Protein Kinase C Dependent Inhibition of Kir3.2 (GIRK2) Channel Activity and Its Molecular Determinants

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATS</td>
<td>Andersen-Tawil Syndrome</td>
</tr>
<tr>
<td>CCD</td>
<td>cortical collecting duct</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DSCR</td>
<td>Down Syndrome Critical Region</td>
</tr>
<tr>
<td>$E_K$</td>
<td>equilibrium potential for potassium</td>
</tr>
<tr>
<td>GABA_B</td>
<td>gamma-amino butyric acid receptor type B</td>
</tr>
<tr>
<td>GIRK</td>
<td>G-protein activated inward rectifying potassium channel</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>$G_\alpha$</td>
<td>G-protein alpha subunit</td>
</tr>
<tr>
<td>$G_\beta_\gamma$</td>
<td>G-protein beta gamma dimer</td>
</tr>
<tr>
<td>$I_{K,\text{Ach}}$</td>
<td>acetylcholine-activated potassium current</td>
</tr>
<tr>
<td>$I_{SK}$</td>
<td>small-conductance potassium current</td>
</tr>
<tr>
<td>$IP_3$</td>
<td>inositol trisphosphate</td>
</tr>
<tr>
<td>$K_{ATP}$</td>
<td>ATP-inhibited potassium current</td>
</tr>
<tr>
<td>$K_{CSA}$</td>
<td>bacterial potassium channel from Streptomyces lividans</td>
</tr>
<tr>
<td>Kir</td>
<td>inward rectifying potassium channel</td>
</tr>
<tr>
<td>$K_v$</td>
<td>voltage-gated potassium channel</td>
</tr>
<tr>
<td>PdBu</td>
<td>phorbol dibutyrate</td>
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</table>
PI3K .......................................................................................................................... phosphatidylinositol 3-kinase
PI4K .......................................................................................................................... phosphatidylinositol 4-kinase
PIP2 ............................................................................................................................ phosphatidylinositol 4,5-bisphosphate
PIP5K ....................................................................................................................... phosphatidylinositol phosphate 5-kinase
PKA ............................................................................................................................ cAMP-dependent protein kinase
PKC ............................................................................................................................ protein kinase C
PMA ............................................................................................................................ Phorbol-12-myristate 13-acetate
PTX ............................................................................................................................ pertussis toxin
ROMK ......................................................................................................................... rat outer medulla potassium channel
SESAME...seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance
SNc ............................................................................................................................. substantia nigra pars compacta
SP ................................................................................................................................. substance P
SUR .............................................................................................................................. sulfonylurea receptor
SVD ............................................................................................................................. snowflake vitreoretinal degeneration
TAL ............................................................................................................................... thick ascending limb
VTA ............................................................................................................................. ventral tegmental area
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Table 1.1. PKC Sensitivity of Kir Channels ........................................................................ 35
PROTEIN KINASE C MEDIATED INHIBITION OF KIR3.2 (GIRK2) ACTIVITY AND ITS MOLECULAR DETERMINANTS

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Inwardly rectifying potassium (Kir) channels are critically important for regulating resting membrane potential in excitable cells, a job underscored by the severe pathophysiology associated with channel dysfunction. While all Kir channels require the activating lipid PIP$_2$, many of these channels have diverse modulatory factors that couple to PIP$_2$-dependent gating. Channels in the Kir3 (GIRK) family, in particular, have several co-activating elements, including G-protein $\beta\gamma$ subunits, ethanol, and sodium. During stimulation of Gq-coupled receptors, downstream activation of Protein Kinase C can phosphorylate and inhibit Kir3 channels, yet the mechanism of inhibition and phosphorylation sites are incompletely understood. We took a combined experimental and computational approach using neuronal Kir3.2 to investigate how phosphorylation at a putative PKC site identified in Kir3.1/3.4 could lead to channel inhibition. Kir3.2 inhibition was found to depend on the phosphorylation state of Ser-196, although mutagenesis data suggest it functions as an allosteric regulator of PKC inhibition. MD simulations identified a molecular switch whereby phosphorylation of Ser-196 recruits a critical gating residue, Arg-201, away from the sodium coordination site Asp-228. Neutralization of
Ser-196 or Arg-201 resulted in less active channels which exhibited increased sensitivity to PKC inhibition. Additionally the interplay of PIP$_2$ and PKC inhibition was examined in depth using homomeric Kir3.2, revealing that increases in channel-PIP$_2$ interactions limit sensitivity to PKC inhibition, whereas low levels of PIP$_2$ increase PKC sensitivity. Neutralization of Ser-196 uncoupled PKC inhibition from this PIP$_2$ dependence. These studies suggest a model whereby PKC inhibition can occur along PIP$_2$-dependent and PIP$_2$-independent pathways, depending on the phosphorylation state of Ser-196.
Chapter 1: BACKGROUND

1.1. Introduction

Inward rectifier potassium (Kir) channels allow precise control of cellular excitability by primarily passing hyperpolarizing currents when the cellular membrane potential is more positive than the equilibrium potential for potassium ions. The inward rectifier family includes channels that are constitutively active (e.g. Kir1, Kir2), as well as channels gated by G-proteins (Kir3), adenosine triphosphate (ATP) (Kir6), and hydrogen ions (Kir4) (Hibino et al., 2010). While constitutively active channels are thought to be refractive to modulation, channels gated by cytoplasmic factors have multiple pathways of activation and inhibition. The focus of this dissertation is on elucidating the molecular mechanism of one specific pathway, PKC-mediated inhibition of G-protein activated inward rectifier potassium channels (GIRKs or Kir3s).

The Kir3 family can be activated by stimulation of G-protein Coupled Receptors (GPCRs) of the pertussis toxin-sensitive (PTX) G\(_{\text{i/o}}\) family. When activated by a bound ligand, the GPCR stimulates hydrolysis of G\(\alpha\)-GTP to G\(\alpha\)–GDP, leading to dissociation of G\(\beta\gamma\) from G\(\alpha\). G\(\beta\gamma\) subunits then directly bind the Kir3 channel to activate it (Hibino et al., 2010; Logothetis et al., 1987; Luscher and Slesinger 2010). There are two presumed pathways of inhibiting channel activity, converging at the G\(_{\text{q}}\)-coupled pathway. Activation of G\(_{\text{q}}\)-coupled GPCRs results in hydrolysis of phosphatidyl-inositol 4,5-bisphosphate (PIP\(_2\)), forming inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG). PIP\(_2\) itself is a coactivator of Kir3 channels,
and is absolutely essential for channel activity (Huang et al., 1998; Zhang et al., 1999).

Reduction of membrane PIP$_2$ levels by $G_q$ activation should therefore directly inhibit Kir3 current. However, $G_q$-coupled receptor activation also increases DAG levels, a potent activator of Protein Kinase C (PKC). PKC activation presents a second means of channel control by inhibiting the Kir3 channel (Keselman et al., 2007; Mao et al., 2004; Stevens et al., 1999). Since PIP$_2$ is an essential activator for Kir channels, we hypothesize that PKC-mediated phosphorylation inhibits channel activity by reducing its affinity for PIP$_2$, in effect changing the level of PIP$_2$ the channel can sense.

1.2. Inward Rectifier Kir channels: Classification and Physiological Roles

The storied history of inward rectifier channels begins with their discovery as an oddly-behaving potassium-selective conductance in skeletal muscle by Bernard Katz in 1949. Unlike the classic voltage-activated delayed rectifier currents from squid axons, this current had the peculiar characteristic of preferentially passing current at potentials negative to the potassium equilibrium potential, $E_K$. This results in currents that are predominantly inward rather than outward, such that the channels can function like molecular one-way diodes or valves. The phenomenon of inward rectification was found to primarily result from blockade by intracellular cations (Mg$^{2+}$) and positively charged polyamines (e.g. spermine). Strongly rectifying potassium channels (called Kir channel for inwardly rectifying K$^+$) are thought to be important in maintaining resting potentials near $E_K$, but do not interfere with normal cellular excitability due to automatic current shut-off at potentials above $E_K$ (Hibino et al., 2010).

With the rapid progress of the molecular age, Kir channel genes were identified and cloned, allowing expression of the encoded genes in isolated, heterologous systems. The Kir channels are classified according to channel structure and homology, and separated into seven
Kir subfamilies, Kir1 thru Kir7, with a channel phylogenetic tree presented in Figure 1.1A. The membrane topology of these channels has historically been inferred from hydropathy plots based on amino acid sequences, although now multiple X-ray crystallographic structures exist. All Kir channels possess a transmembrane domain and a cytoplasmic domain, whereby the transmembrane domain is composed of two helices, M1 and M2, with a pore helix interposed. The channel topology, diagrammed in Figure 1.1B, resembles the voltage-gated potassium channel (Kv) superfamily, where the M1 (or TM1) and M2 (or TM2) helices correspond to S5 (or TM5) and S6 (or TM6) helices in Kv channels.

The Kir1 subfamily is composed of a single member in humans, Kir1.1, also known as ROMK1 for Rat Outer Medullary K+, referring to the tissue of cloning origin. Kir1.1 channels are expressed primarily in renal tissue, and have importance in potassium recycling in the thick ascending limb (TAL) of the loop of Henle as well as the cortical collecting duct (CCD). Although still inwardly rectifying, Kir1.1 channels have comparatively weak rectification (Hibino et al., 2010).

The Kir2 (IRK) subfamily is composed of Kir2.1-2.4, termed classical inward rectifiers owing to strong rectification and a relative lack of cellular modulation. Kir2.1 (IRK1) in particular has a critical role in shaping the late repolarization phase of the cardiac action potential, with predominant expression in ventricular myocytes (Hibino et al., 2010).

G-protein βγ subunits directly bind and activate members of the Kir3 family, also called GIRK for G-protein activated inward rectifier K+ channel. The Gβγ subunits are released from the pertussis toxin-sensitive G/i/o class of G-protein coupled receptors. Tissue-specific Kir3 channels are formed by heterotetrameric assembly, with Kir3.1/3.4 dominating in cardiac cells and Kir3.1/3.2 primarily expressed in neurons. Kir3.1/3.4 channels form the well-documented
I\textsubscript{K,Ach} current in pacemaker and atrial myocytes; they are activated by vagal stimulation, when acetylcholine released from nerve terminals binds the M2 muscarinic receptor and frees G\beta\gamma to bind and activate the channel. Activation of Kir3.1/3.4 hyperpolarizes atrial myocytes, resulting in shortened action potential duration and increased refractory period, effectively reducing heart rate. Neuronal Kir3.1/3.2 channels are responsible for slow inhibitory synaptic potentials, due to stimulation of G\textsubscript{i/o}-coupled receptors like GABA\textsubscript{B} (Gamma-amino butyric acid receptor B), a process resulting in G-protein mediated hyperpolarization. All Kir3 channels are activated by alcohols, and may be involved in neuronal responses to alcoholism (Hibino et al., 2010; Luscher and Slesinger 2010).

Kir4.1 assembles in a heterotetrameric complex with Kir5.1, and is inhibited by intracellular acidification. Evidence of homomeric Kir4.1 also exists, but Kir5.1 does not form functional homomeric channels. Heteromeric assembly seems to affect sensitivity to acidification and increases rectification. Kir4.1 is expressed in glial cells of the central nervous system (CNS), where it is thought to regulate potassium recycling. Kir4.1/5.1 channels are also thought to function as CO\textsubscript{2} sensing elements in the brainstem. Knockout models of Kir4.1 indicate that this channel is important in oligodendrocyte function and myelination (Hibino et al., 2010).

Kir6.1 and Kir6.2 form the ATP-sensitive Kir channel subfamily, with inhibition by ATP and activation by the dinucleotide adenosine diphosphate (ADP). Kir6.1 and Kir6.2 form octameric complexes with SUR subunits, named for binding to the sulfonylurea class of drugs. Kir6.1 is primarily expressed in cardiac tissue, specifically in the coronary artery and endothelium. Kir6.2 is critically involved in glucose-induced insulin release, with primary expression in pancreatic beta cells. Activation of glycolytic pathways increases intracellular
ATP, which inhibits the Kir6.2/SUR complex and depolarizes the beta cell, activating voltage-gated calcium channels, which in turn induce insulin release (Hibino et al., 2010).

Kir7.1 channels are thought to function primarily in potassium transport, with high expression profiles in the choroid plexus, a structure responsible for cerebrospinal fluid secretion, and the retinal pigment epithelium (Hibino et al., 2010). These channels have much lower conductance compared to other Kir channels, ~50 fS, due to a unique methionine residue in the pore region. When mutated to an arginine, the residue in all other Kir channels at the homologous position, Kir7.1 currents display increased conductance (Krapivinsky et al., 1998).

1.3. Knockout models in GIRK channels: Insight into Physiology

Kir3 channel knockouts have shown the diverse impact that these channels have on heart and brain function, primarily. Both Kir3.1 and Kir3.4 knockout mice have reduced carbachol-mediated inward currents in atrial myocytes, consistent with elimination of $I_{K,Ach}$ (Bettahi et al., 2002). Stimulation of pathways leading to lowering of heart rate, like agonism of alpha-1 adrenergic or A1 adenosine receptors, led to a blunted reduction in heart rate in both knockouts. Specific knockouts of Kir3.2 and Kir3.3 have demonstrated a role for functional channels in the brain, especially in drug addiction and pain perception. In locus ceruleus neurons, the opioid met-enkephalin evokes a hyperpolarization due to activation of an outward potassium current. Kir3.2 knockouts have a reduction in opioid-induced hyperpolarization, but display negligible responses in the Kir3.2/3.3 double knockout. These results imply a role for Kir3.2/3.3 heteromers in opioid hyperpolarization in the locus ceruleus (Torrecilla et al., 2002).

There is also evidence for the existence of homotetramers of Kir3.2 in the mammalian brain, specifically in the substantia nigra pars compacta (SNc). Three splice variants of Kir3.2 exist, termed Kir3.2a-c. Kir3.2c contains an additional PDZ-binding domain at the distal C-terminus
not present in Kir3.2a or Kir3.2b. This domain is likely responsible for membrane targeting of Kir3.2c and its heteromeric partners (Luscher and Slesinger 2010). In one example of homomeric function, two splice variants, Kir3.2a and Kir3.2c, colocalize in the mouse SNc, but without evidence of Kir3.1 expression (Inanobe et al., 1999). Human Kir3.2 differs from other mammalian genes by having a single product, which is closest to the Kir3.2c (sometimes called GIRK2A-1) isoform.

1.4. Kir channels gone haywire: Relevance to Disease

The ubiquitous and critical role of inward rectifier channels is underscored by the vast repercussions that dysregulation of these channels has on human disease. Notably, multiple channels including Kir1.1, Kir2.1, Kir4.1, Kir6.1, Kir6.2, and Kir7.1 have been shown to have genetic mutations resulting in multi-organ disorders with a variety of pathologies. Antenatal Barrter Syndrome Type II, characterized by hypokalemia, metabolic alkalosis, and moderate to severe low blood pressure, is caused by a loss of function mutation in Kir1.1 (ROMK1) channels, encoded by the gene KCNJ1. Antenatal Barrter Syndrome mutations in the ROMK1 channel produce their effects by a loss of potassium current in the thick ascending limb (TAL) of the nephron (Figure 1.2) (Simon et al., 1996). This potassium channel normally sends reabsorbed potassium back to the tubule lumen, establishing a high enough luminal potassium concentration to support Na-K-2Cl cotransporter function. Mutations in the coding region of KCNJ1 can affect synthesis, trafficking (Schwalbe et al., 1998), and even PIP2 activation of the channel (Lopes et al., 2002), leading to a reduction of potassium reuptake.

Andersen-Tawil syndrome (ATS), a multi-organ disorder presenting with dysmorphic features, hypokalemic periodic paralysis, and ventricular arrhythmia, results from mutations in the KCNJ2 gene encoding the Kir2.1 channel (Figure 1.2). Like Barrter Syndrome, many of the
mutations found in ATS can affect channel trafficking or interaction with PIP$_2$ (Donaldson et al., 2003; Lopes et al., 2002). Due to the importance of Kir2.1 in late repolarization of the ventricular action potential, defects in Kir2.1 function often manifest as a prolonged QT interval.

Dysregulation of Kir4.1 (encoded by KCNJ10) results in SESAME Syndrome, a multi-symptom, multi-organ disorder that results in seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance (Scholl et al., 2009). Loss of function of Kir4.1 in CNS glial cells and marginal cells of the inner ear can account for many of the phenotypes in SESAME Syndrome (Figure 1.2). Kir4.1 additionally is expressed in several locations in the nephron, such that dysfunction of these channels probably contributes to electrolyte imbalance.

