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Insights into mechanism of alcohol mediated modulation of GIRK channels

A dissertation submitted in partial satisfaction of the requirements for the degree

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in

Biology

by

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Dedication

This dissertation is dedicated to four important women in my life, my grandmothers Bishnu Maya Aryal, and Prithibi Kumari Gautam, my mother Manju Sharma, and my wife Samjhana Rimal Aryal. They have all taught me the importance of love, family and education in their own special way.

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Chapter two was published in Nature Neuroscience. I was the primary author, and the chair of my thesis committee was the senior author. Permission to use this work as a part of my dissertation has been obtained from Hay Dvir, and Senyon Choe, the two other co-authors. I conducted all of the primary work for Chapter three, part of which was submitted as an abstract to be presented at the 54th Annual Biophysical Society meeting in 2010.

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Abstract of the Dissertation

Insights into mechanism of alcohol mediated modulation of GIRK channels

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Ion channels are proteins that control the flow of ions in and out of the cell. They are important in many aspects of human physiology such as muscle contraction, sensory perception, thought and behavior. Ethanol is the most common drug of abuse worldwide. Ethanol modulates numerous ion channels, but the exact mechanism of modulation has been elusive. My dissertation investigates how ethanol and other alcohols modulate the activity of G-protein gated inwardly rectifying potassium (GIRK) channels.

GIRK channels are members of the inwardly rectifying potassium channel family that regulate the resting membrane potential of excitable cells. They are ubiquitously expressed in the brain and the heart. In the neurons, opening of GIRK channel leads to inhibition of neuronal firing. Ethanol activates GIRK channel in neurons. However, exactly how ethanol activates GIRK channels is not known.

In this dissertation, I first used structure-based mutagenesis and electrophysiology to determine that alcohols act on a hydrophobic pocket in the

cytoplasmic domain of GIRK channels resulting in channel activation. To strengthen this finding, I used thiol modifying reagents targeted to the hydrophobic pocket to show that this pocket is important for activation of GIRK channels.

To determine the mechanism of this alcohol mediated activation of GIRK channels, I studied the requirement of G-proteins and PIP_2 and found that alcohol activates GIRK channels in a G-protein independent, but PIP_2 dependent manner. I then studied single channel properties of alcohol activated GIRK channels. I found that alcohols increase the frequency of channel opening. Combining structural models and functional data, I present a novel model for alcohol mediated activation of GIRK channels. In this model, alcohol facilitates spontaneous opening of GIRK channels by binding to the hydrophobic alcohol pocket and opening its cytoplasmic gate. This work contributes to our fundamental understanding of how ethanol modulates ion channels and how GIRK channels are gated.

I. Introduction

The living cell consists of hereditary information and a machinery to transcribe and, translate that information into proteins enabling the cell to divide and form a population of cells that respond to extracellular cues. These cues enable the cells to differentiate into various cell types based on intricate developmental programming. In the brain, these differentiated neuronal cells communicate with each other by a mechanism of cellular excitability. It is this cellular excitability that controls firing of neuronal networks that lead to higher nervous system functions such as sensory perception, movement, memory, and thought. And consequently modulation of this excitability by chemicals such as alcohols leads to alteration in the nervous system functions.

Basis of cellular excitability

Neurons, like all cells, segregate themselves from the environment with a lipid rich plasma membrane that prohibits large molecules and charged ions from readily flowing in and out of the cell. The plasma membrane is inherently polarized due to the presence of negatively charged head groups of phospholipids in the inner leaflet of plasma membrane. Ion channels, along with transporters and pumps, regulate the salt concentrations inside the cell. The inherent polarization of the membrane, along with the regulated difference in salt concentration confers an electrochemical gradient that governs excitability of neurons.

Ion channels are proteins that selectively allow ions to pass through the plasma membrane according to its electrochemical gradient. For example, there is more potassium inside of the cell than outside. If potassium channels open, potassium (K^+) ions will flow out of the cell through the channel to equalize the concentration difference until they reach their reversal potential. At rest, the cell is hyperpolarized (negatively charged), with higher concentration of sodium (Na^+) and chloride (Cl^-) on the outside, and potassium on the inside. If positive ions flow into the cell, via opening of cation permeable ion channels, then the cell becomes depolarized (positively charged). If this depolarization reaches a certain threshold, it leads to firing of an action potential in the neuron in a feed forward mechanism where voltage sensitive sodium channels sense the depolarization, and open, allowing more Na^+ to flow inside the cell, propagating the depolarization along the cell. To terminate this firing, voltage sensitive potassium channels open, leading to outward flow of K^+ to hyperpolarize the cell back to a resting membrane potential.

The basis of neuronal excitability as it relates to ion channels was discovered in many phases. Classical biophysical experiments using intracellular electrodes in giant squid axons led Kenneth S. Cole and colleagues to determine that membrane conductance increases during neuronal firing (Cole, 1934; Cole and Curtis, 1939) . Subsequently, using voltage clamping methods, Hodgkin, Huxley and colleagues determined that Na^+ and K^+ are the primary ions involved in firing of the neuron (Hodgkin and Huxley, 1945, 1952; Hodgkin *et al*., 1952). Later, Hille, Armstrong and colleagues used selective toxins and drugs to block Na^+ and K^+ currents during action potentials, which demonstrated that these conductances are separable and thus might involve selective channels (Armstrong, 1966; Armstrong, 1971; Hille, 1966,

1967, 1970). Ultimately, Jan and colleagues cloned ion channel genes, which led to studying the membrane topology, subunit stoichiometry, and functional properties of ion channels in heterologous expression systems (Jan and Jan, 1997; Papazian *et al*., 1987). Recent endeavors in obtaining the 3D crystal structure of ion channels has led to a structure-based functional approach to determine the mechanism of ion channel function (Doyle *et al*., 1998; MacKinnon, 2003). With the fundamental knowledge of cellular excitability, and the visual insight of the structures of ion channels, we are in a unique position for new discoveries in the field of ion channel biology.

Inwardly rectifying K channels

Channels of the inwardly rectifying potassium family (Kir) conduct K^+ ions into the cell better than out because of physical occlusion of the pore by cytoplasmic polyamines and Mg^{++} (Lopatin *et al*., 1994; Matsuda *et al*., 1987)**.** Therefore, they are not involved during the action potential but contribute to maintaining resting membrane potential. However, opening of these channels at resting membrane potential leads to hyperpolarization of the cell, decreasing excitability. ROMK1 (Kir1.1) and IRK1 (or Kir2.1) were the first inward rectifiers to be cloned; now the family of Kir channels includes Kir1 - Kir7(Ho *et al*., 1993; Kubo *et al*., 2005; Kubo *et al*., 1993a) . Kir channels are characterized by a tetrameric assembly, with subunits having intracellular N- and C- termini with two transmembrane domains, and a pore loop between the transmembrane domains which serves as a K^+ selectivity filter (Doupnik *et al*., 1995; Nichols and Lopatin, 1997). Kir channels differ from classical

voltage-gated K channels in that they lack the four additional transmembrane domains that include the voltage sensor. These Kir channels have evolved different mechanisms of opening and closing, such as sensitivity to pH, phospholipids, ATP and many other modulators. Of importance to my studies, IRK (Kir2.1-Kir2.3) channels are constitutively active and thus have a large basal current (Kubo *et al*., 1993a). GIRK (Kir3.1 to 3.4) channels are typically closed (small basal current) and are activated by Gα i/o coupled G protein coupled receptors (Kubo *et al*., 1993b; Pfaffinger *et al*., 1985). All Kir channel family are directly activated by phospholipids phosphatidylinositol 4,5-bisphosphate (PIP_2) (Huang *et al*., 1998; Schulze *et al*., 2003; Zhang *et al*., 1999). Zhang *et al*. showed that increased affinity of IRK1 for PIP_2 leads to constitutively active channel, while GIRK channels exhibit much lower PIP_2 affinity (Zhang *et al*., 1999) leading to channels with small basal currents.

G-protein gated inwardly rectifying K channels

Four different GIRK channel subunits are expressed in the nervous system (Dascal *et al*., 1993; Karschin *et al*., 1996; Krapivinsky *et al*., 1995; Kubo *et al*., 1993b; Lesage *et al*., 1994). GIRK1, GIRK2 and GIRK3 are expressed in various combinations in different loci of the brain, while the expression of GIRK4 is low in the brain (Karschin *et al*., 1996). GIRK1 and GIRK3 require co-assembly with other GIRK subunits, while GIRK2 subunits can form functional homotetramers (Dascal *et al*., 1993; Jelacic *et al*., 1999; Liao *et al*., 1996; Schoots *et al*., 1999). GIRK channels are activated by inhibitory neurotransmitters that couple to G-protein (Gαi/o) coupled receptors, such as $GABA_B$, $Muscarinic_2$, $5\text{-hydroxytryptamine}_1$, and opioid receptors (Nicoll *et al*., 1990). Upon receptor stimulation, G$\beta\gamma$ subunits of the trimeric G-protein

complex are activated – Gβγ subunits bind directly to and activate GIRK channels (Logothetis *et al*., 1987; Reuveny *et al*., 1994) . Infact, GIRK were one of the first proteins identified that are activated by the Gβγ subunits. At resting membrane potentials, this activation leads to efflux of K^+ that decreases membrane excitability of neurons (Hille, 1992). Consequently, mice lacking GIRK2 exhibit increased spontaneous and pharmacologically induced seizures, as compared to wild-type mice (Signorini *et al*., 1997) and lose a GABA mediated slow inhibitory post synaptic potential that is driven through activation of $GABA_B$ receptor coupled with GIRK channels (Luscher *et al*., 1997).

Crystal structures of inwardly rectifying K channels

Crystal structures of the intracellular domain of GIRK1, GIRK2 and IRK1 (Kir3.1, Kir3.2 and Kir2.1) have been published (Nishida and MacKinnon, 2002; Pegan *et al*., 2006; Pegan *et al*., 2005). These structures were obtained from protein crystals that have the distal N-terminus fused directly to the C-terminus. These cytoplasmic domain assemble in a tetrameric fashion to create a cytoplasmic ion conduction pathway, where the ion conduction path is lined with acidic and hydrophobic residues to accommodate polyamine block which results in inward rectification (Inanobe *et al*., 2007; Nishida and MacKinnon, 2002; Pegan *et al*., 2005). The core structures of Kir3.1 and of Kir2.1 readily superimpose, thus indicating that family members of Kir family have similar structures. Furthermore, a second gate, an intracellular “G loop” has been proposed as a gating ring, because this ring would occlude K^+ ion permeation in a closed state. Recently, crystal structures of full length Kir channels were solved (Nishida *et al*., 2007). This was done by fusing the N- and

C- terminal domains of GIRK1 with the bacterial Kirbac1.3 channel's transmembrane domain (Nishida *et al*., 2007). Upon solving the crystals Nishida *et al*. noticed that there were two distinct conformation states of the cytoplasmic domains. In one of the structures, the "G-loop" was in the "open" position, and in a second structure it was in the "closed" position in which K^+ ions could not pass.

Modulation of ion channels by ethanol

Ethanol is a major drug of addiction and abuse in the US and worldwide. Deaths from alcohol-related cases were over 100,000 per year in the United States, and the National Institute of Alcohol Abuse and Addiction estimated overall economic cost of alcohol abuse per year to be greater than $ 150 billion (NIAAA, (McGinnis and Foege, 1999)). Consumption of ethanol alters central nervous system and cardiovascular system functions, and is metabolized in the liver by the enzyme alcohol dehydrogenase. Abuse of alcohol can lead to addiction and other mental illnesses, cardiovascular diseases, and liver injury. Acute consumption of alcohol leads to mild stimulation that is followed by an overall increase in inhibition of the central nervous system (reviewed in (Deitrich *et al*., 1989)). Historically, ethanol was generally thought to exert its effect by altering the physiochemical properties of membranes, however, new evidence suggests that ethanol exerts its effect by acting directly and specifically on a number of ion channels. $GABA_A$ receptors, NMDA receptors, glycine receptors, acetylcholine receptors, $5HT_3$ receptors, purinergic receptors, voltage gated K channels, TRPV and TRPM, and GIRK channels have all been shown to be sensitive to ethanol in the milimolar range (Benedikt *et al*., 2007; Cardoso *et al*., 1999; Harris, 1999; Kobayashi *et al*., 1999; Lewohl *et al*., 1999; Li *et*

al., 1993; Lovinger *et al*., 1989; Mihic *et al*., 1997; Trevisani *et al*., 2002; Yu *et al*., 1996; Zhou and Lovinger, 1996). This correlates with levels present in an intoxicated person in the 0.1% range (~20 mM blood alcohol)

The alcohol pocket of $GABA_A$ and Gly Receptors

$GABA_A$ and Glycine Receptors are part of the cys loop family of ion channels, which have been extensively studied to implicate a hydrophobic pocket in the transmembrane domain in response to ethanol modulation (Jung *et al*., 2005; Mihic *et al*., 1997). These channels sense the inhibitory neurotransmitters in the brain, GABA or Glycine, and upon opening conduct Cl^- to hyperpolarize the cell. Harris *et al*. first reported that 10 mM ethanol potentiates $GABA_A$ currents in a heterologous system (Harris *et al*., 1995). Furthermore, this group showed that primary alcohols up to dodecanol potentiate $GABA_A$ currents (Dildy-Mayfield *et al*., 1996). Mihic *et al*. subsequently reported that while GlyR channels are potentiated by ethanol, ethanol reduces the activity of GABA ρ1 channels. However, substituting transmembrane domain 2 (TM2) and transmembrane domain 3 (TM3) of GlyR into $GABA_A$ ρ1 channel confers ethanol sensitivity to $GABA_A$ ρ1 channel (Mihic *et al*., 1997). Furthermore, mutating amino acids S270I of TM2 or A291W of TM3 in $GABA_A$ α1 to that $GABA_A$ ρ1 led to significant loss of ethanol mediated potentiation of these channels (Mihic *et al*., 1997). Additional experiments using thiol reactive groups that mimic alcohol at this pocket suggest that presence of hydrophobic group at this site in GlyR potentiates GlyR currents (Mascia *et al*., 2000). It is hypothesized that a physical hydrophobic pocket or a groove exists at the intersection between TM2 and TM3, where ethanol can bind and potentiate GABA or glycine mediated currents. However, there is no

high resolution structural data of this hydrophobic pocket and its interaction with ethanol.

Structure of a hydrophobic pocket of LUSH protein

The first high resolution structure of a hydrophobic pocket of an ethanol bindng protein was recently solved. The dorsophila odorant binding protein LUSH was crystallized with ethanol and other alcohols (Kruse *et al*., 2003). The revealed structure shows that the hydrophobic pocket exists at the intersection of two alpha helices, where hydrophobic amino acids from one helix interact with the hydrocarbons of ethanol, while the –OH group of ethanol interacts with –OH groups from the sidechains of serine and threonine and a carbonyl group from the backbone of the second alpha helix. Furthermore, using NMR spectroscopy this group showed that alcohols stabilize the conformational exchanges inherent to the protein, and that larger alcohols were more effective in stabilizing the conformations (Bucci *et al*., 2006; Kruse *et al*., 2003). These studies show that discrete hydrophobic pockets for alcohol binding exist in proteins, and that alcohols can stabilize conformational changes upon binding.

GIRKs and alcohol

The initial evidence for ethanol mediated activation of GIRK channels came in back-to-back publications in Nature Neuroscience in 1999 (Kobayashi *et al*., 1999; Lewohl *et al*., 1999). Lewohl *et al*. showed that 75 mM ethanol can potentiate $GABA_B$ mediated GIRK channel activation in dissociated cerebellar granual neurons (Lewohl *et al*., 1999). GIRK channels are also activated by ethanol in heterologous

expression system of *xenopus oocytes* (Kobayashi *et al*., 1999; Lewohl *et al*., 1999). Ethanol mediated activation was present with antisense RNA for the G-protein β1, suggesting that ethanol activation is independent of Gβγ activation (Kobayashi *et al*., 1999). Experiments with n-alcohol series, determined that small alcohols, (methanol, ethanol, propanol) were able to activate GIRK1/GIRK2 heterotetramers in an increasing manner whereas larger alcohols such as 1-butanol had a blunted activation, and 1-pentanol inhibited the GIRK channel currents (Kobayashi *et al*., 1999). This result led to the hypothesis that a hydrophobic pocket with discrete size exists in the GIRK channel. Deleting a large portion of the distal C-terminal domain of GIRK2 channels led to loss of activation by ethanol implicating the cytoplasmic domains in being important for ethanol sensitivity (Lewohl *et al*., 1999). However, the site and mechanism for activation of GIRK channel by ethanol has been elusive.

