DEFINING THE MOLECULAR MECHANISMS OF SUBTYPE-SPECIFIC KCNQ2/3 POTASSIUM CHANNEL ACTIVATORS

by

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Abstract

Retigabine is the first approved anti-epileptic drug that acts via activation of voltage-gated potassium channels, targeting KCNQ channels that underlie the neuronal M-current. Retigabine exhibits little specificity between KCNQ2-5, which all contain a Trp residue in the pore domain that is essential for retigabine actions. The retigabine analog ICA 069673 (‘ICA73’) exhibits much stronger effects than retigabine on KCNQ2 channels, including a large hyperpolarizing shift of the voltage-dependence of activation, and roughly two-fold enhancement of peak current. Unlike retigabine, ICA73 exhibits strong subtype specificity for KCNQ2 over KCNQ3, and appears to have a unique mechanism of action, because pore mutations that abolish retigabine action (KCNQ2 Trp236Phe) do not affect ICA73 sensitivity. Based on ICA73 sensitivity of chimeric constructs of the transmembrane segments of KCNQ2 and KCNQ3, this drug appears to interact with the KCNQ2 voltage sensor (S1-S4) rather than the pore region targeted by retigabine. KCNQ2 point mutants in the voltage sensor were generated based on KCNQ2/KCNQ3 sequence differences, and screened for ICA73 sensitivity. These experiments reveal that KCNQ2 residues Phe168 and Ala181 in the S3 segment are essential determinants of ICA73 subtype specificity. Mutations at either position in KCNQ2 abolish the ICA73-mediated gating shift, while retaining retigabine sensitivity. Interestingly, KCNQ2[A181P] mutant channels show little ICA73-mediated gating shift, but retain current potentiation by the drug. When Phe168 and Ala181 are substituted into KCNQ3 ([L198F] and [P211A]), ICA73-sensitivity can be partially rescued. These results demonstrate that retigabine and
ICA73 act via distinct mechanisms, and provide the first insights into channel residues that underlie subtype specificity of KCNQ channel openers. Further mutagenic scanning of the voltage sensor, and screening for potential ICA73 binding residues in solvent accessible pockets have also generated new insights into KCNQ channel function, despite not identifying additional residues essential for ICA73 sensitivity. Taken together, findings presented in this thesis have laid a foundation for further understanding of diverse mechanisms of action of KCNQ potassium channel openers, which may lead to more targeted and rational approaches for drug design.
Preface

I conducted the majority of the experiments and analyzed the data presented in this thesis. Several constructs generated were through the help of our lab technician, Dr. Runying Yang. Data presented in Chapter 3 has been submitted for publication in April 2016, while data presented in Chapter 4 is part of several ongoing studies related to KCNQ openers in Dr. Kurata’s lab.
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<tr>
<td>AIS</td>
<td>axon initial segment</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>VSD</td>
<td>voltage-sensing domain</td>
</tr>
<tr>
<td>PGD</td>
<td>pore-gating domain</td>
</tr>
<tr>
<td>LQTS</td>
<td>long QT syndrome</td>
</tr>
<tr>
<td>SQTS</td>
<td>short QT syndrome</td>
</tr>
<tr>
<td>DFNA2</td>
<td>dominant non-syndromic form of hearing loss type 2</td>
</tr>
<tr>
<td>BFNS</td>
<td>benign familial neonatal seizures</td>
</tr>
<tr>
<td>PNH</td>
<td>peripheral nerve hyperexcitability</td>
</tr>
<tr>
<td>Kᵥ</td>
<td>voltage-gated potassium (channel)</td>
</tr>
<tr>
<td>AED</td>
<td>anti-epileptic drug</td>
</tr>
<tr>
<td>RTG</td>
<td>retigabine</td>
</tr>
<tr>
<td>ICA73</td>
<td>ICA-06973</td>
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Chapter 1: Introduction and Background

1.1 Kv Overview

Potassium channels are fundamental entities in physiology as K⁺ ions are essential for proper functioning of the heart, kidneys, muscles, nerves, and digestive system (Pischalnikova and Sokolova, 2009). These channels conduct K⁺ ions across the membrane, along the electrochemical gradient for K⁺. A variety of cellular processes rely on appropriate K⁺ channel function, including maintenance of resting membrane potential, regulation of cell volume, hormone secretion, and modulation of electrical impulses (Hille, 2001; Pischalnikova and Sokolova, 2009). There are 4 major families of potassium channels: voltage-gated (Kᵥ), Ca²⁺-activated (KᵥCa), inward-rectifying (Kᵢr), and two-pore (Kᵢp2), with Kᵥ being the largest of the families, encompassing 40 genes and further divided into 12 subfamilies (Gutman et al., 2005). As the names may suggest, the families differ by how they are gated, and this diversity enables channel responses to a variety of physiological stimuli (MacKinnon, 2003).

The open probability (Pₒ) of Kᵥ channels depends on the membrane voltage, with channels normally closed at resting voltages around -70 to -90 mV and opened upon membrane depolarization (Sahoo et al., 2014). With this property, Kᵥ channels play significant roles in regulating neuronal and cardiac tissue excitability (Hille, 2001). However, they can also be found in neuroendocrine and endocrine cells, skeletal muscle, placenta, lung, liver, and kidney tissues (Ju and Wray, 2002). In excitable cells, Kᵥ channels can counteract electrical excitation by stabilizing or setting the resting membrane potential; they can also regulate action potential shape.
or frequency (Sahoo et al., 2014). In contrast, K_v channels may also act as K^+ transport vehicles in non-excitable cells (Sahoo et al., 2014). It is not surprising that with their wide range of roles that mutations in K_v channels can result in many severe and inherited diseases such as episodic ataxia type 1, deafness, epilepsy, or cardiac arrhythmia (Pischalnikova and Sokolova, 2009).

1.1.1 Overall Structure of K_v Channels

K_v channels can be composed of α and β subunits, with conduction of K^+ across the lipid bilayers occurring through the integral membrane α subunit (Biggin et al., 2000). A functional K_v channel is tetrameric, with 4 identical or related subunits (MacKinnon, 1991; Li et al., 1992). While tetramerization is promoted by the N-terminus in K_v1-4 channels, it is established by the C-terminus in K_v7 and K_v11 channels (Biggin et al., 2000). Each monomer contains 6 TM helices (S1-S6); the central pore domain is made up of S5 and S6 from all 4 subunits, which is surrounded by 4 VSD’s composed of S1-S4 (Swartz, 2004; Tombola et al., 2005). A small portion of each VSD contacts the pore domain of the neighbouring subunit via its S4-S5 linker that runs underneath the neighbouring subunit (Tombola et al., 2005).

1.1.2 Ion Selectivity of K_v Channels

With their complex yet delicate architecture, K_v channels can conduct K^+ ions with 1000-fold selectivity over Na^+ ions even though their atomic radii are not significantly different: 1.33Å and 0.95Å, respectively (MacKinnon, 2003). The central ion conduction pathway is encircled by the 4 subunits that make up the pore, with each subunit contributing two transmembrane α-helices and a tilted pore helix that runs half way through the membrane, pointing its C-terminal negative ‘end-charge’
dipole towards the ion pathway (Doyle et al., 1998; Zhou et al., 2001). A central water-filled cavity sits near the midpoint of the membrane, where the ion pathway is almost 10Å in diameter (Doyle et al., 1998; Zhou et al., 2001). K⁺ ions are thought to be stabilized at the centre of the membrane by hydration and the negative end-charge of the α-helices directed towards the pathway (Roux and MacKinnon, 1999). While this has been a generally accepted model, it has also been shown that substituting positively charged residues at those central positions did not affect potassium channel function, deeming the pore helix dipole effect to be less prominent (Chatelain et al., 2005).

At the extracellular third of the ion pathway, K⁺ ions are selected by the selectivity filter, which has a conserved signature sequence of GYG or GFG (Doyle et al., 1998; Biggin et al., 2000; MacKinnon, 2003). With 4 evenly spaced layers of carbonyl oxygen atoms and a single layer of threonine hydroxyl oxygen atoms, 4 K⁺ binding sites are formed (MacKinnon, 2003). Since the arrangement is similar to being surrounded by water molecules, K⁺ ions can be dehydrated and bind at low energy cost, with octahedral coordination by 4 oxygen atoms above, and 4 below (MacKinnon, 2003). The high selectivity of K⁺ over Na⁺ occurs because the selectivity structure is dependent on the presence of K⁺; conformational change induced by K⁺ allows stronger binding (MacKinnon, 2003). In addition, the high conduction rate of K⁺ through the channel is due to repulsion that occurs between closely spaced K⁺ ions. 2 ions are present in the filter at a given time; as an ion enters from one side of the filter, repulsion causes another to exit from the opposite side (MacKinnon, 2003).
1.1.3 Kv Channel Gating and Voltage-Sensing

Like K+ selectivity, gating works similarly in all potassium channel families. In response to a stimulus such as a change in membrane voltage for Kv channels, conformational changes in the transmembrane voltage-sensing domain occur to facilitate or trigger pore opening (MacKinnon, 2003). The gate of the channel is located at the intracellular end of the pore and its open and closed conformations are controlled by a conserved glycine gating hinge; the inner helices can obstruct the pore in the closed state and rotate to expand the intracellular diameter in the open state (MacKinnon, 2003; Grottesi et al., 2005). In Kv channels, the gate is linked indirectly to the voltage sensor by the α-helical S4-S5 linker that contacts the S6 helix, allowing coupling of gate conformation to voltage-sensor movement (Grottesi et al., 2005). As the channel opens, charged amino acids on the S4 helix, known as gating charges, move through the membrane electric field and couple electrical work to opening of the channel pore (Schoppa et al., 1992; Sigworth, 1994; Bezanilla, 2000). The total gating charge in Shaker channels is estimated to be approximately 14 elementary charges (the sum of the charged sidechains and the net fraction of the transmembrane voltage difference that they cross) (Schoppa et al., 1992; Aggarwal and MacKinnon, 1996; Seoh et al., 1996). It has been demonstrated that the first 4 arginine residues of the S4 helix (located every third position on the helix), contribute most significantly to this gating charge (Tombola et al., 2005).

When the membrane depolarizes, the voltage sensor in each subunit experiences a voltage-dependent transition from a resting (R) state to an activated (A) state, permitting the pore to open (Bezanilla et al., 1994; Zagotta et al., 1994). As
all 4 subunits transition into the A state, the pore gate opens cooperatively through a concerted transition which is weakly voltage-dependent (Ledwell and Aldrich, 1999). While the full mechanism of the voltage-sensing and gating coupling is still being investigated, it has been suggested that residues at the external side of the pore domain form important contacts that ‘brace’ the voltage sensor domain (Lee et al., 2009b), while a cluster of residues formed between the internal side of the S4 and S5 helices of adjacent subunits are crucial for the concerted opening transition (Soler-Llavina et al., 2006).

1.2 KCNQ Overview

The Kv7 family, also known as the KCNQ family, has 5 known members (Kv7.1-7.5 or KCNQ1-5); they generally function to stabilize a negative resting membrane potential and oppose electrical excitability (Robbins, 2001; Delmas and Brown, 2005). KCNQ channels have a canonical voltage-gated potassium channel structure, a tetramer with six transmembrane domains in each subunit. Unlike many other Kv families, Kv7 subunits lack the N-terminal T1 domain that is responsible for tetramerization in Kv1-4 (Xu et al., 1995; Long et al., 2005). Instead, Kv7 subunits possess a unique tetramerization domain in the C-terminus which has no homology with the T1 domain (Schwake et al., 2006; Howard et al., 2007). In addition, Kv7 subunits share a conserved domain in the proximal C-terminal region near S6, which contains positively charged residues that are thought to bind membrane lipid phosphatidylinositol 4,5 bisphosphate (PIP2) (Delmas and Brown, 2005; Hernandez et al., 2008b).

KCNQ channels are widely expressed in both neuronal and non-neuronal
tissues (Cooper, 2011). While KCNQ2-5 were first discovered in neurons where they play important roles in neurotransmitter-stimulated action potential firing, KCNQ1 was originally recognized for its role in cardiac myocytes that contributes to cardiac action potential repolarization, and is not believed to be significantly expressed in neurons (Jespersen et al., 2005; Mackie and Byron, 2008). It has now been shown that there are also KCNQ channels in vascular smooth muscle cells from several vascular beds where they assist with vascular tone regulation (Mackie and Byron, 2008).