Diseases affecting the eye are associated with mutations in Kir7.1 (encoded by KCNJ13), highlighting its importance in retinal pigment epithelium (Figure 1.2). Snowflake vitreoretinal degeneration (SVD) can arise from a point mutation resulting in the R162W missense mutation, in a putative PIP$_2$-binding region. Kir7.1 channels with this mutation lose potassium selectivity, which is notable in that the mutation is at the membrane-cytoplasmic interface, rather than in the selectivity filter (Hejtmancik et al., 2008). Two mutations of Kir7.1 causing Leber congenital amaurosis have been reported, with one nonsense mutation chopping off the C-terminal domain entirely (Arg166Stop), and another resulting from a cytoplasmic mutant (L241P) (Sergouniotis et al., 2011).

The KCNJ11 gene encodes Kir6.2 ($K_{\text{ATP}}$), a potassium channel expressed in pancreatic β cells that is inhibited by increases in intracellular ATP, and thus is critical for depolarizing β cells to allow glucose-induced insulin release. Kir6.2 forms an octameric complex with its binding partner, the SUR1 subunit, target of sulfonylurea drugs used in diabetes treatment. The homeostatic importance of the Kir6.2 channel becomes clear since either loss of function or
of function phenotypes can be deleterious. Loss of function mutants typically result in hyperinsulinemic hypoglycemia due to unopposed depolarization in the β cell, activating voltage-gated calcium channels. Conversely, gain of function or activating mutations generally lead to a neonatal diabetes phenotype, as expected with reduced glucose-induced insulin release (Figure 1.2). Kir6.2 also has importance in the glucose-sensing mechanisms of brain and skeletal muscle, and the closely related Kir6.1 protects against ischemia in myocardium (Pattnaik et al., 2012).

In the Kir3 (GIRK) channel family, point mutations causing disorders are rare, although mutations altering potassium selectivity have been described. Multiple mutations in KCNJ5, which encodes Kir3.4, have been described in aldosterone-producing adenomas. One particular mutation, T158A, near the selectivity filter, is associated with familial hyperaldosteronism type III, presumably due to persistent depolarization in the adrenal gland and subsequent calcium-induced aldosterone release (Choi et al., 2011). Another mutation, correlating with the predominant expression of Kir3.4 in the heart, results in a variant of Long QT syndrome. This mutant (G387R) was described to produce a loss-of-function phenotype on I_{K,Ach} function in the heart, thus resulting in prolonged depolarization (Yang et al., 2010).

A Kir3.2 mutation naturally occurring in mice, known as weaver, is caused by loss of potassium selectivity, and significant neuronal loss in the cerebellum and substantia nigra occurs due to nonselective cation permeation in these mutant channels (Harkins and Fox 2002; Slesinger et al., 1996). No reported mutations in Kir3.2, encoded by the KCNJ6 gene, are associated with human disease so far. However, the KCNJ6 gene is located on the distal arm of chromosome 21, specifically at 21q22.1, and is thought to be relevant in Down Syndrome, caused by trisomy of chromosome 21. Down Syndrome can be caused by a third copy of a
nominal portion of genes from chromosome 21, the so-called Down Syndrome Critical Region (DSCR). This region encompasses 21q22.1-22.3, in which the KCNJ6 gene is contained. In fact, a recent study by Cooper et al. demonstrated that selective trisomy of Kir3.2 in a mouse model partially recapitulates Down Syndrome phenotypes, manifesting as deficits in fear-conditioned memory and altered synaptic plasticity (Cooper et al., 2012).

Clearly, Kir channel genes fulfill diverse roles in physiology, and disease-causing mutations can result in widespread dysfunction. In several of the above-mentioned diseases, point mutations can lead to alterations in channel-PIP\(_2\) interactions. In the next section, we will briefly explore the PIP\(_2\) dependence of Kir channels and the attempts to identify interacting residues experimentally.

1.5.

**Kir channel gating requires PIP\(_2\)**

PIP\(_2\) composes ~1% of plasma membrane anionic phospholipids, and is by far the most abundant phosphoinositide at the cellular membrane. It can be interconverted into seven different species by various membrane-bound enzymes, summarized in Figure 1.3. Wortmannin, a specific phosphatidylinositol 3-kinase (PI3K) inhibitor at nanomolar concentrations, is also used at high micromolar concentrations to inhibit phosphatidylinositol 4-kinase (PI4K), reducing the available pool of PI4P (phosphatidylinositol 4-phosphate) for conversion into PIP\(_2\) by PIP5K (phosphatidylinositol phosphate 5-kinase). PIP\(_2\) is best known in its role as a precursor lipid for formation of the products DAG and IP\(_3\) after PLC activation. DAG is a specific activator of PKC, while IP\(_3\) acts on the IP\(_3\) receptor to raise cytosolic calcium from endoplasmic reticulum (ER) stores, which can further activate calcium-dependent PKCs.

In the last few decades, plasma membrane PIP\(_2\) has gained new appreciation as a lipid modulator of several ion channels and transporters, of which the list is enormous and continually
expanding (Logothetis et al., 2010). Beginning in 1996, Hilgemann and Ball discovered that the activity of sodium/calcium exchange transporters and $K_{ATP}$ channels can be directly stimulated by exogenous $\text{PIP}_2$. Furthermore, generation of $\text{PIP}_2$ is the reason why application of hydrolyzable ATP to excised patches restores channel currents (Hilgemann and Ball 1996). Soon after, other eukaryotic Kir channels were shown to be activated by $\text{PIP}_2$ (Huang et al., 1998; Sui et al., 1998), although with different affinities (Zhang et al., 1999) and stereospecificity (Rohacs et al., 2003).

Progress was later made in understanding the electrostatic nature of interactions between Kir channels and $\text{PIP}_2$. Neutralization scanning mutagenesis of arginines and lysines to glutamine and cysteine lowered whole-cell currents and reduced apparent $\text{PIP}_2$ binding affinity in some key conserved regions (Lopes et al., 2002). Furthermore, incorporation of charge at specific cysteine mutants via sulfhydryl modifying reagents allowed the identification of residues interacting via electrostatic means. Residues identified by this scan are bolded and marked in blue in an alignment of Kir channels, relative to phosphorylation sites which are boxed and marked in green (Figure 1.4). Numerous positively charged residues were implicated in channel-$\text{PIP}_2$ electrostatic interactions, which could later be mapped onto channel homology models. These maps identified a hotspot for $\text{PIP}_2$-sensitive residues at the proximal N-terminus and C-terminus, including a region known as the CD-loop. Since assessing channel-$\text{PIP}_2$ interactions relied on functional assays of channel activity, it was difficult to discern if specific residue interactions were direct or mediated via allosteric interactions. Still, $\text{PIP}_2$ is now regarded as a necessary cofactor in Kir channels, and channel activity is subject to alterations in $\text{PIP}_2$ levels with cellular signaling pathways.
Equally important, however, are the subtle changes in Kir channel function associated with phosphorylation. Phosphorylation of proteins, the tagging of amino acid residues by the highly negative phosphate anion, is a well-recognized mode of cellular signaling. Some 10,000 proteins, ~30% of the available genes in the human genome, are thought to contain phosphate covalently bound to serine, threonine, or tyrosine residues (Cohen 2002). In addition to having diverse effects on channel synthesis, plasma membrane trafficking, and expression, phosphorylation can mediate nearly instantaneous effects on Kir channel gating, although the molecular mechanism remains elusive. One such phosphorylation pathway concerns the PKC inhibition of Kir channels coupled to Substance P stimulation.

1.6. Regulation of Neuronal GIRKs: excitation of neurons by Substance P

Neuronal G-protein activated inward rectifier (GIRK) currents are critical determinants of cellular excitability. Activation of these channels is well-characterized to be a direct interaction of Gβγ subunits with the channel, as a result of G-protein coupled receptor activation of the Gi/o family. The inhibitory pathway of these channels has historically gained appreciation as well. In multiple neurons, the undecapeptide Substance P (SP) was shown to induce a slow excitatory postsynaptic potential, resulting in increased neuronal excitability. Examination of the excitatory mechanism in voltage-clamp mode of globus pallidus neurons revealed the suppression of an inward rectifier current (Figure 1.5A-B). The current inhibited by Substance P was more prominent at hyperpolarizing potentials, and the reversal potential of the inward current shifted according to the equilibrium potential for potassium, E_K (Figure 1.5 C-D). These results indicated that the channel involved was likely an inward rectifier, conducting primarily potassium ions (Stanfield et al., 1985).
In cultured neurons of the nucleus basalis, SP also induced downregulation of an inwardly rectifying potassium current. This inhibition was readily reversible except in the presence of the nonhydrolyzable GTP analogue GTPγS, implying G proteins are involved in SP-mediated effects. The Substance P mechanism of action was also shown to be insensitive to pertussis toxin (PTX), in stark contrast to the well-recognized PTX-dependence of the stimulation of G-protein activated inwardly rectifying channels (Nakajima et al., 1988). The SP-mediated inhibition of neuronal inward rectifiers was later attributed to PKC activation. Specifically, incubation with the PKC inhibitor staurosporine or injection with the pseudosubstrate inhibitor PKC(19-36) significantly reduced the SP-mediated inhibition. At the single-channel level, in cell-attached patches SP application led to a reversible reduction in channel open probability (Po). When incubated in the phosphatase inhibitor okadaic acid, however, the SP-mediated inhibition became irreversible, implying that dephosphorylation was responsible for reversal of the SP effect (Takano et al., 1995). The single-channel conductance was measured at ~18 pS, lower than the reported conductance for G-protein activated channels (~30 pS) (Kofuji et al., 1995). Although the identity of the channel in this study is unknown, based on location and conductance it is likely to be a member of the Kir2 family, specifically Kir2.3, which has a reported conductance of ~13 pS (Perier et al., 1994).

Focusing exclusively on G-protein activated channels, Velimirovic et al. found that in rat brain locus ceruleus neurons, either somatostatin or met-enkephalin perfusion led to an enhancement of inwardly rectifying currents thru a PTX-sensitive (i.e. G_{i/o}) G-protein mechanism. Consequently, Substance P application inhibited currents preactivated with either somatostatin or met-enkephalin, but this effect was not blocked by PTX. These results indicate
that in locus ceruleus neurons the underlying molecular effector of Substance P is equivalent to the channel activated by PTX-sensitive G-proteins (Velimirovic et al., 1995).

While the Substance P effect on locus ceruleus neurons has not been specifically tested for PKC sensitivity in the same way as the analogous pathway in nucleus basalis neurons, multiple lines of evidence indicate that neuronal G-protein activated channels are sensitive to PKC modulation. First, heterologously expressed Kir3.1/3.2 channels, thought to underlie neuronal G-protein activated currents, were inhibited by phorbol esters which activate PKC, as well as by coexpressed PKC isoforms (Brown et al., 2005). Secondly, endogenous GABA_B-activated currents in hippocampal neurons were inhibited by carbachol (a muscarinic receptor agonist) or the PKC activator PdBu (phorbol dibutyrate), and both effects were blocked by PKC inhibitors (Sohn et al., 2007a; Sohn et al., 2007b). Similarly, in slice preparations of the ventral tegmental area (VTA), SP application resulted in inhibition of baclofen-activated (GABA_B receptor agonist) currents, an effect blocked by either the PKC inhibitor Bisindolyl-maleimide I, the chelation of calcium by BAPTA, the PLC inhibitor U73122, or a NK3 receptor antagonist. Taken together, these results suggest that in VTA neurons SP acts by stimulating NK3 receptors, activating PLC, and activating a calcium-dependent PKC isoform, which results in suppression of a GABA_B-activated inward rectifier potassium channel (Xia et al., 2010).

The results outlined above illustrate the immense progress in elucidating the components involved in inhibition of inward rectifier channels by PKC, but fail to shed light on the molecular determinants of PKC inhibition, notably the channel residue(s) involved in phosphorylation. After a brief overview of PKC classification and mechanism of activation, we will survey the current literature characterizing PKC phosphorylation sites in Kir channels.
1.7. **Protein Kinase C: A diverse kinase family**

The PKC family consists of proteins with structurally related domains involved in kinase activation, and is divided into conventional, novel, and atypical PKCs. The conventional PKCs ($\alpha$, $\beta$I, $\beta$II, and $\gamma$) contain C1A and C1B domains that bind the activator DAG and phorbol esters, as well as a C2 domain responsible for binding phosphatidyl-serine (PtdSer) and calcium. Novel PKCs ($\delta$, $\epsilon$, $\theta$, and $\eta$) have conventional-like C1 domains but differ in having a C2 domain without calcium-binding ability. The atypical PKCs ($\zeta$, $\iota$, $\lambda$) forgo the C2 domain but have a unique C1 domain that can bind anionic phospholipids. All kinases in the PKC family share a highly conserved catalytic domain responsible for phosphorylation of target proteins. Although PKC enzymes are subject to a high degree of constitutive phosphorylation, translocation to the membrane and binding to DAG is considered the major activation paradigm (Wu-Zhang and Newton 2013).

Activation of PKCs can be achieved by phorbol ester drugs which activate PKC in nanomolar concentrations by hijacking the C1 domain that binds DAG, thus facilitating membrane translocation. Phorbol 12-myristate,13-acetate (PMA) is one such drug, a potent tumor promoter which can irreversibly activate PKCs, due to its very low metabolism and high binding affinity. Experimentally, one can examine direct PKC effects by incubating with phorbol esters like PMA or PdBu (phorbol dibutyrate), which does not require activation of upstream PLCs or the resultant PIP$_2$ depletion. Additionally, many enzymes in the PKC family have scaffolding proteins which assist in membrane binding and localization, termed RACKs (Receptor for Activated C Kinase). RACKs exist in a macromolecular complex with PKC and phosphorylation targets, reminiscent of AKAPs (A-kinase anchoring proteins) and PKA (Wu-Zhang and Newton 2013).
1.8. Characterizing PKC inhibition in heterologous cell systems

Native cells can provide information about physiologically relevant modulatory pathways, but discerning the mechanism of these pathways requires studying the respective channels and receptors in isolation. In the following paragraphs we will examine in depth the current literature regarding PKC-dependent phosphorylation and specific residues that are implicated in different Kir channels, with an emphasis on their analogous location in Kir3.2.

In one of the first studies looking at protein kinase modulation of Kir channels, Henry and colleagues tested Kir1.1, Kir2.1, and Kir2.3 for sensitivity to PKC using *Xenopus* oocytes. Perfusion with the PKC activators PMA and PdBu led to an irreversible inhibition of Kir2.3 but not Kir1.1 or Kir2.1. Inhibition of Kir2.3 was partially blocked by preincubation of the oocytes with staurosporine, a blocker of multiple PKC isozymes (Henry et al., 1996). Chimeras of Kir2.3 and Kir2.1 were later employed to identify an N-terminal residue, Thr-53, as necessary for PKC inhibition (for location see Figure 1.4). Astoundingly, a single point mutant at the corresponding residue in Kir2.1, I79T, rendered the channel sensitive to PKC inhibition (Zhu et al., 1999). There has been no biochemical follow-up of this intriguing result, including whether this threonine residue can be phosphorylated *in vivo*. The co-crystal of the closely related Kir2.2 with PIP2 shows that the implicated threonine is located right next to a critical PIP2-binding motif, the RWR (Figure 1.4). Phosphorylation of this threonine would likely disrupt the complex electrostatic interaction network between PIP2 and the RWR motif, perhaps leading to channel inhibition.

The implicated threonine residue is a leucine in all members of the Kir3 family, and thus not a target of phosphorylation. Nevertheless, Kir3 family members are subject to robust inhibition by PKC. Kir3.1, Kir3.2, and Kir3.4 homomeric channels are inhibited by PMA
treatment, as are Kir3.1/3.2 and Kir3.1/3.4 heteromeric channels. The target of phosphorylation is unclear, although Ser-185/Ser-191 has been suggested to be a substrate for PKC phosphorylation in Kir3.1/3.4 channels. Mutation of this residue to alanine in homomeric and heteromeric channels reduced PMA-mediated inhibition. Importantly, this study also demonstrated direct regulation by a catalytic subunit of PKC in excised patches, where treatment of Kir3.1/3.4 channels with PKC led to a reduction in NPo (Mao et al., 2004). In atrial myocytes, native Kir3.1/3.4 channels co-immunoprecipitated with the PKC-specific scaffold protein RACK1, and PKC treatment of excised myocyte patches led to inhibition of ACh-activated Kir currents (Nikolov and Ivanova-Nikolova 2004).

