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# **POSITIVE TRAFFICKING PATHWAYS OF A VOLTAGE GATED POTASSIUM CHANNEL**

A Dissertation Presented

by

Emilee Colleen Connors

to

The Faculty of the Graduate College

of


The University of Vermont

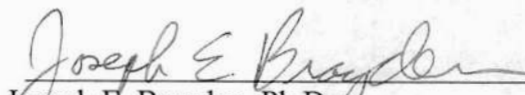
In Partial Fulfillment of the Requirements  
for the Degree of Doctor of Philosophy  
Specializing in Pharmacology

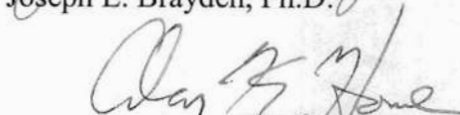
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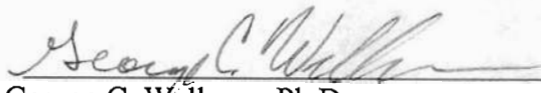
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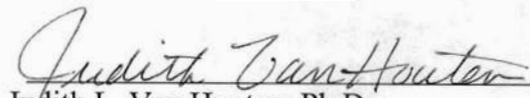
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## ABSTRACT

The voltage-gated potassium channel Kv1.2 is a key determinant of cellular excitability in the nervous and cardiovascular systems. In the brain, Kv1.2 is strongly expressed in neurons of the hippocampus, a structure essential for learning and memory, and the cerebellum, a structure essential for motor control and cognition. In the vasculature, Kv1.2 is expressed in smooth muscle cells where it contributes to the regulation of blood flow. Dynamic regulation of Kv1.2 is fundamental to its role in these tissues. Disruption of this regulation can manifest in a range of pathological conditions, including seizure, hypertension and neuropathic pain. Thus, elucidating the mechanisms by which Kv1.2 is regulated addresses fundamental aspects of human physiology and disease.

Kv1.2 was the first voltage gated ion channel found to be regulated by tyrosine phosphorylation. The ionic current of Kv1.2 is suppressed following tyrosine phosphorylation by a process involving channel endocytosis. Movement of channel away from the plasma membrane involves many proteins associated with the cytoskeleton, including dynamin, cortactin and RhoA. Because trafficking of Kv1.2 away from the cell surface has emerged as the primary mechanism for its negative regulation, we hypothesized that trafficking of the channel to the cell surface could be a mechanism for positive regulation of the Kv1.2 ionic current.

Activation of the cAMP/PKA pathway enhances the ionic current of Kv1.2. We hypothesized that a mechanism for this positive regulation is an increase in the amount of channel protein present at the cell surface. Our data show that cAMP can regulate Kv1.2 surface levels by two opposing trafficking pathways, one PKA-dependent and one PKA-independent. Channel homeostasis is preserved by the dynamic balance between these two pathways. Accordingly, any change in the levels of cAMP causes a net increase in the amount of Kv1.2 present at the cell surface. Specific C-terminal phosphorylation sites of Kv1.2 were identified and shown to have a role in maintaining basal surface channel levels. These findings demonstrate channel trafficking as a mechanism for the positive regulation of the Kv1.2 ionic current.

In addition to Kv1.2 trafficking at the plasma membrane, movement of the channel from the biosynthetic pathway to the cell surface is another checkpoint for its regulation. Here we show that the protein arginine methyltransferase 8 (PRMT8) is able to promote the ER exit of Kv1.2, resulting in an increase in Kv1.2 surface expression. PRMT8 not only promoted surface expression of the high mannose glycosylated form of Kv1.2, characteristic of immature, ER-localized channels, but also enhanced Kv1.2 total protein levels, most likely by decreasing the amount of channel protein available for ER-associated degradation (ERAD). These findings highlight biosynthetic trafficking of Kv1.2 as a crucial part of its regulation and identify a novel role for PRMT8, as a regulator of biosynthetic protein trafficking.

## CITATIONS

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## **CHAPTER 1: LITERATURE REVIEW**

### **1.1. Introduction**

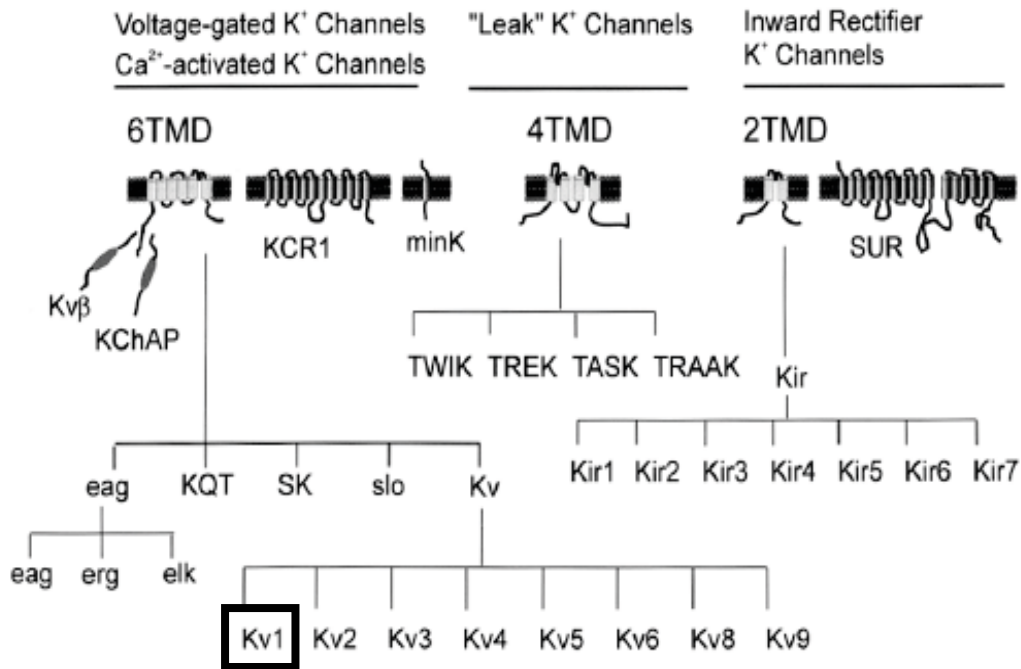
Ion channels are significant components of cellular communication. They connect the cell interior to the extracellular environment by forming a pore in the plasma membrane which ions can pass through. Ion channels regulate the flow of ions in all cell types and are major determinants of the electrical activity that lies at the core of nervous and cardiovascular system function (Kandel ER 2000). In addition, aberrant ion channel function can lead to serious diseases, termed channelopathies (Ashcroft 2006). Cystic fibrosis, myasthenia gravis, Long QT syndrome, seizure activity and hypertension can all be attributed, at least in part, to irregular ion channel function (Lehmann-Horn and Jurkat-Rott 1999; Felix 2000; Buckley and Vincent 2005). Collectively, these pathological conditions emphasize the vital role of ion channels in normal physiological function, and highlight the importance of studying their function and regulation.

### **1.2. Classification of Potassium Channels**

Potassium channels have wide ranging kinetic properties, structure, pharmacology and regulation, making them the largest and most diverse group of ion channels (Coetzee, Amarillo et al. 1999). The unifying feature between all classes of potassium channels is their ability to selectively allow potassium ions to flow across the plasma membrane

(Doyle, Morais Cabral et al. 1998). In general, ion channels were initially categorized by their electrophysiological signature and pharmacology; however the classification of channels is currently based on sequence homology (Alexander, Mathie et al. 2008). There are three major classes of potassium channels (2TM, 4TM and 6TM), named for the number of transmembrane (TM) domains within the pore forming subunits (Figure 1).