Functionally, KCNQ2-5 subtypes form subunits of the low-threshold voltage-gated potassium channel that had been named the “M-channel” (Adams and Brown, 1980; Brown, 1988). They activate at subthreshold potentials, starting at around -60 mV, do not inactivate like many other Kv channels, and generate a steady voltage-dependent outward current as a result (Brown and Passmore, 2009). Moreover, activation of these channels is relatively slow (Brown, 1988). Neuronal KCNQ channels can exert a significant dampening effect on repetitive burst-firing and general excitability of neurons (Brown, 1988). Not surprisingly, KCNQ2 and KCNQ3 channels, the main neuronal players in the family, are expressed densely in the axon initial segments (AIS) where an action potential is initiated; they also co-localize with sodium channels through the binding to the cytoskeletal protein ankyrin G and can thus regulate the action potential threshold (Chung et al., 2006; Rasmussen et al., 2007). Through multiple signaling pathways, neuronal KCNQ channels can control somatic excitability, bursting, and neurotransmitter release throughout the nervous system (Hernandez et al., 2008b).
1.2.1 M-current

KCNQ2-5 members underlie the M-current. The name of this slowly activating and deactivating potassium current is due to its suppression by stimulation of muscarinic acetylcholine receptors now known to be coupled to PIP2 hydrolysis through the Gq signaling cascade (Adams and Brown, 1980; Alfonso et al., 1997; Wang et al., 1998). In the 1960's and 1970's, it was shown that in sympathetic, cortical, and hippocampal neurons, muscarinic acetylcholine receptor agonists induced slow membrane depolarization and decreased potassium conductance (Kobayashi and Libet, 1968; Weight and Votava, 1970; Krnjevic et al., 1971; Kuba and Koketsu, 1976; Dodd et al., 1981). In 1980, the M-current was finally identified as the voltage-sensitive K+ current that was responsible for the decreased potassium conductance after muscarinic receptor stimulation (Adams and Brown, 1980). Activated at relatively negative potentials, around -60 mV, close to the resting potential for many excitable cells, the M-current generates a resting outward current that influences cellular electrical excitability (Mackie and Byron, 2008). As well, this time- and voltage-dependent K+ current does not inactivate and can activate further upon membrane depolarization, acting as a brake for neuronal firing (Hernandez et al., 2008b; Maljevic et al., 2010). While it is still commonly referred to as the M-current, it is now known that the current is not exclusively regulated by muscarinic receptor activation, but also by activation of other G-protein coupled receptors (Mackie and Byron, 2008). In addition, the inhibition of the M-current appears to be an indirect response to the stimulation of these receptors (Marrion, 1997). The activation of Gq receptors leads to the hydrolysis of PIP2 in the membrane catalyzed by phospholipase
This reduction of PIP₂ levels in the membrane has been found to cause closure of the M-channel (Brown and Passmore, 2009). Furthermore, PIP₂ is required for the activation of the channel, with its major binding site being a cluster of basic amino acids in the C-terminus of KCNQ channels (Hernandez et al., 2008b).

1.2.2 KCNQ Channels and Disease

1.2.2.1 KCNQ1

The first member of the KCNQ family, KCNQ1, was identified by positional cloning on chromosome 11p15.5 in families with Long QT syndrome Type I (Wang et al., 1996). It was found that KCNQ1 α-subunits co-assemble with KCNE β-subunits to form functional channels conducting the slow cardiac delayed rectifier K⁺ current, Iₖₛ (Barhanin et al., 1996; Sanguinetti et al., 1996). In the heart, Iₖₛ plays an important role in the repolarization of the cardiac late-phase action potential (Maljevic et al., 2010).

Interestingly, KCNQ1 is the only member of the family that is unable to form hetero-tetramers with other KCNQ subunits (Maljevic et al., 2010). While KCNQ1 can form homomeric channels without the KCNE1 β-subunits, giving rise to fast-activating potassium currents, co-assembly with KCNE1 yields slow-activating currents with increased macroscopic amplitudes (Barhanin et al., 1996; Sanguinetti et al., 1996). The ratio of the co-assembly between KCNE1 and KCNQ1 is still an ongoing debate (Nakajo et al., 2010; Yu et al., 2013; Plant et al., 2014; Murray et al., 2016).

Although KCNQ1 may be most well-known for its activity in the heart, KCNQ1/KCNE1 channels are also expressed in various areas including the stria vascularis of the inner ear, small intestine, pancreas, thyroid gland, forebrain neuronal networks, brainstem nuclei, lungs, GI tract, and the ovaries (Jespersen et al.,
2005; Goldman et al., 2009). In the inner ear, KCNQ1 is thought to conduct K+ current into the scala media, where K+-rich endolymph solution is generated (Jespersen et al., 2005). In the small intestine tip cells, KCNQ1 was found to regulate sodium-coupled glucose uptake (Jespersen et al., 2005). Moreover, KCNQ1/KCNE1 channels are also located in the proximal and distal tubules of the nephron and seem to be important for kidney function (Maljevic et al., 2010).

1.2.2.1.1 Long QT Syndrome

Long QT syndrome (LQTS) affects 1/3000 of the general population and is characterized by an increased duration of the QT interval in the electrocardiogram, a reflection of delayed cardiac repolarization (Robbins, 2001; Zareba and Cygankiewicz, 2008). It can be acquired or congenital, and sufferers usually show few symptoms until certain events such as strenuous exercise, stress, or drug usage (Viskin, 1999). It has the propensity to trigger a characteristic re-entrant arrhythmia (torsades de pointes), which may lead to ventricular tachycardia that can self-terminate or degenerate into lethal ventricular fibrillation (Robbins, 2001). The symptoms can include palpitations, syncope, aborted cardiac arrest, and sudden death or cardiac arrest (Zareba and Cygankiewicz, 2008).

While there are 12 known types of LQTS, the most common type is LQT1, which makes up about 50% of the LQTS patients (Robbins, 2001; Zareba and Cygankiewicz, 2008). Over 240 different known mutations of the KCNQ1 gene have been identified in patients with this disease (Lundby et al., 2010). An autosomal dominant form of LQT1, Romano-Ward syndrome, is caused by the presence of non-functional mutant channels that also inhibit the function of any wild type subunits by preventing
assembly (Chouabe et al., 1997; Wollnik et al., 1997). As well, there is an autosomal recessive type of LQT1, Jervell and Lange-Nielsen syndrome (JLNS). In its heterozygous form, there is weak cardiac dysfunction, but in its homozygous form, there is severe cardiac dysfunction as well as bilateral deafness (Robbins, 2001).

1.2.2.1.2 Short QT Syndrome

At the other end of the spectrum, patients with Short QT syndrome (SQTS) have a constantly short QT interval on their ECG (Zareba and Cygankiewicz, 2008). Although this condition is far rarer than LQTS, patients with no underlying structural heart disease can experience atrial fibrillation, syncopal episodes, as well as sudden cardiac death (Zareba and Cygankiewicz, 2008). One of the 5 types of SQTS, SQT2, is caused by gain-of-function KCNQ1 mutations that increase $I_{Ks}$ and accelerate cardiac repolarization (Zareba and Cygankiewicz, 2008).

1.2.2.2 KCNQ2 & KCNQ3

The KCNQ2 and KCNQ3 genes were discovered using two approaches: screening of a human brain cDNA library with a KCNQ1-derived sequence and positional cloning in families affected by benign familial neonatal seizures (BFNS) (Biervert et al., 1998; Charlier et al., 1998; Singh et al., 1998; Yang et al., 1998). Both KCNQ2 and KCNQ3 subunits have been found in most regions of the brain, including the cortex, cerebellum, basal ganglia, and hippocampus (Maljevic et al., 2010). While the expression pattern of these channels changes during development, they are mainly located at the axon initial segments (AIS) (Devaux et al., 2004; Maljevic et al., 2008).

KCNQ2 and KCNQ3 channels give rise to non-inactivating potassium currents that activate slowly upon depolarization (Maljevic et al., 2010). While KCNQ2
homomeric channels can generate current, KCNQ3 homomeric channels cannot. Heteromeric KCNQ2/KCNQ3 channels produce currents that are greater in magnitude than the homomeric KCNQ2 channels, with possible varying expression ratios of the two subunits (Schroeder et al., 1998). It has been shown that a pore alanine residue (315) in KCNQ3 that is otherwise a threonine in the other KCNQ channels, causes KCNQ3 to be retained in the endoplasmic reticulum, rather than trafficked to the cell membrane (Gomez-Posada et al., 2010). KCNQ3 with the A315T mutation can form homomeric channels and generate currents that are very large in magnitude (Gomez-Posada et al., 2010). These observations have led to the understanding that KCNQ3 channels are intrinsically unstable and therefore depend on heteromerization with KCNQ2 for function (Gomez-Posada et al., 2010). Mutations in KCNQ2 and KCNQ3 can cause BFNS, peripheral nerve hyperexcitability (PNH), and epileptic encephalopathy (Hart et al., 2002; Maljevic et al., 2010; Wang et al., 2015).

1.2.2.2.1 BFNS

BFNS is a rare autosomal dominant epilepsy syndrome with a penetrance of more than 80% (Maljevic et al., 2010). The condition starts within the first days of birth with frequent and often clustered partial and secondary generalized seizures (Maljevic et al., 2010). While these indications disappear spontaneously after a few weeks or months, there is about a 15% risk of recurring seizures later in life for these patients (Maljevic et al., 2010).

Although there are more than 30 known BFNS mutations, the majority are found in KCNQ2, with only 4 in KCNQ3 (Coppola et al., 2003; Neubauer et al., 2008). Various types of mutations in KCNQ2 including truncations, splice site defects, missense,
nonsense, or frame-shift mutations have been found to cause BFNS (Lee et al., 2009a). Pore mutations do not seem to cause a dominant-negative effect on wild-type KCNQ2 channels, but KCNQ2 G271V in the pore has been found to cause infantile seizures (Wang et al., 2015). Not surprisingly, BFNS mutations found in the S4 of KCNQ2 affect channel gating and increase the threshold for channel activation (Maljevic et al., 2010). Mutations found in the C-terminus, on the other hand, tend to impair surface expression by different mechanisms such as reduced protein stability or ability to bind calmodulin, thereby affecting transport to the membrane surface (Maljevic et al., 2010).

Intriguingly, seizures in BFNS typically only occur during the neonatal period. One possible explanation for this phenomenon is that expression of KCNQ2 and KCNQ3 may increase with maturation (Maljevic et al., 2008). In addition, there may be a developmental switch from GABAergic excitatory to inhibitory actions, which may be vital for controlling transient generation of seizures; during the neonatal period, the M-current from KCNQ2 and KCNQ3 channels may be the only available inhibitory channels to act as a brake to neuronal excitability (Okada et al., 2003; Safiulina et al., 2008).

**1.2.2.2 Peripheral Nerve Hyperexcitability (PNH)**

Since KCNQ2 channels are located in both the central and peripheral nervous systems, mutations can also result in PNH (Maljevic and Lerche, 2014). Patients with PNH suffer from spontaneous and continuous muscle overactivity, with undulating movements of the distal skeletal muscle, fasciculations, cramps, and other indications generated by hyperexcitability of peripheral motor neurons (Hart et al., 2002). So far,
only 2 mutations in KCNQ2 have been associated with PNH and they are both found at arginine207 in the S4 voltage sensor (Maljevic and Lerche, 2014).

### 1.2.2.2.3 Epileptic Encephalopathy

Finally, mutations in KCNQ2 have also been found to cause epileptic encephalopathy with cognitive impairment. Patients present with a diversity of syndromes characterized by early occurrence of seizures that are correlated with impaired neurological development; they have pharmaco-resistant neonatal onset of seizures with a strong tonic component (Maljevic and Lerche, 2014). Although by age 3, seizures typically stop, profound intellectual disability and motor impairment persist (Weckhuysen et al., 2012; Milh et al., 2013; Weckhuysen et al., 2013). For this more severe disease, the mutations found so far affect functionally crucial parts of the KCNQ2 channel: the S4 voltage-sensor, pore, and C-terminus (Maljevic and Lerche, 2014). Therefore, it is evident that decreased M-current at the start of life not only can cause seizures, but also affect normal neuromotor development (Maljevic and Lerche, 2014).

### 1.2.2.3 KCNQ4 & KCNQ5

KCNQ4 was cloned by mapping to the DFNA2 locus for a form of nonsyndromic dominant deafness (Kubisch et al., 1999). The majority of KCNQ4 channels have been found in outer hair cells of the inner ear, and specifically the Type I hair cells of the cochlea (Kubisch et al., 1999; Kharkovets et al., 2000). In addition, KCNQ4 is located in the vestibular apparatus and a number of nuclei in the central auditory pathway (Kharkovets et al., 2000). During development, the change in expression pattern of outer hair cells is in parallel with the onset of hearing, alluding to the potential effect
of KCNQ4 on electrical properties of the outer hair cells as well as in lowering intracellular potassium concentrations (Maljevic et al., 2010).

Last but not least, KCNQ5 has been cloned (Schroeder et al., 2000; Lerche et al., 2000). However, not much is known about the channel other than that it forms heteromeric channels with KCNQ2-4. Moreover, no disease has been found to be associated with the gene so far.

More recently, KCNQ 1, 4, and 5 have been found to express in vascular and nonvascular smooth muscles (Greenwood and Ohya, 2009; Stott et al., 2014). It is speculated that these channels can serve as potential therapeutic targets for diseases such as irritable bowel syndrome, constipation, bladder instability, asthma, and pre-term labour due to their role in regulating nonvascular smooth muscle activity (Stott et al., 2014).

1.2.2.3.1 DFNA2

DFNA2 is an autosomal dominant disease that causes hearing loss at high frequencies to occur in patients’ twenties and thirties and can progress to more than 60 dB with middle and low frequencies, within 10 years (Nie, 2008). So far, 8 missense mutations and 2 deletions in the KCNQ4 gene have been found in DFNA2 patients with diverse clinical phenotypes (Nie, 2008). While there are still much to be known about this channel and disease, it has been shown that patients with missense mutations tend to have earlier onset of and all-frequency hearing loss (Nie, 2008). In contrast, patients with deletion mutations seem to have later onset and pure high-frequency hearing loss (Nie, 2008). A possible explanation of the relation of KCNQ4 malfunction to the disease is that loss of KCNQ4 currents may contribute to chronic
K⁺ overload in the outer hair cells, leading to degeneration and progressive hearing loss (Kharkovets et al., 2006). It has been shown that KCNQ channel openers such as retigabine can rescue some DFNA2 mutations, shedding light on potential treatment of DFNA2 and other forms of deafness involving hair cell loss (Leitner et al., 2012).

1.3 PIP₂ Regulation of KCNQ

1.3.1 PIP₂

Since ion channels are expressed in the cell membrane, it is important to note the roles in which phospholipids play in regulating channels. One specific type of phospholipid in the plasma membrane, phosphatidylinositol 4,5-bisphosphate (PIP₂), has been known to bind to and regulate a diversity of ion channels (Hilgemann and Ball, 1996; Hilgemann et al., 2001; Suh and Hille, 2005; Suh and Hille, 2008). An anionic lipid located in the inner leaflet of the surface membrane, PIP₂ makes up a small fraction (<1%) of the entire pool of phospholipids (McLaughlin et al., 2002; Rusten and Stenmark, 2006). It is synthesized in two steps from phosphatidylinositol (PI): sequential phosphorylation steps by PI 4-kinase and PI(4)P5 kinase at the plasma membrane (Falkenburger et al., 2010).