The corresponding residue (Ser-196) was later tested in Kir3.1/3.2 channels in a heterologous HEK293 expression system. In excised patches, purified PKC reversibly inhibited Kir3.1/3.2 channels as well as Kir3.1S185A/Kir3.2S196A. Carbachol stimulation of M3 receptors resulted in equivalent inhibition of wildtype and mutant channels in whole-cell mode. The authors concluded that the residue implicated in Kir3.1/3.4 may not be applicable to Kir3.1/3.2 channels. Additionally, purified C-termini of Kir3.1 and Kir3.2 were assessed for \textit{in vitro} phosphorylation by PKC, but only the Kir3.1 C-terminus led to incorporation of detectable phosphate (Brown et al., 2005).

Reports of Kir1 (ROMK) inhibition by PKC are scarce and often controversial. PMA application inhibits native Kir1 channels that form the small-conductance potassium channel (I_{SK}) on the apical membrane of rat cortical collecting duct cells (CCD) (Wang and Giebisch 1991). In the heterologous \textit{Xenopus} oocyte system, Zeng et al. showed that PMA does not inhibit wildtype Kir1.1 channels but can inhibit channels with reduced apparent PIP$_2$ affinity, exemplified by the S219A and R188Q mutant channels. This implied a role for reduced channel-
PIP\textsubscript{2} interactions as a final common mediator of PKC inhibition. The authors failed to find a direct inhibitory effect of applying the catalytic PKC subunit on Kir1.1\_S219A channels, in contrast to what has been reported for Kir3.1/3.4 and Kir3.1/3.2 channels (Brown et al., 2005; Mao et al., 2004). Instead, they postulated that the inhibition of S219A and R188Q was due to a PKC-mediated reduction in membrane PIP\textsubscript{2} content through an unknown mechanism, rather than via direct Kir1.1 phosphorylation (Zeng et al., 2003). This notion was challenged in a later study, however, that looked more directly at membrane PIP\textsubscript{2} using a FRET-based reporter assay. While G\textsubscript{q}-coupled M1 stimulation led to a reduction in membrane PIP\textsubscript{2}, PMA application did not (Keselman et al., 2007).

A later study looked at more subtle changes in Kir1.1 function, by analyzing the intracellular pH dependence of the channel before and after PMA treatment. PMA treatment induced an alkaline shift of the pKa for inhibition, in effect making the channel more sensitive to intracellular protons. The authors identified a novel threonine residue (Thr-193) as a potential substrate for PKC phosphorylation. When mutated to alanine, the PMA effect on intracellular pH sensitivity was lost. In molecular dynamics (MD) simulations, the distance between phosphorylated Thr-193 and Arg-188 decreases, and consequently Arg-188 is pulled farther from the membrane interface. Although PIP\textsubscript{2} was not included in the simulations, the authors propose that Arg-188 is normally interacting with PIP\textsubscript{2}, and when Thr-193 gets phosphorylated, Arg-188 is pulled away from the PIP\textsubscript{2} molecule (Huang et al., 2012). Sequence alignment with other Kir channels indicates that Thr-193 is unique to Kir1.1, as all other Kir channels do not have a Ser/Thr at this position. Additionally, this study lacks biochemical characterization of the novel phosphorylation site. Arg-188 maps to Arg-201 in the Kir3.2 channel, and Ser-219 aligns with Ser-232 (see Figure 1.4).
Heteromeric channels of Kir4.1/5.1, a potent cellular sensor of acidification with key roles in the eye and brainstem, also exhibited inhibition by PKC activators (Rojas et al., 2007). Kir4.1 homomers were not sensitive to PMA or thymeleatoxin, while Kir4.1/5.1 heteromers were subject to robust inhibition. This inhibition was not found to rely upon endocytosis of active channels, but rather a reduction in channel open probability (Po). The authors found that the purified C-terminal domains of both Kir4.1 and Kir5.1 were subject to phosphorylation by the catalytically active subunit of PKC, but could not identify the specific sites by mutagenesis scanning. Interestingly, the mutant Kir5.1_T174D greatly reduced the sensitivity to PKC inhibition, which was intact in Kir5.1_T174A. This particular residue corresponds to Gln-197 in Kir3.2, located before the PIP2-binding motif KKR, and is either a neutral Gln or positive Lys/Arg in all other channels (see Figure 1.4). The authors conclude that this site is not a target of PKC phosphorylation, but may alter channel function, consistent with its location.

Conversely to the channels examined so far, PKC treatment leads to activation of Kir6.2 (K\textsubscript{ATP}) channels in excised patches, rather than inhibition. This activation seems to rely on changing the dose-dependence of ATP inhibition, essentially reducing the cooperativity of ATP binding. In the presence of low levels of ATP, PKC treatment inhibits the channel, with a crossover of activation/inhibition around 110 \textmu M (Light et al., 2000). Phosphorylation of a single residue, T180, is implicated in this effect, which corresponds to T204 in Kir3.2. Mutation of this residue to alanine or glutamate abrogated the effect of PKC, and led to a loss of \textit{in vitro} phosphorylation in immunoprecipitated Kir6.2 protein. Mutation to either residue also resulted in an increased open time, such that the authors propose that this residue has importance in regulating channel gating as well.
Further complicating matters, an inhibitory effect of PKC phosphorylation of the distal C-terminus of Kir6.2 has been described, at Ser-372 (Aziz et al., 2012). This residue seems to be involved in PKC-dependent internalization rather than direct effects on channel gating. A frequently used construct to study Kir6.2 in isolation rather than as a SUR-containing complex truncates the channel’s distal 26 residues, called Kir6.2ΔC26. This construct does not include Ser-372, which partially explains the predominant activating effect of PKC found by Light et al.

Like most other Kir channels tested, PKC phosphorylation of Kir6.1 has been associated with channel inhibition. Shi et al. identified a distal C-terminus composed of five phosphorylation sites not found in Kir6.2, which mutation analysis identified as important in PKC-dependent inhibition (Shi et al., 2008). Mutation of individual serines to alanine reduced PMA inhibition somewhat, but a channel with all five mutated was rendered completely insensitive to PMA inhibition. Only one of these serines maps to Kir3.2, at Ser-368 (at the distal C-terminus, not included in Figure 1.4). The rest are in an arginine rich region unique to Kir6.1.

The last channel examined is Kir7.1, the channel with the very small conductance found in the retinal pigment epithelium (RPE). The PKC activators OAG and thymeleatoxin inhibit this channel when expressed in Xenopus oocytes. The mutant S201A significantly reduced the effect of OAG. Only Kir3.1 in the Kir3 family contains the corresponding serine residue at Ser-225, whereas all other Kir3 channels have glutamate at this position (Glu-237 in Kir3.2) (Zhang et al., 2008).

This survey of PKC phosphorylation of the Kir channel family (summarized in Table 1.1) indicates that most Kir channels are inhibited by PKC activation, with the exception of reports of activation in Kir6.2. A study by Du et al. looked at a panel of Kir channels and their response to PMA inhibition, where the vast majority of channels were inhibited, including Kir6.2.
Consistently, Kir1.1 and Kir2.1 do not show PMA inhibition, except in cases when channel-PIP2 interactions are decreased (Du et al., 2004). Looking at specific residues implicated in PKC phosphorylation, these studies identify two principal regions likely to be involved: the proximal C-terminus near the base of the M2 helix, and the distal C-terminus. Many of these phosphorylation sites are located nearby residues implicated in PIP2 binding (blue residues in Figure 1.4), such that negatively charged phosphates could alter the complex electrostatic network between the Kir channel and PIP2 (Logothetis et al., 2007). We next turn to the experimental evidence in support of a PIP2 dependence on PKC inhibition in Kir channels.

1.9. Channel-PIP2 interactions underlie PKC inhibition of Kir3 Channels

All Kir channels require the signaling lipid PIP2 for activation, and decreases in membrane PIP2 after Gq-coupled PLC stimulation accounts for inhibition in several Kir channels. In Kir3 channels, some studies have indicated that PKC activation can account for inhibition from Gq-coupled receptors, such that blockers of PKC attenuate Gq-coupled inhibitory pathways (Keselman et al., 2007; Stevens et al., 1999). Since Gq-coupled pathways result in both PIP2 depletion and PKC activation, crosstalk between these pathways converging on a mechanism reducing channel-PIP2 interactions is worth exploring.

The seminal study by Du et al. found that alterations in channel-PIP2 interactions produced by point mutations could in turn alter sensitivity to PKC inhibition. Kir2.1, which is normally resistant to inhibition, gained sensitivity with the mutation R312Q (analogous to R324 in Kir3.2). Conversely, the readily inhibited Kir2.3 channel could be rendered insensitive to PKC by the point mutant I213L (I234 in Kir3.2). Kir3.4* channels also exhibited high sensitivity to PKC inhibition, which was abrogated by coexpression of Gβγ, previously shown to increase channel-PIP2 interactions. These results implied that increased channel-PIP2
interactions, as with Kir2.1, Kir2.3_I213L, and Kir3.4*/Gβγ, reduce or abrogate entirely channel sensitivity to PKC inhibition. Reduced channel-PIP2 interactions, as with Kir2.3, Kir2.1_R312Q, or Kir3.4* alone, increase sensitivity to PKC inhibition. (Du et al., 2004).

In the Kir3.1/3.4 channel expressed in HEK293 cells, PMA inhibition was partially blocked by PIP5K overexpression or by dialysis of exogenous PIP2. Wortmannin pretreatment, which reduces membrane PIP2 levels, led to increased sensitivity to PMA inhibition (Keselman et al., 2007). Similarly in native hippocampal neurons, PIP2 dialysis prevented PKC inhibition of baclofen-activated potassium current (Sohn et al., 2007a). In excised patches, PKC treatment of Kir3.1/3.4 channels resulted in a rightward shift of the dose-dependence of PIP2 activation (Keselman et al., 2007). These results imply that PKC action inhibits Kir3 channels by reducing channel-PIP2 interactions, which would be expected to potentiate inhibition caused by PLC-mediated PIP2 depletion.

To further explore the idea that phosphorylation can alter channel-PIP2 interactions, we next turn to PKA regulation of Kir1.1 and Kir3.1/3.4 pathways, a well-studied phosphorylation paradigm implicated in modulation of channel-PIP2 interactions.

1.10. Mechanistic insight from other phosphorylation pathways: Focus on PKA

The Kir1.1 channel (ROMK1) has been extensively studied for how phosphorylation affects activity. The channel is activated by cAMP-dependent protein kinase (PKA), and the functional PKA phosphorylation sites have been mapped using a combination of biochemical and electrophysiological approaches (Xu et al., 1996). Phosphorylation at S219 and S313 is generally thought to increase channel open probability (Po). Interestingly, one of the antenatal Bartter Syndrome loss-of-function mutations is S219R (Simon et al., 1996), which would abrogate phosphorylation and introduce positive charge at a typically negatively charged site.
Once activated by PKA, the Kir1.1 channel now has a left-shifted PIP$_2$ apparent affinity, consistent with PKA phosphorylation increasing channel-PIP$_2$ interactions. Both the S219A and S313A channel mutants have reduced PIP$_2$ affinity compared to wildtype channel, but the phosphomimetic mutants (S219D and S313D) also have reduced affinity (Liou et al., 1999). It is possible, however, that the phosphomimetic aspartate is not able to adequately mimic the phosphorylated serine. Ser-219 and Ser-313 of Kir1.1 map to Ser-232 and Ser-324 in Kir3.2, respectively, and are next to PIP$_2$-sensitive residues Arg-230 and Arg-324 (see Figure 1.4).

A recent paper has also characterized potential PKA phosphorylation sites, this time in Kir3.1/3.4 channels. Kir3.1/3.4 currents expressed in Xenopus oocytes can be potentiated by injection of cAMP, presumably through a PKA-dependent mechanism. Mutation of Thr-199 in homomeric Kir3.4* had a profound effect, essentially preventing the PKA-dependent potentiation by cAMP. Furthermore, Kir3.4 C-terminal peptides with the T199C mutant had significantly reduced $^{32}$P incorporation after PKA treatment compared to control. The equivalent residue to Thr-199 is present in the crystal structure of Kir3.2 (as Thr-204), but other residues described in this study are in the distal C-terminus of Kir3.1 and Kir3.4, and not present in the Kir3.2 structure (Treiber et al., 2013). Even so, a molecular understanding of how phosphorylation of Thr-199 leads to channel activation is unclear.

In a separate study, Lopes et al. demonstrated that C-terminal peptides of Kir3.1 and Kir3.4 were subject to in vitro phosphorylation by PKA, and whole-cell currents of Kir3.1/3.4 expressed in Xenopus oocytes were reduced after H89 treatment (a PKA inhibitor). Muscarinic inhibition of Kir3.1/3.4, a measure of PIP$_2$ sensitivity, was increased after H89 treatment as well, an effect abolished by heteromeric channels containing putative PKA phosphorylation site mutations (Kir3.1_S221A or S315A, which map to Kir1.1 PKA sites Ser-219 or Ser-313).
Finally, PKA treatment of Kir3.1/3.4 in excised patches resulted in a left-shifted dose-dependence of PIP_2 activation (Lopes et al., 2007). Taken together, these results imply that PKA phosphorylation of Kir3.1/3.4 results in increased apparent PIP_2 affinity, and therefore decreases sensitivity to G_q-mediated PIP_2 depletion. Qualitatively, PKA-mediated increases in PIP_2 affinity resemble the case with Kir1.1, but the molecular explanation of how phosphorylation changes channel-PIP_2 interactions is still lacking.

Lee and colleagues sought to gain a molecular understanding of PKA phosphorylation in the Kir1.1 (ROMK1) channel, at the residue previously shown to be phosphorylated by PKA (Ser-219). To accomplish this, they combined molecular dynamics simulation studies with electrophysiology, specifically focusing on intracellular pH-induced gating. Kir1.1 can be inhibited by decreases in intracellular pH (i.e. acidification) in excised patches, in a manner dependent on PIP_2 affinity. PKA treatment of excised patches can induce a left-ward shift off the pKa for inhibition, essentially making the channel harder to inhibit. The S219A and S313A mutants mimicking a non-phosphorylated state have a right-shifted pKa, approximately 7.2. For structural understanding, the authors first built a homology model based on the KirBac1.1 crystal structure, then used molecular dynamics simulations to investigate the wildtype and Ser-219 phosphorylated channels. Their simulation results indicate that phosphorylation at Ser-219 recruits the nearby Arg-217 residue, ultimately resulting in the movement of Lys-218 closer to the membrane. Although the PIP_2 molecule is not included in their simulation, the authors contend that repositioning of Lys-218 should result in a stronger channel-PIP_2 interaction near this residue (Lee et al., 2008). Further understanding is limited by the lack of explicit PIP_2 in the simulation and experimental testing of its results. Still, this is an important first step in elucidating a structural mechanism for how phosphorylation controls channel gating. In Kir3.2,
Lys-218 is an asparagine, but the nearby Arg-217 is absolutely conserved in all Kir channels (Arg-230 in Kir3.2).

Clearly, PKC inhibition and PKA activation present a reversible, powerful means of controlling channel activity. Evidence points to a mechanism converging on modulation of channel-PIP$_2$ interactions, but the molecular mechanism of how tagging a specific site with the negative phosphate leads to alterations in channel-PIP$_2$ interactions is unclear. Part of the answer to this question may lie in how PIP$_2$ gates a Kir channel, which is explored in the next section.

1.11. A Brief History of Kir Channel Structures

In 1998, a landmark study by MacKinnon’s laboratory, for which he received the Nobel Prize in Chemistry, produced the first crystal structure of a membrane ion channel, the potassium channel KcsA from *Streptomyces lividans* bacteria (Doyle et al., 1998). This crystal structure allowed the determination of the molecular mechanism of potassium channel selectivity, providing a glimpse of potassium ions trapped in the selectivity filter. The KcsA channel monomeric unit is composed of two transmembrane helices (TM1 and TM2) linked together by a pore helix, sharing homology with inward rectifier channels in these regions but of course missing large chunks of the critical intracellular regions. The structure of intracellular regions of similar channels would be solved independently, first by focusing on the Kir3.1 cytoplasmic domain (Nishida and MacKinnon 2002), revealing the molecular underpinnings of inward rectification and block by intracellular cations. Comparison of cytoplasmic domain structures from Kir2.1 and Kir3.1 revealed a putative cytosolic gating element in the βH-βI loop, called the “G-loop” gate because it forms a girdle around the central pore pathway (Pegan et al., 2005).