The 2TM channels are the phylogenetically oldest potassium channels and their structure represents the basic arrangement found in all potassium channels (Yang, Jan et al. 1995). Each subunit is composed of one pore region surrounded by two TM domains. The holochannel is composed of four individual subunits to form a single potassium ion pore (Yang, Jan et al. 1995). Initially termed “anomalous” rectifiers, these channels conduct an inward potassium current at membrane voltages negative to the  $K^+$  equilibrium potential and conduct little, if any, outward current during depolarization (Katz 1949; Lu 2004). The 2TM channel group includes GPCR-activated (Kir3) and ATP-sensitive (Kir6) subtypes. Kir3 or  $K_{ACh}$  channels in cardiac muscle are activated by acetylcholine (ACh) and act to hyperpolarize atrial cells and slow pacemaking (Hille 2001). Kir6 or  $K_{ATP}$  channels, present in pancreatic  $\beta$  cells, neurons and all muscle cell types, hyperpolarize cells by opening when the intracellular concentration of ATP is low (Nichols and Lopatin 1997; Hille 2001). These channels are critical for regulating insulin release and are major targets for diabetic therapy (Loubatieres 1969; Cook and Hales 1984).



**Figure 1: Classification of Potassium Channel Families.** The three classes of potassium channels are based on the structure of the alpha subunits. The largest group contains alpha subunits that have six-transmembrane domains (TMD). A tetramer of these alpha subunits forms a single channel with a single ion conducting pore. Kv1.2 is a member of this group. The second group is the 4TMD potassium leak channels in which four-transmembrane domain containing alpha subunits with two potassium pores each dimerize to form a functional channel. Lastly, the inwardly rectifying channels are tetramers of alpha subunits with two-transmembrane domains and a single pore.

Coetzee et al. **Molecular Diversity of K<sup>+</sup> Channels.** *Ann N Y Acad Sci.* 1999 Apr 30;868:233-85.

The 4TM channels, or tandem pore domain channels, are an unusual class of potassium channels since each alpha subunit contains two pore-forming sequences (Kim 2005). A channel is formed by alpha subunit dimerization, thus the holochannel has a pore similar to channels in other classes. Often termed leak or background channels,



these channels are mostly voltage-independent and can contribute to setting the resting membrane potential and regulating cellular excitability (Cohen, Ben-Abu et al. 2009). They are found in almost all tissues, including neurons, and are thought to be involved both neuroprotection and regulating cell volume (Mathie and Veale 2007). Members of the 4TM channel group can be regulated endogenously by a variety of stimuli including pH, second messengers, temperature, mechanical stretch and also exogenously by general anesthetics (Yost 2003; Cohen, Ben-Abu et al. 2009).

The 6TM channels are the largest class of potassium channels and is further divided into two subclasses, the calcium-activated and the voltage-gated channels. These channels are tetramers of 6TM alpha subunits containing one pore domain each (Jenkinson 2006). Changes in voltage or intracellular calcium cause these channels to open and conduct an outward flow of potassium, down its concentration gradient. Calcium-activated potassium channels include large (big), intermediate and small conductance channels (BK, IK and SK, respectively). IK and SK channels are not voltage dependent, but are calcium sensitive due to their association with calmodulin, a calcium binding protein (Ledoux, Werner et al. 2006). The function of IK channels, mainly expressed in erythrocytes and endothelium, includes the regulation of cell volume and blood pressure (Gardos 1958; Ledoux, Werner et al. 2006). SK channels are predominantly expressed in the nervous system and contribute to the afterhyperpolarization that regulates action potential firing rates (Stocker 2004). BK channels are highly expressed in smooth muscle and neurons and are involved in processes such as vasodilation and neurotransmitter release (Ledoux, Werner et al. 2006;

Cui, Yang et al. 2009). Although BK channels are similar to IK and SK channels because of their calcium sensitivity, they have the added ability to respond to the voltage across the plasma membrane (Cui, Yang et al. 2009). This dual regulation allows BK channels to have a larger role in affecting electrical activity than channels that are activated by either calcium or voltage alone.

Potassium channels activated primarily by voltage are termed voltage-gated potassium channels; nomenclature for these channels is “Kv”, which represents the specificity for the potassium ion (K) and the mode of activation, voltage (v). The Kv1 family proteins were first classified in *Drosophila* with the Shaker phenotype (Papazian, Schwarz et al. 1987; Tempel, Papazian et al. 1987; Schwarz, Tempel et al. 1988; Timpe, Schwarz et al. 1988). Other Kv subfamilies identified in *Drosophila* include Shab, Shaw and Shal, corresponding to Kv2, Kv3 and Kv4, respectively, in humans (Salkoff, Baker et al. 1992). The significance of these channels is discussed below.

Potassium channels can also be classified by their pharmacology. The use of toxins and drugs allows the characterization of channel proteins that contribute to a given current. Both tetraethylammonium (TEA) and 4-aminopyridine (4-AP) are general potassium channel blockers (Hille 2001), however their specificities do not completely overlap (Gutman, Chandy et al. 2005). For example, Kv3.3 is inhibited by both TEA and 4-AP, but another channel, Kv1.2, is relatively insensitive to TEA but sensitive to 4-AP (Gutman, Chandy et al. 2005). Other, more specific blockers allow finer classification. For example, apamin is a relatively specific inhibitor of SK1-3 calcium-activated

potassium channels (Wei, Gutman et al. 2005), and dendrotoxin is a specific inhibitor of Kv1.1 and Kv1.2, but not other channels (Gutman, Chandy et al. 2005).

### **1.3. Physiological Significance of Kv Channels**

The main function of Kv channels is modulating cellular membrane potential by permitting the flow of potassium ions across the plasma membrane in response to changes in membrane potential. Thus, Kv channels are both regulated by, and are regulators of membrane potential. Cellular processes such as excitation-contraction coupling and neurotransmitter release depend on this fundamental role of Kv channels. Clearly, this seemingly simple function of regulating potassium ion flow underlies extremely significant physiological properties.

Action potentials are perhaps the greatest perturbations in membrane potential and it is therefore not surprising that Kv channels have a central role in action potential properties and effects within the cell. Action potentials are created by many different ion channel currents which work in concert to create the action potential waveform. Most Kv channels are closed at resting membrane potential and upon depolarization, they open to repolarize the membrane potential as they draw it closer to the potassium equilibrium potential and farther from the firing threshold. Thus, voltage-gated potassium channels are primary determinants of action potential repolarization.

Importantly, action potential frequency and pattern can be altered by the expression of different ion channels. For example, in auditory neurons, Kv3 channels, which have very rapid kinetics, allow very fast action potential repolarization (Rudy and

McBain 2001). This allows high frequency trains of action potentials necessary to encode auditory information. Additionally, Kv4 channels have been shown to modulate the narrow shape of action potentials in hippocampal pyramidal dendrites by their fast inactivating properties (Kim, Wei et al. 2005).

Just as different potassium channels create functional diversity between cells, the variety of channels expressed within a single cell contributes to regional differences in electrical responsiveness within that cell. For example, Kv1.2 is specifically localized to basket cell terminals in the cerebellum, where it plays a major role in neurotransmitter release but not in action potential repolarization (Southan and Robertson 1998). In those cells, Kv3.1 channels are responsible for action potential repolarization (Southan and Robertson 2000).

The majority of this dissertation focuses on the voltage-gated potassium channel Kv1.2, which is expressed in a variety of brain areas, including the cerebral cortex, the hippocampus, and the cerebellum. Kv1.2 is also expressed in vascular smooth muscle, where it has a central role in the regulation of vascular tone (Thorneloe, Chen et al. 2001; Lu, Hanna et al. 2002). Kv1.2 forms heteromultimers with Kv1.5 in vascular smooth muscle and these channels appear to have a key role in maintaining membrane potential (Kerr, Clement-Chomienne et al. 2001). Channel suppression here could increase the cellular excitability of vascular smooth muscle and lead to the increased vascular tone associated with hypertension.

Therefore, the importance of Kv1.2 is highlighted not only by the normal physiological phenomena it regulates, such as learning and memory (Alkon, Nelson et al.