Importantly, PIP₂ acts as the principal substrate of phospholipases (PLC), which are stimulated by over 50 hormone receptors that couple to the G protein Gq, and some receptor tyrosine kinases (Falkenburger et al., 2010). At the plasma membrane, PLC cleaves PIP₂ into two potent second messengers, lipid diacylglycerol and soluble inositol 1,4,5-trisphosphate (Falkenburger et al., 2010). The latter triggers release of Ca²⁺ from intracellular stores, which leads to further downstream cascades (Falkenburger et al., 2010).
1.3.2 PIP$_2$ Effects on KCNQ Channels

Members of the KCNQ family absolutely require PIP$_2$ for conducting current, as demonstrated in a variety of experiments (Zaydman and Cui, 2014). While it was previously known that the M-current is inhibited by muscarinic receptor activation, it is now believed that this is due to $G_q$-PLC activation leading to PIP$_2$ hydrolysis (Suh and Hille, 2002; Zhang et al., 2003; Suh et al., 2004). Therefore, it is not surprising that PIP$_2$ regulation of KCNQ channels is physiologically important in neurons as it plays a role in neuroexcitability (Delmas and Brown, 2005; Brown et al., 2007). In addition, mutations in KCNQ1 that are associated with Long QT syndrome have been found to disturb PIP$_2$-dependent activation (Park et al., 2005; Logothetis et al., 2010; Zaydman et al., 2013). Therefore, understanding how PIP$_2$ interacts with KCNQ channels is essential for learning further about channel function and mechanisms.

1.3.3 Potential PIP$_2$ Binding Sites

In terms of PIP$_2$ structure, the lipid phosphate group appears to be key in sustaining current, but not the net headgroup charge, the acyl chain length, nor the effects of lipids on membrane curvature (Schmidt et al., 2006). While there seems to be a shared and conserved PIP$_2$ binding site at the VSD-PGD interface (Zaydman and Cui, 2014), PIP$_2$ does not appear to directly affect VSD activation and there is no firm evidence on whether or not PIP$_2$ influences the PGD opening in KCNQ channels (Zaydman and Cui, 2014). At the VSD-PGD interface, charge neutralizing and charge reversing mutations of basic residues decrease channel open probability while simultaneously decreasing apparent affinity to exogenous PIP$_2$ (Hernandez et al., 2008a). Furthermore, experiments have been conducted in KCNQ2 to suggest that
PIP₂ preferentially interacts with the S4-S5 linker during the open state and with the S2-S3 loop in the closed state, both of which are in the VSD-PGD interface (Zhang et al., 2013). Nevertheless, much has yet to be discovered about PIP₂ and its interactions with KCNQ channels; in the meantime, the essential role of PIP₂ must be considered when studying KCNQ in order to yield meaningful results and interpretations.

1.4 KCNQ Channel Openers

Epilepsy is one of the most common and serious chronic neurological disorders, and affects about 1% of the population worldwide (Sander, 2003). This disorder is diverse in presentation and can be generally characterized by abnormal synchronous and rhythmic neuronal activity causing repetitive epileptic seizures (Orhan et al., 2012). Since the discovery of anticonvulsant properties of phenobarbital in 1912, there has been ongoing development of antiepileptic drugs (AEDs), although roughly 30% of all epileptic patients remain resistant to current pharmacotherapy (Brodie and French, 2000; Kwan and Brodie, 2010). To date, several important mechanisms of action of AEDs have been uncovered, including blockage of voltage-gated sodium or calcium channels, enhancement of gamma-aminobutyric acid (GABA)-mediated inhibitory neurotransmission, or attenuation of glutamate-mediated excitatory neurotransmission (Perucca, 2005).

More recently, the fundamental role of KCNQ channels as regulators of neuronal excitability has become more evident, given their ability to maintain the cell’s resting membrane potential and reduce sub-threshold excitability in the brain (Gunthorpe et al., 2012). Therefore, the development of retigabine (RTG) as a first-in-class potassium channel opener AED has shed light on new approaches for treatment
of epilepsy.

1.4.1 Development of RTG

The structure of retigabine, or ezogabine (the US adopted name), was designed based on two drugs known to modulate K,7 channels, linopirdine and flupirtine (Aiken et al., 1995). While the former is a selective K,7 channel blocker and exhibits pro-convulsive and cognitive enhancing effects, the latter is the first K,7 channel enhancer used as an analgesic and shows anticonvulsant properties (Aiken et al., 1995). Retigabine (RTG), chemically identified as ethyl N-[2-amino-4-[4-fluorophenyl]methylamino]phenyl] carbamate, demonstrated the greatest anticonvulsant potency among several structural analogues of flupirtine that were synthesized (Orhan et al., 2012). Developed in the 1980’s, the efficacy of RTG shown in preclinical models supported its use, especially with the discovery and cloning of the new KCNQ family, found to be genetically linked to BFNS (Biervert et al., 1998;Charlier et al., 1998;Singh et al., 1998). Following two large-scale Phase III double-blind placebo-controlled trials in patients with partial (focal) epilepsy, the use of RTG as a drug and KCNQ channels as a drug target for epilepsy were validated (Brodie et al., 2010;French et al., 2011). Finally, the approval of RTG by both the US FDA and European Union for adjunctive treatment of partial onset seizures in adults arrived after more than 20 years of preclinical and clinical research (Gunthorpe et al., 2012). However, at this point, RTG is not yet licensed for other forms of epilepsy or diseases (Orhan et al., 2012).

1.4.1.1 Mechanism of RTG

RTG has been demonstrated through in vitro studies to activate K,7.2/7.3
channels and thereby enhance K⁺ conductance. In electrophysiological experiments, the most pronounced effect of RTG is a marked concentration-dependent hyperpolarizing shift in the voltage-dependent activation curve of KCNQ channels (Gunthorpe et al., 2012). This activation of the M-current results in membrane hyperpolarization towards the potassium equilibrium potential, which can negatively modulate neuronal firing rates and oppose neuronal hyperexcitability as seen in epileptic seizures (Orhan et al., 2012). While RTG can increase K⁺ current by increasing the open probability of KCNQ channels, it does not seem to alter single-channel conductance of individual KCNQ2/3 channels (Gunthorpe et al., 2012). Instead, RTG increases the rate of channel activation and decelerates channel closure (Main et al., 2000; Wickenden et al., 2000; Tatulian et al., 2001). It is important to note that RTG can activate Kᵥ7.2-7.5 channels but not the Kᵥ7.1 cardiac isoform at human therapeutic serum levels, which reduces the risk for potential cardiac adverse effects (Orhan et al., 2012).

Through many studies, the molecular mechanism of RTG has been elucidated. A conserved Trp residue in the S5 of Kᵥ7.2-7.5 members has been found to be required for RTG’s action. A recent study established that the effect relies on a molecule of RTG forming an H-bond with this pore region residue (Kim et al., 2015).

1.4.1.2 Side Effects of RTG

While RTG is the first and best-characterized Kᵥ7 channel opener at the moment, serving as an important chemical tool to study the therapeutic potential of Kᵥ7 channel modulation, it indeed has room for improvement (Tatulian et al., 2001; Tatulian and Brown, 2003). RTG potentiates channels other than KCNQs, such
as GABA-receptors, and has little subtype specificity between different Kv7 family members (Gribkoff, 2003; van Rijn and Willems-van, 2003). Thus, various retigabine side effects have arisen, including undesired effects in the CNS and in smooth muscle. For example, dizziness, somnolence, fatigue, hallucinations, confusion, and speech disorders have been reported in certain patients (Orhan et al., 2012). As well, negative effects on the urological system leading to urinary retention has been a recurring problem in patients (Streng et al., 2004). It has also been reported that RTG can induce blue-grey mucocutaneous discoloration of the skin, nail, oral mucous membrane, conjunctiva, and retina (Garin et al., 2014). Therefore, various new Kv7 channel openers have been synthesized and hopefully new compounds will address shortcomings attributed to RTG.

1.4.2 Other KCNQ Openers

Besides RTG, other compounds have been identified with potentiating activity on Kv7 channels. Since the development of RTG, a number of pharmaceutical companies have used the structure of RTG as a template for constructing new and improved Kv7 openers (Castle, 2010). Several classes of compounds such as oxindole analogues (eg. BMS-204352) are potent Kv7.2-7.5 activators while others like R-L3 activate Kv7.1 (Xiong et al., 2008). As well, fenamates including diclofenac, meclofenamic acid, NH6, and NH29, some widely used NSAIDs that non-selectively inhibit COX-1 and COX-2, are relatively potent Kv7.2/7.3 activators (Munster et al., 2002). Zinc pyrithione (ZnP), used for controlling dandruff and treating psoriasis, has also been shown to be a strong Kv7 activator, potentiating all Kv7 channels except Kv7.3 (Xiong et al., 2007). Icagen has synthesized various pyridinyl benzamide
compounds such as ICA-27243, ICA-110381, and ICA-069673, which appear to selectively open certain Kv7 channels over others (Castle, 2010).

1.4.2.1 New Potential Mechanisms of KCNQ Openers

While it is validated that RTG acts through the Trp residue in the pore region of KCNQ2-5, some of the new KCNQ channel openers appear to act through a different mechanism and binding site. This is suggested as various compounds including ZnPy, NH29, and the Icagen compounds still activate the Trp to Leu/Phe mutant that is insensitive to RTG (Xiong et al., 2007; Peretz et al., 2010; Boehlen et al., 2013; Padilla et al., 2009). Furthermore, a number of these compounds such as NH29 and the Icagen compounds have been suggested to interact with the VSD rather than the pore region of KCNQ (Padilla et al., 2009; Peretz et al., 2010).

1.5 Overview of Chapter 3: Sequence Determinants of Subtype-specific Actions of KCNQ Channel Openers

In this study, an extensive analysis of the compound ICA-06973 (referred to as ICA73 from here onwards) has been done to determine its mechanism of action that selectively activates KCNQ2 channels but not KCNQ3 channels. Through the construction of chimeric channels between KCNQ2 and KCNQ3 as a starting point, we noticed that ICA73 interacted with the VSD of KCNQ2 (in particular, the S3-S4 region) for its effect, rather than with the pore region as RTG does. Using a non-radioactive rubidium efflux assay as a screening tool and whole-cell patch clamping, we sifted through numerous mutants in the S3-S4 to pinpoint the key residues that allow ICA73 to produce its substantial effect on KCNQ2 but not KCNQ3. We identified two mutations in the S3, F168L and A181P, that significantly reduced ICA73 effect. While
the F168L mutation completely abolished the ICA73 effect, the A181P mutation exhibited a surprising effect where the channel loses most of the hyperpolarizing shift by ICA73 but not the current potentiation. In addition, we found that RTG could still activate these two mutants, suggesting two different mechanisms of action for RTG and ICA73. This hypothesis is confirmed as KCNQ2[W236F], a mutant that is insensitive to RTG, can be fully activated by ICA73.

1.6. Overview of Chapter 4: In-depth Analysis of Voltage Sensor Residues Involved in ICA73 Effects on KCNQ2

While Chapter 3 focused on S3-S4 residues in KCNQ2 and KCNQ3 required for ICA73’s dramatic effect on KCNQ2, the first half of the VSD (S1-S2) may also be involved in the effect as seen in the rubidium efflux assay. Since the S1-S2 sequence of KCNQ2 and KCNQ3 differ sizably, Q2 + Q3[S1] and Q2 + Q3[S2] chimeras, in addition to point and cluster mutations were made to tease out potential residues that may contribute to the ICA73 effect. Patch clamp experiments did not find particular residues that were specific to reducing ICA73 effect.

To further decipher how ICA73 may be binding to KCNQ2, molecular simulations were carried out to look at solvent accessible pockets that could make up the potential binding site. Electrophysiology indicated that mutating E130, an important residue in voltage-gated potassium channels that acts as a voltage sensor counter charge, ablates the ICA73 hyperpolarizing shift, but not the current potentiation effect. This observation has not been noted in previous studies. Numerous details in this study as well as the previous chapter have opened new insight on KCNQ channel openers and their heterogeneity as well as how slight
differences in KCNQ2-5 structures can allow drugs to target specific subtypes. This can aid in future drug design which may eliminate non-specific targets and thus reduce side effects.
Chapter 2: Materials and Methods

2.1 KCNQ2 and KCNQ3 Channel Constructs

Mutant channels were derived from human KCNQ2 or KCNQ3 genes (originally in pTLN vector-gifts from Dr. Taglialatela and Dr. T. Jentsh), expressed in pcDNA3.1 (-) plasmid (Invitrogen, Carlsbad, CA). KCNQ3* channels refer to KCNQ3[A315T], carrying a point mutation that allows homomeric expression of KCNQ3 (Gomez-Posada et al., 2010). Chimeras between KCNQ2 and KCNQ3 were constructed using an overlapping PCR method. Flanking primers were used to amplify respective segments of KCNQ2 and KCNQ3. PCR approaches were then used to sequentially combine overlapping fragments until all necessary segments of the chimera were incorporated. The break points for the chimeras generated were as follows. For Q2+Q3[S1-S2] channels, KCNQ2 residues 89-148 were substituted with KCNQ3 residues 117-178. For Q2+Q3[S3-S4] channels, KCNQ2 residues 153-207 were substituted with KCNQ3 residues 183-236. For Q2+Q3[S5-S6] channels, KCNQ2 residues 239-324 were substituted with KCNQ3 residues 268-363. For Q2+Q3[S1] channels, KCNQ2 residues 89-115 were substituted with KCNQ3 residues 117-145. For Q2+Q3[S2] channels, KCNQ2 residues 115-149 were substituted with KCNQ3 residues 145-178. Point mutants in KCNQ2 were constructed using a 2-step overlapping PCR method. All constructs were subcloned into pcDNA3.1(-) using NheI and EcoRI restriction enzymes and verified by Sanger sequencing approaches (Genewiz or University of Alberta Applied Genomics Core).
2.2 Cell Culture and Whole-cell Patch Clamp Recordings

HEK293 cells were cultured in 50 mL polystyrene tissue culture flasks (Falcon) in DMEM (Invitrogen) supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were grown in an incubator at 5% CO₂ and 37 °C. Cells were plated into 6-well plates and co-transfected with plasmids encoding the channel of interest and GFP using jetPRIME DNA transfection reagent (Polyplus Transfection). After 24 hours of incubation with transfection reagent, cells were split onto sterile glass coverslips and electrophysiological experiments were conducted 1 day later.