The first full-length structure of an inward rectifier came from a bacterial source, the KirBac1.1 channel from *Burkholderia pseudomallei* (Kuo et al., 2003). KirBac1.1 is composed
of a four-fold symmetric transmembrane domain similar to KcsA, and a cytoplasmic domain consisting of beta sheets βA thru βM, resembling the isolated cytoplasmic domain structure previously determined. At the base of the transmembrane TM2 helix, an inward facing hydrophobic phenylalanine (F146) lines the pore, forming a barrier to potassium permeation. A glycine residue (G143) approximately one helical turn above F146 presumably forms a hinge point for the TM2 helix to bend, removing F146 block of the permeation pathway. This glycine residue is absolutely conserved among Kir channels, and voltage-gated channels like Shaker and Kv1.2 have a kink-inducing proline at the same position. A structural role for a different highly conserved glycine, located at the halfway point of the TM2 helix, later challenged this hypothesis, and has been implicated in the gating of several ion channels (Jiang et al., 2002), including the Gβγ-mediated activation of Kir3 channels (Jin et al., 2002). Importantly, the KirBac1.1 structure provided evidence for a new gating element, the slide helix, which is located parallel to the plasma membrane and extends perpendicular to the transmembrane domain helices, reminiscent of the S4-S5 linker in voltage-gated potassium channels. While the structure provides a wonderful first look at full-length inward rectifier channels, this prokaryotic channel is functionally inhibited rather than activated by PIP2, as is the case with eukaryotic inward rectifiers (Enkvetchakul et al., 2005). Therefore, the structural information on how PIP2 activates inward rectifier channels was necessarily limited in KirBac1.1.

A chimeric channel structure with a bacterial transmembrane domain (KirBac1.3) and a eukaryotic cytoplasmic domain (Kir3.1) was solved in 2007, resulting in subunits with two gating conformations, termed “constricted” and “dilated” based on whether the G-loop gate was open or closed (Nishida et al., 2007). In the constricted form, sulfur atoms lining the G-loop would prevent a hydrated potassium ion from permeating, whereas in the dilated form, the loop
is wide enough, lined with oxygen atoms from T306 and G307. It is perhaps informative that the crystals were grown in a short-chain analogue of PIP$_2$, however the specific density of the PIP$_2$ head group could not be placed in the structure. For the first full-length eukaryotic Kir channel structure, MacKinnon’s group crystallized chicken Kir2.2. This structure has an open G-loop gate and a closed inner helix bundle crossing gate (Tao et al., 2009). Importantly, chicken Kir2.2 would pave the way toward the first co-crystal of a channel with PIP$_2$.

1.12. A Tale of Two Channels (and PIP$_2$)

The co-crystal structure of chicken Kir2.2 and the short chain analogue DiC8-PIP$_2$ presented for the first time a molecular picture of crystal contacts between the PIP$_2$ molecule and a Kir channel. Scanning neutralization mutagenesis of positively charged residues to glutamine made it clear that positive residues are involved in PIP$_2$ activation and likely PIP$_2$ binding itself, but the exact channel-PIP$_2$ binding interactions remained to be determined. In the co-crystal structure, the head group of PIP$_2$ makes elaborate contacts with positively charged domains of Kir2.2 (Hansen et al., 2011). The 1’ phosphate of PIP$_2$ sits in a nonspecific anionic phospholipid binding site, interacting with backbone atoms of the RWR motif in the slide helix, positioning the fatty acid tail between hydrophobic residues of the channel and the membrane bilayer. More specific phosphatidylinositol binding is found between the 4’ and 5’ phosphates of PIP$_2$ and K183, R186, K188, and K189 of Kir2.2. The last amino acid of the KKR motif, R190, interacts extensively with the cytoplasmic domain but does not form direct interactions with PIP$_2$. This work also sheds significant light on a possible mechanism of PIP$_2$-induced activation, whereby PIP$_2$ head group interaction with the cytoplasmic domain results in an upward translational movement, connecting it to the transmembrane domain. Changes to the G-loop gate were relatively minor, however.
Around the same time, MacKinnon’s group crystallized the nearly full-length Kir3.2 channel (as opposed to the KirBac1.3/Kir3.1 chimeric channel) both in the presence and absence of DiC8-PIP₂ (Figure 1.6A) (Whorton and MacKinnon 2011). The PIP₂-binding site is very similar to the cognate site in Kir2.2, such that the 1’ phosphate interacts with the amide backbone of the KWR motif, and the 4’ and 5’ phosphate interact with positively charged K194, K199, and K200 (see Figure 1.6B). Details of the gating mechanism, however, are in stark contrast. Even in the absence of PIP₂, the cytoplasmic domain is juxtaposed to the transmembrane domain, so a PIP₂-induced upward translation (as in Kir2.2) is unlikely. The interface between the transmembrane and cytosolic domain contains the βC-βD loop (or CD loop), a region containing a residue implicated in sodium binding, Asp-228. When this residue is mutated to the neutral asparagine, the channel no longer requires sodium for full activation (Ho and Murrell-Lagnado 1999; Zhang et al., 1999). Indeed, the crystal structure of the D228N mutant no longer has an electron density for sodium ion at this position.

In both structures of the Kir3.2 channel, whether in the presence or absence of PIP₂, the cytosolic G-loop gate was closed, as was the helix bundle crossing gate. In order to obtain an open G-loop gate structure, MacKinnon’s group systematically mutated several residues at the interface between the transmembrane and cytosolic domains, then tested for activation using the Gᵢ-coupled GPCR M2. Using this methodology, they found that the mutation R201A had almost no activation by the M2 receptor, yet its current was blocked by the Kir3 inhibitor Tertiapin-Q. Interestingly, the R201Q neutralization mutant did not express detectable current.

The R201A mutant structure was crystallized in the presence and absence of PIP₂. The G-loop gate that was closed in the wildtype channel was now in an open conformation, which resulted from a rearrangement of the CD-loop. In the presence of PIP₂, both the helix bundle
crossing and G-loop gates were open. However, in the R201A mutant + PIP$_2$ structure, only two PIP$_2$ molecules were bound per tetrameric channel. The authors state that this is likely a crystallization artifact due to crystal packing, and thus in the normal state all four subunits would have a bound PIP$_2$ molecule. The R201A mutant is predicted to give a constitutively active channel, insensitive to G-protein activation and thus in a fully active state. However, the channel current is greatly diminished compared to the fully activated wildtype Kir3.2 current after M2 receptor activation with carbachol (Whorton and MacKinnon 2011). Thus the physiological relevance of the R201A mutant conformation as “constitutively active” is still an open question.

1.13. PIP$_2$-induced gating: Probable gating motions revealed from MD simulations

The co-crystal structures of Kir channels bound to PIP$_2$ provide valuable insight as to the channel binding pocket, and can differentiate direct interactions from allosteric when combined with experimental data. There is limited information from snapshots of crystal structures of how PIP$_2$ binding induces gating, however. In the case of Kir3.2, for example, very little change is evident from comparing absence and presence of PIP$_2$, yet this channel clearly requires PIP$_2$ for activity. Meng et al. took a computational approach to examine PIP$_2$-induced gating motions in the chimeric Kir3.1 channel, by docking PIP$_2$ to the constricted and dilated conformations (Meng et al., 2012). These simulations implicated a role for the CD-loop in guiding the G-loop gate to an open conformation. Tracking the trajectory from the constricted to the dilated state revealed that the N-terminus forms a βA interface with the βM sheet of an adjacent subunit in the presence of PIP$_2$, whereas in the absence of PIP$_2$ the N-terminus exists as a partially disordered loop. Furthermore, the disordered N-terminal loop constrains CD-loop movement, which in turn locks the G-loop gate in an inactive conformation.
In the simulation, the constricted state with bound PIP$_2$ adopted an intermediate conformation resembling the dilated state. The interaction of PIP$_2$ in the two conformations was dynamic, such that some residues preferentially bound PIP$_2$ in the constricted simulation, while others bound only in the dilated simulation. In the constricted simulation, the PIP$_2$ binding residues were K49 and R52 in the N-terminus, corresponding to K61 and K64 in Kir3.2, as well as R190 in the C-linker helix, which corresponds to R201. In the dilated simulation, PIP$_2$-interacting residues were R66, K183, and R219, which correspond to R77, K194, and R230 respectively (Meng et al., 2012). Comparing with the co-crystal structure in Kir3.2, only K64 in the N-terminus interacts with PIP$_2$, but no interaction is seen for R201 or R230. This indicates that the PIP$_2$ binding site may be dynamic, proceeding through intermediate salt-bridge interactions on the way to an optimized PIP$_2$-binding conformation. Additionally, there may be differences in the gating mechanisms and binding of PIP$_2$ to Kir3.1 vs. Kir3.2, which has not been explored in depth. In support of this, some differences in G$\beta\gamma$ activation between the two channels are evident, where in purified protein systems (bilayers and vesicles, respectively) the chimeric Kir3.1 and full-length Kir3.2 respond differently to G$\beta\gamma$. Chimeric Kir3.1 is functionally inhibited by G$\beta\gamma$ except in the presence of activated G$\alpha$ (Leal-Pinto et al., 2010), whereas Kir3.2 shows activation in the presence of G$\beta\gamma$ alone (Whorton and MacKinnon 2013).

1.14. **Direct Activators of Kir3 Channels: G proteins and Ethanol**

We will now close this chapter by focusing on known activators of Kir3 channels, and the crystallographic or experimental evidence to date regarding the mechanism of activation. Channels in the Kir3 family can be activated directly by G-protein G$\beta\gamma$ subunits, but also by alcohols. Alcohol exerts a direct effect on channel activation independent of G-protein binding, but the two mechanisms of activation may involve similar changes. A recent co-crystal structure
of the Kir3.2 channel with Gβγ has been obtained, indicating that the channel binding interface is composed of the βL-βM loop from one subunit and the βD-βE loop from an adjacent subunit (Whorton and MacKinnon 2013). An independent computational study identified a similar Gβγ binding cleft in Kir3.1* using protein-protein docking algorithms on the Kir3.1 chimera protein structure and Gβγ. Experimentally, Kir3.1 channel activation was found to depend on the size of the side chain at Gβ(L55)γ keeping the βL-M(L333) and βD-E(F243) arms of the cleft apart from one another. Alternatively, the cleft could be kept open electrostatically by the L55E mutation on Gβ acting on E334 and E335 of the βL-M loop (Mahajan et al., 2013).

Synergy of PIP2 and Gβγ has been reported in Kir3 channels, such that binding of Gβγ and subsequent activation seems to stabilize its interaction with PIP2. Channel inhibition by PIP2-antibody is slowed in the presence of Gβγ, while reactivation by PIP2 is enhanced (Huang et al., 1998; Zhang et al., 1999). Aside from Gβγ binding the channel with PIP2 in a ternary complex, which is unlikely given the binding position discussed previously, it is instead more likely that Gβγ binding and activation stabilizes the channel in a conformation that preferentially binds PIP2. A similar allosteric mechanism may be at work in the I213L mutation of Kir2.3, which increases apparent PIP2 binding affinity (Du et al., 2004; Zhang et al., 1999). Limited structural evidence regarding exactly how a switch in hydrophobic residues (Ile to Leu) influences PIP2 affinity exists, but a recent crystal structure of the Kir2.2_I223L mutant in the presence and absence of PIP2 indicates that this mutation may force the G-loop gate into an active, PIP2-bound conformation even in the absence of PIP2 (Hansen et al., 2011).

Interestingly, the putative alcohol binding pocket is located in approximately the same position as the Gβγ binding site. Using a co-crystal structure of the cytosolic domain of Kir2.1 with the bound alcohol MPD (methylpentane-diol), Aryal and colleagues identified a putative
alcohol-binding pocket with marked similarity to the Gβγ binding interface. It is composed of the N-terminus and βL-βM loop from one subunit and the βD-βE loop from the adjacent subunit (Aryal et al., 2009). The molecular pathway of conformational change in ethanol binding is unclear, yet it seems likely that the mechanism is similar to Gβγ activation based on the location of the binding site.

1.15. Sodium-dependent activation of Kir3 channels

The dependence on sodium to fully activate Kir3 channels is well-established, and tremendous progress has been made to identify the sodium coordination site. Sodium-dependent activation is thought to be a compensatory mechanism for increasing hyperpolarizing currents during prolonged activity of excitable cells, which results in increased local sodium concentration. A critical aspartate, Asp-228 in Kir3.2, has been shown to confer sodium sensitivity in mutagenesis studies. Kir3.2, Kir3.3, and Kir3.4 all contain an aspartate at this position, whereas Kir3.1 and most other Kir channels contain an asparagine, except Kir5.1, Kir6.1, and Kir6.2. In the Kir3.2 crystal structure, sodium density is not present in the D228N mutant. Neutralization of this aspartate experimentally increases whole-cell currents, and leads to increased PIP$_2$ apparent affinity, similar to effects of sodium in excised patches (Ho and Murrell-Lagnado 1999). This has led to the hypothesis that sodium functionally neutralizes the negatively charged aspartate, leading to an increase in net positive charge on the channel and increased PIP$_2$ binding (Rosenhouse-Dantsker et al., 2008). Alternatively, the negatively charged aspartate shields an electropositive residue that directly binds to PIP$_2$, such that sodium binding would allow the positive residue to bind PIP$_2$ unhindered.

Two studies have looked at structural mechanisms determining how sodium activates Kir3 channels, with differing potential mechanisms. In Kir3.4, Rosenhouse-Dantsker et al.
applied molecular dynamics simulations to the isolated cytoplasmic domain, modeled on Kir3.1, to investigate how hydrogen-bond networks differ in the presence and absence of sodium. In the absence of sodium, Arg-225 (equivalent to Arg-230 in Kir3.2) of Kir3.4 formed hydrogen bonds with Asp-223 (Asp-228 in Kir3.2), with both residues located in the CD loop. The presence of sodium frees Arg-225 from hydrogen bonding with Asp-223, which presumably allows it to interact with PIP₂. Furthermore, a downstream histidine residue which corresponds to His-233 in Kir3.2, is involved in conferring sodium sensitivity. Kir6.2 is not sodium sensitive, likely due to a methionine instead of a histidine at the equivalent position (Rosenhouse-Dantsker et al., 2008).

The corresponding residue to Arg-230 in Kir2.1, Arg-218, greatly reduces current levels when mutated to the neutral glutamine, and increases the kinetics of PIP₂-antibody inhibition, a marker of reduced channel-PIP₂ interaction. Importantly, tagging an R218C mutant with the positively charged sulphydryl modifying reagent MTSEA both increases channel currents and slows the kinetics of PIP₂-antibody inhibition (Lopes et al., 2002). These results imply that R218C is accessible to intracellular modification, and that incorporation of positive charge can restore channel-PIP₂ interactions.

Crystallographic studies of the isolated Kir3.2 cytoplasmic domain both in the presence and absence of sodium implicate the N-terminal His-69 residue in the sodium coordination site. The mutant H69Q has a greater response to applied PIP₂ than the wildtype channel, but still responds to sodium. The mutant D228N does not retain sodium sensitivity, and has higher PIP₂ response as well. The authors contend that sodium-mediated gating may involve an interaction between the CD loop and the N-terminus, which is stabilized by the presence of sodium (Inanobe et al., 2010). The exact mechanism for how sodium increases channel activity remains
unresolved, although different mechanisms toward the same goal of activation may be at work in Kir3.2 and Kir3.4.