Subsequent studies have examined the role of GIRK channel in ethanol mediated behavior. Mice lacking GIRK2 channels have diminished alcohol mediated analgesia as compared to wild-type mice (Blednov *et al*., 2003). These knockout mice also self-administer more ethanol than wild-type littermates (Blednov *et al*., 2001). However, GIRK2 knockout mice failed to develop conditional place preference for ethanol as compared to wild-type mice (Hill *et al*., 2003) suggesting that GIRK channels are involved, in part, in addiction by alcohol. GIRK3 channels have also been implicated in ethanol-related behavior. The GIRK3 gene in included in a 0.44 Mb region of chromosome 1 that was identified as a qualitative trait locus associated with large effects on withdrawal following ethanol exposure (Kozell *et al*., 2009). Furthermore, it was found that GIRK3 knockout mice exhibit less severe withdrawal from ethanol and other sedatives (Kozell *et al*., 2009). These results suggest the

GIRK channels are involved in multiple ethanol related behavior, such as analgesia, and addiction. In fact, ethanol potentiates a slow inhibitory post synaptic potential (s-IPSP) in midbrain dopaminergic neurons that are implicated in addiction (Federici *et al*., 2009). This s-IPSP is a result of $GABA_B$ receptor mediated activation of GIRK channel. Increasing evidence suggests that GIRK channels are important in alcohol related behaviors.

Although GIRK channels are known to be activated by ethanol, the mechanism of modulation is not known. In this dissertation I will present evidence for direct modulation of GIRK channels by alcohols. Using a recently solved cytoplasmic structure of IRK1 crystallized with a pentanediol as a launching point, structure as a launching point, I have conducted mutagenesis of GIRK2 channels to elucidate a novel hydrophobic pocket in the cytoplasmic domain of these channels as being the site for activation by ethanol and other alcohols. Chapter II is original work which describes these findings and was published as Aryal P, Dvir H, Choe S, and Slesinger PA, A discrete alcohol pocket involved in GIRK channel activation *Nature Neurosci 2009 Aug 12(8) 988-95*. In chapter III, I will describe and new experiments and models that give insight into the mechanism of alcohol mediated activation of GIRK channels. In chapter IV, I will conclude by discussing my findings and giving a future perspective for the fields of GIRK channel and alcohol.

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II. Evidence for a Discrete Alcohol Pocket Involved in GIRK Channel Activation

Abstract

Ethanol modifies neural activity in the brain by modulating ion channels. Ethanol activates G protein-gated inwardly rectifying K^+ channels but the molecular mechanism is not well understood. Here, we used a crystal structure of an inward rectifier containing a bound alcohol and structure-based mutagenesis to probe a putative alcohol-binding pocket located in the cytoplasmic domains of GIRK channels. Substitutions with bulkier side-chains in the alcohol-binding pocket reduced or eliminated activation by alcohols. By contrast, alcohols inhibited constitutively open channels, such as IRK1 or GIRK2 that binds PIP_2 strongly. Mutations in the hydrophobic alcohol-binding pocket of these channels had no effect on alcohol-dependent inhibition, suggesting an alternate site is involved in inhibition. Comparison of high-resolution structures of inwardly rectifying K^+ channels suggests a novel model for activation of GIRK channels via this hydrophobic alcohol-binding pocket. These results provide a tool for developing therapeutic compounds that could mitigate the effects of alcohol.

Introduction

Many ligand-gated ion channels, such as those gated by GABA, N-methyl-D-aspartate, glycine, acetylcholine and serotonin, are responsive to ethanol (EtOH) and other alcohols (Cardoso *et al*., 1999; Lovinger *et al*., 1989; Mihic *et al*., 1997; Zhou and Lovinger, 1996). Initially, alcohol was hypothesized to indirectly alter the function

of channels by changing the fluidity of the lipid bilayer(Harris *et al*., 2008). More recent studies, however, suggest alcohol acts directly through a physical-binding pocket located in the channel protein (Mihic *et al*., 1997; Wick *et al*., 1998). In addition to ligand-gated channels, alcohols also modulate potassium channels (Covarrubias *et al*., 1995; Kobayashi *et al*., 1999; Lewohl *et al*., 1999). For example, EtOH activates G protein-gated inwardly rectifying potassium (GIRK or Kir3) channels (Kobayashi *et al*., 1999; Lewohl *et al*., 1999). Behavioral studies have shown that mice lacking GIRK2 channels exhibit diminished EtOH-dependent analgesia (Blednov *et al*., 2003) and consume more EtOH than wild-type mice (Blednov *et al*., 2001), suggesting a functional role for GIRK channels in response to alcohols *in vivo*.

GIRK channels are also activated following stimulation of G protein-coupled receptors (GPCR), such as m2 muscarinic receptors (m2R). The mechanism of G protein activation has been extensively studied. Agonist binding to the GPCR leads to activation of the pertussis toxin sensitive G protein heterotrimer ($G\alpha\beta\gamma$), allowing the $G\beta\gamma$ subunits to associate directly with the channel and induce channel activation (Reuveny *et al*., 1994; Wickman *et al*., 1994). Mutagenesis and chimeric studies have identified several regions in the cytoplasmic domains of GIRK channels involved in $G\beta\gamma$ binding and activation (Finley *et al*., 2004; He *et al*., 1999; Huang *et al*., 1995; Ivanina *et al*., 2003; Kunkel and Peralta, 1995). Interestingly, pertussis-toxin treatment, which prevents GPCR-mediated G protein activation of GIRK channels, does not prevent alcohol activation (Kobayashi *et al*., 1999). These experiments suggest that alcohol activation occurs through a mechanism distinct from G protein activation.

Similar to GABA-gated ion channels, a physical pocket in the channel with a defined cutoff is postulated to mediate alcohol activation of GIRK channels. That is, alcohols with a carbon chain length up to four carbons (i.e. methanol, ethanol, 1-propanol (1-PrOH) and 1-butanol (1-BuOH)) activate GIRK1/2 heteromeric channels while longer alcohols inhibit the channels (Kobayashi *et al*., 1999; Lewohl *et al*., 1999). This cutoff effect suggests there are physical constraints, possibly linked to the length or hydrophobicity of the alcohol, that determine the sensitivity to alcohol modulation (Kobayashi *et al*., 1999; Wick *et al*., 1998). However, the molecular mechanism underlying alcohol activation of GIRK channels is not known. Mutagenesis studies of GIRK2 channels have implicated the distal C-terminal cytoplasmic domain in activation by alcohol (Hara *et al*., 2001; 1999), but these studies did not reveal a physical alcohol-binding pocket in the channel.

Recently, we described a high resolution structure of the cytoplasmic domains of a G protein-insensitive inwardly rectifying potassium channel (IRK1 or Kir2.1) that contains bound alcohols (Pegan *et al*., 2006). The alcohol, 2-methyl, 2-4-pentanediol (MPD), is bound to four similar solvent accessible hydrophobic pockets, each formed by two adjacent subunits of the tetramer. Intriguingly, this IRK1-bound pocket has features similar to the structure of an odorant alcohol binding protein, LUSH, which was crystallized with EtOH (Kruse *et al*., 2003). In both structures, the alcohol pocket is formed by hydrophobic amino acids and hydrogen bonding polar groups. Thus, the hydrophobic alcohol-bound pocket in IRK1 is a putative site for modulation by alcohols. Because the crystal structure of the cytoplasmic domain of GIRK1 or GIRK2 channels is very similar to that of IRK1 (Inanobe *et al*., 2007; Nishida and MacKinnon, 2002; Pegan *et al*., 2005), we hypothesize that GIRK channels also possess

cytoplasmic hydrophobic alcohol-binding pockets that are involved in alcohol-dependent activation.

Results

Conservation of MPD bound pocket in IRK1 and GIRK2

Recently, we showed that a high resolution structure of the IRK1 cytoplasmic domains contains bound alcohols (which we refer to here as IRK1-MPD)(Pegan *et al*., 2006). The alcohol-binding pocket in the IRK1-MPD complex is formed by hydrophobic amino acid side-chains from three different domains, the N-terminal, the βD-βE ribbon and the βL-βM ribbon (**Fig. 2.1a,b**) (Pegan *et al*., 2005). There are seven amino acids that interact with MPD (Pegan *et al*., 2006). Of these, the hydrophobic side-chains of F47, L232, L245 and L330 and Y337 point toward the pocket. In addition to the hydrophobic environment of the pocket, hydrogen bonds may form between one of the hydroxyl groups of MPD and a hydrogen bonding triangle between backbone carbonyl of P244 and OH group of Y242 via a water and between the second hydroxyl group of MPD and the hydroxyl group of Y337 (Pegan *et al*., 2006). We compared the high-resolution structure of IRK1 with that of GIRK2 (Inanobe *et al*., 2007) and identified the putative alcohol-binding pocket in GIRK2. The hydrophobic pocket in GIRK2 has significant conservation with amino acids that line the pocket in IRK1 and appears large enough to accommodate MPD similar to IRK1 (**Fig. 2.1c,d**).

MPD activates GIRK2 channels similar to primary alcohols

To test whether the hydrophobic pocket in GIRK2 is the site for alcohol-mediated activation, we first investigated whether GIRK2 channels are sensitive to MPD modulation. Whereas primary alcohols up to the size of butanol (1-BuOH; four carbons) activate GIRK1/2 channels (Kobayashi *et al*., 1999; Lewohl *et al*., 1999), the effect of MPD with five backbone carbons was unknown. GIRK2 channels expressed in HEK-293T cells produced a small inwardly rectifying basal K^+ current that was inhibited by extracellular Ba^{++} (**Fig. 2.1e**). Bath application of MPD (100 mM) increased the amplitude of the inwardly rectifying current (**Fig. 2.1e**), indicating that MPD activates GIRK channels. Note also that MPD appeared to inhibit an endogenous voltage-gated outward current at positive potentials (**Fig. 2.1e**, arrow), which is likely a voltage-gated K channel (Covarrubias *et al*., 1995). All three alcohols activated GIRK2 channels at 10 mM and displayed a steep increase in activation around 100 mM (**Fig. 2.2a**). The activation curve for MPD falls between EtOH and 1-PrOH (**Fig. 2.2b**) and does not reach a maximum, similar to previous studies (Kobayashi *et al*., 1999; Lewohl *et al*., 1999). These results show that MPD activates GIRK2 in a similar manner to other small n-alcohols. Interestingly, 1-pentanol (1-PeOH), which also has five backbone carbons like MPD, predominantly inhibits GIRK2 channels (see Supplemental **Fig. 2.S1**). Therefore, a large diol such as MPD activates GIRK2 channels in a similar manner to small primary alcohols, such as ethanol but is different from 1-PeOH (see discussion).

Pertussis toxin treatment does not prevent EtOH activation of GIRK channels, indicating that GPCR coupling to G proteins is not involved (Kobayashi *et al*., 1999). To rule out the possibility that alcohols activate GIRK channels by directly stimulating G protein heterotrimers and liberating $G\beta\gamma$ subunits, we measured the alcohol

response of GIRK2 channels in cells co-expressing a myristoylated form of phosducin (m-Phos) that chelates Gβγ following stimulation of GPCRs (Rishal *et al*., 2005). Compared to controls, carbachol stimulation of m2R evoked smaller GIRK2 currents in cells coexpressing m-Phos (**Fig. 2.2c–e**). All three alcohols, on the other hand, activated GIRK2 channels to the same extent in the presence of m-Phos (**Fig. 2.2d,e**). Thus, alcohol-dependent activation of GIRK2 channels does not appear to require free Gβγ subunits. Together, these results support the interpretation that alcohols directly activate GIRK channels through a physical alcohol-bound pocket.

Role for hydrophobic pocket in alcohol activation

To determine whether the hydrophobic pocket in GIRK2 mediates alcohol activation, we examined the effects of the side-chain volume using an amino acid with a small (Ala) or large (Trp) side-chain (**Fig. 2.3a,b**). Six mutants did not express basal K^+ currents (< –1 $pApF^{-1}$) (**Fig. 2.3b**). In mutant channels engineered with an extracellular hemagglutinin (HA)-tag, we investigated whether the lack of basal current was due to a trafficking defect using confocal microscopy. Four mutants, HA-GIRK2-Y58W, HA-GIRK2-Y58A, HA-GIRK2-L342W and HA-GIRK2-Y349A, expressed on the plasma membrane but did not conduct currents (**Fig. 2.3b**; Supplementary **Fig. 2.S2**). Mutations at GIRK2-I244 impaired expression on the membrane surface (**Fig. 2.3b**). These findings suggest the hydrophobic pocket in GIRK2 is important for channel gating and/or assembly in the absence of alcohol. Four other mutants, GIRK2-L257A, GIRK2-L257W, GIRK2-L342A and GIRK2-Y349W, produced functional channels that were activated by EtOH (**Fig. 2.3c**). However, GIRK2-L257W, displayed significantly smaller EtOH-activated currents

(**Fig. 2.3c**), suggesting that Leu at position 257 in the βD-βE ribbon is a key residue that is required for alcohol-dependent activation of GIRK2 channels.

In the IRK1-MPD structure, L245, which is homologous to L257, is positioned at the base of the pocket, and interacts intimately with MPD. The decrease in EtOH-activated current of GIRK2-L257W raised the possibility that amino acids with bulky side-chains might generally interfere with alcohol activation. We systematically evaluated the effect of substituting twelve different amino acids of increasing molecular side-chain volume in GIRK2-L257. Of the twelve, five expressed (> –1 $pApF^{-1}$) and could be investigated further for possible changes in alcohol-mediated activation (**Fig. 2.4a**). The magnitude and rank order (1-PrOH > MPD > EtOH) for alcohol activation with smaller molecular volume substitutions, such as Ala, Cys and Met, were indistinguishable from wild-type Leu in GIRK2 channels (**Figs. 2.4b,c** and **2.5a**). On the other hand GIRK2-L257Y reduced 1-PrOH and MPD but not EtOH activation while GIRK2-L257W affected EtOH, 1-PrOH and MPD- dependent activation (**Figs. 2.4d,e** and **5a**). Interestingly, 100 mM MPD no longer activated and now inhibited the basal currents for GIRK2-L257W (**Figs. 2.4e** and **2.5a**). For GIRK2-L257Y and GIRK2-L257W, the decrease in alcohol activation was observed at a full range of concentrations (25, 125, 250 mM) for 1-PrOH or MPD (**Fig. 2.5b**), indicating a significant impairment in alcohol sensitivity. In addition to the change in alcohol response, mutations at L257 also reduced the m2R-mediated currents (**Figs. 2.4c-e** and **5a**), indicating that L257 is involved in both alcohol-mediated and Gβγ-mediated activation (see Discussion). Taken together, these results demonstrate that increasing the side-chain volume at L257 leads to a progressive loss in alcohol mediated activation (**Fig. 2.5a** and **inset**). A switch in alcohol activation occurred with

an increase in volume from wild-type Leu (101 $Å^3$) to Tyr (133 $Å^3$) or Trp (168 $Å^3$). In addition, bulky substitutions at L257 affected larger alcohols (MPD) more than smaller alcohols (EtOH), suggesting the molecular volume of the pocket is an important determinant of alcohol specificity.

Because alcohol activation is a property of most types of GIRK channels (Kobayashi *et al*., 1999; Lewohl *et al*., 1999), we reasoned that a homologous mutation in a related GIRK channel would also alter the response to alcohols. To test this idea, we investigated the effects of mutating L252 in GIRK4. We used GIRK4* that contains a S143T mutation in the pore-helix, which enhances channel activity without affecting surface expression (Vivaudou *et al*., 1997). Substituting Ala (26 $Å^3$), Tyr (133 $Å^3$) or Trp (168 $Å^3$) at L252 in GIRK4* channels did not change the basal K^+ currents (**Fig. 2.6a**). Similar to mutations of L257 in GIRK2, Trp and Tyr substitutions in GIRK4* decreased EtOH, 1-PrOH and MPD activation, as compared to L252A, with 1-PrOH now inhibiting GIRK4*-L252W (**Fig. 2.6b–f**). In contrast to GIRK2, however, MPD activation of GIRK4*-L252W was not significantly different from wild-type (**Fig. 2.6e,f**). Mutating GIRK4*-L252 also significantly reduced m2R-activated GIRK currents (**Fig. 2.6c–f**). Thus, the putative hydrophobic alcohol-binding pocket in GIRK4* is important for mediating alcohol activation but GIRK4* may accommodate MPD differently than GIRK2 (see Discussion).

Mutations of MPD pocket do not alter alcohol inhibition

MPD is bound to a hydrophobic pocket in the crystal structure of IRK1, suggesting that MPD might inhibit IRK1 channels like other alcohols (Kobayashi *et al*., 1999; Lewohl *et al*., 1999). Bath applying 100 mM MPD inhibited nearly 50% the

basal inwardly rectifying K^+ current through IRK1 channels (**Fig. 2.7a**). The MPD inhibition was dose dependent and had an IC_{50} of 104 ± 23 mM and a Hill coefficient of 0.93 ± 0.02 (n=8) (**Fig. 2.7b**). We next investigated whether Trp substitutions in the hydrophobic alcohol-binding pocket of IRK1 altered alcohol-dependent inhibition (**Fig. 2.7c**). IRK1-F47W, IRK1-L232W, IRK1-L245W IRK1-L330W but not IRK1-Y337W produced significant basal K^+ current (Ba^{++}-sensitive) (data not shown). Like wild-type IRK1, MPD inhibited the basal currents of mutant channels in a dose-dependent manner. In fact, the IC_{50} for MPD inhibition was indistinguishable among the different IRK1 mutants (**Fig. 2.7d**). Furthermore, IRK1-L245W mutation did not alter inhibition by EtOH, 1-PrOH or 1-BuOH (data not shown). Thus, mutations at the hydrophobic pocket of IRK1 channels do not appear to alter the sensitivity to inhibition by alcohols.