Whole-cell patch clamp recordings were performed using extracellular solution consisting of 135 mM NaCl, 5 mM KCl, 2.8 mM NaAcetate, 1 mM CaCl₂(2H₂O), 1 mM MgCl₂(6H₂O), and 10 mM HEPES, with pH adjusted to 7.4, and intracellular solution containing 135 mM KCl, 5 mM EGTA, and 10 mM HEPES, with pH adjusted to 7.3. Retigabine and ICA069073 were stored as 100 mM stocks in DMSO, and were diluted to working concentrations in extracellular solution on each experimental day. Glass pipette tips were manufactured using soda lime glass (Fisher Scientific) and had open tip resistances of 1-3 MΩ using the standard experimental solutions. Recordings were filtered at 5 kHz and sampled at 10 kHz using a Digidata 1440A (Molecular Devices) controlled by the pClamp 10 software (Molecular Devices). Experimental compounds were purchased from Toronto Research Chemicals (retigabine) or Tocris (ICA-069673).

2.3 Two-electrode Voltage Clamp Recordings

Complementary RNA was transcribed from the cDNA of several constructs using the mMessage mMACHINE Kit (Ambion). Stage V–VI Xenopus laevis oocytes were
prepared as previously described and were injected with cRNA. We used female Xenopus laevis frogs 100 g or greater in size and the oocytes were prepared using a protocol approved by the University of British Columbia Animal Care Committee, in accordance with the Canadian Council for Animal Care guidelines. Oocytes were incubated post injection for 12–96 h at 18 °C before recording. We recorded voltage-clamped potassium currents in standard Ringers solution (in mM): 116 NaCl, 2 KCl, 1 MgCl₂, 0.5 CaCl₂, 5 HEPES (pH 7.4) using an OC-725C voltage clamp (Warner, Hamden, CT). Glass microelectrodes were backfilled with 3 M KCl and had resistances of 0.1–1 MΩ. Data were filtered at 5 kHz and digitized at 10 kHz using a Digidata 1440A (Molecular Devices) controlled by the pClamp 10 software (Molecular Devices).

2.4 Non-radioactive Rb⁺ Efflux Assay

HEK293 cells were plated into 24-well plates and co-transfected with channel of interest, IRK1, and GFP protein, using jetPRIME DNA transfection reagent (Polyplus Transfection). Cells were incubated in the presence of transfection reagent for 24 hours and another day in the presence of regular media. 2 days post transfection, cells were loaded with Rb⁺ loading media (1 mM RbCl in DMEM with 10% FBS and 1% penicillin-streptomycin) for 2 hours. This was followed by washing twice with 0 K⁺ buffer (140 mM NaCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 10 mM HEPES at pH 7.4). Cells were then incubated in 600 μL 0 K⁺ buffer, 100 K⁺ activating buffer (40 mM NaCl, 100 mM KCl, 1.2 mM MgSO₄, 2.5 CaCl₂, and 10 mM HEPES at pH 7.4), or 0 K⁺ buffer supplemented with respective concentrations of ICA 73. 200 μL of assay aliquots were removed at multiple time points (5, 10, and 20 minutes) and cells were lysed with 1%
SDS RIPA lysis buffer following completion of assay. Rb⁺ concentration in supernatant was measured by flame atomic absorption spectroscopy using Aurora Biomed ICR8000 instrument. Amount of Rb⁺ efflux was calculated as a fraction of total Rb⁺ loaded (sum of Rb⁺ remaining in lysed cells and Rb⁺ extruded from cells). Normalized Rb⁺ efflux in drug solution as presented in data is calculated by \( \frac{\text{Efflux}_{\text{with drug}} - \text{Efflux}_{\text{in 0 K⁺}}}{\text{Efflux}_{\text{in 100 K⁺}} - \text{Efflux}_{\text{in 0 K⁺}}} \). Only the 20-minute time point data (mean ± s.e.m.) are presented for simplicity.

2.5 Data Analysis

Voltage dependence of channel activation was fitted with a standard single component Boltzmann equation of the form \( \frac{G}{G_{\text{max}}} = 1/(1 + e^{-(V-V_{1/2})/k}) \) where \( V_{1/2} \) is the voltage where channels exhibit half-maximal activation, and \( k \) is a slope factor reflecting the voltage range over which an \( e \)-fold change in \( P_o \) is observed. Note: channels that exhibit a dramatic response to ICA73 were not fitted.
Chapter 3: Sequence Determinants of Subtype-specific Actions of KCNQ Channel Openers

3.1 Introduction

Retigabine (RTG) is a recently approved anti-epileptic drug currently used as an adjunct add-on treatment for pharmaco-resistant epilepsy (Miceli et al., 2008). Retigabine and its closely related analog flupirtine are currently the only voltage-gated potassium channel openers approved for human use. Their mechanism of action is well-described at the molecular level – they are thought to interact with a Trp sidechain located in the pore-forming S5 segment of KCNQ2-5 channel subunits, and cause a pronounced hyperpolarizing shift in the voltage-dependence of channel activation (Schenzer et al., 2005; Lange et al., 2009). Importantly, RTG exhibits little specificity between KCNQ2-5 channel subunits, and off-target effects in peripheral tissues including the bladder have been a recurring problem in patients (Martyn-St et al., 2012; Brickel et al., 2012). However, RTG has served as an important chemical tool to study the therapeutic potential of KCNQ channel modulation, and as a template for the development of further analogs that may exhibit better subunit selectivity and centrally-restricted actions (Xiong et al., 2008).

Following the recognition of the therapeutic benefit of retigabine, a variety of compounds have been identified that act as KCNQ channel openers (Miceli et al., 2011). Several classes of compounds such as oxindole analogues (e.g., BMS-204352) are potent KCNQ2-5 activators while others like R-L3 activate KCNQ1 (Xiong et al., 2007). As well, fenamates including diclofenac and meclofenamic acid, widely used
NSAIDs that non-selectively inhibit COX-1 and COX-2, are also relatively effective activators of KCNQ2 and KCNQ2/3 heteromers (Munster et al., 2002; Peretz et al., 2007; Peretz et al., 2010). Zinc pyrithione (ZnPy), used for controlling dandruff and treating psoriasis, has also been shown to be a strong KCNQ activator, causing potentiation of all KCNQ channel currents except KCNQ3 (Xiong et al., 2007). Some commercially available compounds have been suggested to exhibit some subtype selectivity, including ML-213, and a family of pyridinyl benzamide compounds including ICA-27243, ICA-110381, and ICA-069673 (Wickenden et al., 2008; Gao et al., 2010; Blom et al., 2010).

With this progress in discovery of KCNQ openers, fundamental principles underlying their actions are beginning to be revealed. For example, we recently demonstrated the requirement for a hydrogen bond donor in the KCNQ3 Trp265 (S5 Trp) side chain that is thought to interact with retigabine (Kim et al., 2015). This chemical property correlated with the polarity of a hydrogen bond acceptor carbonyl found in many KCNQ channel openers. Drugs such as ICA-069673, with very weak polarity at this position exhibit very weak effects on KCNQ3 channels. Also, a close structural analog with much stronger bond polarity (ML-213) exhibited much more potent activation of KCNQ3 (Yu et al., 2011). Given this understanding of the chemical basis of drug action at the putative retigabine binding site, we sought to understand reports of the activating effects of ICA-069673 (and other close analogs) on KCNQ channels. Several reports suggested the possibility that certain KCNQ activators may act on a distinct site present in certain KCNQ subtypes. For example, a family of diclofenac derivatives were shown to be insensitive to mutation of the S5 Trp residue.
that is essential for retigabine actions, and may interact with the VSD rather than the pore region of KCNQ (Padilla et al., 2009; Peretz et al., 2010).

In this study, we have exploited the reported subunit specificity of some retigabine analogs to better define the mechanism of action of these drugs, and to identify sequence determinants that underlie subunit specificity. Our study identifies two nearby residues in the voltage-sensing domain of KCNQ2 that are unique to ICA73-sensitive channels, are required for ICA73 actions, and do not markedly alter channel gating or retigabine sensitivity. These residues demonstrate unambiguously that KCNQ channel openers can act independently of the retigabine binding site with a distinct mechanism. Our study also highlights multi-pronged effects of ICA73, which causes a marked hyperpolarizing shift of KCNQ2, and a dramatic potentiation of current. These two effects can be separated by point mutations in the KCNQ2 voltage-sensing domain.

3.2 Results

3.2.1 Subtype-specific Effects of ICA069673

Retigabine is a non-selective activator of KCNQ2-5 channels, with a well-defined binding site in the pore domain (Lange et al., 2009; Kim et al., 2015). Certain KCNQ activators, including ML-213, ICA 069673 (referred to as ICA73 and ICA81, respectively, from here onwards), and Zn-pyrithione, have been reported to show some subtype specificity, although some reports are conflicting and the determinants of this specificity remain unclear (Xiong et al., 2007; Boehlen et al., 2013). For example, we recently observed that ML-213, described as a KCNQ2-specific activator, could also activate KCNQ3* (KCNQ3[A315T], see Methods) channels with greater
potency than retigabine (Yu et al., 2010; Kim et al., 2015). We continued to investigate the effects of other known KCNQ openers on KCNQ2 and KCNQ3* channels (Fig. 3-1A).

Figure 3-1 ICA069673 exhibits subtype-specificity for KCNQ2 over KCNQ3 channels.

(A) Chemical structures of retigabine and ICA069673 (‘ICA73’). (B,C) Conductance-voltage relationships for KCNQ2 and KCNQ3* were generated using recordings from *Xenopus laevis* oocytes expressing each respective channel. KCNQ2 parameters of activation were (control V1/2 = -37 ± 2 mV, k = 10 ± 1 mV; retigabine V1/2 = -61 ± 1 mV, k = 17 ± 1 mV, n = 5). KCNQ3 parameters of activation were (control V1/2 = -43 ± 1 mV, k = 8 ± 1 mV; ICA73 V1/2 = -50 ± 2 mV, k = 11 ± 2 mV; retigabine V1/2 = -103 ± 1 mV, k = 7 ± 1 mV, n = 5).

We recognized that ICA069673 (‘ICA73’) and retigabine have dramatically different effects on KCNQ2 vs. KCNQ3* channels (Fig. 3-1B,C). Consistent with previous reports, retigabine caused a leftward shift of the V1/2 of activation in both KCNQ2 and KCNQ3* (Schenzer et al., 2005). While the effects of retigabine are more pronounced in KCNQ3*, these findings reflect that retigabine is a relatively non-selective KCNQ channel activator. In contrast, 30 µM ICA73 induced a dramatic hyperpolarizing shift of KCNQ2 channel activation, while even higher concentrations
(100 μM) elicited relatively negligible effects on KCNQ3* (Fig. 3-1C). Based on these effects of ICA73, we set out to investigate specific differences between KCNQ2 and KCNQ3* that underlie subtype-specific drug effects. It is noteworthy that some previous studies have highlighted an alternative binding site in the voltage-sensing domain of KCNQ channels for certain KCNQ openers. However, thus far, potential residues identified that influence drug effects tend to be highly conserved and essential for normal activation gating of Kv channels (e.g. KCNQ2 positions E130, F137, and S4 residues L197, R198, and R201), and do not provide a rationale for understanding subtype specific effects (Tao et al., 2010; Pless et al., 2011; Li et al., 2013b; Brueggemann et al., 2014).

3.2.2 Potentiation and Shift in Voltage-dependent Gating of KCNQ2

It should also be noted that ICA73 exhibits two clear effects on KCNQ2 channels: a shift in the voltage-dependence of activation, and a potentiation of current at voltages where channel activation is saturated. These effects are often not obvious in published work due to a common practice of normalizing conductance-voltage relationships. These multiple effects of ICA73 on KCNQ2 are apparent in exemplar traces (Fig. 3-2A,B), and in conductance-voltage relationships normalized to the peak conductance in control conditions (Fig. 3-2C). These data highlight both the shift in voltage-dependence of activation, and the approximate doubling of peak channel current by ICA73 even at voltages that saturate the conductance-voltage relationship. The maximal ICA73-mediated gating shift is much larger than retigabine - so large that channels cannot be closed completely even with very negative voltage pulses (illustrated by sample tail currents at -130 mV, Fig. 3-2D).
3.2.3 ICA73 Acts within the VSD

We investigated the channel elements responsible for ICA73-selective effects on KCNQ2 over KCNQ3, using a chimeric approach of substituting transmembrane domains in KCNQ2 (ICA73-sensitive) with corresponding (ICA73-insensitive) KCNQ3 sequence (Fig. 3-3A-C). Whole-cell patch clamp experiments revealed that substitution of the KCNQ3 pore (S5-S6) into KCNQ2 largely preserved ICA73...
sensitivity, with persistence of both a large hyperpolarizing gating shift and current potentiation by ICA73 (Fig. 3-3F,I). In contrast, substitution of the KCNQ3 S3-S4 sequence into KCNQ2 rendered channels completely insensitive to ICA73, abolishing both the gating shift and current potentiation effects (Fig. 3-3E,H). Finally, substitution of the KCNQ3 S1-S2 domain caused a reduced ICA73 effect that appeared to preserve current potentiation, but abolish most or all of the drug-mediated shift of activation gating (Fig. 3-3D,G). These results together highlight the voltage-sensing domain (S1-S4) as a likely site for ICA73 subtype selectivity (Padilla et al., 2009), with a particular importance of the S3-S4 segments. These findings also highlight that the gating shift and current potentiation effects may be observed separately, as demonstrated by the Q2+Q3[S1,S2] chimera (Fig. 3-3A,D,G)
Figure 3-3 Substitution of KCNQ3 VSD into KCNQ2 alters channel sensitivity to ICA73.