1.16. Conclusion

In the case of Kir3 channels, activators like PKA phosphorylation, Gβγ, or sodium, seem to converge on promoting channel-PIP2 interactions. Similarly, accumulating evidence indicates that inhibitory PKC phosphorylation likely acts by reducing channel-PIP2 interactions. We can think of the channel as existing in an equilibrium between at least two energy minima or conformations, representing either an inactive or active state. The channel gates (selectivity filter, helix bundle crossing, and G-loop) all need to be in the open conformation for the active state. The active state is promoted by PIP2 binding, whereas conversely the inactive state becomes less energetically favorable in the presence of PIP2. Activators like PKA phosphorylation, Gβγ, or sodium (and possibly ethanol) would then stabilize the active state, which could occur along the same pathway as PIP2 stabilization or independently. Inhibition, like that mediated by PKC phosphorylation, would shift the equilibrium to the inactive state, making the active state less energetically favorable, resulting in reduced channel-PIP2 interactions. In the case of Kir3.2, a fully active conformation is proposed in the context of the R201A mutant (Whorton and MacKinnon 2013), but it is unclear if this is a physiologically relevant conformation. Presumably PKC phosphorylation acts to inhibit Kir3.2 by stabilizing an inactive conformation, making the active conformation less energetically favorable. We explore this idea in detail using putative phosphorylation site-specific mutants and analyzing their impact on PKC inhibition and gating in the next chapters.
Hypothesis

For Kir channels and GIRK channels in particular, phosphorylation presents a reversible, fast means to modulate channel activity. Phosphorylation likely involves pathways of activation or inhibition inherent to channel function, with PIP$_2$ a likely candidate due to its central role. We hypothesize that phosphorylation by PKC manifests its functional effect by reducing channel-PIP$_2$ interactions, and set out to investigate this hypothesis using a combined experimental and computational approach.
<table>
<thead>
<tr>
<th>Channel [Alias]</th>
<th>Functional Effect of PKC</th>
<th>Target residues</th>
<th>In vitro PKC phosphorylation?</th>
<th>System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir1.1 [ROMK1]</td>
<td>Shifts pKa; Inhibits</td>
<td>T193</td>
<td>Yes, multiple</td>
<td>Xenopus; CCD(I_{SK})</td>
</tr>
<tr>
<td>Kir2.1 [IRK1]</td>
<td>None</td>
<td>none</td>
<td>Not tested</td>
<td>Xenopus</td>
</tr>
<tr>
<td>Kir2.2 [IRK2]</td>
<td>Inhibits</td>
<td>none</td>
<td>Not tested</td>
<td>Xenopus</td>
</tr>
<tr>
<td>Kir2.3 [IRK3]</td>
<td>Inhibits</td>
<td>T53</td>
<td>Not tested</td>
<td>Xenopus</td>
</tr>
<tr>
<td>Kir3.1 [GIRK1]</td>
<td>Inhibits</td>
<td>S185</td>
<td>Yes, not site-specific</td>
<td>Xenopus, COS-7, atrial myocytes (I_{K,ACh})</td>
</tr>
<tr>
<td>Kir3.2 [GIRK2]</td>
<td>Inhibits</td>
<td>None</td>
<td>None</td>
<td>Xenopus, Hippocampal neurons (GABA_B), HEK293</td>
</tr>
<tr>
<td>Kir3.4 [GIRK4]</td>
<td>Inhibits</td>
<td>S191</td>
<td>None</td>
<td>Xenopus, atrial myocytes (I_{K,ACh})</td>
</tr>
<tr>
<td>Kir4.1/5.1</td>
<td>Inhibits</td>
<td>None</td>
<td>Yes, not site-specific</td>
<td>Xenopus, HEK293</td>
</tr>
<tr>
<td>Kir6.1 [KATP]</td>
<td>Inhibits</td>
<td>S354, S379, S385, S391, S397</td>
<td>Yes, multiple</td>
<td>HEK293</td>
</tr>
<tr>
<td>Kir6.2 [KATP]</td>
<td>Activates; Stimulates channel internalization; Inhibits</td>
<td>T180; S372</td>
<td>Yes</td>
<td>HEK293; Xenopus</td>
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<tr>
<td>Kir7.1</td>
<td>Inhibits</td>
<td>S201</td>
<td>Not tested</td>
<td>Xenopus</td>
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Figure 1.1. Kir channel phylogeny and topology. A. Phylogenetic tree of Kir channels. B. General topology of a Kir channel, as compared to a Kv channel. Adapted from Hibino et al., 2010.
Figure 1.2. Channelopathies of Kir Channels. Schematic of Kir channels with tissue-specific expression and recognized channelopathies. Up arrows indicate gain of function, while down arrows indicate loss of function mutations. Modified from Pattnaik et al., 2012.
Figure 1.3. Schematic of phosphoinositide interconversion and PIP$_2$ hydrolysis. The phosphatidylinositol (PtdIns) precursor can be interconverted into seven different phosphorylated species by phosphatidylinositol kinases and phosphatases. Wortmannin inhibits PI3K and PI4K at high micromolar concentrations. PLC hydrolysis of PIP$_2$ results in DAG and IP$_3$. DAG activates PKC directly, but increases in calcium via IP$_3$ action also activate calcium-sensitive PKCs.
Figure 1.4. Alignment of selected eukaryotic Kir channels. PIP2-binding residues determined experimentally are colored in blue. Potential phosphorylation sites are boxed and colored in green. Structural domains are highlighted by a gray background. HBC: helix bundle crossing; SF: selectivity filter. Relevant PIP2 sites in Kir3.2 are: K64, R77, K90, W91, R92, K194, K199, K200, R201, R230, R240, and R324. R201, R230, R240 and R324 do not bind PIP2 in the crystal structure. Relevant phosphorylation sites are: S196 (PKC in Kir3.1/3.4), T204 (PKA in Kir3.1/3.4, PKC in Kir6.2), S232 (PKA in Kir1.1), S326 (PKA in Kir1.1). Channel numbering is according to the human isoform, except for Kir3.2, which is mouse.
Figure 1.5. Neuronal excitation by Substance P results from inhibition of an inwardly rectifying potassium current. Substance P application to globus pallidus neurons results in a depolarization attributed to a reduction in inward potassium current. A. In current-clamp mode, Substance P evokes a reversible depolarization. B. In voltage-clamp mode, Substance P application results in a temporary reduction of inward rectifying currents. C. SP-sensitive current is inward rectifying and reversal potential shifts according to $E_K$. D. SP-sensitive conductance is half-maximal at $E_K$. Adopted from Stanfield et al., Nature 1985.
Figure 1.6. Kir3.2 structure and location of PIP$_2$-binding residues. A. Tetrameric Kir3.2 channel with differential coloring of monomers. B. Close-up of PIP$_2$ binding site. C-linker contains the K199 and K200 in the KKR motif. Adapted from Whorton et al., 2011.
Chapter 2. MATERIALS AND METHODS

2.1. Molecular Biology

For expression in *Xenopus* oocytes, the following vectors were used: pGEMHE (for Kir3.1 and Kir3.4), pXoom (for Kir3.2), and pSD64TF (for Ci-VSP). Point mutations were introduced using standard *Pfu*-based mutagenesis technique according to the QuikChange protocol (Stratagene) and mutations were verified by sequencing (Genewiz). Plasmids were linearized prior to *in vitro* transcription using a commercially available T7 mRNA transcription kit, with the exception of pSD64TF, which used SP6 mRNA transcription kit.

2.2. Reagents

Wortmannin, PMA, Mg-ATP and Bis-indolylmaleimide II (Bis) were purchased from Sigma. DiC8-PIP$_2$ was purchased from Avanti. Alkaline phosphatase (Calf intestinal phosphatase or CIP) was from New England Biolabs.

2.3. Oocyte preparation and injection

Oocytes from *Xenopus laevis* were surgically removed and subjected to collagenase treatment according to standard protocols. Once defolliculated, oocytes were transferred to OR2 solution supplemented with Ca$^{2+}$ and Penicillin/Streptomycin antibiotics. Stage V or VI oocytes were injected with 50 nL of cRNA resuspended in DEPC water. For wortmannin treatments, oocytes were incubated in wortmannin-containing OR2 at 50 uM final concentration for two hours prior to experiments. For Bis treatment, 30 nL of 150 uM Bis (diluted in ND96K) was
injected into oocytes two hours prior to experiments. Oocytes were also incubated in 5 uM Bis (in OR2 media).

2.4. Two Electrode Voltage Clamp

Borosilicate glass electrodes were pulled using a Flaming-Brown micropipette puller and filled with a 3M KCl solution containing 1.5% agarose. Resistances were kept between 0.3 and 1.0 MΩ. Currents were recorded 1-3 days after injection using a GeneClamp 500 amplifier (Axon Laboratories). A voltage ramp protocol was used to monitor inward current, from -80 mV to +80 mV at a holding of 0 mV. Specialized Ci-VSP protocols were used to monitor impact of PIP2 depletion on channel current. To obtain the percentage of PMA inhibition, the PMA-inhibited current at -80 mV (I_{max} - I_{post-PMA}) was divided by the maximal current at -80 mV (I_{max}) prior to PMA application. In channels with very small currents (less than 1 uA, as in Kir3.2WT or Kir3.2_1234L_196Q), the raw current was first barium-subtracted to exclude endogenous inward currents from the analysis, which were typically 200 to 300 nA at -80 mV. Barium subtraction was then applied to all members of the test group. The ND96K (HK) solution used to monitor inward current contained (in mM): 96 KCl, 10 HEPES-K, 1 MgCl2, 1.8 CaCl2, pH 7.4. ND96 (LK) contained: 96 NaCl, 2 KCl, 5 HEPES-Na, 1 MgCl2, 1.8 CaCl2, pH 7.4. PMA was dissolved in DMSO to make a 10 mM stock, and diluted into ND96K for a 300 nM final concentration. For barium block, a solution of 5 mM BaCl2 in ND96K was perfused.

2.5. Inside-out Macropatch

The vitelline membrane was manually peeled using forceps to gain access to the oocyte plasma membrane. Pipettes were pulled using a Flaming-Brown micropipette puller and then fire-polished to give a final resistance of 0.5 to 2 MΩ. Currents were acquired at 10 kHz and
filtered at 2 kHz using pClamp software and the Axopatch 200A amplifier (Axon Instruments). Inward currents were monitored using a ramp protocol from -80 mV to +80 mV, with holding potential at 0 mV. Following gigaseal formation, the pipette was pulled away from the membrane to achieve the inside-out patch, and perfused directly using a multi-barrel gravity-driven perfusion apparatus. Pipette solution (in mM): 96 KCl, 10 HEPES, 1.8 CaCl$_2$, 1 MgCl$_2$, 1 NaCl, pH 7.4. Bath solution (in mM): 76 KCl, 20 NaCl, 5 EGTA, 10 HEPES, pH 7.4. For MgATP solution, 100 mM MgATP in dH$_2$O was added to the bath solution for a final concentration of 2 mM, then the pH was adjusted to 7.4. For AP experiments, 100 U/mL AP was made in the bath solution with 10 mM MgCl$_2$. DiC8-PiP$_2$ was added to this same solution without AP.

2.6. Molecular Dynamics

For simulations, systems were set up similar to Meng et al., 2012. Briefly, a channel tetramer was generated from the crystal coordinates of PDB code 3SYA, corresponding to Kir3.2 with DiC8-PiP$_2$ and sodium. The acyl chains of native PiP$_2$ (20:4, Arachidonyl-stearyl) were manually built with Discovery Studio onto the crystal PiP$_2$ headgroup. The tetramer was inserted into a POPC membrane via the INFLATE script. The system was solvated (SPC model) and ionized (0.15 KCl) in GROMACS, then sodium ions and potassium ions were manually added to the sodium site and the selectivity filter, respectively, by superimposition using the 3SYA structure. The 53a6 GROMOS forcefield was altered to include parameters for PiP$_2$ and phosphoserine (SEP). After minimization using a steepest descent algorithm, two equilibration runs were executed with progressively weaker position restraint (1000 then 10) on heavy atoms, followed by a 100 ns production run with no restraints. Simulation analysis was run using GROMACS, Simulaid, and VMD programs.
2.7. Protein Expression and Purification

Mouse Kir3.2NC cytoplasmic domain was cloned into a modified pET-28b vector (the prokaryotic pSMT3 small ubiquitin-related modifier (Sumo)/His-tag fusion system). The His-SUMO fusion protein was expressed in *E. coli* strain Rosetta2(DE3) (Novagen). The sequence of the Kir3.2 cytoplasmic domain (excluding the His-SUMO tag) used for expression is as follows (with the linked N-terminus underlined):

RKIQRYVRKDGKCNVHHGNVREKRAETLVFSTHAVISMRDGKLCLMFRVGDLRNSHIV RASIRAKLIKSQTSEGFIPLNQTDINGVYYTGDDRLFLVSPLISHEINQQSPFWEISKAQ LPKEELIVVILEGMVEATGMCQARSSYITSEILWGYRFUPVLTLEDGFYEVDFYNDHET

This N-terminal His-SUMO tag fusion protein fragment was expressed, solubilized, and purified on a nickel column as previously described (Zhang et al., 2012). Briefly, Kir3.2NC protein was expressed in IPTG auto-induction media and shaken overnight at 30 °C. The bacterial pellet was resuspended in lysis buffer containing a protease inhibitor cocktail and sonicated to release soluble protein, then centrifuged and the pellet was discarded. The supernatant was loaded onto a pre-equilibrated Ni-NTA column, and the bead-lysate mixture was gently resuspended for one hour at 4 degrees C. The beads with bound protein were loaded back onto the column, which was then washed with 20 mM imidazole-containing lysis buffer for 10 column volumes. Protein was eluted with 500 mM imidazole. To cleave the His-SUMO tag, ubiquitin-like protease 1 (Ulp1) was added to the protein sample and dialyzed in 150 mM KCl, 20 mM Tris, pH 8.5; 10% sucrose, and 1 mM TCEP overnight at 4 °C. After digestion, proteins were subjected to a second affinity chromatography step to remove the cleaved His-SUMO fragment. The flow-through was collected and concentrated to ~1 mL using Amicon Ultra.
conical spin tubes. An SDS-PAGE gel was run to assess purity, and concentration was quantified (0.3 mg/mL) using the OD280 with calculated extinction coefficient.

2.8. *In Vitro* PKC Phosphorylation

PKC phosphorylation was carried out according to the manufacturer’s instructions (Promega). Briefly, a 4X PKC buffer was prepared containing (in mM): 80 HEPES, 6.8 CaCl₂, 40 MgCl₂, 4 DTT, 0.6 Na-ATP, pH 7.4. Phosphatidyl-serine (PS) was dried under nitrogen gas, then PKC buffer was added to the dried lipid and sonicated in a bath sonicator to achieve a final PS concentration of 2.4 mg/mL. For the PKC reaction, 30 ug of purified Kir3.2NC in 1X PKC buffer was incubated with 1 uL (25 ng) of PKC and 300 nM PMA for 1.5 hours at 30 degrees C. Reaction was terminated by adding 4X SDS sample buffer. Samples were boiled and loaded onto an SDS-PAGE gel. The gel was stained with Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen) according to the manufacturer’s instructions. Briefly, the gel was fixed overnight with 50% methanol, 10% acetic acid solution, washed 4 x 15 min with MilliQ water, and stained for 2 hours with ProQ PhosphoStain. The staining step and all steps thereafter were performed in the dark. The gel was destained 4 x 30 min with a solution containing 50 mM Na-Acetate (pH 4.0) and 20% acetonitrile. Then the gel was washed 2 x 10 min with MilliQ water prior to visualization on a Typhoon fluorescent image scanner set to excitation at 532 nm and emission at 560 nm.

2.9. Statistics

All error bars represent the standard error of the mean (SEM). Statistical significance was assessed using Student’s t-test assuming unequal variance between samples in Origin 8.5 (OriginLab). Statistical significance was set at p < 0.05, denoted by an asterisk * in figures. For
Ci-VSP mediated current inhibition, a single-component exponential function was fit to the descending phase of the data and the tau was extracted. For normalized current data, barium-subtracted currents for each group were divided by the mean of the control group. All experiments were repeated in at least two separate batches of oocytes.
3.1. Introduction

Mutagenesis studies by Mao et al. in Kir3.1 and Kir3.4 identified a critical serine residue (S185 in Kir3.1 and S191 in Kir3.4) implicated in PKC-mediated phosphorylation. When the corresponding serine was mutated to alanine or aspartate, PMA inhibition was significantly reduced. Additionally, inhibition in excised patches by purified PKC was no longer effective. These results led the authors to conclude that Ser-185/Ser-191 was the major PKC phosphorylation site in Kir3.1/3.4 heterotetrameric channels (Mao et al., 2004).

We sought to identify a structural mechanism for how phosphorylation might affect gating by using a structure-function approach in the Kir3.2 channel, for which the full-length crystal structure is available with and without PIP₂ (Whorton and MacKinnon 2011). The PKC site identified in Kir3.1 and Kir3.4 corresponds to Ser-196 in Kir3.2, and is located at the base of the TM2 helix (see Figure 3.1A). The side chain of Ser-196 lines the permeation pathway, located one helical turn below the helix bundle crossing residue F192. The G-loop gate, another cytosolic gating element, is located directly below Ser-196. In addition, residues forming salt-bridge interactions with PIP₂ are nearby (Figure 3.1B), including K194 (upstream), K199, and K200 (both downstream). All of these elements make Ser-196 a prime target for gating modification by phosphorylation.
Given the exciting position of this residue, we sought to test the PKC inhibition of Kir3.2 at the analogous position to Kir3.1/Kir3.4 S185/S191. Our experiments utilized channels expressed in Xenopus oocytes, followed by PMA perfusion and quantification of the resulting inhibition. We also employed molecular dynamics simulations with in silico channel mutants to identify protein-protein network interaction changes mediated by mutation of specific residues.