IRK1 channels are constitutively open, producing a large basal K^+ current. We speculated that alcohols might therefore inhibit GIRK channels engineered to be constitutively open. We introduced a high affinity PIP_2 site shown previously to produce a large basal current (Zhang *et al*., 1999; Zhou *et al*., 2001) (GIRK2-PIP_2). In contrast to wild-type GIRK2, GIRK2-PIP_2 exhibited large basal currents as expected (–530 ± 197 $pApF^{-1}$, n=5). Application of 100 mM MPD inhibited the basal K^+ current of GIRK2-PIP_2 (**Fig. 2.7e**). Similar to IRK1, we hypothesized that mutating L257 to Trp in GIRK2-PIP_2 would have no effect in alcohol mediated inhibition. Accordingly, GIRK2-PIP_2-L257W produced large Ba^{++}-sensitive currents (–363 ± 182 $pApF^{-1}$, n=6) that were inhibited by MPD, similar to GIRK2-PIP_2 channels (**Fig. 2.7f**). We conclude that the hydrophobic alcohol-binding pocket in IRK1 or GIRK2 is not involved in alcohol-dependent inhibition. Furthermore, these results show that constitutively open inwardly rectifying K^+ channels are not activated further by alcohols.

While investigating alcohol modulation of GIRK4*, we discovered that 1-BuOH activated GIRK4*, in contrast to inhibition of GIRK4 wild-type (Supplementary **Fig. 2.S3**) or GIRK1/4 heterotetramers (Kobayashi *et al*., 1999; Lewohl *et al*., 1999). GIRK4 and GIRK4* differ by a point mutation (S143T) in the pore-helix(Vivaudou *et al*., 1997) suggesting that S143T may regulate sensitivity to alcohol inhibition. To assess whether this site is generally involved in alcohol-mediated inhibition of GIRK channels, we introduced a Thr at the equivalent Ser (S148T) in GIRK2-PIP_2 channels. As predicted, GIRK2-PIP_2-S148T significantly shifted the IC_{50} for MPD-dependent inhibition compared to GIRK2-PIP_2 (**Fig. 2.7f**). Taken together, these experiments implicate amino acids in the pore-helix in regulating the extent of alcohol-dependent inhibition and support the conclusion that the cytoplasmic alcohol-binding pocket mediates alcohol-dependent activation, but not inhibition, of GIRK channels.

Discussion

Based on high-resolution channel structures and functional mutagenesis, we have identified a physical site for alcohol-mediated activation of GIRK channels. Amino acid substitutions that increased the molecular side-chain volume at a conserved Leu in the βD-βE ribbon of the hydrophobic pocket of GIRK2 decreased alcohol mediated activation of GIRK channels (see **Fig. 2.8a**). In particular, two substitutions, Trp and Tyr, at the Leu in the hydrophobic pocket of GIRK2 (L257) and GIRK4* (L252) channels produced a progressive loss in alcohol activation. For GIRK4*, Ala substitution increased the amplitude of alcohol-activated currents. Thus, increasing or decreasing the volume of the pocket by altering the amino acid side-

chain produced changes in alcohol activation. Similarly, the size of the putative alcohol-binding pocket in $GABA_A$ α1 and glycine receptors is important for determining modulation by alcohol and other small anesthetics. Increasing the bulkiness of amino acids in the putative alcohol-binding pocket of these channels eliminates the modulation by EtOH (Mihic *et al*., 1997) or isofluorane (Jenkins *et al*., 2001). By contrast, decreasing the size of amino acids in the same region of the decanol-*insensitive* GABA ρ1 receptors now enables potentiation by decanol(Mihic *et al*., 1997; Wick *et al*., 1998). Taken together with our findings, these studies support a model that physical pockets of defined dimensions can be probed with mutations that change the dimension of the alcohol-binding sites. Using the IRK1-MPD structure as a guide, we estimate the volume of the hydrophobic alcohol binding pocket of GIRK channels to be ~250 $Å^3$, which is large enough to accommodate bulky alcohols like MPD (~130 $Å^3$, **Fig. 2.8b**). A Trp mutation in the pocket would decrease the volume that could potentially occlude larger alcohols (**Fig. 2.8b**). In addition, the sensitivity to activation may be different between GIRK2 and GIRK4* channels. For example, Trp substitution in GIRK2 eliminated MPD activation, revealing inhibition of current. In GIRK4*, Trp substitution eliminated 1-PrOH activation but showed only a significant decrease in MPD activation when comparing Ala with Trp substitutions. One possible explanation is that MPD fits differently in the alcohol pocket of GIRK4*, which could be revealed in a complex of MPD and GIRK4 structure.

The observations that mutations at the hydrophobic pocket did not alter alcohol dependent inhibition of IRK1, and that S148T mutation (but not L257W) in GIRK2-PIP_2 decreased alcohol-dependent inhibition suggest that GIRK channels possess two different sites for alcohol modulation. In the Kirbac1.3 structure, the Ser

is located in the pore helix of Kirbac1.3 where there is no space for alcohol (Supplemental **Fig. 2.S4**), suggesting that S148 regulates the sensitivity to inhibition but does not form the binding site. Alcohols might interfere with ion permeation or possibly with gating at the transmembrane domains, similar to voltage-gated K channels (Covarrubias *et al*., 1995). Another possibility is that alcohols inhibit the channel by altering the fluidity of the lipid membrane (Harris *et al*., 2008) and/or decreasing interactions with phospholipid phosphatidylinositol bisphosphate (PIP_2) which is required for channel function(Huang *et al*., 1998).

Interestingly, whereas 1-octanol inhibits GIRK channels, co-application of 1-octanol with EtOH has no effect on EtOH-mediated activation, also raising the possibility of second site for inhibition (Lewohl *et al*., 1999). The net effect of alcohol modulation in GIRK channels would be therefore determined by the relative potencies of activation and inhibition. In support of this, we found that bath application of 1-PeOH inhibited GIRK2 channels but induced a large current immediately after washout (Supplementary **Fig. 2.S1**), revealing two components of alcohol modulation. It is notable that the dominant effect of MPD on GIRK is activation. A functional difference between diols and primary alcohols has been reported previously for NMDA channels, where long chain diols, but not primary alcohols, are more potent modulators of NMDA currents (Peoples and Ren, 2002). The presence of two sites for alcohol modulation also suggests that ascribing a cutoff number for alcohol activation of GIRK channels would not be accurate, in contrast to the determination of the cutoff number for $GABA_A$ channels (Dildy-Mayfield *et al*., 1996).

Two different types of alcohol-bound protein structures have been solved previously, the enzymatic/catalytic alcohol dehydrogenase (ADH) and a non-catalytic

Drosophila odorant-binding protein, LUSH. In ADH, primary alcohol is coordinated with Zn^{2+} in a hydrophobic pocket, where it catalyzes the oxidation of alcohol to aldehyde (Ramaswamy *et al*., 1994; Svensson *et al*., 2000). Mutagenesis studies in the pocket indicated the bulkiness of side-chains in the hydrophobic pocket determines alcohol specificity (Weinhold and Benner, 1995). The high-resolution structure of LUSH in complex with small alcohols showed that in addition to hydrophobic interactions, a network of hydrogen bonds help stabilize alcohol in the LUSH alcohol-binding pocket (Kim *et al*., 1998; Kruse *et al*., 2003; Thode *et al*., 2008). The hydrophobic pocket in IRK1-MPD has many of the same features of these alcohol-binding pockets. First, hydrophobic amino acids form the pocket and interact intimately with hydrocarbons of the alcohol (**Fig. 2.1b**). In GIRK2, mutations of L257 to bulkier amino acids significantly reduce or lead to loss of alcohol-mediated activation. This finding is consistent with the role of hydrophobic side-chains in determining the size of the alcohol-binding pocket. Second, the IRK1-MPD structure indicates that hydrogen-bonds form between MPD and the backbone carbonyl of P244, Y242 via a water and a hydroxyl of Y337 (**Fig. 2.1**) (. At the homologous position for IRK1-Y242, GIRK2 contains a Phe (F254), which indicates that this hydrogen-bond triangle may not be essential. Additionally, we found that MPD mediated activation was not affected by Y349W mutation at the homologous position of IRK1-Y337) (Supplemental **Fig. 2.S5**). Therefore it is possible that the carbonyl group of Pro in the βD-βE ribbon is the linchpin that stabilizes alcohol in the pocket via hydrogen-bonding. Unnatural amino acid mutagenesis (Lu *et al*., 2001) would be needed to further establish the importance of this H-bond interaction in stabilizing alcohol.

Though specific alcohol-induced conformational changes in the channel protein remain to be determined, our structural analysis between IRK1-MPD structure and that of chimeric KirBac1.3-GIRK1 structure provides some new clues into channel gating (Nishida *et al*., 2007; Pegan *et al*., 2005). Two different conformational states of GIRK have been described: a putative open state, due to the open position of the G-loops in the cytoplasmic gate (Nishida *et al*., 2007; Pegan *et al*., 2005) (GIRK1-*open*, **Fig. 2.8c**) and a putative closed state (GIRK1-*closed*, **Fig. 2.8c**). We aligned the IRK1-MPD structure with these two different Kirbac1.3-GIRK1 structures, and discovered that IRK1-MPD structure aligns better with the GIRK1-*open* in the hydrophobic alcohol-binding pocket (**Fig. 2.8c, zoom**). By contrast, the alignment with GIRK1-*closed* structure shows striking differences in the hydrophobic alcohol pocket. In particular, the side-chains from F46 in the N-terminal domain, L246 in the βD-βE ribbon and L333 in the βL-βM ribbon fill the hydrophobic pocket of the putative closed state of GIRK1. In the open state, however, structural rearrangements of F46, L246, L333 and F338 occur that enable MPD to now fit in the pocket.

Based on our mutagenesis data and structural analyses, we propose a tenable model for alcohol activation of GIRK channels. At rest, GIRK2 channels undergo infrequent structural rearrangements in the pocket that correlate with the open and closed positions of the channel's cytoplasmic gates, the G loops (Nishida *et al*., 2007; Pegan *et al*., 2005) and M2 transmembrane domains (Jin *et al*., 2002; Sadja *et al*., 2001; Yi *et al*., 2001) (**Fig. 2.8c**). Alcohol entering the pocket could then stabilize the open conformation, leading to alcohol activated currents. Bulky substitutions at L257/L252 of GIRK channels, located at the base of the alcohol pocket, would hinder alcohols from filling the pocket. Alcohols might also displace

other amino acids that fill the hydrophobic pocket in the closed state (**Fig. 2.8c**), promoting the open state. Previous studies have suggested that Gβγ dependent activation of GIRK channels involves increases in PIP_2 affinity (Huang *et al*., 1998; Zhang *et al*., 1999). Similarly, changes in PIP_2 binding may also be involved in alcohol-dependent activation of GIRK channels. Studies in the future will need to investigate the molecular relationships between movement of the N-terminal domain, βD-βE and βL-βM ribbons within the hydrophobic pocket, PIP_2 interactions, and the channel gates.

The alcohol-binding pocket may be also involved in Gβγ-dependent activation. Mutation of a conserved Leu (GIRK2-L344, GIRK4*L339, GIRK1-L333, **Fig. 2.8c**) to Glu in the βL-βM ribbon of GIRK channels attenuates Gβγ activation (Finley *et al*., 2004; He *et al*., 1999; Ivanina *et al*., 2003). We found that mutations in the βD-βE (L257) ribbon that altered alcohol-dependent activation also reduced Gβγ-dependent activation (**Figs. 2.4** and **2.5**). Together, these results suggest that conformational changes in the βD-βE and βL-βM structural elements, along with the N-terminal domain (Riven *et al*., 2003; Sarac *et al*., 2005), may be central to both alcohol- and Gβγ-dependent activation. Intriguingly, hydrophobic amino acids in the G protein Gβ subunit have been implicated in GIRK channel activation (Ford *et al*., 1998), which perhaps interact directly with the alcohol-binding pocket in GIRK.

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from Hay Dvir, and Senyon Choe, the two other co-authors. P.A.S. and P.A. designed the experiments and analyzed the data. P.A. conducted the molecular cloning, electrophysiology and imaging experiments. H.D. and P.A. collaborated on structural analysis and figure production. H.D. conducted modeling experiment. P.A., H.D., and P.A.S co-wrote and revised the manuscript. P.A.S and S.C. supervised the project.

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Materials and Methods

Molecular Biology and Cell Culture

GIRK2c (for clarity, we refer to GIRK2c as 'GIRK2' in this study), GIRK4, IRK1, m2 muscarinic receptor (m2R) and m-Phosducin (m-Phos) were subcloned into pcDNA3.1 vector (Invitrogen) m-Phos cDNA was kindly provided by Dr. Nathan Dascal (Rishal *et al*., 2005). GIRK4* contains a S143T mutation in the pore-helix (Vivaudou *et al*., 1997). Point mutations were introduced by Quickchange site directed mutagenesis kit (Stratagene). GIRK2-PIP_2 mutant was created by overlap-

PCR method (Ho *et al*., 1989). Briefly, a region of GIRK2-D228-L234 was replaced with the homologous region of IRK1-N216-L222; this region contains seven amino acids in the βC-βD region implicated previously in PIP_2 binding (Zhang *et al*., 1999; Zhou *et al*., 2001) (we refer to this mutant as GIRK2-PIP_2). All mutations were confirmed by DNA sequencing. HEK-293T cells were cultured in DMEM supplemented with 10% fetal bovine serum, and 1X Glutamax (Invitrogen) in a humidified 37 ^{o}C incubator with 5% CO_2. Cells were plated in 12 well dish, and transiently transfected with DNA using Lipofectamine 2000 (Invitrogen). Cells were re-plated to 12-mm glass coverslips coated with poly-D-Lysine (20 μg/ml) 12-24 hr after transfection.

Detection of channels expressed on membrane surface

GIRK2c and mutant channels were engineered with an extracellular hemagglutinin (HA) epitope inserted between I126 - E127 for immunohistochemical detection with anti-HA antibodies (Clancy *et al*., 2007). HEK-293T cells were transfected with 0.5 μg of channel cDNA and examined 24-48 hr after transfection. Cells were washed with 1X Dulbecco's Phosphate Buffer Saline (DPBS; Invitrogen), fixed with 2% para-formaldehyde (PFA) in 1X DPBS for 10 min and rinsed with 1X DPBS (at 22-25 °C). To label surface channels, cells were incubated with blocking buffer (3% BSA in 1X DPBS) for 1 hr and then with anti-HA mouse antibody (1:400 in blocking buffer; Covance) for 2 hr at 22 °C. To label cytoplasmic channels, cells were rinsed with 1X DPBS, permeabilized with 0.25% TritonX-100 (Sigma) in blocking buffer for 10 min at 22 °C and incubated with blocking buffer for 1 hr. Cells were then incubated with rabbit anti-GIRK2 (1:200 in blocking buffer; Alomone) for 2 hrs.

Following rinses in 1X DPBS, cells were incubated with fluorescent secondary antibodies, anti-mouse Alexa-647 and anti-rabbit Alexa-488 (1:300; Invitrogen) for 1 hr in the dark. Cells were rinsed with 1X DPBS, mounted on microscope slides using Progold anti-fading reagent (Invitrogen), and both fluorophores imaged with a Leica TSC SP2 AOBS laser confocal microscope.

Whole-cell patch-clamp electrophysiology

HEK-293T cells were transfected with 0.2 μg of channel cDNA and 0.04 μg of eYFP cDNA to identify transfected cells. For some experiments, 0.8 μg of m2R and 0.8 μg of m-phosducin cDNA were also transfected. Whole-cell patch clamp recordings were performed 24-72 hr after transfection. Borosilicate glass electrodes (Warner Instruments) of 5-7 mΩ were filled with intracellular solution (130 mM KCl, 20 mM NaCl, 5 mM EGTA, 2.56 mM K_2ATP, 5.46 mM $MgCl_2$, 0.30 mM Li_2GTP, and 10 mM HEPES, pH 7.4). Extracellular 20K solution contained 20 mM KCl, 140 mM NaCl, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM HEPES (pH7.4). Alcohols (0.1 – 300 mM), carbachol (5 μM), or $BaCl_2$ (1 mM) were diluted into the 20K solution and applied directly to the cell with the rapid valve-controlled, perfusion system (Warner Instruments, VC6, MM-6 manifold). All chemicals for electrophysiology were purchased from Sigma-Aldrich. Whole-cell patch-clamp currents were recorded using Axopatch 200B (Molecular Devices; Axon Instruments) amplifier. Currents were adjusted electronically for cell capacitance and series resistance (80–100%), filtered at 1kHz with an 8-pole Bessel filter and digitized at 5kHz with a Digidata 1200 interface (Molecular Devices: Axon Instruments). Currents were elicited with voltage ramp protocol, from –100 mV to +50 mV, delivered at 0.5 Hz. Currents were

measured at –100 mV and converted to current density ($pApF^{-1}$) by dividing by the membrane capacitance. Basal K^+ currents (Ba^{++}-sensitive) were quantified at –100 mV by applying 1 mM $BaCl_2$ in 20K and measuring the amplitude of the Ba^{++}-inhibited current. Alcohol- and carbachol-modulated currents were measured by averaging current from two consecutive sweeps upon reaching steady state, and subtracting the mean basal current before and after the application of the modulator. Pooled data are presented as mean ± s.e.m. and evaluated for statistical significance ($P < 0.05$) using a one-way ANOVA, followed by Bonferroni multiple comparison post-hoc test. To determine the $IC_{50,}$ the inhibition curves were fit with the Hill equation y=1/(1+[x/c]^b), where y is fraction current remaining, x is the concentration of alcohol, b is the Hill coefficient and c is the IC_{50}, the concentration of alcohol that produces 50% inhibition.