(A-C) Cartoon representations of chimeric channels of KCNQ2 (grey) and KCNQ3 (red). (D-F) Conductance-voltage relationships for (D) Q2+ Q3[S1,S2] (control V1/2 = -9 ± 1 mV, k= 8 ± 1 mV; ICA73 V1/2 = -22 mV ± 5 mV, k = 5 ± 1 mV, n = 5), (E) Q2 + Q3[S3-S4] (control V1/2 = -40 ± 3 mV, k=11 ± 1 mV; ICA73 V1/2 = -39 ± 2 mV, k = 10 ± 1 mV, n = 4), and (F) Q2+ Q3[S5-S6] (control V1/2 = -25 ± 7 mV, k= 11 ± 2 mV; n = 5) normalized to peak conductance in control. (G-I) Sample current traces for G) Q2+ Q3[S1,S2], (H) Q2 + Q3[S3-S4], and (I) Q2+ Q3[S5-S6] illustrating the lack of ICA73 effect when S3-S4 of KCNQ3 is substituted into KCNQ2. In all panels, error bars represent s.e.m.
3.2.4 Identification of Amino Acid Determinants of ICA73 Subtype Specificity

In order to identify residues in the KCNQ2 voltage-sensing domain that confer ICA73 specificity, we used sequence alignments of KCNQ2 and KCNQ3 (Fig. 3-4A) to guide mutagenesis of KCNQ2 (to corresponding KCNQ3 residues) at positions throughout S3 and S4. In addition, due to a large number of residue differences in S1 and S2, we generated two additional chimeras with either S1 or S2 from KCNQ3 substituted into KCNQ2. We employed a non-radioactive Rb⁺ efflux assay to screen these mutants for ICA73 sensitivity (Li et al., 2013a). Since ICA73 effectively activates KCNQ2 channels even at very hyperpolarized potentials (Fig. 3-2C), ICA73-mediated Rb⁺ efflux in hyperpolarizing solutions was used to identify mutants that retain ICA73-sensitivity. Using this approach, we identified the KCNQ2 F168L and KCNQ2 A181P mutations as highly perturbative to ICA73 sensitivity (Fig. 3-4B). It is also noteworthy that the S1 chimera and S2 chimera both retained significant ICA73 sensitivity (in contrast to the S1-S2 chimera described in Fig. 3-3). This finding suggested to us that there may be important interactions between S1 and S2 that play a role in ICA73 sensitivity (Fig. 3-3D,G), but that swapping individual KCNQ2 and KCNQ3 residues may not be a powerful approach to identify these differences. We have focused the remainder of our study on the S3-S4 residues identified in this initial scan, as these are previously unrecognized determinants of subtype specificity of KCNQ activators.
Figure 3-4 Rubidium efflux screen identifies two S3 mutations that diminish ICA73 response.

(A) Alignment of KCNQ2-5 sequences. Differences between KCNQ2 and KCNQ3 were used to identify candidate non-conserved residues in the VSD that may be essential for ICA73 sensitivity. (B) A rubidium efflux assay was used to scan ICA73 responses from mutants as indicated. Normalized ICA73 response was calculated as the ratio of the response in ICA73 (in hyperpolarizing solution), to the maximal rubidium efflux observed in depolarizing solution (see Materials and Methods). (C) Structural representation of a molecular model of a single KCNQ2 subunit highlighting all candidate residues screened (red), F168L and A181P (blue), and Trp236 (yellow).
3.2.5 Aromatic Residues at KCNQ2 Residue 168 Preserve ICA73 Sensitivity

KCNQ2[F168L] channels generated functional currents with activation kinetics and voltage-dependence similar to wild-type KCNQ2 (Fig. 3-5). However, ICA73 had virtually no effect on these channels (Fig. 3-5A-C), consistent with data from Rb⁺ efflux experiments. The F168L mutation abolished both the ICA73-mediated hyperpolarizing gating shift, and current potentiation (Fig. 3-5B). Also, ICA73 had virtually no effect on kinetics of channel closure (Fig. 3-5C). We tested additional aromatic substitutions at position 168 (Fig. 3-5D,E). Conductance-voltage relationships suggest that the aromatic substitutions preserve potentiation and gating effects of ICA73, although the magnitude of ICA73 effects was not as pronounced in the F168Y mutant (this mutant also caused an intrinsic gating shift in the absence of drug, Fig. 3-5D,E). This essential residue is located in an interesting region at the cytoplasmic side of the S3 transmembrane helix (Fig. 3-5F). In previously published models of the KCNQ2 open state (Miceli et al., 2011; Gourgy-Hacohen et al., 2014), F168 is in close proximity with a conserved lysine (KCNQ2 K219) in the S4-S5 linker, and a conserved arginine (KCNQ2 R213) at the cytoplasmic side of the S4 segment. It is also possible that F168 could come into close contact with other S4 charged sidechains in alternative voltage sensor configurations.
Figure 3-5 Functional characterization of KCNQ2[F168L] mutant channels.

(A) Exemplar patch clamp current recordings of KCNQ2[F168L]. Cells were held at -80 mV, and pulsed between -140 mV and +40 mV for 2 s, with tail currents measured at -20 mV. (B) Conductance-voltage relationships of KCNQ2[F168L]. The conductance-voltage relationship in ICA73 is normalized to the peak conductance in control conditions for each individual cell (data are mean ± s.e.m.) (control V1/2 = -13 ± 2 mV, k= 9 ± 1 mV; ICA73 V1/2 = -10 ± 7 mV, k = 15 ± 4 mV, n = 4). (C) Time constants (τ) of channel closure were measured by pulsing to a range of negative voltages from a holding potential of +20 mV; inset, sample tail currents of KCNQ2[F168L] at -130 mV. (D,E) Conductance-voltage relationships of KCNQ2[F168Y] (control V1/2 = -7 ± 5 mV, k= 19 ± 3, n = 4) and KCNQ2[F168W] (control V1/2 = -29 ± 9 mV, k= 17 ± 2 mV, n = 3), collected as in panel (A). (F) Structural model of the KCNQ2 open state, depicted as a ‘bottom view’ of the intracellular side of the channel transmembrane domain. KCNQ2 residue F168 is in close proximity with R213 and K219, suggesting possible interactions that may underlie the role of F168.
3.2.6 Proline at Position 181 in KCNQ2 Disrupts ICA73 Effects

Surprisingly, with more detailed characterization, we observed that the KCNQ2[A181P] retained pronounced potentiation of peak current, but a much smaller shift of voltage-dependent activation (Fig. 3-6A,B). This behavior was similar to the Q2+Q3[S1,S2] chimera (Fig. 3-3A,D,G), illustrating once again that the gating shift and current potentiation effects do not necessarily coincide. Tail current kinetics also illustrate channel susceptibility to the drug, although ICA73-mediated deceleration of channel closure is not as dramatic as observed in WT KCNQ2 (Fig. 3-6C, Fig. 3-2D). Since prolines can break alpha-helical structures, we tested additional substitutions of either a bulky residue (Leu, high helical propensity), or a small and flexible residue (Gly, low helical propensity, but still amenable to alpha-helical configuration), at position 181. ICA73 had a significant effect on both mutants (Fig. 3-6D), suggesting that A181 may not be essential for drug binding or a helical configuration of S3, but the proline present in KCNQ3 may deform the top of the S3 helix (Fig. 3-6F) and alter the channel response to ICA73.
Figure 3-6 Functional characterization of KCNQ2[A181P] mutant channels illustrates the separable nature of current potentiation and gating shift.

(A) Exemplar patch clamp current recordings of KCNQ2[A181P]. (B) Conductance-voltage relationships of KCNQ2[A181P]. The conductance-voltage relationship in ICA73 is normalized to the peak conductance in control conditions for each individual cell (data are mean ± s.e.m.) (control V1/2 = -20 ± 2 mV, k= 11 ± 1 mV; ICA73 V1/2 = -45 ± 3 mV, k = 2 ± 2 mV, n = 5). (C) Time constants (τ) of channel closure were measured by pulsing to a range of negative voltages from a holding potential of +20 mV; inset, sample tail currents of KCNQ2[A181P] at -130 mV. (D,E) Conductance-voltage relationships of KCNQ2[A181G] (control V1/2 = -10 ± 6 mV, k= 11 ± 2 mV, n = 3) and KCNQ2[A181L] (control V1/2 = -18 ± 8 mV, k= 12 ± 1 mV, n = 3), collected as in panel (A). (F) Molecular model of the voltage-sensing domain of KCNQ2 channels in the open state. Residue A181 is located on the extracellular side of the S3 segment.
3.2.7 ICA73-insensitive Mutations Preserve Retigabine Sensitivity

Experiments thus far suggest that ICA73 and retigabine likely act via different binding sites and different mechanisms. However, it should be noted that there have been no direct binding studies of KCNQ activators to these channels, and it can be challenging to distinguish drug binding from other effects on channel gating or mechanisms that couple drug binding to the channel gate. To further investigate the effects of the A181P and F168L mutations, and the effects of retigabine versus ICA73, we compared the effects of these drugs on various mutations known to influence KCNQ activators. KCNQ2[W236F] mutant channels have disrupted retigabine sensitivity, but retain sensitivity to ICA73 (Fig. 3-7A). KCNQ2[A181P] or [F168L] mutant channels, shown here to exhibit disrupted ICA73 sensitivity, retain sensitivity to retigabine (Fig. 3-7B,C). Taken together with the relatively innocuous effects of these mutations on channel gating in the absence of activator compounds, these data suggest that ICA73 and retigabine act on different channel sites, and that the A181P and F168L are not intrinsically disruptive of channel structure or activation gating (since channels gate relatively normally, and retain sensitivity to the established channel activator retigabine).
Figure 3-7 ICA73-insensitive mutants do not perturb retigabine sensitivity.

(A-C) Conductance voltage relationships for (A) retigabine-insensitive mutant KCNQ2[W236F] (control $V_{1/2} = -45 \pm 1$ mV, $k = 8 \pm 1$ mV; retigabine $V_{1/2} = -47 \pm 2$ mV, $k = 8 \pm 1$ mV, $n = 5$), and the ICA73-insensitive mutants (B) KCNQ2[F168L] (control $V_{1/2} = -30 \pm 3$ mV, $k = 10 \pm 2$ mV; retigabine $V_{1/2} = -53 \pm 5$ mV, $k = 14 \pm 2$ mV, $n = 5$) and (C) KCNQ2[A181P] (control $V_{1/2} = -45 \pm 4$ mV, $k = 12 \pm 2$ mV; retigabine $V_{1/2} = -71 \pm 8$ mV, $k = 10 \pm 2$ mV, $n = 4$), in control conditions or 100 µM retigabine as indicated. Note that the KCNQ2[F168L] and [A181P] mutations exhibited variable channel rundown in the presence of retigabine (no potentiation as observed with ICA73), so their conductance-voltage relationships have been normalized to 1 for clarity.

3.2.8 ICA73-sensitivity can be Transferred to KCNQ3

We investigated whether the presence of KCNQ2 S3 residues F168 and A181 can introduce ICA73 sensitivity into KCNQ3. We substituted the residues back into KCNQ3 at corresponding positions L198F and P211A. While we were not able to express the KCNQ3[L198F] alone, we detected functional currents from KCNQ3[P211A] as well as KCNQ3[L198F/P211A] channels. Both channels behaved similarly as wild type KCNQ3 in the absence of ICA73, yielding large non-inactivating currents in control conditions (Fig. 3-8A,B). Interestingly, both constructs also responded to ICA73 much more strongly than wild type KCNQ3, as the conductance-voltage relationships were shifted by about 25-30 mV. This effect is milder than what is observed with KCNQ2 channels, and no consistent current potentiation by ICA73
was observed. Taken together, these results propose that there are other factors involved in the ICA73 effect but that the residues we identified are indeed crucial players.

![Figure 3-8 ICA73 effect can be partially rescued by substitution of F168 and A181 into KCNQ3.](image)

(A-C) Conductance-voltage relationship of (A) KCNQ3* (control V1/2 = -52 ± 4 mV, k = 6 ± 2 mV, n = 5; 100 µM ICA73 V1/2 = -61 ±3 mV, k = 8 ± 2 mV, n = 5), (B) KCNQ3* P211A (control V1/2 = -53 ± 3 mV, k = 5 ± 1 mV, n = 4; 100 µM ICA73 V1/2 = -77 ± 6 mV, k = 8 ± 1 mV, n = 4), and (C) KCNQ3* L198F/P211A (control V1/2 = -54 ± 3 mV, k = 7 ± 1 mV, n = 5; 100 µM ICA73 V1/2 = -80 ± 3 mV, k = 12 ± 2 mV, n = 5) normalized to peak conductance in given condition. In all panels, error bars represent s.e.m.