1998), but also by potentially life-threatening conditions that can be attributed, in part, to aberrant functioning of the channel. Seizures (Lambe and Aghajanian 2001), neuropathic pain (Ishikawa, Tanaka et al. 1999; Kim, Choi et al. 2002; Yang, Takimoto et al. 2004), and multiple sclerosis (Black, Waxman et al. 2006; Howell, Palser et al. 2006) are among these diseases. Furthermore, a recent publication suggests that auto-antibodies against Kv1.2 and its family members may be associated with a wide range of human pathologies, including neuromyotonia (Kleopa, Elman et al. 2006) and epilepsy (Kim, Kim et al. 2007).

It is well established that a mutation within the Kv1.1 potassium channel, which can exist as Kv1.1/Kv1.2 heteromultimeric channels in cerebellum, contributes to cerebellar dysfunction associated with Episodic ataxia (Brandt and Strupp 1997). Episodic ataxia type 1 (EA-1) is a neurological disorder that is caused mainly by mutations in the gene encoding the Kv1.1 channel subunit. At least nineteen mutations in the gene encoding for Kv1.1 have been identified in patients with EA-1. These mutations all lead to a loss of function of Kv1.1, thus reducing potassium ion permeability. The physiological manifestations of decreased Kv1.1 function are longer action potentials, reduced repolarization and ultimately increased cellular excitability. These effects cause the symptoms of this hereditary disease, which include loss of muscle coordination (ataxia), rippling muscle contractions (myokymia), seizures, dizziness and blurred vision (Rajakulendran, Schorge et al. 2007). The occurrence of EA-1 is rare, however, it is frequently used as a model to study other disorders such as epilepsy and migraines. Therefore, elucidating the molecular mechanisms involved in Kv channel regulation

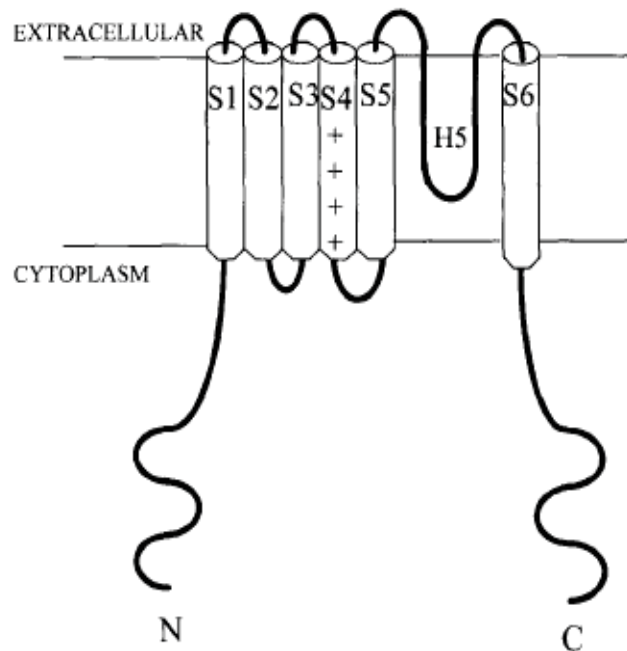
contributes to the understanding multiple physiological processes with strong clinical relevance.

#### **1.4. Kv1.2 Structure**

Kv1 channel structure is critical for normal localization, regulation and function of the protein. Much of what is known about the significance of Kv1.2 function has been deduced from its intricate structure. Indeed, the resolution of the Kv1.2 crystal structure by the McKinnon lab in 2005 was a major advance in the understanding of Kv channel structure and function (Long, Campbell et al. 2005). Kv1 family channels are formed by tetramerization of individual alpha subunits. Channels can be tetramers of the same alpha subunit or heterotetramers formed by different combinations of Kv1 family members. As mentioned above, the formation of a complete channel by the grouping of various alpha subunits increases the functional diversity of the potassium channel family. Specific functional domains of Kv1 channels have been categorized, including the T1 domain, six transmembrane domains (S1-S6), extracellular domains, voltage sensing domain, pore forming domain and an intrinsically disordered C-terminus (Figure 2).

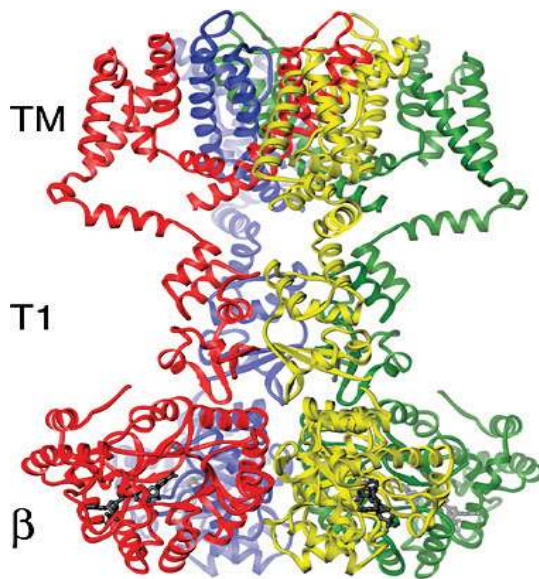
The tetramerization (T1) domain is formed from the N-terminal portions of the four alpha subunits (Kreusch, Pfaffinger et al. 1998). Sometimes termed the “hanging gondola” (Kobertz, Williams et al. 2000), the T1 domain is depicted in the Kv1.2/Kvβ2 crystal structure (Figure 3). It is critical for channel tetramerization, gating, beta subunit binding, and localization of the channel. Besides the voltage sensing domain and selectivity filter of Kv channels, the T1 domain is the most highly conserved domain. The

sequence and structure of this domain reveals a polar interface between subunits and it is this property that permits tetramerization of only channels that have a similar polar interface (i.e. channels within the same sub-family) (Minor, Lin et al. 2000). Mutational analysis of the T1 domain polar interface revealed that this area affects channel gating by a change in conformation (Minor, Lin et al. 2000). The degree of stability of the T1 tetramer may thus alter Kv1.2 gating. The T1 domain serves as a platform for beta subunit binding, which therefore can confer axonal localization of the channel (Gu, Jan et al. 2003).



**Figure 2: Kv1 alpha subunit domains.** S1-S6 depict the six transmembrane domains. S4 is the voltage sensing domain and the potassium pore is formed between S5 and S6. Both the N- and C-termini hang into the cytoplasm and are major sites of post-translational regulation.

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**Figure 3: Kv1.2/Kvβ2 structure complex.** Each color represents one of four alpha/beta subunit complexes. TM is the transmembrane domain region of the alpha subunits, where the potassium pore is located. The T1 domain is formed by the joining of the four N-termini of each alpha subunit. This domain hangs below the transmembrane domain. The beta subunits attach to the alpha subunits in a 1:1 ratio.

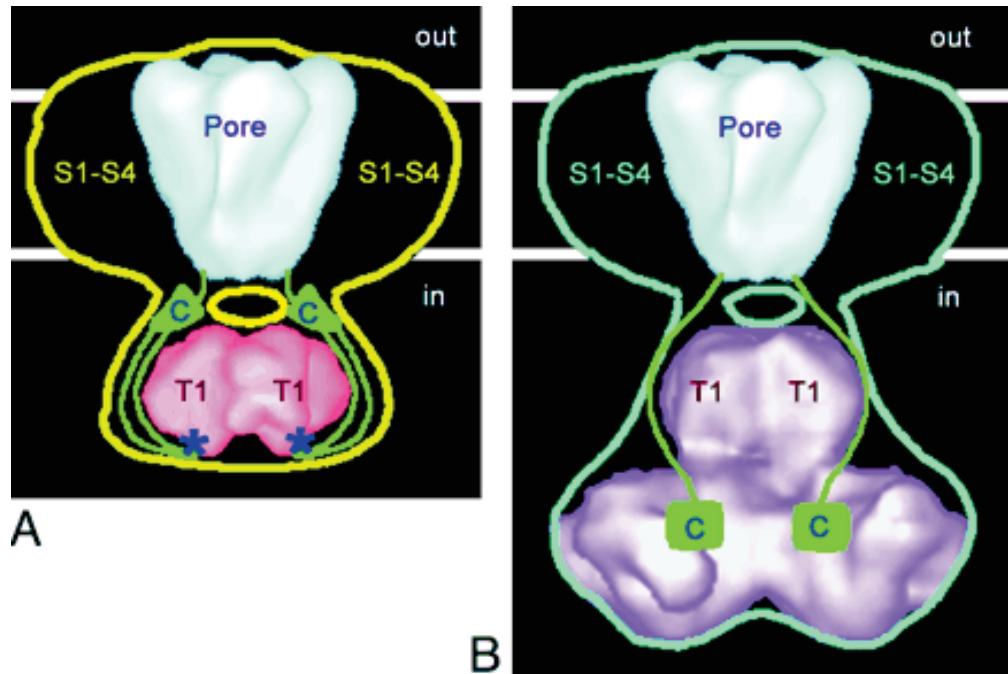
From: Long et al. **Crystal Structure of a Mammalian Voltage-Dependent *Shaker* Family K<sup>+</sup> Channel.** *Science*. 2005. 309, 897-902. Reprinted with permission from AAAS.