Structural Analysis

Molecular representations were made using PyMOL (DeLano Scientific) with PDB files 2GIX (IRK1-MPD), 2E4F (GIRK2) and 2QKS (Kirbac1.3-GIRK1). The cavity of the IRK1-MPD pocket was calculated using CASTp server(Dundas *et al*., 2006) with 1.4 Å probe radius. The model of IRK-L245W hydrophobic pocket was determined by optimizing the best rotamer position for Trp using PyMOL software. Molecular volume estimates for amino acid side-chains were based on reported values(Harpaz *et al*., 1994).

Figures

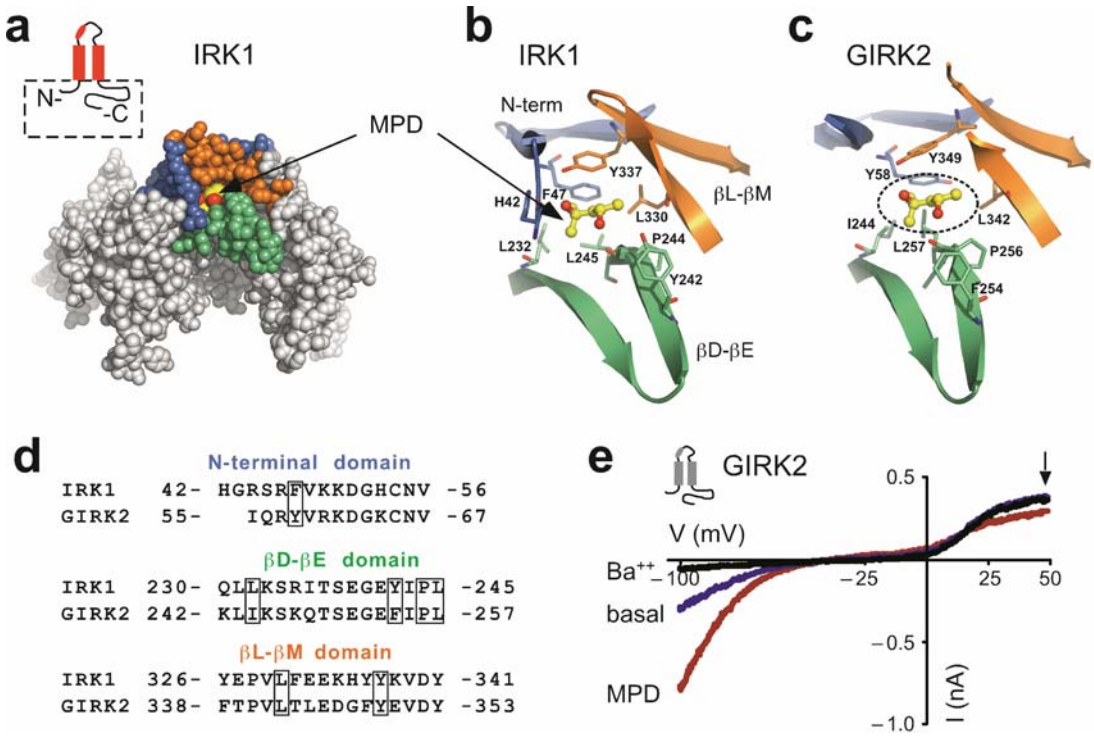

Fig. 2.1 A conserved alcohol-binding pocket in IRK1 and GIRK2 channels. a) CPK representation of the cytoplasmic domains from two subunits of IRK1 in complex with an alcohol, MPD (PDB: 2GIX). The pocket for MPD is formed by three structural elements: the N-terminal domain (blue) and the βD-βE ribbon (green) from one subunit, and the βL-βM ribbon (orange) from an adjacent subunit. Inset, schematic of IRK1 (red) shows the major structural elements of the subunit including pore loop and helix, two transmembrane domains, and N- and C-terminals used in the structure (dashed box). **b,c)** Detailed structural views of amino acids forming the hydrophobic alcohol pocket of IRK1 with MPD (ball and stick) (b) and a putative hydrophobic alcohol pocket in GIRK2 (PDB: 2E4F) (c). Amino acid residues shown in stick format are colored according to the domain they originate from; as MPD is shown in ball-and-stick format. The putative position of MPD in the GIRK2 (dashed circle) was obtained by superposition of two adjacent cytoplasmic domains from IRK1 structure and corresponding subunits from GIRK2 structure. **d)** Sequence alignment for the three domains comprising the hydrophobic alcohol pocket in IRK1 and GIRK2 channels. Boxes indicate amino acids that form hydrophobic and hydrogen-bond interactions in IRK1-MPD, and are conserved in GIRK2. 'HG' in the N-terminal domain of IRK1 originates from the polypeptide linker in the IRK1-MPD structure. **e)** Current-voltage plots for GIRK2 channels recorded in the presence of 20K (blue), 20K plus 1 mM Ba^{++} (black) or 20K plus 100 mM MPD (red). Currents were elicited by voltage ramps from –100 mV to +50 mV. MPD-induced current was 246% ± 27% (n=5, mean and s.e.m.) of basal K^{+} current (Ba^{++} sensitive).

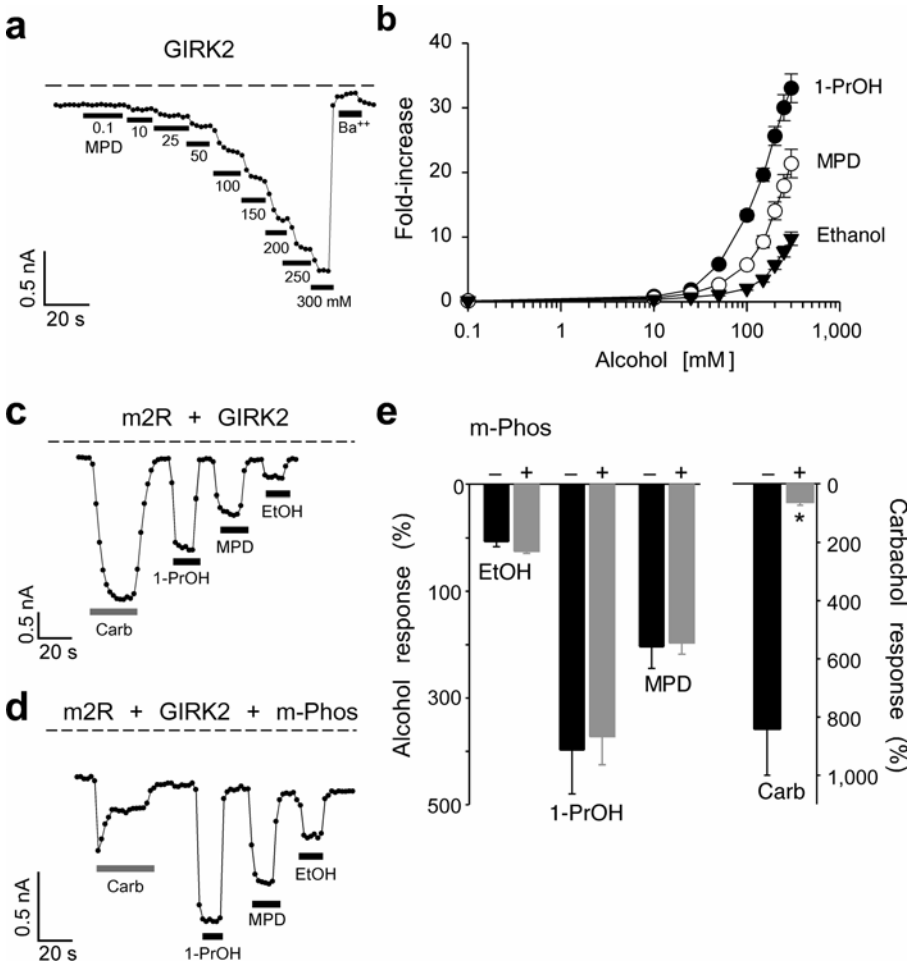

Fig. 2.2 **MPD activates GIRK2 in a manner similar to other alcohols. a)** The inward current through GIRK2 channels plotted as a function of time (at –100 mV) shows the response to the increasing concentrations of MPD and to 1 mM Ba^{++}. Dashed line shows zero current level. **b)** Dose-response curves are shown for MPD (n=6), 1-PrOH (n=6), and EtOH (n=6). The fold-increase was calculated by normalizing to the basal K^{+} current (Ba^{++}-sensitive). **c-e)** Chelating Gβγ with m-Phos attenuates m2R- but not alcohol-mediated activation of GIRK2. Current responses recorded at –100 mV are shown for m2R/GIRK2 (c) or m2R/GIRK2/m-Phos (d) in response to 100 mM 1-PrOH, 100 mM MPD, 100 mM EtOH, or 5 μM carbachol. **e)** Bar graphs show the mean percentage alcohol and carbachol responses (± s.e.m.), normalized to the Ba^{++}-sensitive basal current, in the absence (solid, n=4) or presence of m-Phos (grey, n=7). Asterisks indicate statistical significant difference from wild-type (P < 0.05).

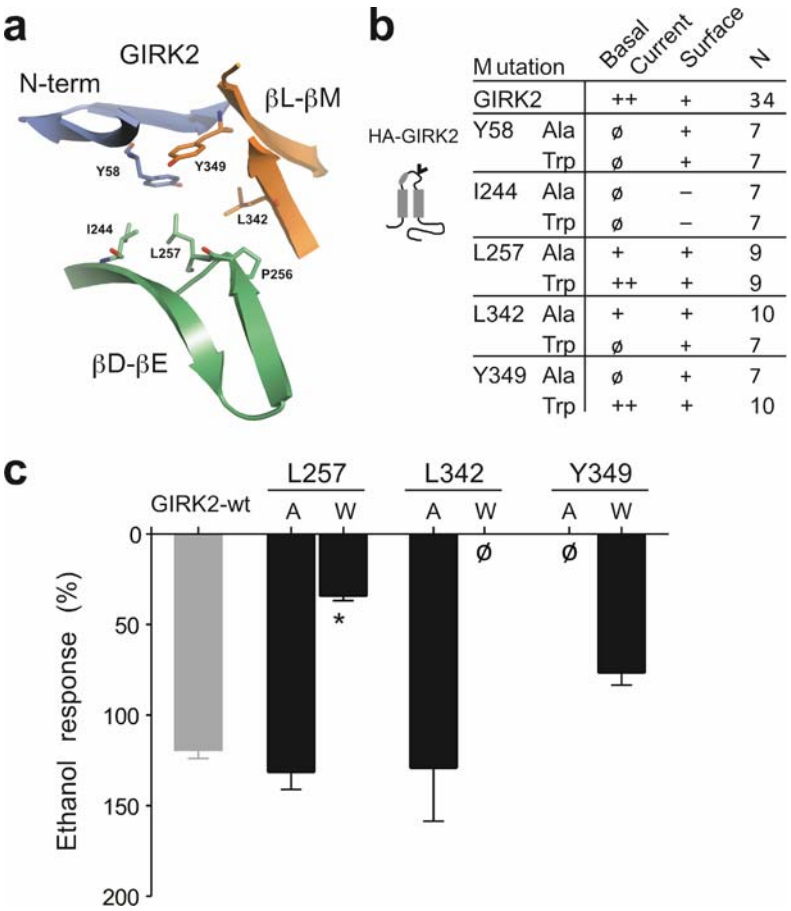

Fig. 2.3 Ala/Trp scan of the hydrophobic alcohol-binding pocket in GIRK2. **a)** Ribbon structure shows amino acids that line the hydrophobic alcohol pocket in GIRK2. **b)** Summary table of Ala/Trp mutagenesis. Basal K^+ currents (Ba^{++}-sensitive) were divided into three groups; < –1 $pApF^{-1}$ (ø), –1 to –5 $pApF^{-1}$ (+) and > –5 $pApF^{-1}$ (++) (n = number of recordings). Surface expression on the plasma membrane was assessed in separate experiments with HA-tagged channels; detected on the surface (+) or detected only in cytoplasm (–). See Supplemental **Fig.2.S1**. Schematic shows location of HA tag ('v') in GIRK2 (grey). **c)** Bar graph shows the mean ethanol percentage response, normalized to the basal K^+ current, for different mutant channels (± s.e.m.). L257W showed a significant statistical decrease in EtOH response (*P < 0.05 vs. wild-type

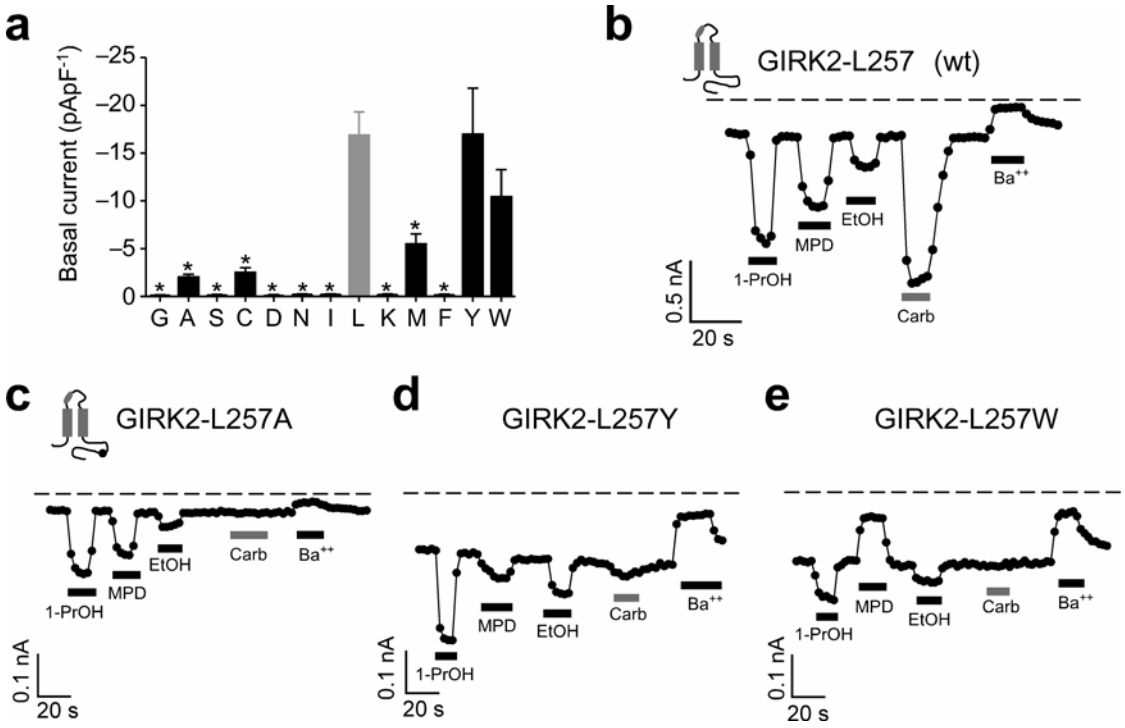

Fig. 2.4 Comprehensive mutagenesis of GIRK2-L257 in βD-βE ribbon of hydrophobic alcohol-binding pocket reveals changes in alcohol- and Gβγ-activated currents. a) Bar graph shows the mean (± s.e.m.) amplitude of basal K^+ current (Ba^{++}-sensitive) for substitutions of increasing molecular side-chain volume at GIRK2-L257: Gly (n=7), Ala (n=9), Ser (n=7), Cys (n=8), Asp (n=7), Asn (n=6), Ile (n=7), Leu (wt; n=34; grey bar), Lys (n=7), Met (n=8), Phe (n=7), Tyr (n=9) and Trp (n=9). **b-e)** Inward K^+ currents measured at –100 mV for wild-type GIRK2 (b) and the indicated GIRK2-L257 mutants (c-e) in response to 100 mM 1-PrOH, 100 mM MPD, 100 mM EtOH, 5 μM carbachol, or 1 mM Ba^{++}. Inset shows the approximate position of the C-terminal mutation.