3.3 Discussion

The development of retigabine and flupirtine as ‘first-in-class’ voltage-gated potassium channel openers has led to efforts to further develop and understand the pharmacophore of this emerging drug class (Bentzen et al., 2006; Xiong et al., 2008; Gao et al., 2010; Peretz et al., 2010; Blom et al., 2010; Kim et al., 2015). Retigabine exhibits little subtype specificity between KCNQ2-5, so a more detailed understanding of the mechanism of action of KCNQ openers may contribute to the development of more specific or potent analogs. It is now recognized that the lack of subtype specificity of retigabine arises because of its essential interaction with a S5 Trp residue that is conserved in retigabine-sensitive KCNQ2-5 subunits (Lange et al.,
Several KCNQ openers have been reported to exhibit some subtype selectivity but the mechanism and sequence determinants underlying this specificity have been unclear (Padilla et al., 2009; Yu et al., 2011; Brueggemann et al., 2014). In addition, the effects of KCNQ openers are often studied in heteromeric KCNQ channel mixtures, so it is unclear whether individual KCNQ subtypes may differentially contribute to drug sensitivity.

In this study, we exploited dramatic differences in ICA73 effects in KCNQ2 vs. KCNQ3 to identify specific residues that are essential for ICA73 subtype specificity. ICA73 induces a very large hyperpolarizing shift of the KCNQ2 (but not KCNQ3) conductance-voltage relationship, together with a large potentiation of current magnitude – both effects far exceed what is typically reported for retigabine. Using a chimeric approach, we identified S3 residues A181 and F168 in KCNQ2 that are essential for normal ICA73 sensitivity (Figs. 4-6). Interestingly, our findings highlight that certain mutations (KCNQ2[A181P] or the Q2+Q3[S1,S2] chimera) dissociate gating shift effects from effects on current potentiation.

Previous investigations have reported voltage sensor-mediated effects of certain KCNQ openers (Padilla et al., 2009; Peretz et al., 2010; Gao et al., 2010). For example, a class of diclofenac-derived compounds was reported to interact with conserved voltage-sensing residues in KCNQ2 (Peretz et al., 2010). Also, a chimeric study suggested that the voltage sensor plays an important role in mediating the effects of ICA73 and other related compounds (Padilla et al., 2009). Lastly, recent work has demonstrated that mutation of KCNQ2 residues E130 and F137 influence sensitivity to ICA73 and other closely related compounds (Li et al.,
However, these residues are highly conserved residues among voltage-gated potassium channels, serving important roles as voltage sensor counter charges (E130) or as the ‘gating charge transfer center’ (F137) (Papazian et al., 1995; Tao et al., 2010; Pless et al., 2011; Pless et al., 2014). Mutations at these positions often have significant effects on channel function, and cannot account for the ICA73 subtype specificity for KCNQ2 over KCNQ3 (since both channels have identical residues at these positions).

Our study identifies KCNQ2 F168 as an essential determinant of ICA73 sensitivity. KCNQ2[F168L] mutant channels were completely unresponsive to ICA73, exhibiting no hyperpolarizing shift or current potentiation in the presence of the drug (Fig. 5). Based on our findings thus far, an aromatic side chain at this position appears to be required for ICA73 sensitivity. In molecular models of KCNQ2, the F168L position is located at a convergence of many positively charged sidechains including a voltage-sensing arginine (R213), and a lysine (K219) in the S4-S5 linker. The requirement for an aromatic sidechain may involve this proximity to positively charged side chains, or may be related to positioning of S3 near the membrane interface. In any case, both Lys219 and Arg213 are almost certainly conformationally mobile during channel gating and we are pursuing a more detailed understanding of the interactions between these residues with F168 in different channel states.

The KCNQ2 A181P mutation had a very unexpected outcome on ICA73 response: the drug-mediated hyperpolarizing gating shift was virtually abolished, although the current potentiation effect was preserved (Fig. 6). This observation highlights the importance of monitoring both of these distinct parameters when
characterizing KCNQ openers – as noted earlier, conductance-voltage relationships are often normalized, and this data transformation may mask the current potentiating effect of ICA73 or other openers (particularly when it occurs together with a large gating shift). Since the current potentiation effect of ICA73 persists in KCNQ2[A181P], we speculate that this mutation does not abolish drug binding, but rather alters how drug interactions influence the gating process. Supporting this suggestion is that ICA73 interaction clearly decelerates channel closure, despite not significantly changing the voltage-dependence of channel opening. We also observed that ICA73 sensitivity was preserved with a variety of substitutions at position A181, suggesting that the native alanine may not directly contribute to binding. Rather, the presence of a proline (as in KCNQ3) may alter the nature of the response to the drug, rather than altering binding. Importantly, there may be additional residues/mechanisms generating subtype specificity, as KCNQ5 has also been reported to be insensitive to ICA73, but shares the essential alanine and phenylalanine residues identified in our study (Brueggemann et al., 2014). This is further confirmed since substituting these two residues into KCNQ3 only partially endowed ICA73-sensitivity.

Taken together, our findings demonstrate that despite sharing structural similarities with retigabine, ICA73 acts via an entirely different mechanism of action. Specific residues distant from the putative retigabine binding site are able to influence ICA73 effects on KCNQ2, and underlie subtype specificity of the drug for KCNQ2 over KCNQ3. Moreover, mutations of these residues in the voltage sensor domain have multi-pronged effects on ICA73 actions, as some are able to specifically abolish the effects of ICA73 on voltage-dependence, while preserving current
potentiation. Our findings demonstrate unambiguously that KCNQ openers should be
classified into at least two subgroups based on their primary site of action (which can
be determined based on differential effects of the KCNQ2[W236L/F] or [F168L]
mutations). Ongoing investigation of the multi-pronged effects of KCNQ openers
acting on the voltage sensor will hopefully lead to a deeper understanding of the
general principles underlying the actions of these drugs.
Chapter 4: In-depth Analysis of Voltage Sensor Residues Involved in ICA73 Effects on KCNQ2

4.1 Introduction

With the emergence of potassium channel activators as a novel class of potential drugs for treating neuronal and smooth muscle disorders, it is becoming apparent that there are at least two classes of compounds that act through distinct mechanisms on the KCNQ M-channel family. While the only approved drugs on the market, retigabine and flupirtine, have been shown recently to act through a hydrogen-bond with the conserved S5 Trp residue in KCNQ2-5 channels (Kim et al., 2015), various other compounds have been found to act in a different manner. In particular, a few compounds from the diclofenac family and pyridinyl benzamides manufactured by Icagen have been shown to act independently of the S5 Trp residue, likely acting through the VSD instead (Padilla et al., 2009; Peretz et al., 2010). However, there has been very little understanding of potential binding residues in the VSD, particularly in terms of residues that may allow some drugs to exhibit subtype specificity.

In Chapter 3, we demonstrated that ICA73 exhibits strong subtype specificity, activating KCNQ2 but not KCNQ3 channels, and we investigated the structural determinants of this specificity. Using a chimeric approach, we pinpointed the site of action of ICA73 to be in the VSD and focused on the S3-S4 region since swapping the KCNQ3 S3-S4 region into KCNQ2 completely abolished ICA73 effect. Screening mutagenesis allowed us to identify two residues in the S3 segment of KCNQ2, F168
and A181, that strongly influence the subtype specificity of ICA73 effects. Importantly, substituting these residues back into KCNQ3 (L198F and P211A), respectively, endowed ICA73 sensitivity to KCNQ3 channels.

While our electrophysiology techniques allowed us to determine the specific residues involved in ICA73 effect, it is difficult to distinguish residues directly involved in binding from those that are involved in transducing binding to a functional effect. Unexpectedly, we noticed that there are two separable effects that ICA73 exerts on KCNQ2 channels. First, the drug induces a dramatic hyperpolarizing gating shift. Second, ICA73 causes increased current magnitude by approximately at least 2-fold, at the plateau of the conductance-voltage relationship. The separation of these two outcomes is evident in the KCNQ2[A181P] channel, where the leftward shift by ICA73 in the conductance-voltage relationship is minimal but current potentiation is preserved.

In this chapter, I sought to investigate ICA73 interactions with the voltage sensor in more detail. We revisited the S1-S2 region of the VSD since the Q2 + Q3[S1,S2] chimera had a similar profile as KCNQ2[A181P], using a scanning mutagenesis approach. Although mutagenesis in this region did not identify further residues that abolish ICA73 effects, we noticed several residues that either altered native channel function or specific aspects of ICA73 effects. In addition, we observed further evidence for separable effects of ICA73. Finally, we investigated KCNQ2 residue K219. This position was highlighted briefly in Chapter 3, because molecular models of KCNQ2 open and closed states predict that K219 is conformationally mobile and comes into close proximity with KCNQ2 F168 in the channel open state.
Our findings suggest an important role for this residue in channel opening, and may lead to further investigations of how this fits into the ICA73 actions. While we were unable to single out residues that are responsible for ICA73 specificity in this chapter, the experiments have given us insight on overall channel function and laid the foundation for future studies.

4.2 Results

4.2.1 Characterizing the Influence of the S1-S2 Segments on ICA73 Sensitivity

In Chapter 3, we highlighted that the KCNQ2[A181P] mutant channel exhibited an unexpected selective reduction of the ICA73-mediated gating, while preserving drug effects on current potentiation. This phenotype was also observed in our initial electrophysiological characterization of the Q2+Q3[S1,S2] chimeric channel. Therefore, we have undertaken a more detailed characterization of the S1 and S2 segments in terms of their influence on ICA73 effects. We noted that the S1 and S2 segments of KCNQ2 and KCNQ3 diverged significantly, whereas the S3 and S4 segments had very few sequence differences. Aiming to possibly narrow down essential sequences for normal ICA73 sensitivity, we constructed more specific chimeras by swapping only the S1 or S2 segment of KCNQ2 and KCNQ3. We observed that both of these chimeric channels retain ICA73 sensitivity (Fig. 4-1B,C). However, the Q2 + Q3[S2] construct exhibited a marked reduction in the hyperpolarizing shift by ICA73 while preserving the current potentiation.
Figure 4-1 S1-S2 domain and ICA73 sensitivity.

Conductance-voltage relationships for (A) Q2 + Q3[S1-S2] (control V1/2 = -9 ± 1 mV, k = 8 ± 1 mV; ICA73 V1/2 = -22 mV ± 5 mV, k = 5 ± 1 mV, n = 5), (B) Q2 + Q3[S1] (control V1/2 = -9 ± 2 mV, k = 13 ± 1 mV, n = 4), and (C) Q2 + Q3[S2] (control V1/2 = -27 ± 1 mV, k = 18 ± 4 mV, n = 5; ICA73 V1/2 = -62 ± 3 mV, k = 7 ± 1 mV, n = 4) normalized to peak conductance in control. (D) Sequence alignment between KCNQ2 and KCNQ3 in S1-S2 region. In all panels, error bars represent s.e.m.

4.2.2 ICA73 Effect on S1 Point Mutations

Despite the Q2 + Q3[S1] construct appearing to show full sensitivity to ICA73 (Fig. 4-1B), we investigated the effects of substituting of individual residues (or clusters of neighbouring residues) from KCNQ3 into KCNQ2 (Fig. 4-2). As one might expect, most of the mutants were quite responsive to ICA73, exhibiting a hyperpolarizing gating shift as well as current potentiation. However, certain noteworthy differences stood out at some positions. Some residue substitutions exhibited a less dramatic gating shift relative to WT KCNQ2 (eg. S110A, S105G),...
highlighted by near complete channel closure at hyperpolarizing voltages. Other mutants (eg. Y98L) exhibited significantly greater potentiation relative to WT KCNQ2. Interestingly, the KCNQ2[I115F] construct (Fig. 4-2H) exhibited a clear hyperpolarizing shift by ICA73 but a much smaller current potentiation effect relative to WT KCNQ2.

![Figure 4-2 Characterizing S1 point mutations and ICA73 effect.](image)

(A-H) Conductance-voltage relationships for (A) Q2 F93L/I94L (control V1/2 = -30 ± 8 mV, k = 12 ± 1 mV, n = 3), (B) Q2 Y98L (control V1/2 = -33 ± 4 mV, k = 10 ± 2 mV, n = 3), (C) Q2 L102I (control V1/2 = -38 ± 3 mV, k = 14 ± 1 mV, n = 5), (D) Q2 S105G (control V1/2 = -27 ± 1 mV, k = 9 ± 1 mV, n = 3), (E) Q2 V108I (control V1/2 = -31 ± 6 mV, k = 12 ± 1 mV, n = 5), (F) Q2 S110A (control V1/2 = -21 ± 8 mV, k = 12 ± 1 mV, n = 4), (G) Q2 F112L/S113T (control V1/2 = -26 ± 4 mV, k = 13 ± 2 mV, n = 3), and (H) Q2 I115F (control V1/2 = -42 ± 8 mV, k = 12 ± 2 mV, n = 4) normalized to peak conductance in control. (I) Molecular model of voltage-sensing domain of KCNQ2 channels highlighting S1 residues screened (yellow) and I115 (blue). In all panels, error bars represent s.e.m.
4.2.3 ICA73 Effect on S2 Point Mutations

We also investigated the S2 segment because the Q2 + Q3[S2] chimera had a reduced response to ICA73 (Fig. 4-1C). Again, we screened each position where KCNQ2 and KCNQ3 differed. To our surprise, none of these mutations severely hindered the ICA73 effect (Fig. 4-3), although there were some notable consequences of some mutants. The KCNQ2[T133A] (Fig. 4-3H) mutant had a very right-shifted conductance-voltage relationship relative to WT KCNQ2 and other mutant constructs, but both features of the ICA73 effect persisted. Also, the KCNQ2[G124D] (Fig. 4-3C) mutant channel exhibited much greater current potentiation. Overall, based on the sequence-motivated electrophysiological screening of S1 and S2 mutants, we were unable to find specific residues essential for ICA73 sensitivity, with the possible exception of KCNQ2[I115F] (Fig. 4-2H). Further experiments are necessary to look at different aspects the mutations may have on channel function; for example, comparing $V_{1/2}$ values to amount of shift in conductance-voltage relationships or magnitude of current potentiation. At this point, it appears that the two residues in S3, F168L and A181P, as discussed in Chapter 3, are the ones that specifically influence ICA73 effect.
Figure 4-3 Characterizing S2 point mutations and ICA73 effect.