3.2. Results

3.2.1. PMA inhibition of homomeric GIRK channels

We first examined the homomeric channel mutants in the Kir3.x family, using previously described mutations that enhance homomeric activity, known as Kir3.1* (F137S) (Chan et al., 1996), Kir3.4* (S143T) (Vivaudou et al., 1997), and Kir3.2* (E152D) (Yi et al., 2001). We perfused 300 nM PMA, a membrane-permeable PKC activator, and quantified the extent of inhibition for Kir3 homomeric channels. To determine the extent of inhibition, the PMA-sensitive current ($I_{\text{max}} - I_{\text{post-PMA}}$) was divided by the current prior to PMA application ($I_{\text{max}}$), and expressed as a percentage (see Figure 3.2A, left panel). Consistent with the results in Mao et al., Kir3.4*_S191A and Kir3.1*_S185A both had significantly reduced PMA inhibition compared with their respective channels (Kir3.1*S185A 46.8 +/- 0.8% vs. Kir3.1* control 80 +/- 1.4%, Figure 3.2B; Kir3.4*S191A 57 +/- 2.1% vs. Kir3.4* control 88.3 +/- 0.7%, Figure 3.2A). The phosphomimetic Kir3.4* S191D exhibited 10 +/- 5.6% inhibition, resulting in a significant reduction compared to Kir3.4* S191A, as reported by Mao et al. (Figure 3.2A). We also tested the Kir3.1*_S185D mutant, which displayed 67.6 +/- 8.4% PMA inhibition, and was not statistically significant from Kir3.1* control (Figure 3.2B), in contrast to Kir3.1*_S185A. This mutant was not tested in Mao et al. for PMA inhibition.
In the Kir3.2* channel, PMA inhibition of the phosphomimetic S196E mutant was not significantly different from Kir3.2* control (Kir3.2* 27 +/- 5.4% vs. Kir3.2* S196E 19.5 +/- 5.2 %, Figure 3.2C). Surprisingly, the Kir3.2* S196Q neutralization mutant had significantly higher PMA inhibition (53.8 +/- 4.2%) than either Kir3.2* control or S196E. These results indicate that Kir3.2* has increased PMA inhibition after neutralization of Ser-196, compared to Kir3.1* or Kir3.4*, which have the opposite effect, reduced PMA inhibition.

We then compared the current levels by normalizing to the homomeric control channel. Both Kir3.1* S185A (4.0% of control) and Kir3.1* S185D (2.6% of control) resulted in significant reductions in current level compared to Kir3.1* control (Figure 3.3B). Currents of the mutants were essentially undetectable, requiring longer expression times than Kir3.1* for detectable current (as in Figure 3.2B). Kir3.4* S191A (98 +/- 16.7%) had similar current levels to Kir3.4* control, while Kir3.4* S191D had significantly diminished currents (11.5 +/- 0.3%) (Figure 3.3A). In the Kir3.2* channel, the S196Q mutant was 20 +/- 1.8% of control, while the Kir3.2* S196E mutant was closer to Kir3.2*, at 69.0 +/- 3% (Figure 3.3C). These results indicate that residue substitution at this position in different Kir3 channels is heterogeneous. Kir3.4* follows a pattern consistent with the phosphomimetic residue playing a role in PMA inhibition, namely that the S191D mutant has significantly reduced currents, while S191A does not change current level. Kir3.1*, on the other hand, has significant current reduction after substitution by either Ala or Asp. For Kir3.2*, the neutralization mutant S196Q significantly reduces currents, while phosphomimetic S196E has currents that are closer to control.
3.2.2. MD Simulations predict a switch mechanism dependent on phosphorylation of Ser-196

We hypothesized that the channel may be constitutively phosphorylated at position Ser-196, and that neutralization or dephosphorylation will affect gating behavior. To further examine how phosphorylation at this residue affects gating, we ran molecular dynamics simulations using the crystal structure of the Kir3.2 channel with bound PIP$_2$ (PDB code 3SYA), and performed mutations at Ser-196 in silico using a background mutation of E152D as with our experimental results. We first analyzed protein salt-bridge interactions, comparing S196Q and S196E mutants. In the S196Q mutant, a strong salt-bridge interaction persists between Asp-228 and Arg-201 of each subunit. Over the course of the simulation in S196E, the negatively charged Glu-196 recruits Arg-201 away from Asp-228. The carbon-to-carbon distance averaged over the four channel subunits is depicted in Figure 3.4 for both S196Q and S196E. Consistent with the salt-bridge results for S196E, the average distance for Arg-201/Asp-228 increases (from ~4 Å to ~8.1 Å), while simultaneously the distance for Arg-201/Glu-196 decreases (from ~10 Å to ~6.5 Å), allowing interaction. In the S196Q mutant, the average distance for Arg-201/Asp-228 and Arg-201/Gln-196 does not change appreciably over the 100 ns simulation, and furthermore does not show the same coordination as S196E (compare Figure 3.4A with Figure 3.4B).

Inspection of the trajectories as well as an 80 ns snapshot of the two simulations (Figure 3.4C-D) indicates that in S196E, Arg-201 moves away from Asp-228 and swings upward to form interactions with Glu-196. In S196Q, Arg-201 firmly interacts with Asp-228 for the duration of the simulation.
Given that S196Q has a neutral charge and S196E would have a strongly negative charge, we performed the same simulations on a channel with either Ser-196 (E152D control) or Phosphoserine-196. The pattern of salt-bridge interactions was consistent with our previous results for the S196Q and S196E mutants. Only the phosphorylated serine (SEP) forms salt-bridge interactions with Arg-201, recruiting the residue away from Asp-228 as shown in the average distance plots and an 80 ns snapshot (Figure 3.5).

As mentioned earlier, the Ser-196 residue is located approximately one helical turn from a key gating residue Phe-192, which forms the helix bundle crossing gate. We examined the intersubunit distance of this gate by calculating the minimal distance of residue-residue interactions. Interestingly, the S196E mutant adopts a half-open conformation at the helix bundle crossing, whereby the intersubunit A-B distance increases, but the intersubunit C-D distance does not (Figure 3.6B). In the S196Q mutant both intersubunit F192 distances remain stable, and the gates maintain a closed conformation (Figure 3.6A).

3.2.3. Mutation of Arg-201 affects gating and PMA inhibition

The outcome of the simulations suggested a switch mechanism whereby phosphorylation at Ser-196 can recruit Arg-201 away from Asp-228. The mutant Kir3.2_R201A has been crystallized, demonstrating a putative open G-loop structure, as well as a half-open helix bundle crossing gate in the presence of PIP2. It has been suggested to adopt a fully active state resembling Gβγ binding (Whorton and MacKinnon 2011; Whorton and MacKinnon 2013). We found that in the Kir3.2* background, the R201A mutant produces very small currents, nearly indistinguishable from background (Figure 3.7B). An additional mutation D228N (Kir3.2*_D228N_R201A) potentiates the current more than 20-fold, but the currents are still significantly reduced compared to either D228N alone or Kir3.2*. On its own, the mutant Kir3.2*_D228N

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potentiates Kir3.2* currents approximately twofold (Figure 3.7A). This result suggests that mutation of R201 is detrimental to channel activity, and that the negatively charged Asp-228 greatly reduces channel activity.

Given that the S196Q mutant has increased PMA inhibition as well as diminished currents, we tested the PMA inhibition of the Kir3.2*_D228N_R201A mutant, finding that it was significantly increased compared to Kir3.2*_D228N alone (47.5 +/- 3.6% for Kir3.2*_D228N_R201A vs. 19 +/- 3.9% for Kir3.2*_D228N, Figure 3.7C). These results indicate that the putative interaction of Arg-201 with phosphorylated Ser-196 is important for both channel activity and PKC inhibition.

3.2.4. Global changes in phosphorylation affect PIP2-induced gating

We proceeded to functionally test the channel for phosphorylation at Ser-196 by perfusing the channel with a nonspecific phosphatase in an excised inside-out macropatch. When the patch is excised, PIP2 levels rapidly decrease due to endogenous lipid phosphatase activity, manifesting as channel rundown. We used Mg-ATP to reactivate the channel and then perfused calf intestinal phosphatase (CIP, 100 U/mL) reconstituted in the Mg-ATP solution. Perfusion of the phosphatase led to rapid channel inhibition, which recovered upon washout (Figure 3.8A). Recovery of the current by Mg-ATP is thought to be dependent on PIP5K activity, so it is possible that the phosphatase can nonspecifically decrease PIP2 levels, perhaps by dephosphorylating PIP5K. We tested more directly how channel dephosphorylation affects PIP2-induced gating by perfusing DiC8-PIP2. Perfusion of DiC8-PIP2 after CIP treatment resulted in greater current recovery compared to DiC8-PIP2 alone. This was more pronounced with 5 μM PIP2 than 50 μM PIP2, suggesting that CIP treatment changes channel affinity for
These results imply that nonspecific dephosphorylation of Kir3.2 results in greater PIP$_2$ activation, possibly by changing PIP$_2$ affinity, although this was not explicitly tested.

### 3.3. Discussion

Our study thus far has identified a residue interaction triangle in the Kir3.2 channel, where the key players are Ser-196, Arg-201, and Asp-228. Phosphorylation of Ser-196 presents a toggle switch, which recruits Arg-201 away from Asp-228. This in turn results in increased channel activation and lower sensitivity to PMA inhibition. Disruption of the SEP-196/Arg-201 interaction, either by S196Q or R201A, results in a channel with greatly reduced currents, as well as higher sensitivity to PMA inhibition.

The mutagenesis and simulation data imply that phosphorylation can change both gating and PKC inhibition, yet biochemical identification of phosphorylation at Ser-196 remains to be determined. This work further implies that changes at allosteric sites can affect functional inhibition by PKC, casting doubt on the work of Mao et al. in identifying the functional site of phosphorylation in Kir3.1/3.4 channels. Although it is still possible that the sites in Kir3.1/3.4 (S185/S191) and Kir3.2 (S196) are different, the mutagenesis data on homomeric channels seems to suggest otherwise. While alanine mutants Kir3.1* _S185A and Kir3.4* _S191A have less overall inhibition than their wildtype counterparts, the aspartate mutants differ. Kir3.1* S185D has higher inhibition, similar to Kir3.1* control, but Kir3.4* S191D has even lower inhibition than G4*S191A. These data suggest that in terms of PMA inhibition, at least one other phosphorylation site is involved besides Kir3.1 _S185/Kir3.4 _S191. One caveat in these studies regards the low current levels of the Kir3.1* _S185 mutants, such that much longer expression times were required. This resulted in greater variability of PMA inhibition than compared to Kir3.4*, for example. The conclusions of Mao et al. in regard to Kir3.4*
phosphorylation at Ser-196 are therefore still applicable, but with the specific case of Kir3.1* Ser-185, the data are inconclusive. Our mutagenesis data strongly suggest that Ser-196 is not directly phosphorylated in the context of PMA inhibition, but rather acts as an allosteric regulator.

The mutagenesis results in Kir3.2* suggest a dependence of PKC-mediated inhibition on phosphorylation at an allosteric site, Ser-196 (see Figure 3.9). When Ser-196 is phosphorylated, the helix bundle crossing gate is stabilized and the channel is less sensitive to PKC inhibition. Phosphorylated Ser-196 competes with Asp-228 for the critical Arg-201. In the absence of phosphorylation, Asp-228 tightly interacts with Arg-201, stabilizing the closed state. Interestingly, the R201A mutant has been used to obtain the crystal structure of Kir3.2 with an open G-loop gate. In the presence of PIP2, this mutant is thought to have a partially open helix bundle crossing gate as well. Presumably due to packing of crystal contacts, only two PIP2 molecules are bound per tetramer, such that one monomer has an open helix bundle crossing and its partner maintains a PIP2-deficient structure. Therefore, the R201A mutant has been described as a constitutive, fully active channel (Whorton and MacKinnon 2011). However, the current levels of Kir3.2*_R201A are drastically reduced compared to the Kir3.2* control. Neutralization of Asp-228 with the D228N mutant rescues Kir3.2*_R201A currents more than twenty-fold, much greater than the effect of D228N alone on Kir3.2* (approximately two-fold). The Kir3.2*_D228N_R201A double mutant has increased sensitivity to PKC inhibition compared to the Kir3.2*_D228N control.

The potentiation effect of the D228N mutation on Kir3.2*_R201A indicates that Asp-228 likely inhibits the channel in the absence of the Arg-201 side-chain. Asp-228 is a critical residue for sodium potentiation, whereby sodium binding shields the negatively charged aspartate from
positive PIP$_2$-interacting residues (Ho and Murrell-Lagnado 1999; Inanobe et al., 2010; Whorton and MacKinnon 2011). A likely candidate for interaction is Arg-230, located two residues away on the CD-loop. Simulations in a homology model of Kir3.4 indicate that sodium coordination prevents interaction between Asp-223 and Arg-225 in Kir3.4, the homologous residues to Asp-228 and Arg-230 (Rosenhouse-Dantsker et al., 2008). In the co-crystal of Kir3.2 and PIP$_2$, Arg-230 does not form interactions with PIP$_2$, but it is possible that Arg-230 interacts with Asp-228 in some intermediate PIP$_2$-binding state. Evidence from MD simulations in the Kir3.1-chimera structure points to this possibility, where the equivalent residue (Arg-230 equivalent) makes PIP$_2$ contacts in the dilated conformation intermediate state only (Meng et al., 2012).

The mutagenesis data regarding current levels and PMA inhibition strongly suggest that Ser-196 is normally phosphorylated in Kir3.2 expressed in *Xenopus* oocytes. This has not been tested explicitly, however. A phospho-antibody for Ser-196 does not exist, but there are indirect methods to identify phosphorylation at this site. For example, immunoprecipitates of Kir3.2* and Kir3.2* _S196Q or S196E could be compared, and there should be a reduction in incorporated $^{32}$P in the mutants when compared to control.

In the absence of phosphorylation at Ser-196, it seems unlikely that Ser-196 (an uncharged, polar residue) would function equivalently to Glu-196 (a negatively charged residue). Since the principal difference between Gln and Glu is the introduction of negative charge, we believe that Ser-196 is likely in a phosphorylated state. It remains to be determined whether Kir3.1 and/or Kir3.4 also might be constitutively phosphorylated at this position, confounding the mutagenesis results on PKC inhibition in Mao et al.

Finally, experiments in the excised inside-out patch configuration indicate that Kir3.2* channels respond differently to PIP$_2$ after global dephosphorylation by nonspecific phosphatase.
treatment. Importantly, different phosphorylation signals likely converge to determine channel-
PIP$_2$ affinity. In the Kir1.1 channel, PKA phosphorylation increases channel-PIP$_2$ affinity and
results in higher channel activity (Liou et al., 1999). PKA phosphorylation in Kir3.1/3.4
channels also can activate channels, likely by increasing channel-PIP$_2$ affinity (Lopes et al.,
2007). In the case of Kir3.2* channels, the balance of phosphorylation before CIP treatment is
inhibitory, as dephosphorylation results in larger PIP$_2$-induced currents. Testing specific
phosphorylation site mutants with this paradigm will be instrumental in determining how
phosphorylation at specific sites contributes to channel-PIP$_2$ interactions. Interestingly, an
examination of I$_{K,ach}$ currents and heterologously expressed Kir3.1/3.4 channels demonstrated
that G$\beta\gamma$-dependent activation was abolished after treatment with PP1 phosphatase. The
phosphorylation site(s) could not be pinpointed, but the phosphatase effect was dependent on the
distal C-terminus of Kir3.1 channels (Medina et al., 2000). This also indicates that changes in
global phosphorylation can affect channel activation, this time by the activator G$\beta\gamma$.

