels as therapy for arrhythmia” Poster presentation on the 29th Nov at the Australian Physiological Society/Australian Society for Biophysics meeting at Adelaide.

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2010 Divya Mehra, Dirk van Helden, Bjorn Knollmann and Derek Laver; “Flecainide blocks Ca\(^{2+}\) release channels associated with CPVT induced cardiac arrhythmias” abstract selected for poster presentation on the 31st Jan. at Australian Neuroscience Society / Australian Physiological Society scientific meeting at Sydney.

2010 Divya Mehra, Hyun Seok Hwang, Huiyong Yin, Sung I Kim, Bjorn C. Knollmann, Derek Laver; “Propafenone and flecainide inhibit cardiac ryanodine receptors and prevent catecholaminergic polymorphic ventricular tachycardia (CPVT) in mice” at the 20th World Congress of the International Society for Heart research due on 13th May.

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2012 Awarded the Travel Scholarship Grant at the Gordon Research Conference: Excitation Contraction coupling meeting Switzerland in June.

2010 Awarded Travel Grant at the Australian Physiological Society/Australian Society of Biophysics meeting, Adelaide.

2010 Awarded the Travel Grant at the International Society of Heart Research Conference at Kyoto, Japan.

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<td>ATP</td>
<td>adenosine-triphosphate</td>
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<tr>
<td>BAPTA</td>
<td>1,2- bis(2-aminophenoxy) ethane- N,N,N’-N’- tetraacetic acid</td>
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<tr>
<td>CICR</td>
<td>Ca$^{2+}$ - induced Ca$^{2+}$ release</td>
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<tr>
<td>CASQ2</td>
<td>calsequestrin</td>
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<tr>
<td>CH$_3$O$_3$S</td>
<td>methanesulfonate</td>
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<tr>
<td>EC-coupling</td>
<td>excitation-contraction coupling</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione disulfide</td>
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<tr>
<td>IC$_{50}$</td>
<td>half-inhibiting concentration</td>
</tr>
<tr>
<td>K$_i$</td>
<td>half-inhibiting concentration</td>
</tr>
<tr>
<td>mM</td>
<td>minimolar (mmol/l)</td>
</tr>
<tr>
<td>ms</td>
<td>millisecond</td>
</tr>
<tr>
<td>NaF</td>
<td>sodium fluoride</td>
</tr>
<tr>
<td>NaN$_3$</td>
<td>sodium azide</td>
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<tr>
<td>NCX</td>
<td>Na$^+$ / Ca$^{2+}$ exchanger</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar (nmol/l)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>P$_o$</td>
<td>open probability</td>
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<td>PC</td>
<td>1-palmitoyl-2-oleoyl-sn-Glycero-3-phosphocholine</td>
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<td>PE</td>
<td>1-palmitoyl-2-oleoyl-sn-Glycero-3-phosphoethanolamine</td>
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<tr>
<td>pS</td>
<td>picosiemens</td>
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<tr>
<td>RyR2</td>
<td>cardiac ryanodine receptor</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
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<tr>
<td>s$^{-1}$</td>
<td>1/second</td>
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### Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SAN</td>
<td>sinoatrial node</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic/endoplasmic reticulum Ca(^{2+})-ATPase</td>
</tr>
<tr>
<td>TES</td>
<td>N-tris[hydroxymethyl-2-aminoethanesulfonic acid]</td>
</tr>
<tr>
<td>T(_o)</td>
<td>mean open time</td>
</tr>
<tr>
<td>T(_c)</td>
<td>mean closed time</td>
</tr>
<tr>
<td>(\mu l)</td>
<td>microliter</td>
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<tr>
<td>(\mu M)</td>
<td>micromolar ((\mu)mol/l)</td>
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Abstract

The cardiac ryanodine receptors (RyR2) are the calcium release channel in the sarcoplasmic reticulum (SR). Mutations in RyR2 or calsequestrin are known to cause Catecholaminergic polymorphic ventricular tachycardia (CPVT), an arrhythmia occurs in periods of emotional stress or exercise. Previous work showed that a Class I anti arrhythmic drug, flecainide blocked RyR2, thus reducing the spontaneous Ca\(^{2+}\) release that causes arrhythmia in CPVT. Tetracaine is a classical RyR2 blocker and it might be expected that block of RyR2 via tetracaine could potentially be a solution to arrhythmia due to SR overload. However, contrary to this tetracaine is pro-arrhythmic. This project investigates the mechanisms of RyR2 inhibition by anti arrhythmic drugs with a view to identifying inhibitory mechanisms that are antiarrhythmic. I investigated RyR2 inhibition including nine Class I agents-Na\(^+\) channel blockers, Class II-\(\beta\) blocker carvedilol, Class IV-Ca\(^{2+}\) channel blocker verapamil and K201 (JTV519).