(A-K) Conductance-voltage relationships for (A) Q2 K120T/S121V (control V1/2 = -35 ± 5 mV, k = 12 ± 1 mV, n = 5), (B) Q2 E123G (control V1/2 = -26 ± 6 mV, k = 12 ± 1 mV, n = 3), (C) Q2 G124D (control V1/2 = -22 ± 5 mV, k = 14 ± 1 mV, n = 3), (D) Q2 A125W (control V1/2 = -22 ± 7 mV, k = 11 ± 1 mV, n = 3), (E) Q2 Y127L/I128L (control V1/2 = -20 ± 3 mV, k = 11 ± 1 mV, n = 3), (F) Q2 I131T (control V1/2 = -32 ± 2 mV, k = 10 ± 1 mV, n = 3), (G) Q2 V132F (control V1/2 = -29 ± 9 mV, k = 10 ± 1 mV, n = 3), (H) Q2 T133A (control V1/2 = -22 ± 2 mV, k = 21 ± 1 mV, n = 3), (I) Q2 V135F/V136I (control V1/2 = -28 ± 4 mV, k = 14 ± 2 mV, n = 5), (J) Q2 V139A (control V1/2 = -25 ± 2 mV, k = 11 ± 2 mV, n = 5), and (K) Q2 Y141F/F142A/V143L (control V1/2 = -36 ± 1 mV, k = 11 ± 1 mV, n = 3) normalized to peak conductance in control. (L) Molecular model of voltage-sensing domain of KCNQ2 channels highlighting S2 residues screened (yellow), G124 (blue), and T133 (blue). In all panels, error bars represent s.e.m.
4.2.4 Potential Binding Pocket Residues and ICA73 Effects

Since our mutagenesis and electrophysiology experiments cannot distinguish between the binding vs. effect of ICA73 on KCNQ2, we were interested in deciphering the possible binding site of ICA73. Molecular simulations of solvent accessible pockets in the KCNQ2 structure highlighted several potential binding residues for ICA73 (Fig. 4-4). We mutated each of these residues to alanine and tested ICA73 effects (Fig. 4-4). The Q2 R201A mutation (Fig. 4-4H) resulted in a channel that was almost constitutively open in the absence of ICA73; addition of ICA73 further enhanced the opening of the channel at very hyperpolarizing potentials, but did not dramatically potentiate currents. This effect may arise because R201 is one of the crucial positive charges in the voltage sensor, thus neutralizing the charge at this position may destabilize the resting state of the voltage sensor, allowing the channel to open with little voltage-dependence. The dramatic relative stabilization of the open state, even in the absence of ICA73, likely underlies the weaker ICA73-mediated potentiation observed in the R201A mutant channel. More interestingly, we observed a second point mutation that duplicates the effects of the KCNQ2[A181P] mutant. Specifically, the KCNQ2[E130A] mutation (Fig. 4-4C) almost completely abolished the ICA73-mediated hyperpolarizing gating shift, but retained current potentiation.
Figure 4-4 ICA73 effect on potential binding pocket residues.

(A-I) Conductance-voltage relationships for (A) Q2 C106A (control V1/2 = -43 ± 7 mV, k = 16 ± 1 mV, n = 3), (B) Q2 L107A (control V1/2 = 5 ± 2 mV, k = 14 ± 1 mV, n = 3), (C) Q2 E130A (control V1/2 = -8 ± 3 mV, k = 15 ± 2 mV, n = 4; ICA73 V1/2 = -39 ± 3 mV, k = 11 ± 1 mV, n = 4), (D) Q2 I134A (control V1/2 = -52 ± 1 mV, k = 14 ± 1 mV, n = 3), (E) Q2 S179A (control V1/2 = -16 ± 1 mV, k = 18 ± 2 mV, n = 3), (F) Q2 V182A (control V1/2 = 1 ± 7 mV, k = 15 ± 2 mV, n = 3), (G) Q2 R198A (control V1/2 = -9 ± 1 mV, k = 10 ± 1 mV, n = 3), (H) Q2 R201A (n = 5), and (I) Q2 Q204A (control V1/2 = -32 ± 3 mV, k = 10 ± 2 mV, n = 3) normalized to peak conductance in control. In all panels, error bars represent s.e.m. (J) Molecular model of KCNQ2 channel showing potential binding pocket residues (yellow) and highlighting E130 (blue) and R201 (blue).
4.2.5 KCNQ2 I115F may have Reduced Current Potentiation by ICA73

As mentioned above, the KCNQ2[I115F] construct appeared to exhibit a significantly reduced ICA73-mediated currently potentiation, and we have highlighted this experimental finding in Fig. 4-5. We consistently observed very small increases in current magnitude with addition of ICA73 relative to WT KCNQ2 (Fig. 5-5A,B,F,G). We have included cell-by-cell plots of current magnitude at 20 mV before and after drug for both WT KCNQ2 (Fig. 5-5C) and KCNQ2[I115F] (Fig. 5-5D) to illustrate this trend. Located at the top of the S1-S2 linker (Fig. 5-5E), position 115 may play a role in facilitating the current potentiation we observe with ICA73 on most KCNQ2 constructs. As illustrated in Shaker, there is a link between the pore region and S1 that may be an important determinant of coupling between the pore and voltage sensor (Lee et al., 2009b). Further studies need to be conducted to clarify and validate this observation.
Figure 4-5 Current potentiation by ICA73 may be reduced in KCNQ2[I115F].

(A,B) Conductance-voltage relationships of (A) KCNQ2 wild type (control $V_{1/2} = -40 \pm 5$ mV, $k = 5 \pm 1$ mV, $n = 5$) and (B) KCNQ2[I115F] (control $V_{1/2} = -42 \pm 8$ mV, $k = 12 \pm 2$ mV, $n = 4$) normalized to peak conductance in control. (C,D) Plots of current magnitude at 20 mV with and without 30 µM ICA73 of sample individual cells for (C) KCNQ2 wild type and (D) KCNQ2[I115F]. (E) Molecular model of top view of voltage-sensing domain highlighting I115 (blue). (F,G) Exemplar patch clamp current recordings of KCNQ2[I115F] in (F) control and (G) with 30 µM ICA73.
4.2.6 KCNQ2[E130A] Abolishes the ICA73-mediated Shift but not Current Potentiation

One of the suggested potential binding pocket residues, E130 is a highly conserved residue among voltage-gated potassium channels that acts as a voltage-sensor counter charge (Fig 4-6E) (Papazian et al., 1995; Tao et al., 2010; Pless et al., 2011; Pless et al., 2014). Previous studies have suggested that mutations at E130 abolished the effects of ICA73 and closely-related compounds (Li et al., 2013b; Brueggemann et al., 2014). However, our study carefully distinguished the gating shift and potentiation effects of ICA73, revealing that while the hyperpolarizing shift by ICA73 is mostly eliminated, the current potentiation is still present in KCNQ2[E130A] channels (Fig. 4-6A,B). As mentioned in Chapter 3, this is a feature of the data that appears to have often been overlooked due to common practices of normalizing conductance-voltage relationships to peak current in the given condition. Looking at the kinetics of this construct, we also notice that in the presence of ICA73, channel closure is decelerated (Fig. 4-6C), suggesting that ICA73 most likely still binds to the channel, but the mutation alters the structure in a way that eliminates the hyperpolarizing shift of voltage-dependence.
Figure 4-6 ICA73-mediated shift is abolished in KCNQ2[E130A] which preserves current potentiation.6

(A) Exemplar patch clamp current recordings of KCNQ2[E130A]. (B) Conductance-voltage relationship of KCNQ2[E130A] (control V1/2 = -8 ± 3 mV, k = 15 ± 2 mV, n = 4; ICA73 V1/2 = -39 ± 3 mV, k = 11 ± 1 mV, n = 4) normalized to peak conductance in control. (C) Time constants (τ) of channel closure were measured by pulsing to a range of negative voltages from a holding potential of +20 mV; inset, sample tail currents of KCNQ2[E130A] at -130 mV. (D) Sequence alignment highlighting conserved E130 in numerous Kv channels.
4.2.7 KCNQ2 K219 may Serve Important Role in Channel Opening

Based on our previous finding that the KCNQ2[F168L] mutant is completely unresponsive to ICA73, we investigated the role of residue K219 that is positioned in close proximity to the F168 in the open state of KCNQ2. Moreover, in molecular models of KCNQ2 activation, there is considerable motion of K219 relative to F168, with these two sidechains only suggested to approach one another very late in the activation sequence. We hypothesized that the positive charge of K219 may form a stabilizing interaction with the π-electrons in the aromatic ring of Phe. Mutating K219 to either an alanine or arginine significantly perturbed channel function; current magnitude in control conditions was very small for both mutants (Fig. 4-7A,B). Interestingly, ICA73 addition caused dramatic potentiation of current in both KCNQ2[K219A] and [K219R] (Fig. 4-7A,B). While arginine is also positively charged, we speculate that the charge density on the lysine may be optimal for the stabilizing interaction and thus the K219R mutant behaves similarly to the K219A mutant. Additional experiments should be conducted to elucidate the specific role of K219 in terms of interactions with F168 and ICA73 effects.
Figure 4-7 Examining the potential role of KCNQ2 K219 in channel opening.

(A,B) Exemplar patch clamp current recordings of KCNQ2[K219A] in (A) control and (B) with 30 µM ICA73. (D,E) Exemplar patch clamp current recordings of KCNQ2[K219R] in (D) control and (E) with 30 µM ICA73. (C,F) Conductance-voltage relationships of (C) KCNQ2[K219A] (control V1/2 = -3 ± 1 mV, k = 12 ± 2, n = 3) and (F) KCNQ2[K219R] (control V1/2 = -21 ± 1 mV, k = 11 ± 2 mV, n = 3) normalized to peak conductance in control. (G) open and closed states of KCNQ2 highlighting K219 and F168 in bottom and side views.
4.3 Discussion

There is growing recognition of the therapeutic potential of potassium channel activators on neuronal diseases such as pain and epilepsy as well as smooth muscle diseases. As the mechanisms of action of retigabine and flupirtine have been unravelled, there is more interest in improving the design of this drug class for better specificity. However, retigabine and flupirtine remain the only KCNQ activators (or Kv channel activators) approved for widespread human use. Certain compounds including ICA73 have shown strong subtype specificity. In the previous chapter, we demonstrated through chimeric and mutagenesis approaches that ICA73 interacts with the VSD rather than the retigabine binding site in the pore region. Particularly, two residues in the S3 of KCNQ2, F168 and A181, are important for ICA73 specificity. Mutations at these positions significantly reduce drug effect on the channel. More importantly, we showed that the ICA73 effect has two components that may be separable: first, a hyperpolarizing gating shift, and second, an increase in current magnitude.

In this chapter, we further investigated different regions of the KCNQ2 and KCNQ3 voltage sensor, in order to better understand how ICA73 interacts with and influences KCNQ2. We performed a detailed analysis of the S1-S2 region of the VSD, motivated by chimeric studies showing that replacement of the KCNQ2[S1-S2] with corresponding sequence of KCNQ3 abolished the ICA73-mediated gating shift. After screening each residue that differs between KCNQ2 and KCNQ3, we found none that altered the ICA73 effect substantially. Since the S1-S2 region in KCNQ2 and KCNQ3
differ significantly, the chimeric construct Q2 + Q3[S1,S2] may have an altered structure which results in the differential response to ICA73.

Although the S1-S2 screen did not suggest any specific residues that ICA73 may interact with, we noticed that KCNQ2[I115F] may have a weakened response to ICA73. While ICA73 induces a pronounced hyperpolarizing shift to KCNQ2[I115F], there appears to be minimal potentiation of currents. This is distinct from what we reported for the KCNQ2[A181P] mutant, again demonstrating separable effects of ICA73. Data on this particular observation is preliminary but it is likely that more experiments can tease out the importance of the I115 residue and its possible role in facilitating current potentiation by ICA73. We believe that its location at the top of the S1-S2 linker in the extracellular domain may contribute to this role (Lee et al., 2009b).

To further deduce the effects of ICA73 and its possible interactions with KCNQ2 channels, we examined the KCNQ2 structural model, identifying potential residues that make up solvent accessible cavities where ICA73 may bind. We noted that all of these residues are found in both KCNQ2 and KCNQ3, and many are highly conserved among voltage-gated potassium channels. Thus, mutating some of these residues to alanine altered the channel function. For example, KCNQ2[R201A] lost its sigmoidal conductance-voltage relationship as the channel was essentially open at very hyperpolarized potentials even in control conditions. However, addition of ICA73 still increased the magnitude of current of this construct, suggesting that the mutation likely did not damage the ICA73 interaction with the channel. We also identified KCNQ2[E130A] which was a mutation shown in other studies to abolish ICA73 effect (Li et al., 2013b; Brueggemann et al., 2014). However, we observed that
current potentiation was preserved, a feature which had not been discussed in previous studies. Given these persistent ICA73-mediated effects, we do not attribute this residue as an essential ICA73 binding residue. Rather, we suspect that effects observed in the A181P and E130A mutations may be largely explained by a shift in the state-dependence of ICA73 binding (see Chapter 5: General Discussion).