Each Kv1 alpha subunit has six transmembrane domains that span the plasma membrane (S1-S6). The first extracellular domain of Kv1.2, between S1 and S2, is the only site of channel glycosylation. Kv1.1-Kv1.5 all possess this N-linked glycosylation site. This post-translational modification can alter channel trafficking and function, as discussed below.



The S4 portion of the channel contains the voltage sensing domain. Evenly spaced, positively charged amino acids are able to detect changes in voltage which subsequently cause a conformational change within the channel allowing potassium ions to flow through the pore. The pore forming domain is between S5 and S6 and is termed the P-loop. In 1955, Hodgekin and Keynes predicted that potassium ions move through the channel in a single file, with multiple ions occupying the channel at any one time (Hodgkin and Keynes 1955). The molecular basis for this was confirmed by x-ray crystallography studies from the MacKinnon Lab, depicting the architecture of the narrowest part of the pore, the selectivity filter, a structure common to all potassium channels (Doyle, Morais Cabral et al. 1998). The selectivity filter ensures that only K<sup>+</sup> ions enter the pore.

The C-terminus of each alpha subunit hangs into the cytoplasm and has been termed “intrinsically disordered”. Interestingly, the presence or absence of the beta subunit can cause a conformational change of the C-terminus (Figure 4). The location of the N- and C- termini in the cytoplasm is critical for channel regulation because they are major targets for modifications and protein-protein interactions involved in channel regulation, as discussed below.



**Figure 4: Model of Kv1 channel C-termini conformational change in the presence or absence of Kvβ2.** (A) C-termini fold back on the T1 domain in the absence of Kvβ2. (B) The addition of the beta subunit causes the C-termini to unfold into an extended conformation.

Sokolova et al. **Conformational changes in the C terminus of Shaker K<sup>+</sup> channel bound to the rat Kvbeta2-subunit.** *Proc Natl Acad Sci U S A.* 2003 Oct 28;100(22):12607-12. Epub 2003 Oct 20.

Kv1 family channels can associate with accessory proteins termed Kvβ subunits, which have profound effects on both biophysical properties and trafficking of the channels. β subunits associate with alpha subunits in a 1:1 stoichiometry; thus there are four β subunits attached to a tetramer of alpha subunits. There are three members of the Kvβ family (Kvβ1-3), with Kvβ1 and Kvβ2 having the most pronounced effects on channel function. β1 subunits can affect Kv1.2 channel gating (Rettig, Heinemann et al. 1994). The N-terminus of β1 subunits can act as an inactivation ball, changing the

kinetics of Kv1.2 to a more rapidly inactivating channel (Rettig, Heinemann et al. 1994).  $\beta 2$  subunits do not induce inactivation of Kv1.2; instead, the predominant function of Kv $\beta 2$  is to alter channel trafficking (Shi, Nakahira et al. 1996; Campomanes, Carroll et al. 2002). Binding of  $\beta 2$  subunits can promote surface expression of Kv1 family channels and accurate axonal targeting, as mentioned above. It has been suggested that beta subunit binding promotes biosynthetic maturation of the channel and channel stability (Shi, Nakahira et al. 1996; Manganas and Trimmer 2000). Interestingly, the crystal structure of Kv1.2/Kv $\beta 2$  revealed the presence of the cofactor NADP<sup>+</sup>, suggesting aldo-reductase activity of Kv $\beta 2$ . The NADP<sup>+</sup> binding site is critical for proper axonal localization and possibly functions to couple the redox state of the cell to excitability (Gulbis, Mann et al. 1999).

### **1.5. Kv1.2 Regulation**

The molecular mechanisms behind Kv1.2 regulation are complex and involve the integration of many signaling pathways. Ion flow can be regulated at the plasma membrane by changes in biophysical properties of the channel pore and by channel trafficking to and from the cell surface. Kv1.2 function is coordinated by both of these mechanisms, which involve intricate interplay between protein-protein interactions and post-translational modifications. That Kv1.2 contributes to electrical excitability in a variety of cell types, including neurons, emphasizes the importance of both positive and negative regulation of the channel.

This trafficking is a coordinated movement and is highly regulated. As Kv1.2 mRNA is translated, the T1 domains of four alpha subunits unite to form a tetrameric holochannel. As mentioned above, the tetramer can be of four identical alpha subunits or any four alpha subunits belonging to the Kv1 family. Assembly of the channel is within the Endoplasmic Reticulum (ER) and it is during this assembly process that the beta subunits bind the channel. Exit from the ER serves as a check point, preventing trafficking of irregular channels to the cell surface. This is a critical step as the exit of misfolded channels could have detrimental dominant negative effects within the cell. This quality control system is dependent on a number of factors, including folding, assembly, glycosylation and interaction with ER chaperone proteins, like calnexin (Khanna, Myers et al. 2001; Manganas and Trimmer 2004).

ER export and retention are major mechanisms by which the cell regulates the amount of channel that reaches the cell surface (Ma and Jan 2002). Both ER export and retention motifs have been identified within the sequences of potassium channels. For example, a C-terminal VXXSL motif within Kv1.4 can serve as an ER export motif (Li, Takimoto et al. 2000). Signals such as the one on Kv1.4 can be masked by phosphorylation of the motif or protein-binding to hide the signal and thus disrupt normal ER exit. ER exit is also dependent on the glycosylation status of the channel and calnexin binding (Khanna, Lee et al. 2004; Manganas and Trimmer 2004). The various ER export and retention motifs among Kv1 alpha subunits increase the functional diversity between heteromultimers.

From the ER, the channel is normally transported to the Golgi apparatus where maturation of the channel occurs by modification of the glycosylation chain. In a study in which the glycosylation state of the channel was manipulated, effects on channel gating, trafficking and stimulated action potentials were apparent (Watanabe, Zhu et al. 2007). Glycosylation can alter the activation and deactivation properties of the channel as well as C-type inactivation. Decreased channel glycosylation caused a reduced expression of channels at the cell surface but did not alter protein stability. Finally, the physiological consequences of modulating Kv1.2 glycosylation, and thus channel function, were shown to include altered waveforms and interspike intervals of stimulated action potentials (Watanabe, Zhu et al. 2007). Collectively, these results clearly depict an essential role for glycosylation in normal channel function.

Once the channel is at the cell surface it functions to regulate the membrane potential of the cell; originally, all channels were thought to be primarily regulated at the plasma membrane by changes in their biophysical properties. Serine/threonine kinases were long thought to be the main regulators of ion channels. For example, previous studies in *Xenopus* oocytes show that application of Protein Kinase A (PKA) altered the single channel properties of Kv1.2 to cause an increase the channel's ionic current (Huang, Morielli et al. 1994). In addition to being regulated by PKA, Kv1.2 is also regulated by Protein Kinase C (Tsai, Morielli et al. 1997).