RyR2 was isolated from sheep heart, incorporated in lipid bilayers and investigated by single-channel recordings in presence of diastolic Ca\(^{2+}\) (100 nM cytoplasmic) and systolic Ca\(^{2+}\) (100 \(\mu\)M cytoplasmic). All drugs showed inhibition from both cytoplasmic and luminal sides of the membrane consistent with the ability of the drugs to permeate through the bilayer. Two inhibition modes on RyR2 with distinct kinetics were detected,

1) induction of brief closed events with a mean duration of \(\sim 0.5-4\) ms referred to as the fast block,

2) induction of long closed events with a mean duration of \(\sim 20-600\) ms referred as the slow block.

Binding rates for both forms of block were proportional to concentration and unbinding rates were concentration independent, consistent with bimolecular binding. Drug binding was strongly voltage-dependent (more potent at positive membrane potential) consistent with movement of the cation form of the drug in the trans membrane electric field.

All drugs showed fast block RyR2 but they varied substantially in their potency. The association rates of the drugs fell into two broad classifications. Group A: Flecainide, propafenone, quinidine, encainide, verapamil, K201 and carvedilol have a fast association (exceeding a threshold of \(2 \mu\text{M}^{-1}\text{s}^{-1}\)). The association rates for Group B: mexiletine, procainamide, disopyramide, pilscainide and tocainide lie below this threshold. Drugs in Group A were seen in previous studies to reduce SR Ca\(^{2+}\) release while those in Group B did not. Moreover, the

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potency of drug block of RyR2 correlated with their IC$_{50}$ for Ca$^{2+}$ wave suppression, spontaneous Ca$^{2+}$ wave rate and the proportion of animals with ventricular tachycardia after exercise.

Fast block for Class I drugs and K201 was to a substate exhibiting 20-50% of full open state conductance. Procainamide and verapamil caused a blocked state, with conductance indistinguishable from baseline. K201 causes two substates with conductances of 40 and 30 % of the open state (+40 mV). Kinetics of block was consistent with substate (or substates in the case of K201) due to binding of one molecule and complete block by a second molecule.

Only flecainide, carvedilol, K201 and verapamil showed slow block. The slow block was amplified by conditions that cause lower levels of channel open probability such as the case with 1 mM Mg$^{2+}$ or Ca$^{2+}$ in the cytoplasm. The binding rate for slow block increased proportionally with channel closed probability indicating a high preference for the closed state of the channel. The duration of the slow block seen for carvedilol, verapamil and K201 (~ 40 ms) was very different to that for flecainide (500 ms).

Class I agents are use-dependent blockers of the Na$^+$ channel as they preferentially bind to these channels in their inactivated (closed) state. Activation of Na$^+$ channels (i.e. their use) leads directly to their inactivation. This work shows that flecainide, K201, verapamil and carvedilol also bind to the RyR2 in its closed state. However, in the physiological context, use of the RyR2 (i.e. Ca$^{2+}$ release) depends on the channel being open so that use of the RyR2 results in loss of drug effect even though in both cases, the drug binds preferentially to the closed channel. The term inverse-use dependence may be used to describe drugs in Group A with respect to their RyR2 inhibition.

This study illustrates two mechanisms: fast and slow block of RyR2 that are specific for each class of drug. Our results show that Mg$^{2+}$, at physiological concentrations, makes flecainide, K201, carvedilol and verapamil a more potent inhibitor of RyR2 by inducing the slow inhibition mechanism. At +40 mV, slow mechanism for all four agents have ~ two-fold lower IC$_{50}$ (41, 10, 17 and 45 µM, respectively) than the fast mechanism (70, 24, 30, 90 µM, respectively). Differences lie in the off rate of flecainide compared to tetracaine, a classical RyR2 blocker. Flecainide off rate is 2 s$^{-1}$ and tetracaine is 20 s$^{-1}$. This could have a direct implication on the control of RyR2-mediated SR Ca$^{2+}$ release and on how the release is terminated on a beat to beat basis given the self-regenerating nature of Ca$^{2+}$ induced Ca$^{2+}$ release.