Finally, we investigated K219, a residue that we hypothesized may form a stabilizing interaction with F168 in the KCNQ2 open state. We proposed that the positive charge of lysine at this position played an important role, possibly forming an interaction with the aromatic π-electrons of F168. Surprisingly, we recorded little current from both KCNQ2[K219A] and KCNQ2[K219R] in control conditions, far smaller than other constructs tested; however, application of 30 μM ICA73 induced a large hyperpolarizing gating shift and a dramatic current increase. While the alanine is uncharged, we expected the positively charged arginine to provide at least a partial rescue of the WT phenotype, but this did not turn out to be the case. It is possible that the delocalized positive charge in the arginine residue is insufficient to mimic the presence of a lysine, or perhaps the positive charge on the lysine is oriented in a way that provides a more optimal interaction with F168. Future experiments should address this observation and investigate the R213 residue that is also located in the same region, possibly interacting with F168. As more details on ICA73 and KCNQ2 unfold, better understanding of the channel structure and the mechanism of action of ICA73 can lead to improved drug design with minimal side effects.
Chapter 5: General Discussion

Development of KCNQ channel activators:

While there are over 20 anti-epileptic drugs on the market, approximately 30% of patients are resistant to current pharmacotherapy (Brodie and French, 2000; Kwan and Brodie, 2010). Thus, the development and approval of retigabine, the first anti-epileptic drug that acts as a voltage-gated potassium channel opener, has stirred up interest in the therapeutic potential of this new drug class. By increasing the activity of KCNQ2/3 channels around the resting membrane potential, retigabine can have a hyperpolarizing influence on membrane voltage, and negatively modulate neuronal firing rate (Orhan et al., 2012). However, retigabine can also activate other members of the KCNQ channel family, including KCNQ4 and KCNQ5 which are prominently expressed in smooth muscle and the ear rather than the brain. This lack of specificity arises because retigabine's site of action is a conserved Trp residue present in the S5 segment of KCNQ2-5 channels. In addition to lacking subtype specificity, retigabine affects other receptors such as GABA-receptors (Gribkoff, 2003; van Rijn and Willems-van, 2003), and this may also contribute to various side effects that have arisen. Patients have experienced recurring problems involving urinary retention, dizziness, and blue-grey mucocutaneous discoloration, to name a few (Streng et al., 2004; Orhan et al., 2012; Garin et al., 2014). Therefore, there is a need for ongoing improvement of this first-in-class drug.

Numerous drugs with KCNQ channel opener activity have been synthesized. Many of the designs (including ICA73 characterized in this study) are based on the
structure of retigabine. As more compounds are tested, it is becoming apparent that several may act differently from retigabine. Rather than targeting the pore region of KCNQ channels, it has been shown that some Icagen compounds and NH29 from the diclofenac family may act on the VSD (Padilla et al., 2009; Peretz et al., 2010). However, the mechanism of action of these compounds are mostly still unknown.

In this concluding chapter, I hope to highlight three fundamental points related to the mechanism of action of ICA73. First, ICA73 has a ‘two-pronged’ effect on drug sensitive channels: it causes a hyperpolarizing gating shift, in addition to a pronounced potentiation of peak current. These effects are markedly greater than the effects of retigabine, but only in certain KCNQ channel types (eg. KCNQ2, but not KCNQ3). Second, ICA73 acts on a unique binding site, distinct from the retigabine binding site. Lastly, the gating and potentiation effects of ICA73 appear to be ‘separable’ in the sense that I have generated point mutants that can exhibit one effect in the absence of the other (particularly, potentiation in the absence of a gating shift). I will review the evidence and implications of these features of ICA73 modulation of KCNQ2.

**Large subtype-selective actions of ICA73**

In Chapter 3, we demonstrated that ICA-069673 (ICA73), a pyridinyl benzamide, selectively activates KCNQ2 over KCNQ3 channels. It shifts the voltage-dependence of activation of KCNQ2 channels to very hyperpolarized potentials. The ICA73-induced hyperpolarizing shift in the conductance-voltage relationship is so large that we cannot fully characterize the magnitude of this effect, simply because we cannot voltage-clamp cells to a sufficiently negative voltage to fully close channels
in the presence of ICA73. I also showed that by normalizing the conductance-voltage relationship to the peak conductance in control conditions, it is apparent that ICA73 induces a ~2-fold increase in current magnitude that is not observed with retigabine. These marked differences from retigabine provided an initial clue that ICA73 acts via a distinct mechanism from retigabine.

I exploited the differences between ICA73 effects on KCNQ2 and KCNQ3 to investigate the site of action of ICA73. A chimeric approach was used to first narrow down the importance of the VSD, and particularly the S3-S4 region. Next, I employed mutagenesis and adapted a non-radioactive Rb+ efflux assay to screen for residues essential for ICA73 effects on KCNQ2. I identified two residues, F168 at the bottom of S3, and A181 at the top of S3, which significantly reduced ICA73 effects. These findings are a significant step forward, as they are the first specific residues reported to influence subtype selectivity of a KCNQ channel activity.

**Detailed characterization of positions underlying subtype specificity**

I used whole-cell patch clamp recordings to characterize the effects of mutations at KCNQ2 positions F168 and A181. The F168L mutation rendered the channel completely insensitive to ICA73. I also determined that an aromatic residue at position 168 was sufficient for ICA73 effect since the KCNQ2[F168Y] and [F168W] mutants were both responsive to ICA73. Mutation of KCNQ2 A181 yielded a very unexpected phenotype, where the current magnitude of KCNQ2[A181P] channel was potentiated by ICA73 (similar to wild type KCNQ2), but the ICA73-mediated hyperpolarizing shift was abolished. We hypothesized that the proline residue at the corresponding position in KCNQ3 may alter the helical structure of S3, and thereby
alter the interaction of ICA73 with the channel. KCNQ2[A181G] and [A181L] were both responsive to ICA73, suggesting that the alanine at position 181 likely plays a ‘permissive’ role for ICA73 binding/effect, rather than making essential contact for drug binding. Since channel closure of KCNQ2[A181P] is significantly slowed by ICA73 (in the absence of a shift in voltage-dependent gating), it appears that binding does occur but fails to translate into an effect on the voltage-dependent gating mechanism. Importantly, substitution of these residues back into KCNQ3 (L198F and P211A, respectively), could rescue some ICA73 effect on the otherwise insensitive KCNQ3 channel. However, it should be noted that the hyperpolarizing shift was much smaller than in KCNQ2, and the current magnitude is not potentiated in the presence of ICA73.

*Scanning the VSD reveals that ICA73-sensitivity is very tolerant of mutation*  

Although the two residues we identified in the S3 show promising evidence of being involved in ICA73 sensitivity and subtype specificity, I further investigated the influence of VSD mutations to fill the gap of unknowns about the mechanism of action of ICA73. In Chapter 4, I revisited the S1-S2 region of the VSD to test whether other residues may influence ICA73 sensitivity; this was motivated by my finding that the Q2 + Q3[S1,S2] chimera had a similar behavior towards ICA73 as the KCNQ2[A181P] mutant (strong current potentiation despite no gating shift). However, after thorough screening of all residues that differ between KCNQ2 and KCNQ3 in the S1-S2 region, I could not identify particular residues that were important for ICA73 specificity. Nonetheless, several mutations resulted in channels that behaved differently in the absence of ICA73, and KCNQ2[I115F] surprisingly exhibited a dramatic
hyperpolarizing shift by ICA73, but the current potentiation was much smaller compared to wild type KCNQ2. This observation further supports our observation that ICA73 exhibits two separable effects on KCNQ2 channels.

We also examined a potential binding site for ICA73 based on published molecular models and the presence of a solvent accessible pocket in a KCNQ2 structural model (Guiscard Seebohm, personal communication). Screening numerous residues did not yield any with notable effects on ICA73 sensitivity. However, I noticed a few residues that altered the native function of the channel (in the absence of drug). For example, KCNQ2[R201A] was constitutively open even in the absence of drug, most likely due to the lack of positive charge liberating the voltage sensor. In addition, KCNQ2[E130A], a mutation previously reported to abolish the effect of ICA73 and similar compounds (Li et al., 2013b; Brueggemann et al., 2014), was found to abolish the ICA73 hyperpolarizing shift, while retaining current potentiation by ICA73. This was an important result because it suggests that the E130A mutation does not cause the channel to become completely unresponsive to ICA73 and that binding may very well still occur. Since E130 is a highly conserved residue among most voltage-gated potassium channels, acting as a voltage sensor counter-charge (Papazian et al., 1995; Tao et al., 2010; Pless et al., 2011; Pless et al., 2014), neutralizing the charge alters the channel function and may indirectly influence ICA73 effects. Also, this residue cannot account for ICA73 specificity for KCNQ2 (KCNQ3 channels share this residue, as do most other Kv channels).

My experimental characterization of the KCNQ2[E130A] highlights an important shortcoming of many studies investigating KCNQ channel openers.
Specifically, there has been a common practice of normalizing conductance-voltage relationships (see (Padilla et al., 2009; Peretz et al., 2010; Boehlen et al., 2013; Li et al., 2013b)), leading to an incomplete representation of the full effect of the drugs (and the impact of mutations). For example, the KCNQ2 [E130A] mutation has previously been reported to abolish ICA73 sensitivity (Li et al., 2013b; Brueggemann et al., 2014), but my findings clearly illustrate that at least certain aspects of ICA73 effects persist in these channels. I would argue that the drug must still be binding to KCNQ2[E130A] channels, but that the effects of drug binding are not translated normally to the gating machinery of the channel.

In addition, I investigated the K219 residue in KCNQ2, since molecular models suggest that it comes in close contact with the F168 residue during the channel open state. Whole-cell patch clamp experiments of KCNQ2[K219A] and [K219R] showed interesting results in which channels yielded minimal current in control conditions, but were dramatically potentiated upon addition of ICA73. Although these observations suggest that an interaction between F168 and K219 is not essential for ICA73 sensitivity, these residues may play an important role in regulation channel activity.

**Overall significance and future directions:**

Taken together, findings presented in Chapters 3 and 4 demonstrate an extensive analysis of a potassium channel opener, ICA73, with a distinct activity profile from retigabine. I utilized the unique properties of this compound to decipher the differences between KCNQ2 and KCNQ3 channels, which may lead to better understanding of subtype differences within the KCNQ family. My studies are the first
to define individual residues that underlie subtype specific effects of KCNQ channel openers. I also demonstrate unambiguously that ICA73 (and likely other closely related openers) act via a wholly different binding site and mechanism than the more widely studied KCNQ opener retigabine. Lastly, I have demonstrated that the effects of ICA73 (gating shift, and potentiation) can be dissociated with certain mutations in the VSD, and this likely has important consequences for understanding the mechanism of action of KCNQ channel openers.

Extending beyond these results, there are still numerous questions remaining that should be pursued. Firstly, the stoichiometry of ICA73 binding to KCNQ channels is unknown, and could be investigated by engineering tetrameric KCNQ2 channels with varying numbers of subunits carrying either the F168L or A181P mutations. These experiments would allow us to study the stoichiometry and cooperativity of drug effect; for example, is one molecule of ICA73 acting on one wild type monomer of KCNQ2 sufficient to generate the full ICA73 effect, or are four molecules required to bind for a full drug effect? It would be interesting to compare this result to the stoichiometry of retigabine on KCNQ3 channels, which may provide further insights into differences between ICA73 and retigabine actions on KCNQ2 channels.

As well, more investigation of the KCNQ2 I115 residue could help answer the questions of how ICA73 potentiates KCNQ2 currents, a trait not reproducibly seen with retigabine. One possibility is that the location of I115 at the top of the S1-S2 linker serves an important role in coupling the voltage sensor to the pore, as suggested previously (Lee et al., 2009b). Identifying the potential role this residue may play in facilitating current potentiation by ICA73 can again help us understand
the mechanism of action of ICA73. Similarly, it would be worth examining the K219 and R213 residues which may play crucial roles in interacting with F168 to facilitate channel opening that can be reinforced by ICA73.

Finally, the two separable effects of ICA73 (potentiation and gating shift), has sparked a hypothesis regarding the potential state-dependence of binding of ICA73. This appears to be especially important for understanding the unusual conductance-voltage relationships of KCNQ2[A181P], Q2 + Q3[S1,S2], and KCNQ2[E130A], where ICA73 causes a minimal gating shift, but dramatically potentiates currents. These results indicate that the mutations most likely did not prevent ICA73 from interacting with the channel (since currents are still potentiated). However, the mutations may have altered the state-dependence of binding. We hypothesize that ICA73 may preferentially bind to and stabilize the activated state of voltage sensor in wild type KCNQ2 channels, and this would underlie a change in voltage sensor equilibrium in the presence of ICA73. In the case of the mutants mentioned above, I suggest that this state-preference is lost, and that ICA73 can bind the activated and resting voltage-sensor conformations with similar energetics. As a result, the voltage-dependence of channel opening should be barely altered, but the open probability of channels bound by ICA73 in the open state could still be increased, thereby potentiating overall current magnitude. Further experiments to test this hypothesis can provide more insight on how ICA73 and other similar compounds work. This developing understanding will be especially important because in drug design, state-dependent interactions of drugs with their targets can be an advantageous property, allowing for more specific targeting of hyperactive receptors (Tao et al., 2006). Thus, these
experiments will hopefully lay a valuable foundation for further understanding of the mechanism of action and potential therapeutic uses for KCNQ channel activators.

**Conclusion:**

This thesis has revealed novel aspects of KCNQ channel activators as well as details of structural and functional elements of KCNQ channels. Together, the findings can aid in the improvement of drug design as it is now clear that there are multiple mechanism of actions of compounds acting on M-channels. Importantly, with deeper understanding of the differences between KCNQ subtypes, the aim for more specific therapeutic targets can hopefully be achieved.
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