The discovery of the first voltage-gated ion channel, Kv1.2, to be regulated by tyrosine phosphorylation widened the field of possible cellular systems that might influence voltage-gated channels (Huang, Morielli et al. 1993). In those studies, G

protein-coupled, m1 muscarinic acetylcholine receptor activation induced tyrosine phosphorylation of Kv1.2. This modification led to suppression of the channel's ionic current (Huang, Morielli et al. 1993). Since then, other activators of tyrosine kinases have been found to regulate Kv1.2. These include other G protein coupled receptors (Stirling, Williams et al. 2009) and receptor tyrosine kinases (Tsai, Morielli et al. 1997). One of the key phosphorylation sites within Kv1.2 required for this process is the N-terminal tyrosine 132. Tyrosine phosphorylation mediated regulation of Kv1.2 was a novel discovery, which linked cellular excitability to tyrosine phosphorylation dependent signaling. Other Kv1 channels have since been shown to be regulated by tyrosine phosphorylation, including Kv1.3 and Kv1.5 (Holmes, Fadool et al. 1996; Holmes, Fadool et al. 1996).

Interestingly, the physical mechanism for tyrosine phosphorylation induced suppression of Kv1.2 was identified as channel trafficking, not altered channel biophysics (Nesti, Everill et al. 2004). Therefore, not only was tyrosine phosphorylation of Kv1.2 completely unique, but the mechanism by which it regulated channel function was also novel. M1 muscarinic receptor activation causes tyrosine phosphorylation and subsequent endocytosis of Kv1.2. This physical removal of channel from the plasma membrane leads to suppression of the Kv1.2 ionic current (Nesti, Everill et al. 2004). Accordingly, resolving the proteins and pathways involved the regulation of Kv1.2 trafficking is an important area of research, some of which is included in this dissertation.

Kv1.2 interacts with a plethora of proteins which coordinate its trafficking to and from the plasma membrane and its cellular localization. Many proteins involved in

regulating Kv1.2 are associated with the actin cytoskeleton, such as dynamin, cortactin, and RhoA. Dynamin is a GTP-binding protein that functions during multiple stages of endocytosis, including pinching newly formed vesicles from the plasma membrane and targeting vesicles to designated locations. Dynamin has been identified as a critical player in tyrosine phosphorylation induced Kv1.2 endocytosis (Nesti, Everill et al. 2004). Overexpression of a dominant negative (GTP-ase deficient) form of dynamin blocks channel endocytosis and current suppression (Nesti, Everill et al. 2004).

Cortactin can directly bind Kv1.2 (Hattan, Nesti et al. 2002). This dynamic interaction depends on the state of tyrosine phosphorylation of the channel, such that when the channel becomes tyrosine phosphorylated the cortactin-Kv1.2 interaction is diminished. A study on the functional domains of cortactin revealed that the cortactin-dynamin interaction was not necessary for channel endocytosis (Williams, Markey et al. 2007). The actin-regulatory domains of cortactin, however, were shown to be critical for Kv1.2 endocytosis. Specifically, mutations of cortactin's C-terminal tyrosines and F-actin binding region, which disrupt its ability to activate Arp2/3 and further regulate actin, are necessary for proper channel regulation (Williams, Markey et al. 2007).

Tyrosine phosphorylation mediated suppression of Kv1.2 involves RhoA, a small GTPase of the Ras family that is known to regulate the cytoskeleton (Cachero, Morielli et al. 1998). Inhibition of RhoA, either pharmacologically with C3 exoenzyme or by overexpressing a dominant negative form of RhoA, blocks GPCR mediated channel suppression. Thus, RhoA activity is inversely correlated with Kv1.2 activity. Until recently, the mechanism by which RhoA activity can cause channel suppression was

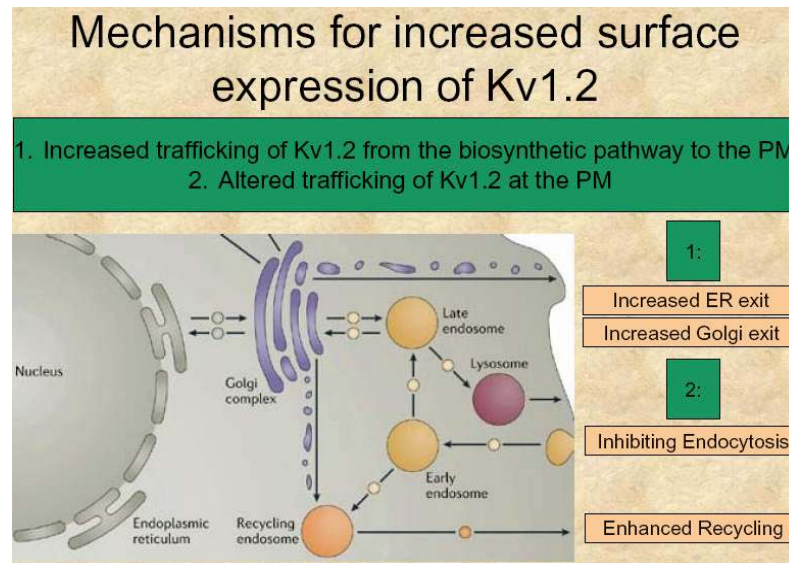
unknown. Stirling et al. identified channel trafficking as the mechanism, identifying RhoA as a modulator of both basal channel turnover and stimulated channel endocytosis. RhoA downstream effectors, LIM-Kinase/cofilin were also shown to be involved in regulating Kv1.2, further connecting the channel to actin dynamics (Stirling, Williams et al. 2009).

Kv1.2 oscillates at the plasma membrane between cycles of endocytosis and recycling back to the cell surface, resulting in a net steady-state level of surface channel (Nesti, Everill et al. 2004). This basal turnover of the channel is cholesterol-dependent (Stirling, Williams et al. 2009). Kv1.2 endocytosis can also occur through receptor-induced channel internalization, which occurs by a clathrin-dependent mechanism (Stirling, Williams et al. 2009). The mechanisms of the two pathways of Kv1.2 endocytosis involve cortactin and RhoA, both of which play discrete roles in each pathway. The F-actin binding domain of cortactin is implicated in both receptor-mediated and steady state endocytosis of the channel, whereas its Arp 2/3 activation domain is necessary for receptor-induced Kv1.2 endocytosis only (Williams, Markey et al. 2007). RhoA, and its downstream effectors, are involved in both the steady-state and the receptor-mediated endocytosis. RhoA is required for the internalization step of the clathrin-dependent, receptor-mediated endocytosis and the recycling step of the cholesterol-dependent, steady-state Kv1.2 trafficking (Stirling, Williams et al. 2009).

Endocytosis has been established as a negative regulator of Kv1.2, however, it is possible that it can positively regulate the channel as well. Positive trafficking can be defined as movement of the channel that causes a net increase of channel expression at



the plasma membrane (Figure 5). Inhibiting endocytosis, stimulating recycling pathways or inhibiting channel degradation could cause a net increase of channel at the surface. Another mechanism for positive trafficking is the movement of newly synthesized or ER retained channel to the cell surface. Little is known about the positive trafficking of Kv1.2 and some questions include: What are the players and pathways that stimulate positive trafficking of Kv1.2? Are the signaling pathways involved in positive trafficking the same as those involved in negative trafficking? Is positive trafficking encoded for within the sequence of the channel? Is positive trafficking of Kv1.2 a major mode of overall channel regulation? The work presented in this dissertation begins to address these questions.



**Figure 5: Mechanisms for increased surface expression of Kv1.2.** Positive trafficking of Kv1.2 can occur by (1) increasing trafficking along the biosynthetic pathway to the plasma membrane and by (2) altering the trafficking of the channel at the plasma membrane to yield a net increase of channel at the cell surface.