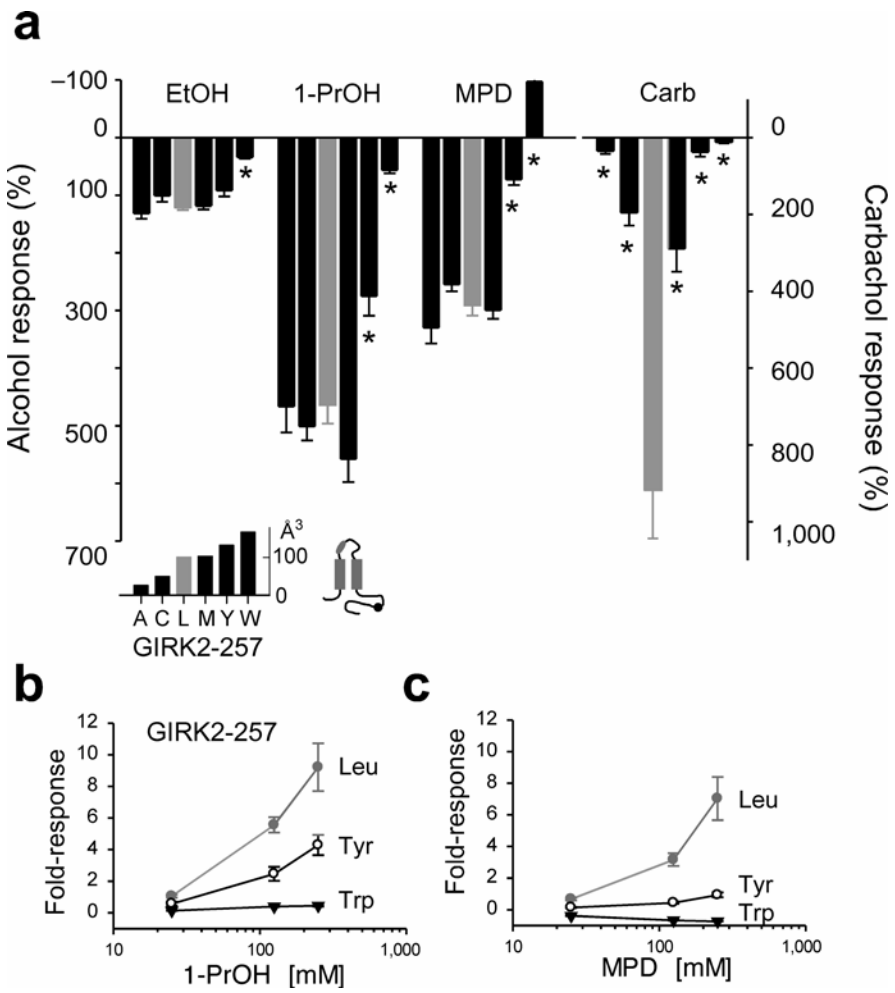

Fig. 2.5 Reduced alcohol activation with increasing bulkiness of amino acid substitutions at GIRK2-L257. a) Bar graph shows the mean percentage response to different alcohols and carbachol (± s.e.m.), normalized to the basal K^+ current (Ba^{++}-sensitive). Upward response indicates inhibition. Amino acid substitutions are arranged by increasing side-chain volume (Å^3, see inset). Asterisk indicates significant statistical difference ($P < 0.05$ vs. Leu). **b,c)** Dose-response curves are shown for GIRK2-L257, GIRK2-L257Y and GIRK2-L257W channels for 1-PrOH **(b)** and MPD **(c).** Note suppression of alcohol activation at all concentrations tested.

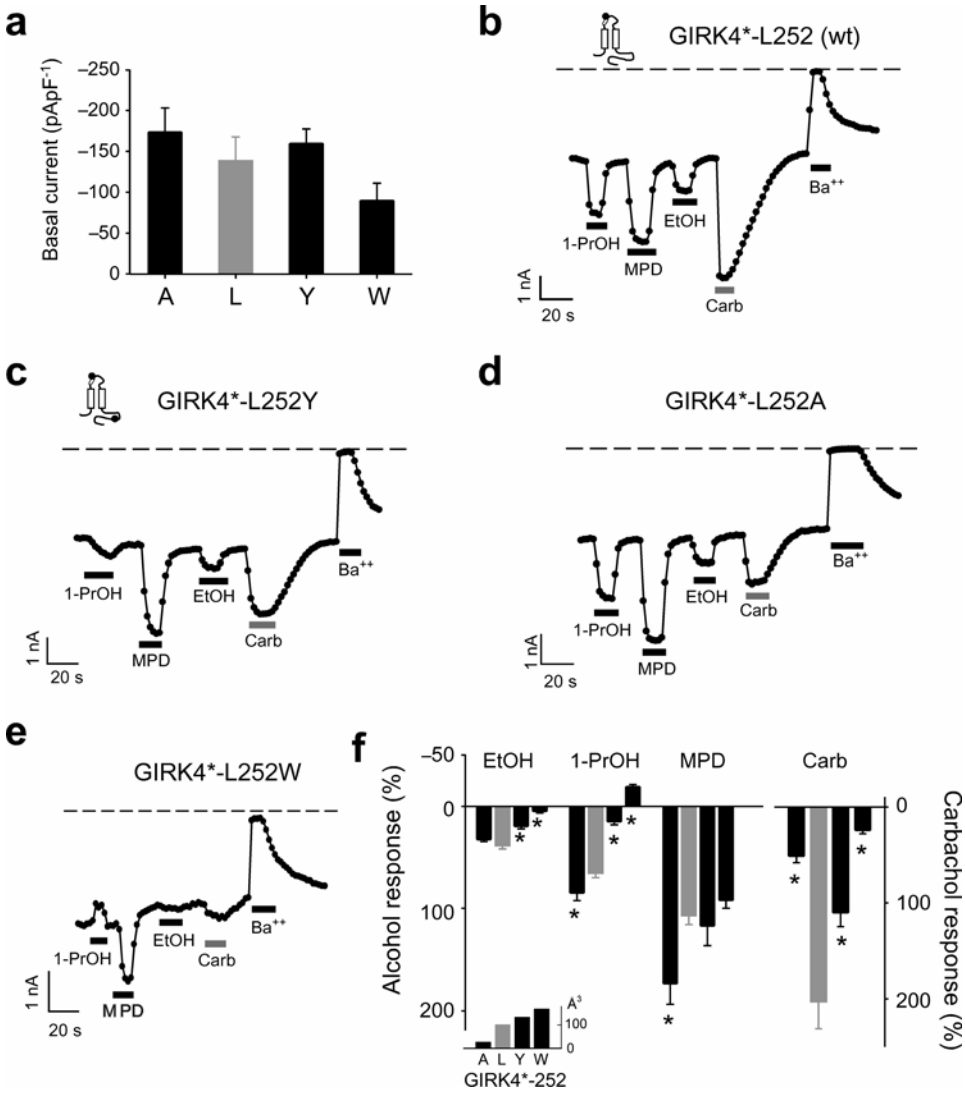

Fig. 2.6 Mutations in the hydrophobic alcohol-binding pocket of GIRK4* alter alcohol-activated currents. a) Mean basal K^+ currents (Ba^{++}-sensitive) measured for Ala (n=8), Leu (wt; grey bar, n=8), Tyr (n=8), and Trp (n=8) substitutions at GIRK4*-L252. There are no statistical differences in basal currents (P > 0.05 vs. Leu). **b-e**) Inward K^+ currents measured at –100 mV for GIRK4* (**b**) and different GIRK4*-L252 mutants (**c-e**) in response to 100 mM 1-PrOH, 100 mM MPD, 100 mM EtOH, 5 μM carbachol, or 1 mM Ba^{++}. **f**) Bar graphs show the mean percentage responses to different alcohols and carbachol, normalized to the basal K^+ current (Ba^{++}-sensitive). Amino acid substitutions are arranged by increasing side-chain volume ($Å^3$) (see inset). Asterisk indicates significant statistical difference ($P < 0.05$ vs. Leu). Channel schematics show the approximate position of the pore-helix (white ellipse) mutation, for making GIRK4*, and the C-terminal mutation (black circle). All values are mean ± s.e.m

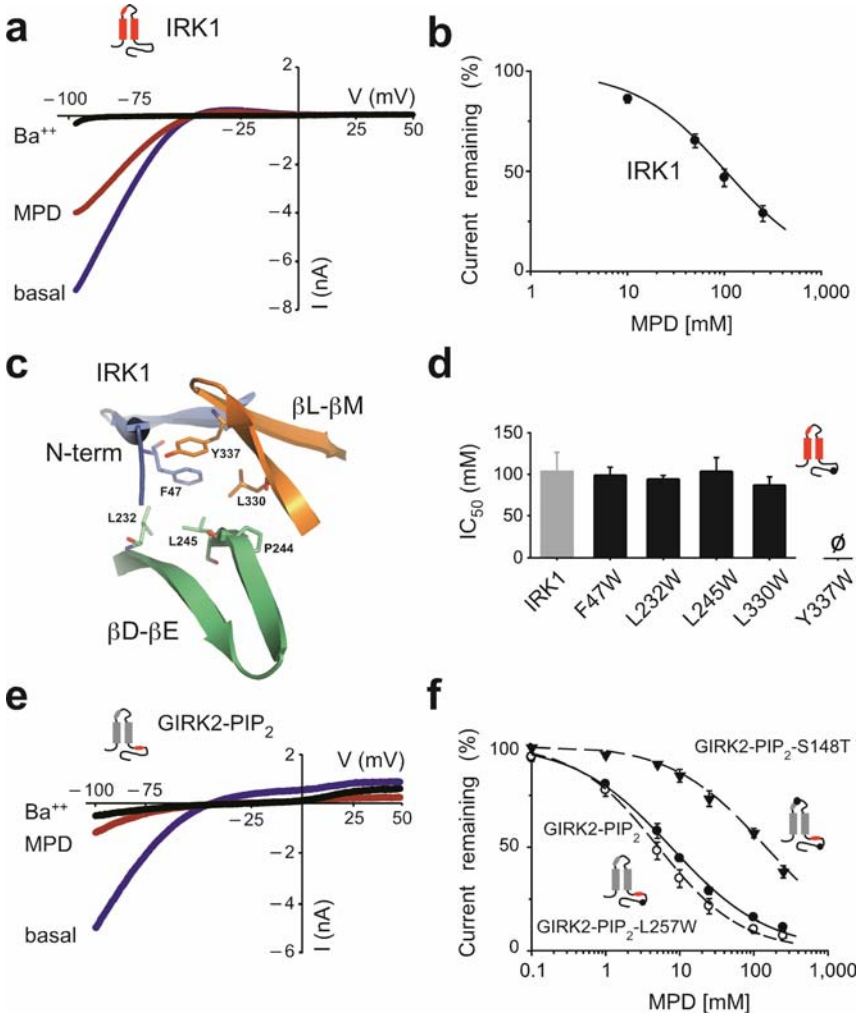

Fig. 2.7 Mutations in the hydrophobic alcohol-binding pocket of IRK1 have no effect on alcohol-dependent inhibition. a) Current-voltage plots for IRK1 channels are shown for 20K (blue), 20K plus 1 mM Ba^{++} (black) or 20K plus 100 mM MPD (red). MPD inhibited the basal K^+ current (Ba^{++}-sensitive) by 53.1%± 4.1% (n=8). **b)** Dose-response curve is shown for MPD inhibition of IRK1 channel. Smooth curve shows best fit using the Hill equation, with an IC_{50} of 104 ± 23 mM and Hill coefficient of 0.93 ± 0.03 (n=8). **c)** Structural view of amino acids that line the hydrophobic alcohol pocket in IRK1. **d)** Bar graph shows mean IC_{50}'s for MPD-dependent inhibition of IRK1 (n=8), IRK1-F47W (n=7), IRK1-L232W (n=7), IRK1-L245W (n=6), IRK1-L330W (n=6). There is no statistical difference compared to wild-type IRK1 (P > 0.05). **e)** Current-voltage plots are shown for GIRK2-PIP_2 (GIRK2 engineered with high affinity PIP_2 binding domain from IRK1) channels recorded in the presence of 20K (blue), 20K plus 1 mM Ba^{++} (black) or 20K plus 100 mM MPD (red). **f**) Dose-response curves for MPD-dependent inhibition of GIRK2-PIP_2 (solid circle), GIRK2-PIP_2-L257W (open circle) and GIRK2-PIP_2-S148T (solid triangle). Smooth curves show best fit using the Hill equation and having IC_{50}'s and Hill coefficients of 7.7 ± 1.0 mM and 0.66 ± 0.03 (n=5) for GIRK2-PIP_2, 5.2 ± 1.0 mM and 0.77 ± 0.04 (n=5) for GIRK2-PIP_2-L257W, and 147.0 ± 31.5 mM and 0.67 ± 0.05 (n=6) for GIRK2-PIP_2–S148T. All values are mean ± s.e.m.

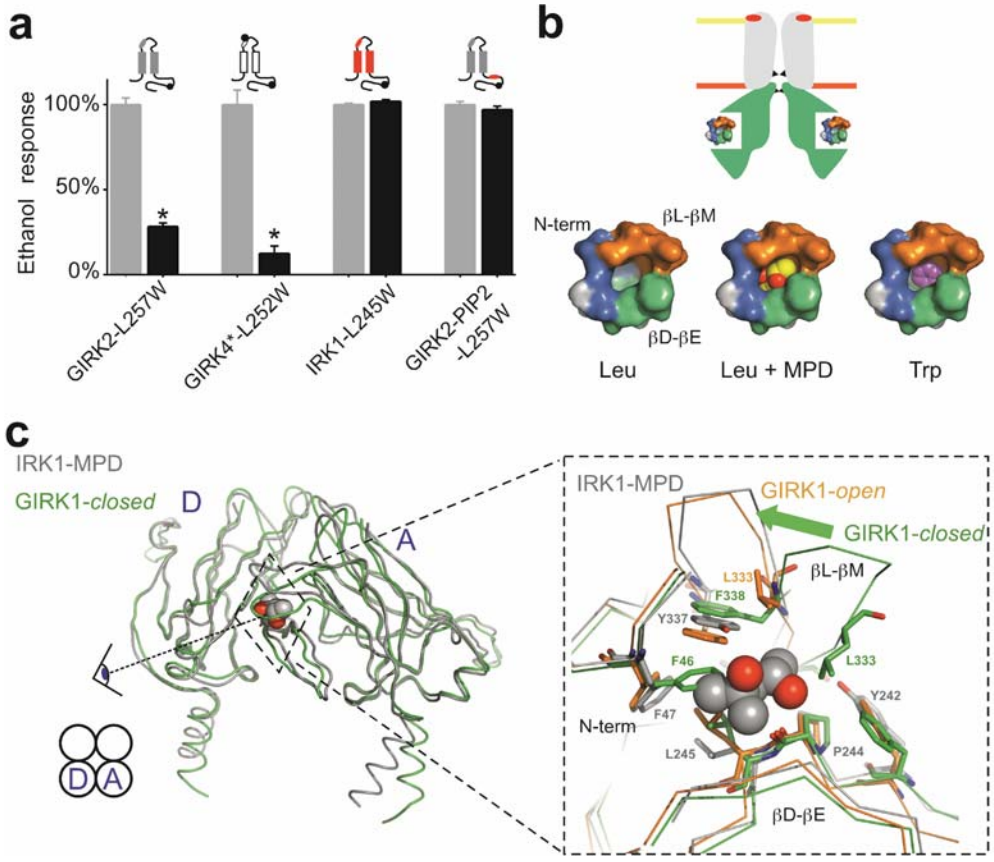

Fig. 2.8 Model for alcohol-dependent activation of GIRK channels. a) Bar graph shows the mean percentage EtOH response (activation or inhibition normalized to wild-type) for a Trp mutation in four different channels, GIRK2-L257W (n=9), GIRK4-L252W (n=8), IRK1-L245W (n=8) and GIRK2-PIP_2-L257W (n=5). Only mutations in alcohol-binding pocket of wild-type GIRK channels affect the response to alcohol. **b)** Top, schematic of inward rectifier shows location of alcohol-binding pocket in cytoplasmic domains, two gates (G-loop and M2 transmembrane; black triangles) and pore-helix region (red ellipse). PIP_2 is enriched in lower leaflet of bilayer (orange). Below, molecular surface representations of the alcohol pocket without (Leu), with MPD (Leu+MPD) and modeled with L257W (Trp), using the IRK1-MPD structure as a guide. **c)** Left, alignment of the putative closed state of GIRK1 chimeric channel (GIRK1-*closed*; green) (PDB:2QKS) with the IRK1-MPD structure (grey) (PDB:2GIX). Spaghetti structures show two adjacent cytoplasmic subunits (subunits D and A) and the hydrophobic alcohol pocket at the cytoplasmic subunit interface. Right, zoom shows alignment of the N-terminal domain, βD-βE and βL-βM ribbons from the IRK1-MPD (grey), GIRK1-*open* (orange) and GIRK1-*closed* (green) structures. IRK1-MPD aligns better with the putative open state of GIRK1. Note the significant displacement in the βL-βM beta ribbon element (arrow) and the side-chains of hydrophobic amino acids in the two structures. GIRK1-*closed* but not GIRK1-*open* has a collapsed alcohol-binding pocket, due to interaction and rotation of F46 (IRK1-F47), L246 (IRK1-L245) and F338 (IRK1-Y337). GIRK1-L333 in the βL-βM domain, implicated previously in Gβγ gating of GIRK channels(Finley *et al*., 2004; He *et al*., 1999; Ivanina *et al*., 2003), is shown for reference.