In summary, we have identified a likely constitutively phosphorylated site in the Kir3.2
channel at Ser-196. Phosphorylation of this residue leads to increased gating and reduced
sensitivity to PMA inhibition in homomeric Kir3.2*. MD simulations suggest that
phosphorylated Ser-196 interacts with Arg-201, pulling Arg-201 away from Asp-228.
Neutralization of either Ser-196 or Arg-201 results in diminished activity and increased
sensitivity to PMA inhibition. Surprisingly, nonspecific phosphatase treatment of Kir3.2*
resulted in increased PIP$_2$ activation, suggesting that the global phosphorylation signal output is
inhibitory.
Figure 3.1. Ser-196, a putative PKC phosphorylation site, is located in a key position to affect gating behavior. A. Location of Ser-196 in relation to the F192 helix bundle crossing gate and the G-loop gate. B. Slight rotation along Y-axis depicting nearby PIP2-binding residues: K194, K199, and K200. K64 from N-terminus is also depicted.
Figure 3.2. PMA Inhibition of Kir3.4*(S143T), Kir3.1*(F137S), and Kir3.2*(E152D) and serine mutants. A. Left, time course of PMA inhibition for Kir3.4* and S191D with time points measured indicated by arrows. Right, summary PMA inhibition data for Kir3.4* and S191 mutants. **, S191D has a statistically significant difference from S191A at p<0.05. B. Left, time course for Kir3.1* and S185D. Right, summary data for Kir3.1* and S185 mutants. C. Left, time course for Kir3.2* and S196Q. Right, summary data for Kir3.2* and S196 mutants. *, p <0.05 compared to control.
Figure 3.3. Normalized currents for neutralization and phosphomimetic serine mutants. A. S191D has reduced current compared to Kir3.4* control or S191A. B. Both S185A and S185D have reduced currents compared to Kir3.1* control. C. S196Q has reduced currents compared to Kir3.2* and S196E, which has a small decrease from Kir3.2* control. **, S196E has a statistically significant increase over S196Q at p<0.05. *, p<0.05 compared to control.
Figure 3.4. Comparison of simulation results for S196Q and S196E in Kir3.2(E152D) background. A. Simulation time-course for average distance per subunit for Arg-201(CZ)/Gln-196(CD) and Arg-201(CZ)/Asp-228(CG). B. Time-course for Arg-201(CZ)/Glu-196(CD) and Arg-201(CZ)/Asp-228(CG). C. 80 ns snapshot of MD simulation depicting Gln-196, Arg-201, and Asp-228. Phe-192 is shown as a space-filling model. D. 80 ns snapshot depicting Glu-196, Arg-201, and Asp-228.
Figure 3.5. SEP196 mimics the switch mechanism found in S196E. A. Timecourse for Ser-196(CB)/Arg-201(CZ) and Arg-201(CZ)/Asp-228(CG). B. Timecourse for SEP-196(P)/Arg-201(CZ) and Arg-201(CZ)/Asp-228(CG). C. 80 ns snapshot depicting Arg-201, Ser-196, and Asp-228. D. 80 ns snapshot depicting Arg-201, SEP196, and Asp-228.
Figure 3.6. Intersubunit helix bundle crossing distance at F192 increases in S196E, but not S196Q. A. Time-course for S196Q for A-B and C-D intersubunit minimal distances. B. Time-course for S196E, showing increase in A-B distance. C. 80 ns snapshot showing F192 A-B distance in S196Q. Chain A is colored in red and chain B is colored in blue. D. 80 ns snapshot for F192 A-B distance in S196E.
Figure 3.7. Neutralization of R201 results in low currents with increased sensitivity to PMA in Kir3.2* D228N background. A. Kir3.2* D228N has higher currents than Kir3.2* control. B. Kir3.2* R201A D228N has reduced currents compared to Kir3.2* D228N. **, Kir3.2* R201A D228N has significantly higher currents than Kir3.2* R201A at p<0.05. C. PMA inhibition is increased in Kir3.2* D228N R201A compared to Kir3.2* D228N control. *, p<0.05 compared to control.
Figure 3.8. Alkaline phosphatase (CIP) treatment of excised inside-out patches. A. AP treatment of Kir3.2* reactivated by MgATP inhibits channel function, but is recovered by MgATP. B. AP pretreatment of Kir3.2* results in higher PIP2-induced activation level at 5 uM DiC8-PIP2. C. Same experiment as B, but with 50 uM PIP2.
Figure 3.9. Schematic of PKC inhibition depending on phosphorylation state of Ser-196 and salt-bridge formation with Arg-201. In PKC sensitive channels, Q196 or dephosphorylated S196 cannot interact with R201, and is stabilized by D228. In PKC insensitive channels, R201 interacts with E196 or phosphorylated S196, stabilizing the open state of the helix bundle crossing gate.
Chapter 4. PKC INHIBITION IN KIR3.2 IS DEPENDENT ON CHANNEL-PIP2 INTERACTIONS

4.1. Introduction

PIP2 is a universal activator of inward rectifier Kir channels, and the Kir3 channel family is no exception. In the absence of PIP2, there is no Kir3 channel activity, such that the channel is in a persistently closed state. Inhibition of PIP2-dependent channels can occur by PIP2 hydrolysis, brought about by Gq-coupled receptors, which activate phospholipase C (PLC). PIP2 hydrolysis reduces local levels of PIP2, requiring resynthesis, but also increases the concentration of its byproducts, inositol trisphosphate (IP3) and diacylglycerol (DAG). While IP3 releases intracellular calcium, membrane-localized DAG activates PKC. The mechanism of PKC inhibition is unclear, but evidence suggests that the final common mediator is a reduction in channel-PIP2 interactions.

Kir channel apparent affinity for PIP2 can be measured using a water-soluble analog called DiC8-PIP2. The PIP2 dose response for different Kir channels can then be measured, as well as the effect of individual point mutants. In this assay, the Kir2.1 channel has higher PIP2 affinity than the closely related Kir2.3 channel. The dose response is an indirect measurement, however, and cannot rule out allosteric effects of mutations. One such allosteric site is Leu-222. The L222I has a right-shifted PIP2 dose response is Kir2.1, just as the I223L has a left-shifted dose response in Kir2.3 (Zhang et al., 1999). It is unclear how a simple leucine to isoleucine
change, a relatively conservative mutation, can have such dramatic effects on PIP$_2$ apparent affinity, although these residues are located very close to a critical structural element implicated in PIP$_2$-mediated gating, the CD-loop (Meng et al., 2012). Strictly speaking, measurements of PIP$_2$ dose-response rely on channel activity, and as such are not pure measurements of binding affinity. We use the term apparent affinity to distinguish from actual binding affinity, which would need to be measured biochemically.

Du and colleagues examined two Kir2 channels, one that is normally inhibited by PKC (Kir2.3) and one that is not (Kir2.1). In the case of Kir2.3, the point mutation I223L which increases apparent PIP$_2$ affinity resulted in a loss of PKC inhibition. A separate mutation, R312Q, reduces apparent PIP$_2$ affinity in the Kir2.1 channel, likely by neutralizing a positive PIP$_2$-binding residue. The mutant channel Kir2.1R312Q gained PKC inhibition, whereas the control was not inhibited (Du et al., 2004). These results suggested PKC inhibition could be a matter of fine-tuning PIP$_2$ affinity, and that PKC phosphorylation exerts a functional effect only on channels with sufficiently reduced PIP$_2$ affinity.

Turning to Kir3 channels, Keselman et al. demonstrated that PKC application to an excised patch containing Kir3.1/3.4 channels shifts the dose-dependence of PIP$_2$ for the channel to the right. This indicates that after PKC phosphorylation, the channel has a lower apparent PIP$_2$ affinity (Keselman et al., 2007). We proceeded to test whether manipulations that increase or decrease channel-PIP$_2$ interactions can affect PKC inhibition in the homomeric Kir3.2 channel.
4.2. Results

4.2.1 PKC Inhibitor blocks PMA inhibition

We first set out to examine in homomeric Kir3.2 the impact of increasing or decreasing channel-PIP2 interactions on PKC inhibition. We confirmed that PMA effects were due to PKC activation by using the pan-PKC inhibitor Bis-indolylmaleimide II (called Bis), which has been used in multiple studies concerning Kir channel inhibition (Du et al., 2004; Keselman et al., 2007). As shown in Figure 4.1, both currents from Kir3.2_E152D and Kir3.4_E147D (a homomeric mutant with the analogous mutation in Kir3.4) were inhibited by 300 nM PMA, which was blocked by 5 uM Bis pretreatment.

4.2.1. Lowering PIP2 levels increases PMA inhibition

One puzzling result from our previous study was the relatively low inhibition of Kir3.2* compared to either Kir3.1* or Kir3.4* (Figure 4.2A). We hypothesized that the low level of PMA inhibition in Kir3.2* may be due to enhanced channel-PIP2 interactions, and proceeded to lower PIP2 levels. Wortmannin treatment of oocytes at high micromolar concentrations is commonly used to inhibit Type III PI4K (Balla et al., 2008). PI4K is critical for maintaining PIP2 levels, as it phosphorylates phosphatidylinositol at the 4’ position, which is rapidly converted to PIP2 by PIP5K. We observed that after wortmannin treatment, PMA inhibition of Kir3.2* was significantly increased (from 23.6 +/- 2.3% before to 66 +/- 1.4% inhibition after, Figure 4.2B).

Since Kir3.2* could be converted into a channel with increased sensitivity to PKC inhibition by lowering PIP2, we sought to determine if the current-enhancing mutation E152D works by increasing PIP2 affinity compared to Kir3.2 alone. We used Ci-VSP, a voltage-

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activated PIP$_2$ 5-phosphatase, to examine the kinetics and recovery of both Kir3.2* and Kir3.2 wildtype, a relative measure of channel-PIP$_2$ affinity (An et al., 2012; Rodriguez-Menchaca et al., 2012). We used a voltage protocol designed to activate Ci-VSP submaximally, whereby Ci-VSP is activated at +10 mV and current levels are monitored by brief pulses to -80 mV. Recovery is analyzed by holding at -80 mV following a one minute +80 mV depolarization protocol that fully inhibits channel current (Figure 4.3A). A single exponential function was fit to the descending curve of Ci-VSP inhibition shown in Figure 4.3B, and the Tau of inhibition was compared for Kir3.2* and Kir3.2 (Figure 4.3C). The Tau of inhibition of Kir3.2* (~8.0 s) was significantly slower compared to Kir3.2 wildtype (~2.5 s) channels. Additionally, the recovery of Kir3.2* was qualitatively faster than Kir3.2 wildtype (Figure 4.3D), but complex kinetics prevented a simple tau analysis. These results for Kir3.2 wildtype channels, faster Ci-VSP inhibition and slower recovery, are consistent with a reduced PIP$_2$ affinity compared to Kir3.2* channels.

### 4.2.2. Increases in channel-PIP$_2$ affinity reduces PKC inhibition

Since lowering PIP2 levels results in increased sensitivity to PMA inhibition, we also tested whether increasing channel-PIP$_2$ interactions would result in reduced sensitivity to PMA inhibition. To do this, we utilized two mutations known to increase channel current and impact apparent PIP$_2$ affinity: D228N and I234L. The D228N mutation renders the Kir3.2 channel insensitive to sodium activation (Inanobe et al., 2010; Rosenhouse-Dantsker et al., 2008; Zhang et al., 1999), and the I234L mutation allosterically strengthens channel-PIP$_2$ interactions in a variety of Kir channels (Du et al., 2004; Zhang et al., 1999). We compared D228N and I234L mutants in the Kir3.2* background, finding that I234L had the largest effect on current level (~3.3-fold enhancement), shown in Figure 4.4A. Since the PMA inhibition of Kir3.2* was
already low, we tested PMA inhibition of D228N and I234L in the wildtype background. Only the I234L mutant had significantly reduced PMA inhibition compared to Kir3.2 control (21 +/- 2.8% for Kir3.2_I234L vs. 45.6 +/- 6% inhibition for Kir3.2 control), whereas Kir3.2 and D228N inhibition were similar, summarized in Figure 4.4B. Importantly, the PMA inhibition of Kir3.2_I234L was significantly higher (45.4 +/- 6.8%) in wortmannin treated oocytes, consistent with a PIP2-dependent effect.

4.2.3. Neutralization of Ser-196 uncouples PKC inhibition from PIP2-dependence

We tested the Ser-196 mutants for PIP2 dependence as well using S196Q and S196E. As shown in Figure 4.5A, wortmannin treatment led to significantly increased inhibition in Kir3.2* (from 27 +/- 5.4% control to 63.9 +/- 3.5% wortmannin treated) and Kir3.2*_S196E (from 19.4 +/- 5.2% control to 53 +/- 2.9% wortmannin treated), but did not significantly affect the already high inhibition of Kir3.2*_S196Q (from 54 +/- 4.2% control to 65 +/- 4.5% wortmannin treated). In the wildtype Kir3.2_I234L background (Figure 4.5B), S196Q had significantly greater PMA inhibition (61.8 +/- 1.9%) than either control (24.7 +/- 4.3%) or S196E (33.9 +/- 2.6%), similar to wortmannin-treated Kir3.2_I234L_S196E (61.9 +/- 1.9%). These results indicate that the PKC inhibition of the S196Q mutation is unresponsive to changes in PIP2 levels. The similar PIP2 dependence of the S196E mutant and the Kir3.2 channel further provide functional evidence that Ser-196 is likely phosphorylated in the oocytes, as the two residues are functionally equivalent.

4.3. Discussion

These results partially recapitulate the findings of Du et al., further showing that PKC inhibition is dependent on channel-PIP2 interactions and can be fine-tuned in Kir3.2 by increasing or lowering ambient PIP2. Notably, the charge on Ser-196 can uncouple PKC
inhibition from dependence on channel-PIP2 interactions. In the case of neutralizing S196Q, increasing channel-PIP2 interactions with the I234L mutant is not sufficient to reduce PKC inhibition, which is higher than Kir3.2_I324L control. S196E maintains a dependence on channel-PIP2 interactions similar to wildtype, suggesting that phosphorylated Ser-196 is resistant to PKC inhibition, but in a PIP2-dependent manner.

We propose a model that incorporates both PIP2 and the phosphorylation state of Ser-196 in PKC inhibition (see Figure 4.6). Kir3.2 is resistant to PKC inhibition in cases of high PIP2 and when Ser-196 is phosphorylated. When Ser-196 is not phosphorylated, as with the S196Q mutant, then the channel is readily inhibited, even when PIP2 levels are high. Interestingly, this requirement of Ser-196 phosphorylation seems unique to Kir3.2 in the Kir3 family, as similar mutations in Kir3.1 and Kir3.4 resulted in diverse PKC inhibition phenotypes. The earlier data from Kir3.4* presents the best case for Ser-191 phosphorylation involved in PKC inhibition, as only the phosphomimetic S191D mutant had reduced currents and reduced PMA effect. It would be interesting to see if lowering PIP2 levels would increase the inhibition of Ser-191 mutants as well. One study looked at the PIP2 dependence of PKC inhibition in Kir3.4, finding that both a PIP2-enhancing chimera and the point mutation Kir3.4_I229L reduced the extent of PKC inhibition compared to Kir3.4* control (Zhang et al., 2004). This finding is consistent with our study and the results of Du et al. in Kir2 channels (Du et al., 2004).

Characterization of exactly how channel-PIP2 interactions determine PKC inhibition will necessitate the identification of residues directly phosphorylated by PKC. Early mutagenesis studies identified a potential site in Kir2.3, T53, which is located right next to the critical PIP2-binding motif RWR, responsible for salt bridge interactions with the 1’ phosphate of PIP2 in Kir2.2 and Kir3.2 channels. Kir2.1, a channel that is resistant to PKC inhibition, has an Ile at the
corresponding position, but mutation to Thr could render the channel sensitive to PKC (Zhu et al., 1999). The negatively charged phosphothreonine would likely have a significant impact on PIP$_2$ binding, perhaps by forming salt-bridge interactions with the nearby RWR motif. Evidence of direct phosphorylation at Thr-53 has remained elusive, however. Additionally, the results of Du et al. whereby reduction of channel-PIP$_2$ interactions (i.e. the R312Q mutant) render the Kir2.1 channel sensitive to PKC inhibition indicate that additional phosphorylation sites are likely involved.

Some controversy exists in the literature as to whether PKC inhibition operates by phosphorylating the channel directly or by causing a nonspecific reduction in PIP$_2$ levels. Multiple studies have shown in excised patches that catalytic PKC treatment of Kir3 channels results in a reduction in channel open probability (Brown et al., 2005; Mao et al., 2004; Nikolov et al., 2004). PKC treatment of Kir3.1/3.4 channels resulted in a right-shift of the dose-dependence of DiC8-PIP$_2$ activation, indicating that the channel is likely to be directly regulated by PKC. Examination of PIP$_2$ levels measured by FRET of PH-PLC domains in HEK293 cells found no effect of PMA on PIP$_2$ levels but a robust effect of M1 receptor activation (Keselman et al., 2007). A recent study in Xenopus oocytes implicated PMA activation of PKCβII as a mechanism of increasing PIP$_2$ levels, rather than reducing them (Chen et al., 2011). In this case, an increase in PIP$_2$ levels would be expected to partially block PKC inhibition, instead of facilitating it. Resolving whether Kir3 channels are regulated directly by PKC will probably require reconstitution in purified systems, as with the Kir3.1 chimera or full-length Kir3.2 protein.