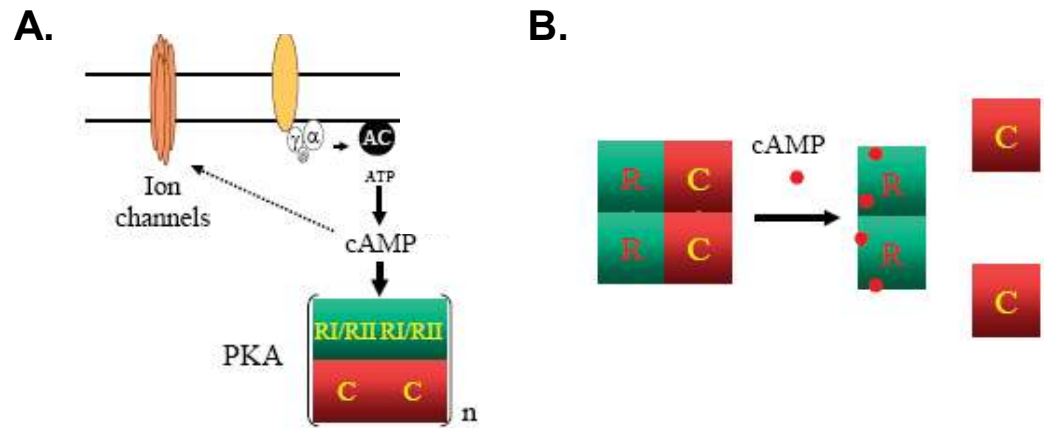
Image adapted from and reprinted by permission from Macmillan Publishers Ltd : Stow et al. **SNAREing immunity: the role of SNAREs in the immune system.** *Nature Reviews Immunology*. 2006.

## 1.6. cAMP/PKA Signaling

The cyclic AMP/Protein Kinase A (cAMP/PKA) pathway is a canonical signaling pathway that has many roles in cellular physiology. Activation of G-protein coupled receptors, such as the beta-adrenergic receptor, leads to activation of adenylate cyclase by  $G_{\alpha_s}$ . Adenylate cyclase converts ATP into cAMP, which can affect a number of downstream effectors including PKA. In its inactive state, PKA is composed of two regulatory subunits and two catalytic subunits. cAMP binds to the PKA regulatory subunit (two cAMP molecules per PKA regulatory subunit) and causes a conformational change, allowing the PKA catalytic subunits to be released (Figure 6). PKA generally phosphorylates substrates that have a consensus sequence of RXXS/T. Serine/Threonine phosphorylation is a reversible modification since enzymes called phosphatases, such as PP2A, are able to dephosphorylate substrates by hydrolysis. Both direct phosphorylation of ion channel proteins and phosphorylation of proteins that regulate channel function can generate potent changes in cellular excitability.

Stimulation of the cAMP/PKA pathway enhances the ionic current of Kv1.2 (Huang, Morielli et al. 1994). One of the mechanisms for this increase is by altering the channel's biophysical properties in a PKA-dependent manner. Application of PKA increases in the amount of time single channels spend in a higher conductance state (Huang, Morielli et al. 1994). Originally, this response was shown to be dependent on a PKA consensus sequence in the N-terminus of the channel (Threonine-46) and it was suggested that PKA directly phosphorylates Kv1.2 (Huang, Morielli et al. 1994). Recent

work however, has provided strong evidence that the C-terminal serine S449, and not T46, is a target for phosphorylation by PKA (Yang, Vacher et al. 2007; Connors, Ballif et al. 2008; Johnson, El-Yazbi et al. 2009).



**Figure 6: Diagram of the cAMP/PKA pathway.** (A) ATP is converted into cAMP when adenylate cyclase is activated by a G $\alpha_s$ . cAMP has many downstream effectors, one of which is Protein Kinase A. (B) The catalytic subunits of PKA are released from the regulatory subunits when cAMP binds and triggers a conformational change.

Skålhegg et al. **Specificity in the cAMP/PKA Signaling Pathway. Differential Expression, Regulation, and Subcellular Localization of Subunits of PKA.** *Frontiers in Bioscience*. 2000, Aug 1;5: D678-693.

In addition to modifying Kv1.2, cAMP/PKA signaling is also involved in the positive trafficking of a variety of membrane proteins. For example, endocytosis of the cystic fibrosis transmembrane conductance regulator (CFTR) is inhibited by the cAMP/PKA pathway (Prince, Workman et al. 1994). Activation of this pathway also increases the trafficking of aquaporin-2 and the amiloride-sensitive sodium channel (ENaC) to the plasma membrane (Kuwahara, Fushimi et al. 1995; Butterworth, Edinger

et al. 2005). These examples of cAMP/PKA induced trafficking led to the hypothesis that cAMP/PKA may regulate the trafficking of Kv1.2. This hypothesis was supported by the data showing that cAMP/PKA signaling was involved in Kv1.2 regulation and that Kv1.2 is negatively regulated by trafficking away from the plasma membrane (Huang, Morielli et al. 1994; Nesti, Everill et al. 2004). Chapter Two of this dissertation explores the involvement of the cAMP/PKA pathway in trafficking of Kv1.2.

### **1.7. Protein Arginine Methyltransferase 8**

Arginine methylation is a post-translational modification catalyzed by protein arginine methyltransferases (PRMTs). The physiological significance of arginine methylation is best understood in the nucleus where it has important roles in modulating the activity of histones and gene regulatory proteins (Lee, Teyssier et al. 2005). Arginine methylation also occurs in the cytoplasm; however its effects there have been largely unexplored. Recently, a neuronally-expressed, plasma membrane-localized protein arginine methyltransferase (PRMT8) was discovered (Lee, Sayegh et al. 2005). Despite its distinct localization, the biological role of PRMT8 is virtually unknown.

As a type I methyltransferase, PRMT8 catalyzes the formation of monomethyl-arginine (MMA) and asymmetric dimethyl-arginine (ADMA) (Lee, Sayegh et al. 2005). MMA is the addition of one methyl group upon the terminal nitrogen of the substrate's arginine side chain and ADMA is the addition of two methyl groups upon a single terminal nitrogen. Arginine methylation is a covalent modification that results in a

bulkier side group, altered hydrogen bonding and increased hydrophobicity. Arginine methylation does not, however, change the positive charge of the arginine. (Boisvert, Chenard et al. 2005).

Physiological consequences of arginine methylation include transcriptional regulation, RNA processing, altered signal transduction and DNA repair (Bedford and Clarke 2009). The methylation of transcription factors, co-activators, co-repressors and especially histones implicates PRMTs as key modulators of epigenetics. Some PRMT substrates are associated with RNA processing, and thus arginine methylation plays a major role in regulating RNA translation, splicing, localization, and stabilization (Bedford and Clarke 2009). Protein-protein interactions can be either disrupted or enhanced by arginine methylation and thus have major consequences for signal transduction. Some signaling pathways shown to be effected by arginine methylation include insulin signaling, glucose metabolism and estrogen signaling (Bedford and Clarke 2009). Noticeably, PRMT effects are wide-ranging and further studies should only broaden the physiological significance of these proteins.

There are currently at least ten members of the human family of protein arginine methyltransferases. PRMT8 is unique within this family due to its highly specialized N-terminus. The N-terminus of PRMT8 confers its localization and is also involved in its methyltransferase activity and scaffolding function. PRMT8 is the only methyltransferase specifically targeted to the plasma membrane via a myristoylation site within its N-terminus. It is the only known methyltransferase to be targeted to the plasma membrane. Also located within the N-terminus of PRMT8 are two proline-rich domains that interact

with proteins harboring SH3 domains, including FYN and PRMT2 (Sayegh, Webb et al. 2007). Furthermore, this N-terminus has been implicated as an autoinhibitory domain, as removal of this domain increases the methyltransferase activity of this enzyme (Sayegh, Webb et al. 2007).