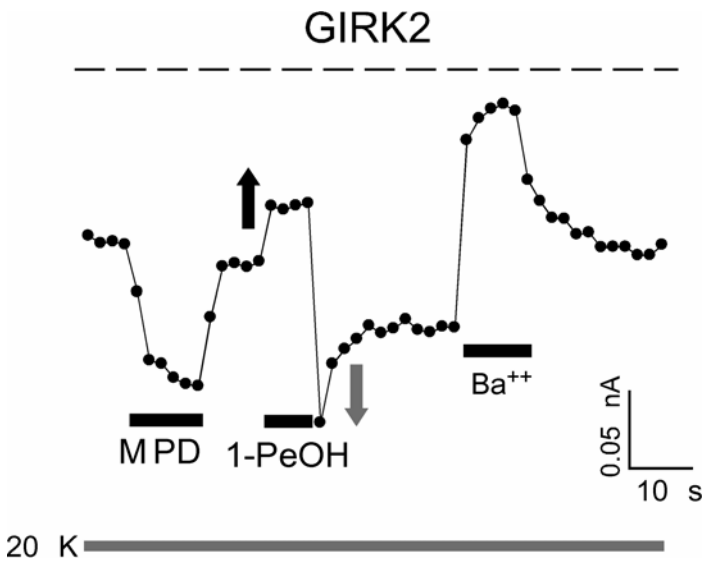

Fig. 2.S1 Dual modulation of GIRK2 by 1-PeOH. Example of inward K^+ current for GIRK2 measured at –100 mV in response to 25 mM MPD or 1-PeOH. 1-PeOH inhibits (black arrow) the basal current and reveals a potentiated current (grey arrow) immediately following washout of 1-PeOH. If rate of deactivation is slower than that for unblocking, then the potentiated current reflects activated GIRK2 channels.

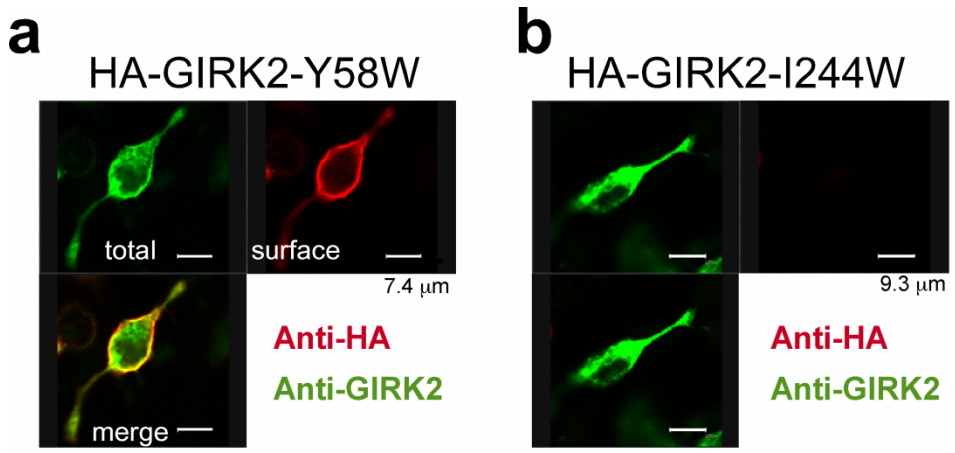

Fig. 2.S2 Assay to detect surface expression of mutant channels. HA-tagged channels were transfected in HEK cells and immunostained with anti-HA antibodies (unpermeabilized), followed by permeabilization and immunostaining with anti-GIRK2 antibodies. **a)** HA-GIRK2-Y58W expressed on the surface of unpermeabilized cells (red) as well as in the cytoplasm (green). **b)** GIRK2-I244W was only detected in permeabilized cells (green), indicating no surface expression.

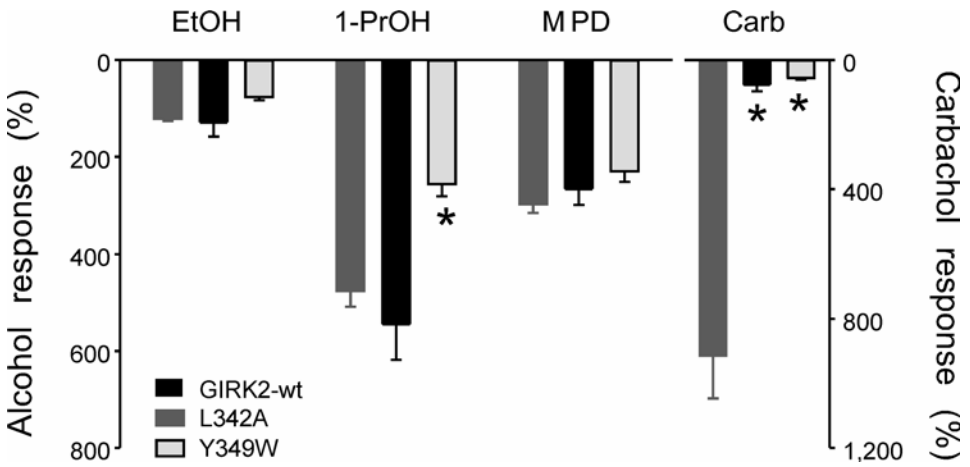

Fig. 2.S3 Mutations L342 and Y349 in the βL-βM loop of GIRK2 reduce both alcohol and/or m2R-activated currents. Bar graphs show the mean response (%) for GIRK2 (dark grey, n = 34), GIRK2-L342A (black, n = 10) and GIRK2-Y349W (light grey, n = 10) in response to 100 mM 1-PrOH, 100 mM MPD, 100 mM EtOH, or 5 μM carbachol, normalized to Ba^{++}-sensitive basal current. **P* < 0.05 vs. wild-type. Substitutions at A, F, Y or W at L342 and A, T, L, F at Y349 produced little or no Ba^{++} sensitive basal currents (< –1 $pApF^{-1}$).

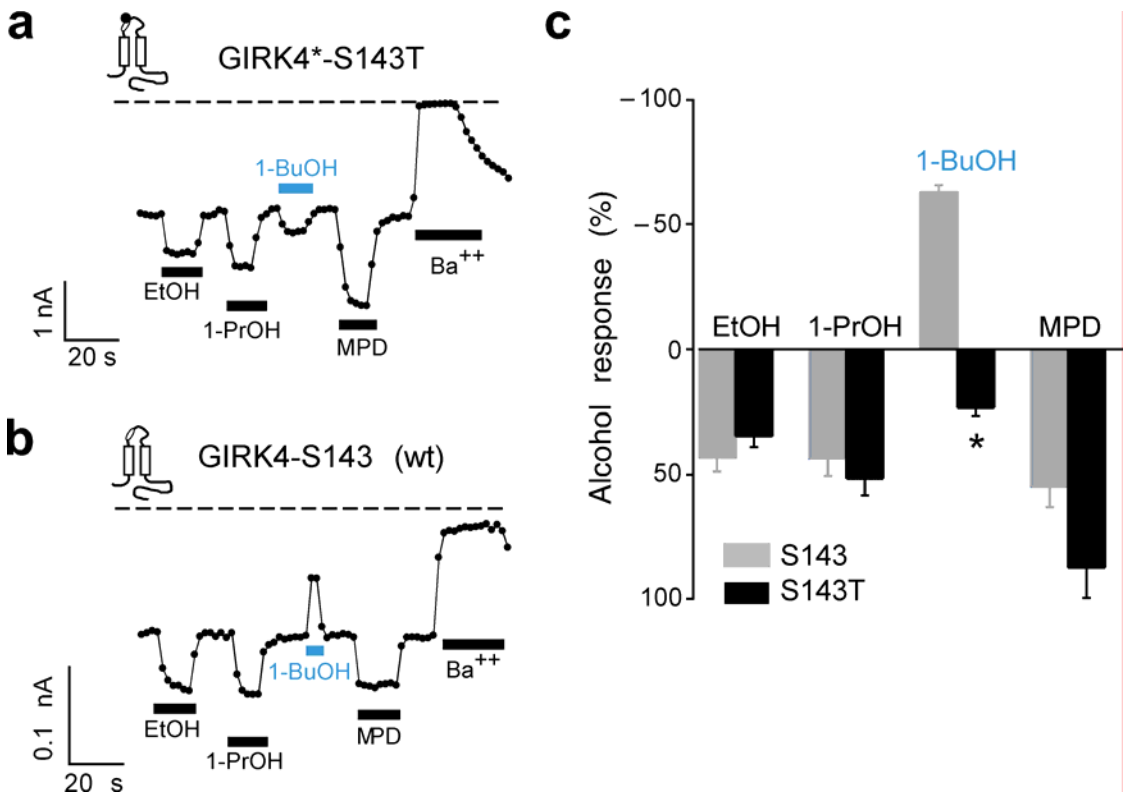

Fig. 2.S4 Mutation in GIRK4 pore-helix converts 1-BuOH from an inhibitor to activator. a-b) Examples of currents for GIRK4* (S143T) **(a)** and GIRK4 wild-type (wt) **(b)** channels measured at –100 mV in response to 50 mM EtOH, 50 mM 1-PrOH, 50 mM 1-BuOH or 50 mM MPD or 1 mM Ba^{++} in 20K solution. The Ba^{++}-sensitive basal currents were significantly larger for GIRK4*-(S143T) (–174 ± 25 $pApF^{-1}$, n = 7) than for wild-type GIRK4 (–9.57± 2.21 $pApF^{-1}$, n = 7). **c)** Bar graph shows mean percentage alcohol response normalized to Ba^{++}-sensitive basal current (n = 6-7).

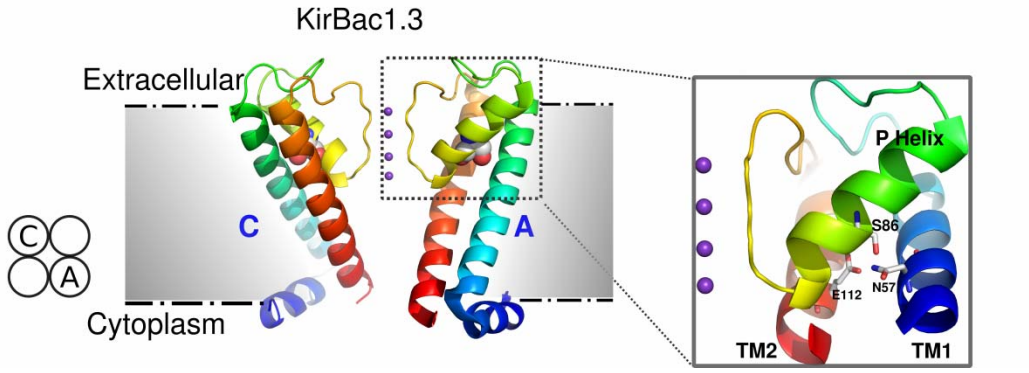

Fig. 2.S5 Site for regulating sensitivity to alcohol-dependent inhibition. S86, which is the amino acid homologous to S148 in GIRK2, is located in the pore-helix of KirBac1.3. Two opposing subunits (labeled A and C) are shown from the putative closed state of the Kirbac1.3-GIRK1 structure (PDB: 2QKS) and with four K^+ in the selectivity filter. Inset, zoom shows S86 from the pore-helix interacts with amino acids N57 and E112 from M1 and M2 transmembrane domains, respectively. There is no appreciable space to accommodate alcohol, suggesting this site influences an inhibition site located elsewhere in the channel.

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III. Insights into mechanism of alcohol mediated activation of GIRK channels

Abstract

Alcohols act on a hydrophobic pocket at the cytoplasmic domain of GIRK channels to activate the channels, however the mechanism of activation is not known. Here I use three novel approaches to gain insight into mechanism of alcohol mediated activation of GIRK channels. First, I show that thiol reactive reagents that have hydrophobic moieties activate GIRK2 channels when targeted to the hydrophobic pocket. This experiment strengthens our finding that this hydrophobic pocket is important for GIRK channel activation. Second, using a chemical biology approach to deplete PIP_2, I show that this phospholipid is required for alcohol mediated activation of GIRK channels. Third, using single channel recordings, I show that there is an increase in frequency of opening of GIRK channels when alcohol is applied. Work described in this chapter contributes to our fundamental understanding of how GIRK channels are activated by alcohols.

Introduction

Consumption of ethanol alters the normal activity of the brain by acting on numerous proteins including ion channels; This leads to a general inhibition of brain. GIRK channels are expressed ubiquitously in the brain, and are activated by ethanol and other alcohols. Activation of GIRK channels by ethanol would lead to hyperpolarization of neurons and would contribute to the general inhibition. Ethanol activates GIRK channels in neurons and in heterologous expression systems,

however the mechanism of activation was previously not known. We have recently described in detail a hydrophobic pocket in the cytoplasmic domain of GIRK channels that when mutated led to loss of alcohol dependent activation of the channel (Chapter II). This led us to conclude that ethanol acts directly on GIRK channels to activate the channel. Here I will present new evidence from experiments with MTS reagents, depletion of PIP_2 and single channel analysis of alcohol mediated activation to support this conclusion and elaborate on a model for alcohol mediated activation of GIRK channels involving this hydrophobic pocket.

Results

Targeting MTS reagents to the alcohol pocket

Evidence for an alcohol pocket in GIRK channels was based on structural similarities between the alcohol bound hydrophobic pocket of IRK1 and that of GIRK2. Increasing the bulkyness of a conserved Leucine at hydrophobic pocket of GIRK2 and GIRK4 channels led to loss of alcohol mediated activation of this channel, leading to the conclusion that this pocket is important for alcohol mediated activation (see chapter II). Another method to validate this finding is to introduce thiol modifying reagents that contain hydrophobic groups at this pocket to determine whether this would lead to activation of GIRK currents. I hypothesize that MTS reagents with hydrophobic will mimic alcohol mediated activation of GIRK channels.

Methylthiosulfonate (MTS) is a thiol reactive group, and has been used extensively in studying the topology of ion channels (Akabas et al., 1994; Akabas et

al., 1992; Holmgren et al., 1996). MTS reagents act on free thiol groups, such as the side chain of cysteine. To study the effect of chemical modification, we first needed to determine whether wild-type GIRK2 channels are sensitive to MTS reagents. We chose F-MTS (benzyl methylthiosulfonate) because this MTS reagent contains a bulky hydrophobic benzyl group. Previous studies with GIRK1 and GIRK2 suggested wild-type channels are sensitive to MTS reagents (Guo et al., 2002). Therefore, we wanted to examine the effect of F-MTS on wild-type GIRK2 channels. **Fig. 3.1a** shows that wild-type GIRK2 channels are potently inhibited by 10 μM F-MTS. GIRK2 contains four cysteines in the cytoplasmic domains (none is near the pocket). We then mutated the four intracellular cysteines (C66V; C190T; C220S and C332V) in GIRK2 (we refer to this as GIRK2$^{(Cys-)}$). GIRK2$^{(Cys-)}$ shows both ethanol and MPD activation (100 mM), like wild-type GIRK2. Importantly, GIRK2$^{(Cys-)}$ shows a small increase in basal currents in response still to F-MTS (**Fig. 3.1b**). This small increase in basal current was not reversed by the reducing agent DTT, suggesting perhaps a secondary effect of the MTS reagent.

To introduce cysteine at the hydrophobic pocket, I first studied the L257C mutant in the GIRK2$^{(Cys-)}$ channels, and discovered that the F-MTS had no effect on basal or alcohol activated currents(data not shown). L257 is at the bottom of the hydrophobic pocket, and is most likely not accessible to the reactive methylthiosulfonate group of F-MTS. We then examined S246, because it is located at the lip of the GIRK2 hydrophobic pocket, and the –OH group points into the pocket in the structure of GIRK2 channels (**Fig. 3.2a**) (Inanobe et al., 2007). GIRK2$^{(Cys-)}$-S246C shows both ethanol and MPD-activated currents (**Fig. 3.2b**). Exposure to 10 μM F-MTS leads to a robust and significant increase in the basal GIRK current which

was partially reversed by addition of the reducing agent DTT (**Fig. 3.2c**). In addition, I observed that the rank order for activation is altered. Whereas the MPD mediated activation was larger than ethanol mediated activation before F-MTS, the MPD mediated activation is significantly blunted after the F-MTS application. Furthermore, this change in rank order was also reversed by the application of DTT (**Fig. 3.2d**). Similar changes in basal current and rank order of activation were seen with application of Tyrosine-MTS, but not with the membrane impermeant MTSET (data not shown)

GIRKs, PIP_2 and alcohol

Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a negatively charged phospholipid that is concentrated at the cytoplasmic leaflet of the plasma membrane (see review (Hilgemann et al., 2001)). This phospholipid is known to regulate ion channels such as the KCNQ, TRP, and the Kir channel family (Huang et al., 1998; Rohacs et al., 2005; Suh and Hille, 2002). Huang et al. showed that depletion of PIP_2 leads to a rundown of IRK and GIRK currents, which is recovered by application of PIP_2 to the cytoplasmic side of a excised patches containing these channels (Huang et al., 1998). This rundown can also be caused by addition of PIP_2 antibody that would chelate PIP_2 away from the channels. IRK1 channels are constitutively active, and have a strong affinity for PIP_2, evidenced by a relatively slow rundown of channel currents after addition of PIP_2 antibody from the cytoplasmic side (Huang et al., 1998). This is in contrast to a rapid rundown of GIRK channel currents in the presence of the

PIP_2 antibody. I hypothesize that PIP_2 is needed for activation of GIRK channels by alcohol.