In the next chapter we will attempt to identify PKC phosphorylation sites in Kir3.2 by utilizing purified protein and mass spectrometry. Once identified, the location of the PKC
phosphorylation site(s) in the context of PIP$_2$-interacting residues might help explain how PKC inhibition couples to reductions in channel-PIP$_2$ interactions.
Figure 4.1. PMA perfusion inhibits homomeric Kir3.2(E152D) and Kir3.4(E147D) currents in a PKC-dependent manner. A. Time course of PMA inhibition in control Kir3.2(E152D). B. Time course of PMA inhibition in Kir3.2(E152D) after Bis pretreatment. C. Time course of PMA inhibition in control Kir3.4(E147D). D. Time course of PMA inhibition in Kir3.4(E147D) pretreated with Bis.
Figure 4.2. Kir3.2* has lower PMA inhibition relative to Kir3.1* or Kir3.4*, which can be increased by PIP2 depletion. A. Kir3.2* has significantly reduced PMA inhibition compared to either Kir3.4* or Kir3.1*. B. Wortmannin pretreatment increases PMA inhibition of Kir3.2*. *, p<0.05 compared to control.
Figure 4.3. Kir3.2*(E152D) has slower Ci-VSP mediated inhibition and faster recovery vs. Kir3.2 wildtype. A. Left, voltage protocols (in mV) used for inhibition (top) and recovery (bottom). Right, sample inhibition traces for Kir3.2*(E152D) (top) and recovery (bottom). B. Kir3.2*(E152D) displays faster current inhibition compared to Kir3.2 wildtype. C. Tau of inhibition for Kir3.2* is significantly slower than wildtype. *,p<0.05 compared to control. D. Kir3.2* has faster recovery than Kir3.2 wildtype at -80 mV.
Figure 4.4. Kir3.2 currents are inhibited by PMA in a PIP<sub>2</sub>-dependent manner. A. D228N and I234L mutants increase Kir3.2* current, as expected with an increase in PIP<sub>2</sub> affinity. **, I234L has significantly higher current than D228N at p<0.05. B. Kir3.2_I234L has reduced PMA inhibition compared to either Kir3.2_D228N or Kir3.2 wildtype control. **, Wortmannin treated Kir3.2_I234L has significantly increased PMA inhibition compared to Kir3.2_I23L control. *, p<0.05 compared to control.
Figure 4.5. S196Q does not exhibit PIP$_2$ dependence for PMA inhibition. A. Both Kir3.2* and Kir3.2*_S196E have increased PMA inhibition after wortmannin treatment, but Kir3.2*_S196Q does not. B. S196Q in Kir3.2_I234L background has significantly increased currents compared to Kir3.2_I234L control, similar to Kir3.2_I234L_S196E after wortmannin treatment. *, p<0.05 compared to control group.
Figure 4.6. Model for PKC Inhibition of Kir3.2 depending on Ser-196 phosphorylation state and PIP$_2$ concentration. The functional effect of PKC phosphorylation is high inhibition when Ser-196 is not phosphorylated. When Ser-196 is phosphorylated, engaging Arg-201, and if PIP$_2$ concentration is high, PKC inhibition is low. If PIP$_2$ concentration is low, PKC inhibition is high even if Ser-196 is phosphorylated. Presumably the PKC phosphorylation site(s) conferring inhibition is located in the cytoplasmic domain, or at least at a site different from Ser-196.
Chapter 5. CHARACTERIZATION AND FUNCTIONAL ROLE OF PKC-DEPENDENT RESIDUES IN KIR3.2

5.1. Introduction

Clearly, homomeric Kir3.2 is responsive to PMA inhibition in a PKC-dependent manner, yet the phosphorylation sites of PKC on the channel remain unknown. Our data thus far indicate that disruption of phosphorylation of Ser-196 could not occlude PMA inhibition, since the S196Q mutant exhibited increased PMA inhibition. Furthermore, Kir3.2 sensitivity to PKC inhibition can be modulated by increasing or decreasing ambient PIP2 levels. The mechanism of how PKC phosphorylation can be modulated by PIP2 will require identification of the phosphorylation sites in the context of Kir3.2 structure. Additionally, no studies to date have identified Kir3.2 to be directly phosphorylated by PKC. We proceeded to purify the cytosolic domain of Kir3.2 and see if PKC can phosphorylate the channel in vitro. Additionally, putative phosphorylation sites in Kir3.2 were analyzed for their impact on PMA-mediated inhibition.

5.2. Results

5.2.1. Kir3.2 is phosphorylated by PKC in vitro

The cytosolic domain of Kir3.2 has several PKC consensus sites in its primary sequence. We examined the phosphorylation potential of Kir3.2 by using a phosphorylation search algorithm, NetPhosK (Blom et al., 2004). The algorithm identified 11 potential serines and 12
potential threonines for phosphorylation. We excluded two sites from further analysis due to their extracellular location. Further kinase-specific characterization of the remaining sites indicates three potential sites that were not tested in Kir3.4* by Mao et al.: T51, T85, S326, and S366 (Figure 5.1).

We first confirmed that purified PKC could phosphorylate the cytoplasmic domain of Kir3.2. The Kir3.2 cytoplasmic domain is composed of an N-term/C-term concatemer and has been previously crystallized (Inanobe et al., 2007; Inanobe et al., 2010), matching well to the full-length Kir3.2 channel. The cytosolic domain, called Kir3.2NC, was tagged with a SUMO-His N-terminus, expressed in E. coli bacteria, and purified by nickel immobilized metal affinity chromatography (IMAC, see Chapter 2: Methods). This protein was incubated with purified PKC and ran on an SDS-PAGE gel stained with Pro-Q Diamond Phosphoprotein Gel Stain (Rusinova et al., 2009). As shown in Figure 5.2A, the Kir3.2NC protein incubated with PKC was significantly darker than the Kir3.2NC protein alone, indicating the presence of either phosphoserine or phosphothreonine. Figure 5.2B shows the total protein by Coomassie colloidal blue stain, which was similar, indicating the increased signal was from phosphorylation. We used purified Histone III-S as a positive control, showing that the Histone III-S incubated with PKC also had a higher phospho-signal.

Comparison of mass spectra of PKC-treated Kir3.2NC vs. untreated control identified a phosphopeptide containing pSer-238. Ser-238 lies in a key region to affect PIP2-dependent gating, the βD strand. The nearby positively charged Arg-240 has been shown to reduce channel-PIP2 interactions when the corresponding residue, Arg-228, is neutralized in Kir2.1 (Lopes et al., 2002).
5.2.2. PMA Inhibition Screen of Kir3.2 Ser/Thr Residues

We tested S238A in the background of Kir3.2*_D228N_S196Q, which gave consistently high PMA inhibition and expression. However, the S238A mutant showed strong inhibition by PMA, similar to the control, shown in Figure 5.3. Currents of S238A were significantly reduced compared to Kir3.2* control (data now shown), whereas Kir3.2*_S238D and Kir3.2*_S238E had no detectable current. Two additional mutations tested in the Kir3.2*_D228N_S196Q background, S366A and S388A, had significant PMA inhibition that was not different from the control. The T51A and T364A mutants in the Kir3.2*_D228N_S196Q background also exhibited high sensitivity to PMA inhibition (data not shown).

Additionally, we tested mutations at a PKA site previously identified in Kir3.4 (Treiber et al., 2013), which corresponds to Thr-204 in Kir3.2 (Figure 5.4A). Unexpectedly, both T204A (29.2 +/- 3.5%) and T204D (49.5 +/- 2.8%) mutations resulted in significantly increased PMA inhibition compared to Kir3.2* alone (18.6 +/- 2.8%), shown in Figure 5.4C. T204D had significantly higher PMA inhibition than either T204A or Kir3.2* control. Comparing the current levels, T204D was significantly reduced (29.2 +/- 1.6%) compared to T204A (88.8 +/- 5.8%), which was similar to Kir3.2* control (Figure 5.4B).

5.3. Discussion

The prevalence of PKC consensus sites in Kir3.2, as well as general phosphorylation sites not specific to PKC, indicates the likelihood of multiple phosphorylation sites involved in functional PKC inhibition. This study presents evidence for the first time that PKC phosphorylates the Kir3.2 cytoplasmic domain. Importantly, this domain is a concatemer of the N-term/C-term cytoplasmic domains, which has been crystallized in the presence and absence of sodium (Inanobe et al., 2010). This cytoplasmic domain matches well with the full-length Kir3.2
structure. Previously, *in vitro* phosphorylation with PKC has been observed with the isolated C-terminal domain of the Kir3.1 and Kir3.4 channels (Brown et al., 2005; Thomas et al., 2006), but not the Kir3.2 channel, implying that N-terminal elements may be necessary for PKC phosphorylation.

PKC phosphorylation of the cytoplasmic domain of Kir3.2 and subsequent mass spectrometry identified Ser-238 as an *in vitro* PKC phosphorylation site. The S238A mutant was significantly inhibited by PMA, to the same degree as the Kir3.2* control. We cannot rule out Ser-238 as a potential site involved in PMA inhibition, as other phosphorylation sites may compensate for loss of Ser-238. Normalized current levels suggest that Ser-238 may be constitutively phosphorylated in Kir3.2 channels, as the neutralization mutant S238A had significantly reduced currents. However, both phosphomimetic S238D and S238E mutants had no detectable current in the Kir3.2* background, preventing further analysis.

Alignment of eukaryotic Kir channels indicates that the respective Ser residue is present in Kir3.2, 3.3, and 3.4, but not Kir3.1. Additionally, Kir6.1 and Kir6.2 have a Ser and a Thr at the same position. Ser-238 is located in the βD strand, immediately adjacent to a key region implicated in PIP2-mediated gating, the CD-loop, also the location of Asp-228 (the sodium coordination site) and Ile-234 (mutation to Leu increases PIP2 affinity). The arginine in the phosphorylation motif SxR, Arg-240, corresponds to Arg-228 in Kir2.1, and its mutation affects PIP2 sensitivity in this channel.

We mutated a residue implicated in PKA mediated gating, Thr-204 (Treiber et al., 2013), and found increased PMA sensitivity in the mutants. The phosphomimetic T204D in particular gave reduced current levels and increased PMA inhibition, reminiscent of S196Q or R201A mutants. It is possible that T204D could attract Arg-201 away from Ser-196, thus leading to the
S196Q-like phenotype. The hydroxyl group of Thr-204 is located in close proximity (~3.8 Å) to the C-Zeta atom of Arg-201, which contains the guanidinium group. The functional relevance of T204A is not easily explained, but could reflect an allosteric effect on Arg-201 placement as well. Thr-204 is conserved in all eukaryotic Kir channels, except for Kir7.1 which has a Ser at the corresponding position. Although the analogous residue to Thr-204 has been characterized in Kir3.4 channels (Treiber et al., 2013), the effect of PKA phosphorylation at this residue remains untested in Kir3.2 channels. Additionally, this residue is a hotspot for phosphorylation in other Kir channels, and is implicated in PKC activation of Kir6.2 (Light et al., 2000), rather than inhibition. The proximity of this residue to PIP2-binding residues in the C-linker helix (i.e. the KKR motif) is very intriguing and warrants further exploration.

In summary, we have shown for the first time direct in vitro phosphorylation of the Kir3.2 cytoplasmic domain by PKC, and identified a potential PKC-sensitive phosphorylation site Ser-238 by mass spectrometry. This serine lies next to a key region implicated in PIP2-mediated gating, the CD-loop, and is two residues away from a residue putatively involved in channel-PIP2 interactions, Arg-240. In addition, we examined how a putative PKA site identified in Kir3.4 channels affects PKC inhibition, finding that the charge on this residue can allosterically regulate inhibition. Allosteric regulation at this site supports the idea that residues near the helix bundle crossing can couple to PKC-dependent inhibition, likely by engaging in a molecular tug of war for the crucial Arg-201.
Figure 5.1. Primary sequence of mouse Kir3.2 with consensus phosphorylation sites. Bold: predicted phosphorylation sites; underline: predicted PKC sites; gray background: tested in Kir3.4*; red: not accessible (extracellular).

1  MTMAKLTESMTNVLEGDSMDQDVESPVAIHQPKLPQARDDLPRHISRD
51- TKRKIQRYVVKDGKCNVHGKVRYLTDIFTTLVDKWRFNLLIFVM
101- VYTVTWLFFGMIWWLIAYIRGMDHIEDPSWTPCVTNLNGFVSAFLFSIE
151- TETTIGYRYVITDKCEPIILLILQSVLGSIVNAFMVCMGFKISEQPKK
201- RAETLVFSTHAVISMRDGKLCLMFVGRDLRNSHIVEASIRALKLISQTS
251- EGEFIPLNQTDINVGYTGDDRLFLVSPLISSHEINQSPFWEISKAQLP
301- KEELEIVVILEGMVEATGMCQARSSYITSEILWGRFTPVLTLEDGFYE
351- VDYNFHETYETSTPDSLSAKELAELANRAELPLSWSVSKNQHAELETE
401- EEKENPEELTERNG
Figure 5.2. Phosphorylation of Kir3.2 cytosolic domain by purified PKC. A. Phosphoprotein stain of Histone control and Kir3.2NC. B. Coomassie stain of same gel indicating total protein.
Figure 5.3. PMA Inhibition of Kir3.2\textsuperscript{*}D228N_S196Q phosphosite mutants. S238A, S366A, and S388A all demonstrated significant PMA inhibition.
Figure 5.4. Effect of T204 mutations on current and PMA inhibition. A. Location of T204 from Kir3.2/PIP2 crystal structure (PDB code 3SYA) in relation to R201 and PIP2. B. Normalized currents for Kir3.2*, T204A, and T204D. C. PMA inhibition of Kir3.2*, T204A, and T204D. *, p<0.05 compared to control group. **, T204D is significantly increased compared to T204A at p<0.05.
Chapter 6. CONCLUDING REMARKS

We set out to determine how PKC phosphorylation of a particular residue in a Kir channel couples to the end goal of inhibition, hypothesizing that a reduction in channel-PIP$_2$ interactions would accomplish this goal. We mutated residues thought to be targets of PKC phosphorylation in Kir3.1/3.4 with the intent of investigating these mutations for their impact on channel-PIP$_2$ interactions. Immediately it became clear that the mutagenesis approach to blindly identifying PKC sites can lead to erroneous conclusions. In the context of Kir3.1* and Kir3.4*, there is a discrepancy between the PMA inhibition of Ser-to-Ala mutants and Ser-to-Asp mutants, while neither residue can be phosphorylated. On the other hand, Kir3.2* residues display the opposite paradigm: the phosphomimetic Ser-to-Glu acts like the wildtype, while the neutral Ser-to-Gln increases PMA inhibition. This study shows that specific point mutations can have far-reaching effects on gating and allosteric coupling to PKC inhibition.

It is prudent, then, to address this problem in multiple ways, which is why we pursued a mass spectrometry approach to identify phosphorylation sites directly from the *in vitro* phosphorylated cytoplasmic domain of Kir3.2. This project could be extended to the full-length Kir3.2, which would provide PKC with a more physiological target; alternatively, the phosphorylation of heterologously expressed Kir3.2 could be investigated using immunoprecipitation techniques coupled with mass spectrometry, by comparing before and after PMA or PKC treatment.
After identification of the phosphorylation site(s) and subsequent mutagenesis to confirm their relevance, we can use atomistic MD simulations to identify changes in salt-bridge and hydrogen bonding networks that would likely occur after phosphorylation. This iterative approach should prove useful in identifying the impact of phosphorylation of membrane proteins for which a crystal structure is available, or a suitable homology model.

Perhaps most intriguingly, this study raises the possibility that Kir3.2 is phosphorylated at a residue (Ser-196) interposed between two of the gating elements, the helix bundle crossing gate and the G-loop gate. It would be useful to have a phospho-antibody to this residue in order to investigate its phosphorylation state in native systems. Given the close proximity of Ser-196 to the plasma membrane, it is possible that Ser-196 is constitutively phosphorylated prior to tetrameric assembly, perhaps in the ER or Golgi. The access of phosphatases would be another question, asking if dephosphorylation of Kir3.2 at this residue can increase their PKC sensitivity.

Finally, we turn to PIP$_2$, which is absolutely necessary for channel activity. Mutations which alter channel-PIP$_2$ interactions have already been shown to underlie several known channelopathies, with severe impact on constitutively active channels like Kir1.1 (Barrter) and Kir2.1 (Andersen-Tawil). Some naturally occurring mutations are also thought to change phosphorylation motifs in several ion channels, so-called phosphorylopathies (Gentile 2012). Clearly, the regulation of channel function is complex and multifactorial, such that cases of low PIP$_2$ or high PIP$_2$ levels can have a significant impact on the functional effect of phosphorylation. Knowing how specific phosphorylation sites can affect channel-PIP$_2$ interactions may lead to an important revelation for other PIP$_2$-sensitive channels, of which the list is continually expanding.
Literature Cited


Appendix 1. Mutations tested without detectable current

In Kir3.2* (E152D) Background:

- S196D/C/F/G/R/L
- Q197R
- T317A
- S232A/D/E
- S326E
- S238D/E
- T85A
VITA

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