A recent study identified a number of PRMT8 binding partners (Pahlich, Zakaryan et al. 2008). These protein-protein interactions offer critical insights to the potential function of PRMT8. PRMT1 was among the proteins identified, and further research supports PRMT8-PRMT1 dimer formation (Lee, Sayegh et al. 2005). PRMT8 and PRMT1 are highly homologous, except for the specialized N-terminus of PRMT8, which confers its plasma membrane localization. The binding of PRMT1 to PRMT8 may serve to localize PRMT1 activity (Lee, Sayegh et al. 2005). Also on the list of identified PRMT8 binding partners was the RNA-binding protein, Ewing's sarcoma (EWS). The binding of the EWS protein to PRMT8 was shown to be independent of its methylation state (Pahlich, Zakaryan et al. 2008). These findings imply that addition to its methyltransferase abilities, PRMT8 may serve as a scaffold, targeting specific proteins to the plasma membrane. Lastly, PRMT8 was shown to bind the cytoskeletal proteins, actin and tubulin (Pahlich, Zakaryan et al. 2008). If PRMT8 functions as an adaptor protein, its interaction with the cytoskeleton could suggest a role for PRMT8 in protein trafficking.

PRMT8 is highly expressed in neuronal brain tissue, specifically in the hippocampus and cerebellum (Taneda, Miyata et al. 2007). This is unique as other arginine methyltransferases are ubiquitously expressed. This anatomical localization overlaps with that of Kv1.2. In addition, Kv1.2 and PRMT8 are both targeted to the

plasma membrane. Since many of the details involved in the regulation of Kv1.2 by cytoskeleton-dependent trafficking mechanisms have been resolved, we used Kv1.2 as a tool to explore potential roles of PRMT8 on protein trafficking. Chapter Three of this dissertation explores mechanisms by which PRMT8 regulates protein trafficking.

## **1.8. Summary**

The focus of this dissertation is on the regulation of the voltage-gated potassium channel Kv1.2, including how post-translational modifications and protein-protein interactions affect its trafficking and localization. Chapter two, previously published in the *Journal of Biological Chemistry*, explores the regulation of Kv1.2 trafficking by the cAMP/PKA pathway (Connors, Ballif et al. 2008). Until this work was published, it was unclear whether positive regulation of the channel could involve acute trafficking at the plasma membrane. We show that Kv1.2 surface levels are altered by distinct PKA-dependent and PKA-independent effects. In addition, we identify phosphorylation sites in the C-terminus of the channel which prove to be essential for channel trafficking and function. Importantly, this research established innovative links between the cAMP/PKA signaling pathway and membrane excitability.

The second half of this thesis is focused on trafficking changes induced by the protein arginine methyltransferase, PRMT8. The function of this neuronally expressed, plasma membrane-localized methyltransferase is virtually unknown. This work identifies protein arginine methyltransferase 8 as a key regulator of Kv1.2 trafficking and suggests

that it may play a large and yet completely unexplored role in protein trafficking. In so doing, this research identifies arginine methyltransferases as potential drug targets for a myriad of ion channel related diseases. Therefore, the work in this dissertation provides a fundamentally new perspective on the cellular mechanisms governing ion channels and the many physiological processes they influence.



**CHAPTER 2: HOMEOSTATIC REGULATION OF KV1.2 POTASSIUM  
CHANNEL TRAFFICKING BY CYCLIC AMP**

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Running title: cAMP Regulation of Kv1.2 Trafficking

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## **2.1. Abstract**

The Shaker family potassium channel, Kv1.2, is a key determinant of membrane excitability in neurons and cardiovascular tissue. Kv1.2 is subject to multiple forms of regulation and therefore integrates cellular signals involved in the homeostasis of excitability. The cyclic AMP/protein kinase A (PKA) pathway enhances Kv1.2 ionic current; however, the mechanisms for this are not fully known. Here we show that cAMP maintains Kv1.2 homeostasis through opposing effects on channel trafficking. We found that Kv1.2 is regulated by two distinct cAMP pathways, one PKA-dependent and the other PKA-independent. PKA inhibitors elevate Kv1.2 surface levels, suggesting that basal levels of cAMP control steady-state turnover of the channel. Elevation of cAMP above basal levels also increases the amount of Kv1.2 at the cell surface. This effect is not blocked by PKA inhibitors, but is blocked by inhibition of Kv1.2 endocytosis. We conclude that Kv1.2 levels at the cell surface are kept in dynamic balance by opposing effects of cAMP.

## **2.2. Introduction**

The voltage-gated potassium channel, Kv1.2, plays an important role in modulating membrane potential, and its effects are associated with a wide range of human pathologies. In the brain, Kv1.2 is associated with seizure activity (Lambe and Aghajanian 2001; Brew, Gittelman et al. 2007). In dorsal root ganglion neurons, Kv1.2 plays a central role in nociception and is involved in the generation of neuropathic pain (Ishikawa, Tanaka et al. 1999; Kim, Choi et al. 2002; Yang, Takimoto et al. 2004). In

cardiac and vascular smooth muscle, Kv1.2 activity is associated with increased vascular tone and hypertension (Wang, Juhaszova et al. 1997; Cox 2002; Hong, Weir et al. 2004). Kv1.2 activity within the cell is not static, but is instead subject to both positive and negative regulation (Huang, Morielli et al. 1994; Nesti, Everill et al. 2004). Disruption of that regulation may underlie some of the pathologies associated with Kv1.2 function. Therefore, elucidating the mechanisms of Kv1.2 regulation is essential for understanding a broad range of pathological conditions.

A key modulator of Kv1.2 function is the cyclic AMP/protein kinase A (PKA) signaling pathway. Stimulation of  $\beta_2$ -adrenergic receptors causes a potent and reversible enhancement of Kv1.2 ionic current. Part of the mechanism for this increase is alteration of the channel's biophysical properties by PKA (Huang, Morielli et al. 1994); activation of PKA increases the time that single channels spend in higher conductance states. Mutational analysis revealed that an N-terminal threonine (Thr-46) within a PKA consensus sequence is required for the response to PKA, suggesting direct phosphorylation of the channel protein at this site (Huang, Morielli et al. 1994). In addition to positive regulation by PKA, Kv1.2 can be negatively regulated by tyrosine phosphorylation (Huang, Morielli et al. 1993). Activation of muscarinic acetylcholine receptors, for example, elicits tyrosine phosphorylation of Kv1.2 and the resultant suppression of its ionic current (Huang, Morielli et al. 1993). Tyrosine phosphorylation of Kv1.2 also elicits endocytosis of the channel, and this endocytosis is a mechanism for current suppression (Nesti, Everill et al. 2004). This process is dependent upon the proteins dynamin and cortactin, both of which are well known for their roles in

endocytosis (Herskovits, Burgess et al. 1993; Chen, Wang et al. 2006; Williams, Markey et al. 2007). Therefore, tyrosine kinase-dependent trafficking is an important mechanism for regulation of Kv1.2 activity. It is not known whether trafficking plays an equally important role in Kv1.2 regulation by cAMP/PKA.

Trafficking of a variety of membrane proteins is modulated by the cAMP/PKA pathway. Elevation of cAMP inhibits the rapid endocytosis of the cystic fibrosis transmembrane conductance regulator, thus increasing the amount of channel at the cell surface (Prince, Workman et al. 1994). The mechanisms by which cAMP affects the cystic fibrosis transmembrane conductance regulator are varied and may involve both PKA-dependent and PKA-independent mechanisms (Howard, Jiang et al. 2000; Chang, Di et al. 2002; Pereira, Parker et al. 2007). Phosphorylation of aquaporin-2 by PKA causes channel insertion into the plasma membrane (Kuwahara, Fushimi et al. 1995); however, this can also occur via a PKA-independent mechanism (Valenti, Procino et al. 2000; Lu, Sun et al. 2004). Furthermore, increasing cAMP enhances trafficking of the amiloride-sensitive sodium channel (ENaC) to the plasma membrane, and defects in this trafficking are associated with Liddle syndrome (Butterworth, Edinger et al. 2005). Therefore, the cAMP/PKA pathway is an important regulator of plasma membrane protein trafficking. Furthermore, the mechanisms involved are varied; in some cases, cAMP modulates trafficking via PKA, but in other cases, cAMP works independently of PKA.