To determine whether PIP_2 is required for alcohol mediated activation of GIRK2 channels, I used a chemical biology method to deplete PIP_2. This method utilizes the heterodimerization of protein domains from FK506 and mTOR (Suh et al., 2006). The LDR protein contains the rapamycin binding motif of FK506 that is fused to a lipid anchoring domain Lyn_{11} while the CF-Inp54p protein has CFP fused to rapamycin binding domain of mTOR which then is fused to Inp54p, an inositol polyphosphate 5 phosphatase. When rapamycin is present in cells the CF-In54p will translocate, and deplete PIP_2 from the plasma membrane. To demonstrate that this system works in HEK293T cells I co-transfected LDR and the control CF protein which lacks the Inp54p. The CF protein is localized throughout the cytoplasm (**Fig. 3.3a**). Addition of 50 μM rapamycin translocates the CF protein to the plasma membrane, as seen by concentration of CFP at the plasma membrane (**Fig. 3.3b**). When GIRK2 containing cells are co-transfected with LDR and CF, addition of 50 μM rapamycin has no effect on basal and alcohol induced currents (**Fig. 3.3c**). However, when GIRK2 containing cells are cotransfected with LDR and CFIn54P, addition of 50uM rapamycin leads to a rapid loss of 85% of GIRK2 basal currents, along with an 85% loss in alcohol induced currents demonstrating that localization of CFIn54p to the plasma membrane leads to a robust loss in basal and induced GIRK currents (**Fig. 3.3d**).

Single channel analysis of alcohol mediated activation of GIRK channels

I can utilize single channel analysis to further understand the mechanism of activation of GIRK channel by alcohols. At the single channel level, a macroscopic current I is defined as $I=NP_o i$, where N= number of channels, $nP_o i$, where n is number of channels, P_o is the open channel probability and *i* is the unitary conducatance. An increase in macroscopic currents can occur by one of to three means 1) increase in the number of channels, this is in principle by insertion of ion channels to the plasma membrane. 2) Increase in open probability, which is measured by the time spent by an ion channel in the open state. There are several components of open channel probability; one is the frequency of opening, if the frequency of opening increases, the more likely the channel is in the open state. Second is the increase in mean open times, the longer the channel stays open, the larger the open channel probability. 3) Increase in unitary conductance occurs when more ions are conducted leading to increase single channel currents. Previous work shows that 100 mM ethanol increases the open probability but not the single channel conductance of GIRK1/2 channels. However, the mean open time was not measured in this study (Kobayashi et al., 1999).

To determine the single channel kinetic of GIRK2 channels in the presence of alcohol, I recorded single channel currents of GIRK2 in the absence and presence of 200 mM 1-propanol (**Fig. 3.4a**). The number of events detected for nine 45 second recordings were 3656 for basal currents and 8976 for 1-Propanol application. There is a PIP_2 infrequent basal opening of GIRK2 channels with the nP_o being 0.004±0.002, and when 1-Propanol was present the nP_o increased to 0.01±0.004. The average increase in nP_o was 2.75 fold when 1-Propanol was applied (**Fig. 3.4b**). However the further analysis of normalized mean open times showed that the time

constant τ did not change in the presence of the 1-propanol (**Fig. 3.4c**). Therefore, alcohol such as 1-Propanol increases the frequency, but not the mean open times of GIRK2 single channel currents.

Discussion

Targeting MTS reagents to the alcohol pocket

Work with MTS reagents show that targeting a hydrophobic MTS reagent to the GIRK2 hydrophobic pocket leads to channel activation. This suggests that the F-MTS behaves as a tethered alcohol, whose hydrophobic moiety interacts with the hydrophobic pocket to activate the channel. Secondly, the benzyl moiety changes the size of the pocket, interfering with activation by the larger MPD. Importantly, both the increase in basal currents and change in rank order are reversed by DTT. This finding validates the model; the hydrophobic pocket is important for GIRK channel activation. However, further experiments are needed to determine structural changes that occur at the hydropobic pocket that leads to activation of GIRK2 currents by F-MTS and alcohols.

GIRKs PIP_2 and alcohol

Experiment using the Rapamycin system to deplete PIP_2 demonstrates that PIP_2 is required for activation of GIRK2 currents by alcohol. This conclusion confirms the hypothesis that alcohol mediated activation is dependent on interaction of PIP_2. This finding is similar to the findings showing that after PIP_2 rundown, addition of

Gβγ cannot recover GIRK channel currents, however addition of PIP_2 after Gβγ application led to robust GIRK channel currents, arguing that a Gβγ mediated activation of GIRK channel requires PIP_2 (Huang et al., 1998). Furthermore, Zhang et al. showed that application of Gβγ leads to a significantly slower rundown kinetics, suggesting that Gβγ increases the affinity of GIRK channel to PIP_2 (Zhang et al., 1999). Similar studies are needed to determine whether alcohols increases the affinity of GIRK channels to PIP_2, which subsequently leads to opening of the channel

Swapping a sequence of 38 amino acids from IRK1 to GIRK4* led to GIRK channels that are constitutively active channels that and have a high affinity for PIP_2 indicating (Zhang et al., 1999). This swapped domain contains the βCβD loop, which contains positively charged amino-acids that would be positioned near the cytoplasmic leaflet of the plasma membrane (Pegan et al., 2005). In fact a refined version of this chimera, in which only four amino acids were swapped from IRK1 to GIRK2 in the βCβD domain led to GIRK2 channels that were constitutively active with a large basal current (Aryal et al., 2009). We have used this mutant, GIRK2-PIP2, to show that alcohols, such as MPD does not potentiate GIRK channel currents (**Fig. 2.7**). Taken together, I conclude that PIP_2 is required for alcohol mediated activation of GIRK channels, and when GIRK2 channels are tightly bound to PIP_2 there is no further activation of GIRK2 currents. Therefore, PIP_2 binding is an essential step in activation of GIRK channels by alcohol.

Single channel analysis of alcohol mediated activation of GIRK channels

The increase in frequency of GIRK2 channel opening by alcohol is translated to the enhancement macroscopic GIRK currents. There is spontaneous opening of GIRK2 channels in the absence of alcohol; addition of alcohol facilitates this spontaneous opening. This could be achieved by decreasing the energy barrier for conformational changes at the hydrophobic pocket. Further experiments are needed to determine how the frequency of channel opening is increased. The concentration dependence of alcohol, and the temperature dependence on the frequency of opening should be investigated. Furthermore, studies of single channel kinetics of hydrophobic pocket and GIRK2-PIP_2 mutant is needed to determine the range of single channel behavior upon loss of activation and constitutive activation of GIRK2 channels respectively. Lastly, alteration in single channel kinetics during MTS-mediated activation could determine whether the mean open time is prolonged when the MTS-reagents are tethered to the hydrophobic pocket.

Acknowledgments

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Materials and methods

MTS reagent modifications:

MTS reagents were obtained from Toronto Research Chemicals. Hydrophobic MTS reagents were resuspended in DMSO at 100 mM concentration. Resuspended MTS reagents were diluted to 10 μM, EtOH and MPD were diluted to 100 mM, and DTT was diluted to 10mM final concentration in 20K extracellular solution (Aryal et al., 2009). GIRK2 cDNA was transfected into HEK293T and whole cell currents were measured while holding membrane voltage at -100 mV and were sampled at 250 μs. Currents were analyzed using Clampfit 8 software. MTS reagents were applied for 10 seconds and allowed to reach steady state for additional 10 seconds. Percent activation was measured by measuring currents before MTS application and 20 seconds after MTS application and after DTT application. Ratio of MPD to Ethanol activated currents was obtained by dividing MPD activated currents by Ethanol activated currents.

Depletion of PIP_2

LDR, CF and CFIn54p constructs were kind gifts from Dr. T. Meyer. 0.8 μg of these constructed were co-transfected with GIRK2 and whole cell patch clamp recordings were conducted as described previously (Aryal et al., 2009). Rapamycin was kind gift from Dr. M. Montminy. 50 mM Rapamycin was made up in Ethanol, and was then directly diluted into 20K solution giving the final concentration of 50 μM Rapamycin. Translocation of CF proteins were imaged before and after application of 50 μM Rapamycin using TILL photonics imaging system with a Nikon TE2000 microscope and DPSS 422 nm laser as described previously (Fowler et al., 2007).

Single channel measurements:

We studied HEK293T cells transfected with the cDNA for GIRK2. Cell-attached single-channel recordings were conducted with high 150K in the internal and external solutions (in mM: 140 KCl, 2 MgCl2, 10 HEPES/Na (pH 7.2), 5 EGTA/K (pH 7.4, ~10 KOH)). The high K solution zeroes the resting membrane potential so that the patch potential is known more accurately. Single-channel currents were recorded at -80 mV. Because of the very low probability of opening it is possible that multiple GIRK2 channel currents were recorded, however, only patches with one conductance state were analyzed. Single-channel recordings were sampled at 10 kHz at -80 mV for 1 minutes of baseline activity and then 1 minutes with 200 mM 1-propanol in the bath. Single channel kinetics from 8 different cells where the last 45 seconds of basal and1-propanol applied recordings were used for analysis Currents were filtered at 1-2 kHz, open and closed channel times were derived from idealized traces (1/2 threshold criterion) using Clampfit 9.0 software. Open and closed time histograms were fit with one exponentials using Maximum Likelihood Estimate method of fitting and assessed for goodness of fit.

Figures

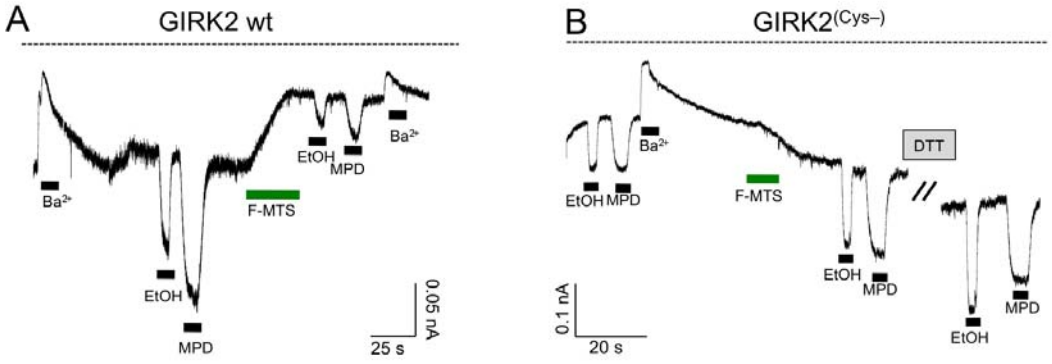

Fig. 3.1 Effect of MTS modification on wild-type GIRK2 and GIRK2$^{(Cys–)}$ channels. A) Current response measured at –100 mV in response to 100 mM EtOH or MPD before and then after exposure to 10 μM F-MTS. F-MTS inhibits wild-type GIRK2 channels. 1 mM Ba^{++} shows Ba-sensitive basal current **B)** Current response measured for GIRK2$^{(Cys–)}$ as described in A. GIRK2$^{(Cys–)}$ shows little response to F-MTS. The small increase in basal current is not reversed by DTT (10 mM).

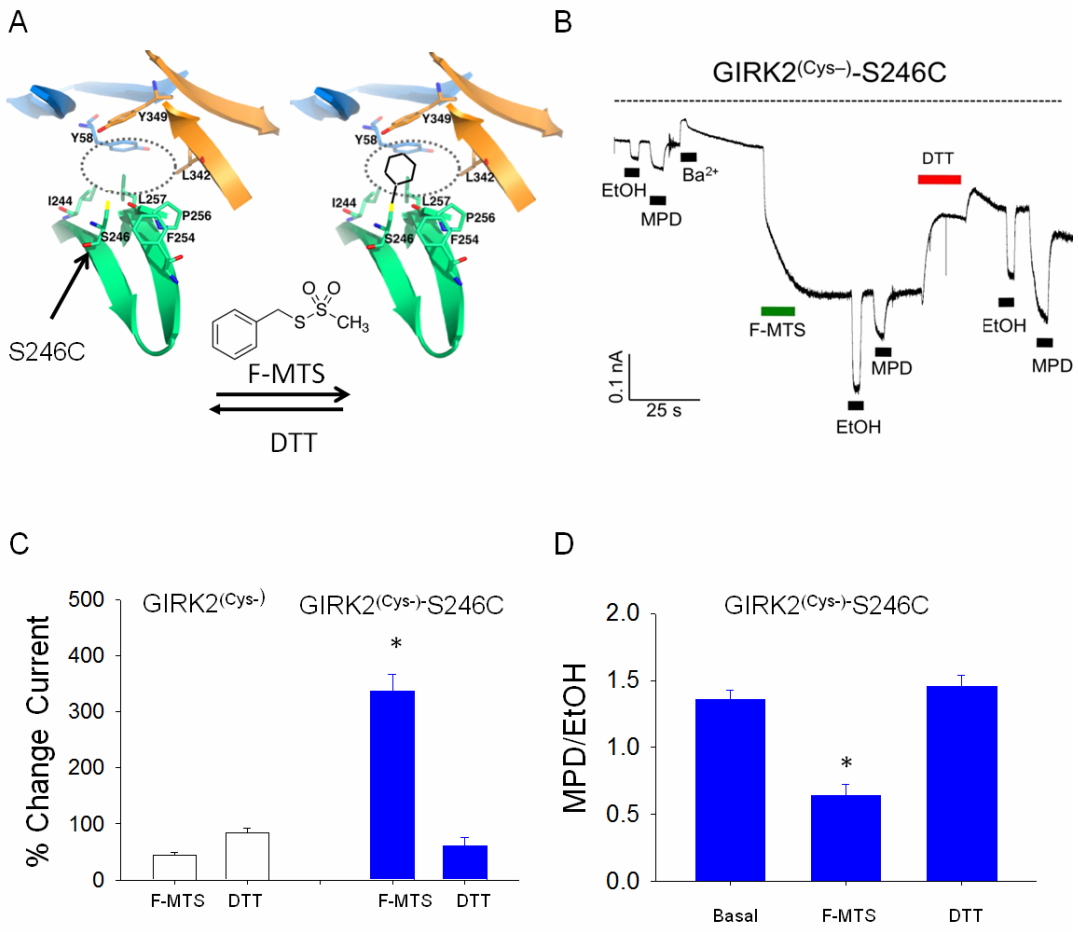

Fig. 3.2 F-MTS modifies GIRK2$^{(Cys–)}$-S246C. A) Left: ribbon structure shows the position of S246C at the lip of GIRK2 hydrophobic pocket. Center: Molecular representation of F-MTS. Right: prediction of structure of hydrophobic pocket upon F-MTS binding. **B)** Response of GIRK2$^{(Cys–)}$-S246C to 100 mM EtOH or MPD before or after exposure to 10 μM F-MTS (A). Note increase in basal current with F-MTS and change in EtOH vs MPD response. DTT (10 mM) partially reversers the F-MTS induced change in current. **C)** Bar graph shows mean percentage increase in basal current following F-MTS for GIRK2$^{(Cys–)}$ and GIRK2$^{(Cys–)}$-S246C channels n=5 . DTT does not reverse increase in basal current for GIRK2$^{(Cys–)}$ but does for GIRK2$^{(Cys–)}$-S246C. **D)** Bar graph shows mean ratio of MPD and Ethanol activated currents. Addition of FMTS leads to significant loss of MPD/Ethanol ratio n=5 (*P < 0.05 ANOVA)

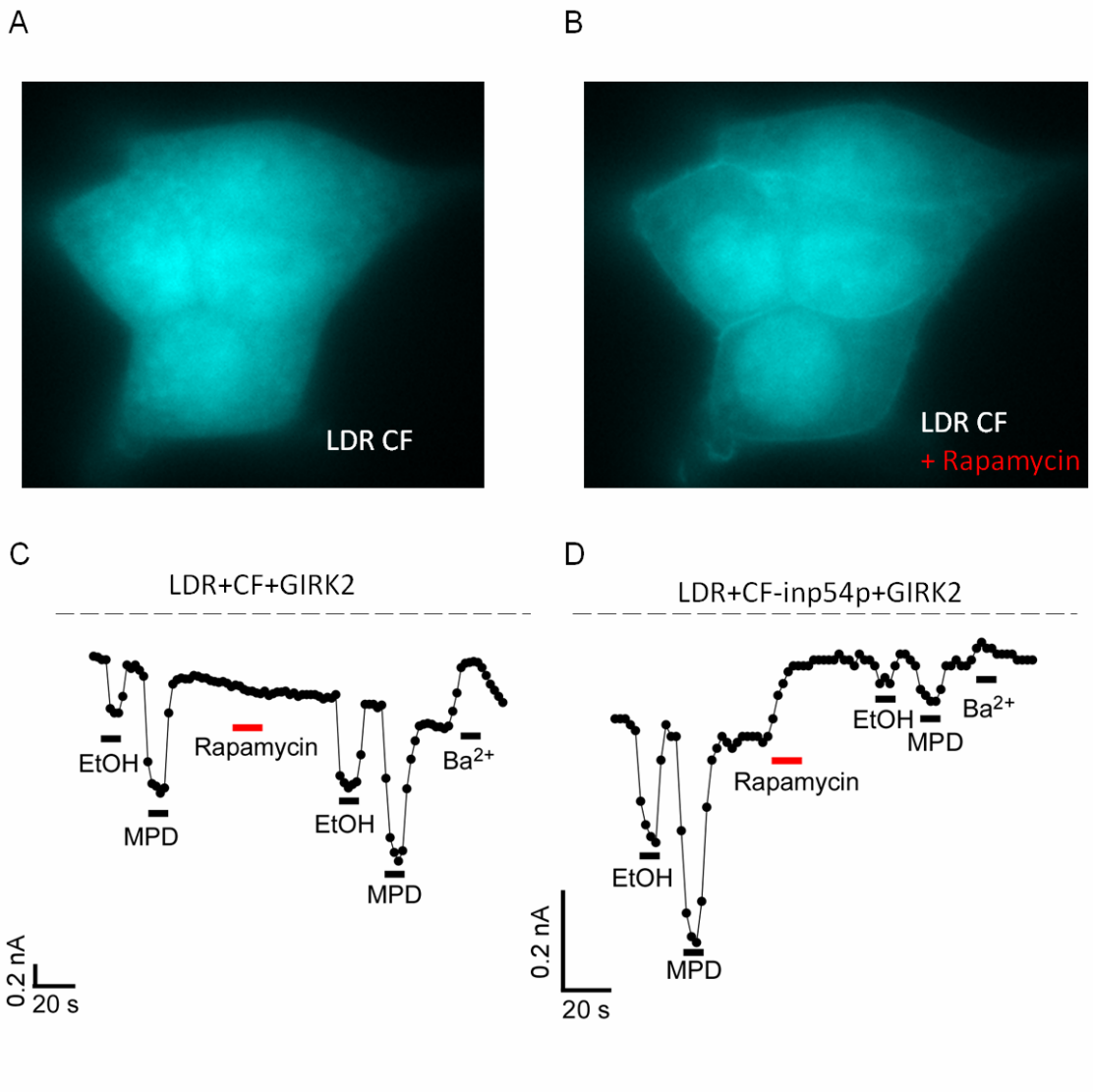

Fig. 3.3 Effects of PIP_2 depletion on basal and alcohol induced currents. A) Eppifluroscent image of HEK293T cells transfected with LDR and CF shows CF protein is distributed throughout the cytoplasm **B)** CF protein is concentrated to the plasma membrane upon addition of 50uM Rapamycin **C)** addition of rapamycin has no effect on GIRK channel current in cells transfected with LDR and CF **D)** Addition of rapamycin leads to loss of basal and alcohol activated GIRK current in cells transfected with LDR and CF-in54p. CF-in54p depletes PIP_2 from the plasma membrane.

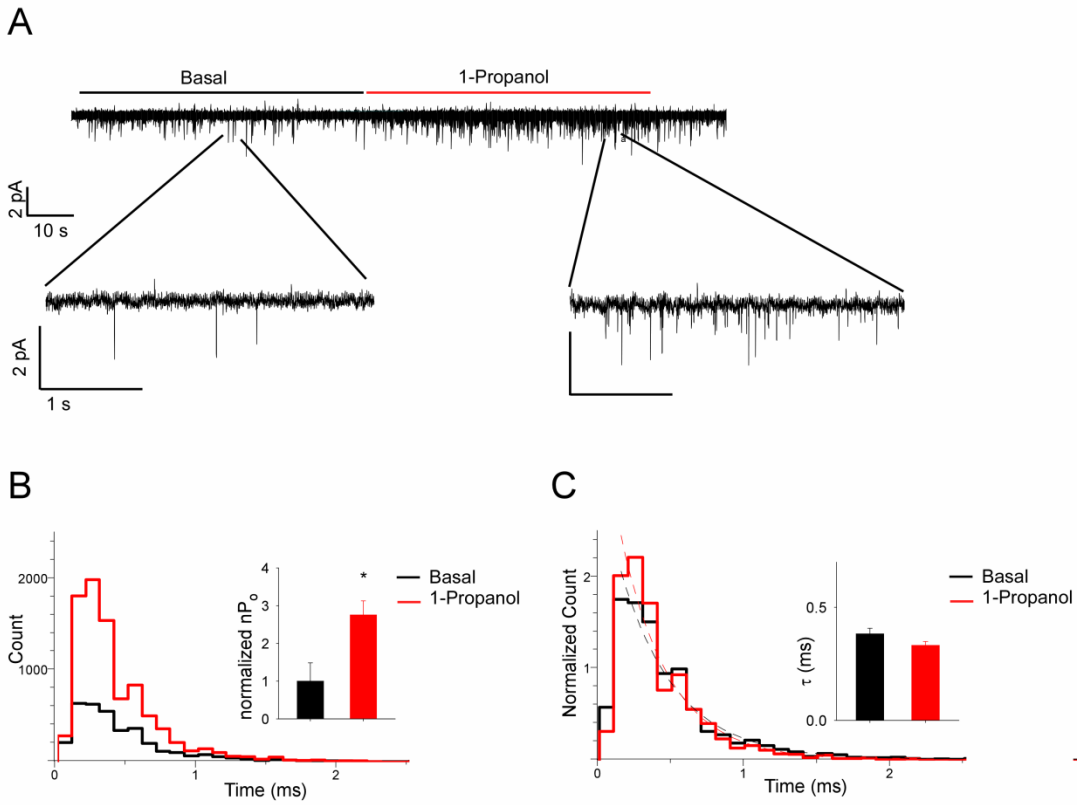

Fig. 3.4 Alcohol increases nP_o of GIRK channels at a single channel level: A) top: a typical single channel recording of GIRK2 channels in HEK293T cells without (basal) and with 1-propanol. Application of propanol markedly increases GIRK channel activity. Bottom: 3 second window of single channel activity of basal and 1-propanol induced GIRK channel activity note the increase in events. **B)** Histogram of GIRK channel openings from nine 45 seconds recording of basal and 1-propanol activated GIRK currents. Inset: average nP_o of 1-propanol activated GIRK currents normalized to basal current. ($P<0.05$ t-test) **C)** Normalized histogram of basal and 1-propanol mediated openings fitted with one exponential. The mean open times are not different between two groups: Time constant tau is not significantly different between basal and 1-Propanol mediated opening events.

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IV. Discussion

A model for alcohol mediated activation of GIRK channels

Alcohols activate GIRK channels by directly acting on hydrophobic pockets that are located at the cytoplasmic domain. Structural alignment of IRK1-MPD with GIRK1 “open” and GIRK1 “closed” shows that IRK1-MPD aligns well with GIRK1 “open” at this hydrophobic pocket, whereas hydrophobic amino-acids in GIRK1 “closed” would occlude an alcohol, such as MPD to bind to the pocket (**Fig. 2.8**). There are two gates in the cytoplasmic region of channels that would occlude K^+ when closed, one is at the G-loop (Nishida *et al*., 2007; Pegan *et al*., 2005), and the second is at cytoplasmic side of the transmembrane region, where four alpha helix of TM2 domains of the tetrameric channel intersect (Doyle *et al*., 1998; Nishida *et al*., 2007; Sadja *et al*., 2001; Yi *et al*., 2001). The two gates are in close proximity to one another, near the PIP_2 binding site thus these three structural elements could couple to each other. Therefore, these elements could be components of a single cytoplasmic gate.

From the alignment of IRK1-MPD to GIRK1 closed and GIRK1 open, we can infer that IRK1-MPD structure that resembles a hydrophobic pocket of an alcohol-bound and open channel. At rest, there could be an intrinsic but infrequent conformation change at the hydrophobic pocket, that leads to opening of the channel, alcohols acting at the hydrophobic pocket could then facilitate the conformation change which leads to increasing in frequency of channel opening. Channel opening requires PIP_2 and conformation changes at the G loop and TM2 cytoplasmic gates

that allow potassium ions to flow in and out of the cell. (See **Fig 4.1** for a cartoon model)

It should be noted that one assumption that is placed in this model is that these are in fact the open and closed conformation of GIRK channels. This assumption is based on the fact that GIRK1 "open" conformation has the G-loop in a position that would allow conduction of K^+ (Pegan *et al*., 2005). Furthermore, the GIRK1 "open" channels were co-crystallized with a detergent molecule nonylglucoside, which was located at a putative PIP_2 binding site. This co-cyrstalization stabilizes amino-acids that are known to interact with PIP_2 (Nishida *et al*., 2007). Moreover, the TM2 gate is closed in both of the Kir-Bac 3.1 GIRK1 chimeric channel structure and could be the result of the fusion of the cytoplasmic domain of an eukaryotic Kir channel with the transmembrane domains of a bacterial Kir channel (Nishida *et al*., 2007). Crystal structures of full length GIRK channel with co-crystalized with and without alcohol could in determining the exact nature of open and closed conformations.

Convergence of alcohol and Gβγ activation at the hydrophobic pocket.

GIRK channels are activated by G-proteins and alcohol. The mechanism of activation of this channel has not been fully elucidated. I show that all of the substitutions made at that conserved Leucine at the βD-βE domain of the hydrophobic pocket leads to loss of loss of Carbachol mediated activation (Aryal *et al*., 2009). Furthermoe, the βL-βM domain, which is a part of the hydrophobic pocket, has been implicated in Gβγ mediated activation (Finley *et al*., 2004; He *et al*., 1999;

Ivanina *et al*., 2003). Together, these studies suggest that this hydrophobic pocket is important for G-protein and alcohol mediated activation. Therefore, similar conformational changes could occur at the pocket during alcohol and Gβγ mediated activation. Using fluorescent resonance energy transfer (FRET) Rivan *et al*. has shown that the N- and C-terminal domains of adjacent GIRK channel subunits increase in FRET, hence move closer, during Gβγ meditated activation (Riven *et al*., 2003). The hydrophobic pocket in GIRK channels is at the interface of adjacent subunits (Aryal *et al*., 2009). I predict that similar increase in FRET would occur upon alcohol mediated activation of GIRK channels. Future studies will have to utilize molecular rulers such as advanced fluorescent imaging techniques, and chemical modifications such as crosslinking to monitor the conformation change at this pocket.

Alcohol binding motifs

The hydrophobic pocket in GIRK channel is at water accessible subunit interface in the cytoplasmic domain. The pocket is made up of three different domains, a hairpin domain from the N-terminus, and two beta loop domains from the C-terminus. This pocket is markedly different from that of the putative $GABA_A$ pocket and the crystallized alcohol-bound LUSH pocket (Harris *et al*., 2008; Kruse *et al*., 2003). In the LUSH pocket hydrophobic pocket is at interface of two alpha helix domains where hydropbobic interactions occur between hydrophobic amino acids of the protein and the hydrocarbons of ethanol (Kruse *et al*., 2003). Furthermore, hydrophilic interactions occurs between the –OH group of alcohol and hydrogen bond donating groups from sidechains –OH of serine and threonine, as well as from a

carbonyl group from the backbone (Kruse *et al*., 2003). The hydrophobic pocket in inwardly rectifying K channels are formed by hydrophobic sidechains from three different domains, which interact with the hydrocarbons of alcohol, whereas the hydrophilic interaction is between the –OH group of alcohol and a carbonyl group of a proline(Aryal *et al*., 2009; Pegan *et al*., 2005). The carbonyl group of a proline (L256 in GIRK2) followed by a hydrophobic group, such as a leucine (L257 in GIRK2) could serve as a general alcohol binding motif. The increased structural rigidity and kink that is induced by a proline could serve to stabilize the H-bond donating carbonyl group, whereas the hydrophobic motif of Leucine could interact with hydrocarbons of alcohol. Although, the IRK1-MPD structure has been useful to investigate alcohol dependent activation of GIRK channels, an ethanol-bound GIRK channel structures would enhance our understanding of how this small alcohol interacts with the hydrophobic pocket.

Summary

This dissertation describes a novel mechanism of alcohol mediated activation of GIRK channels. I provide evidence that alcohols, such as ethanol, activate GIRK channels by acting on a hydrophobic pocket that is located at subunit interfaces of cytoplasmic domain. This activation is $G\beta\gamma$ independent, requires PIP_2 and increases the frequency of single channel opening. Moreover, I present evidence using thiol modifying reagents that targeting hydrophobic moieties to the pocket leads to activation of GIRK channels. Furthermore, I provide a tenable model of GIRK channel

activation in which alcohols facilitates opening of the channel by acting at the hydrophobic pocket.

Future perspective

It is my hope that the work I have presented in this dissertation provides the ion channel field a new model of how GIRK channels open. The outstanding question here is: what are exact conformational changes that occur at this hydrophobic pocket and at the cytoplasmic gates during channel activation? Novel techniques will be needed to monitor small conformation change at the hydrophobic pocket and at the gates during GIRK channel activation by alcohol. Furthermore, NMR studies that can determine structural changes in the presence of alcohol at this pocket can also be utilized. Future studies are also needed to determine the interplay between the hydrophobic pocket and PIP_2 binding domains, and the cytoplsamic gate. It is also important to determine whether $G\beta\gamma$ mediated activation and alcohol mediated activation involve similar conformational changes at the hydrophobic pocket and the cytoplasmic gates. Innovative thinking and development of new tools will lead to exciting new findings that stem from the work presented here.

Figures

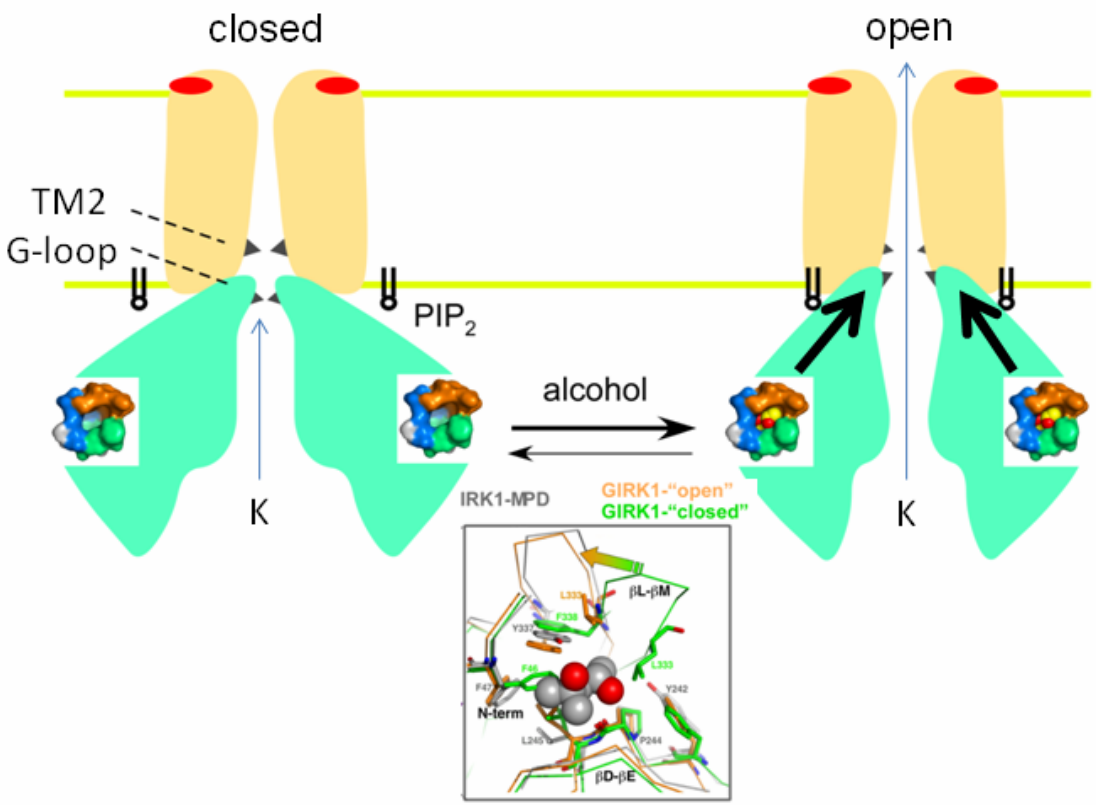

Fig. 4.1 **A model of mechanism of GIRK channel activation**. Left, cartoon of GIRK channels in the closed conformation, with no PIP_2 interaction and the TM2 and G-loop gates occluding K^+ from flowing through. Right, cartoon of GIRK channels in the open conformation with increased PIP_2 interaction, and TM2 and G-loop gates in the open configuration, and alcohol bound to the hydrophobic pocket. At rest, there is an intrinsic but infrequent opening of GIRK channels. Alcohols acting at the hydrophobic pocket could then facilitate the opening of GIRK channels Bottom: There is a structural rearrangement at the hydrophobic pocket, during channel opening. The hydrophobic pocket is comprised of N-terminal domain, the βD-βE domain, and βLβM domain at a subunit interface. How structural rearrangements at the hydrophobic pocket is translated into opening of the channel gates yet to be determined, and is designated by black arrows drawn on the open conformation.

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