Here, we show that the cAMP/PKA pathway influences Kv1.2 trafficking. Our experiments reveal distinct PKA-dependent and PKA-independent effects on Kv1.2

surface levels. Our findings suggest this occurs through cAMP-dependent modulation of channel endocytosis. Low cAMP levels maintain basal PKA activity, which enhances constitutive Kv1.2 endocytosis, and high cAMP levels inhibit constitutive endocytosis of the channel independently of PKA. We conclude that homeostasis of Kv1.2 expression in the plasma membrane is dependent on a critical balance of cAMP levels within the cell.

### 2.3. Experimental Procedures

**Materials:** Antibody directed against the first extracellular loop of Kv1.2 (anti-Kv1.2e) was developed with assistance from BIOSOURCE International (Camarillo, CA). Anti-phosphorylated RXX(S/T) antibody was purchased from Cell Signaling (Danvers, MA), and anti-Kv1.2 and anti-Kv $\beta$ 2 monoclonal antibodies from the University of California (Davis, CA). A rabbit anti-Kv1.2 antibody raised against a peptide region within the carboxyl terminus of Kv1.2 (anti-Kv1.2p) was developed with assistance from Cocalico (Reamstown, PA). Alexa Fluor-conjugated secondary antibodies and phalloidin (Invitrogen) were used for immunofluorescence. Non-targeting siRNA (D-0012106) and cortactin siRNA (M-010508) were purchased from Dharmacon (Lafayette, CO). Isoproterenol, propranolol, and isobutylmethylxanthine were purchased from Sigma; myristoylated PKA inhibitor peptide was from BIOMOL International (Plymouth Meeting, PA); and all other reagents were from EMD Biosciences (San Diego, CA) unless noted otherwise.

**Tissue Culture:** Two different stable cell lines were used: clonal human embryonic kidney 293 cells stably expressing M1 muscarinic acetylcholine receptors (HEK-M) and

the HEK-M clonal cell line also stably expressing Kv1.2 $\alpha$  and Kv $\beta$ 2 (HEK-K). Cells were cultured as reported previously (Nesti, Everill et al. 2004). Confluent cultures were plated to a low density (25,000 cells/cm<sup>2</sup>) onto poly-D-lysine-coated tissue culture plates (Corning Glass Works, Corning, NY) and subsequently placed in serum-free medium overnight. Following stimulus treatment where appropriate, cells were metabolically poisoned with 0.5% sodium azide for 30 min at 37 °C to block endocytosis and then processed for flow cytometry or Western blot analysis as described previously (Nesti, Everill et al. 2004).

**Transient Transfection of HEK Cells:** Dynamin was expressed using pEGFP-N1. Dynamin K44A was generously provided by Dr. Mark A. McNiven. GFP was expressed using pEGFP-N1 (Clontech). Kv1.2 $\alpha$  and Kv $\beta$ 2 were expressed using the pRK5 mammalian expression vector. Mutagenesis primers were purchased from Operon Biotechnologies (Huntsville, AL), and mutagenesis was performed with the Stratagene QuikChange site-directed mutagenesis kit (La Jolla, CA). HEK-M or HEK-K cells were transiently transfected with Kv1.2, mutant Kv1.2 subunit, Kv $\beta$ 2 subunit, GFP, or dynamin K44A using the TransIT-293 liposomal transfection reagent (Mirus, Madison, WI) as per the supplied protocol. siRNA was transiently transfected with calcium phosphate.

**Detection of Kv1.2 Surface Levels by Flow Cytometry:** Surface Kv1.2 was labeled with a rabbit polyclonal antibody (anti-Kv1.2e) directed against an extracellular epitope within the channel. Secondary labeling was done with a fluorescently conjugated goat anti-rabbit IgG (Southern Biotech, Birmingham, AL). Kv1.2 surface levels were quantified as the number of cells emitting at 667 nm with fluorescence intensity above a

threshold value determined using control IgG-labeled cells. For experiments requiring transient transfection, GFP was used as a marker, and emission at 667 nm was taken only from cells with green fluorescence. Flow cytometry was done with the Easycyte single laser flow cytometer (Guava Technologies, Hayward, CA). Analysis of cell populations and histograms was done with FCS Express and WinMDI softwares, respectively.

**Alkaline Phosphatase Treatment:** Cell lysates were centrifuged at 20,000  $\times g$  for 4 min at 4 °C, and the resulting supernatant was ultracentrifuged at 95,000  $\times g$  for 10 min at 4 °C. The supernatant was incubated with calf intestinal alkaline phosphatase (20 units) for 60 min at 37 °C in a buffer designed to support alkaline phosphatase activity (100 mM NaCl, 10 mM Tris, 10 mM MgCl<sub>2</sub>, pH 8.2) or a buffer designed to inhibit alkaline phosphatase activity (100 mM NaCl, 10 mM Tris, 10 mM EDTA, pH 4.3).

**Immunoprecipitation and Immunoblot:** Cells were washed with ice-cold phosphate-buffered saline and lysed in radioimmune precipitation assay buffer (50 mM Tris, 150 mM NaCl, 11 mM EDTA, 0.25% deoxycholate, 1% Nonidet P-40, 10% glycerol, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM Na<sub>4</sub> BAPTA, 1 mM dithiothreitol, protease inhibitors (Sigma, catalog no. P8340), phosphatase inhibitors (Calbiochem, catalog nos. 524624 and 524625), pH 8.0). The lysate was centrifuged at 20,000  $\times g$  for 4 min, and the resulting supernatant was ultracentrifuged at 95,000  $\times g$  for 10 min. Kv1.2 was immunoprecipitated from the ultracentrifuge supernatant using anti-Kv1.2p antibody. Bound proteins were eluted from the beads and then separated by SDS-PAGE. Western blotting detection of Kv1.2 was done with anti-Kv1.2 monoclonal antibody. Blots were imaged and quantified with the Odyssey infrared imaging system (Li-Cor, Lincoln NE).

**Electrophysiology:** HEK-K cells were grown on poly-D-lysine-coated 35-mm tissue culture dishes as described above, but were not treated with sodium azide. Kv1.2 ionic current was measured using a whole-cell voltage clamp. The pipette solution contained 120 mM KCl, 3.69 mM CaCl<sub>2</sub> (60 nM free Ca<sup>2+</sup>), 94 μM MgCl<sub>2</sub> (100 nM free Mg<sup>2+</sup>), 5 mM BAPTA, 5 mM EDTA, 5 mM NaHEPES, and 5 mM glucose, pH 7.1; the bath saline contained 100 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.8 mM MgCl<sub>2</sub>, 23 mM glucose, and 5 mM NaHEPES, pH 7.4. The experimental chamber was maintained at 35 °C. Maximum series resistance error was calculated to be <4% and was not compensated. Currents were evoked with a family of 75-ms voltage steps in 10-mV increments from -70 to +50 mV. Recordings were made immediately after break-in to limit spontaneous current rundown. Data collection was done with an Axopatch 200D amplifier interfaced via a Digi-Data 1322A D/A converter to a Windows-based computer running the pCLAMP data acquisition program Clampex (Molecular Devices, Sunnyvale, CA). Data analysis was performed with the pCLAMP data analysis program Clampfit (Molecular Devices) and with Origin (Microcal, Northampton, MA).

**Immunofluorescence:** Immunofluorescence imaging was performed as described previously (Nesti, Everill et al. 2004). Images were acquired with the DeltaVision deconvolution restoration microscopy system (Applied Precision, Issaquah, WA).

**Mass Spectrometry:** A Coomassie-stained band corresponding to Kv1.2 was excised, diced, and subjected to in-gel digestion with sequencing grade modified trypsin (6 ng/μl; Promega, Madison, WI) in 50 mM ammonium bicarbonate overnight at 37 °C. Peptides were extracted with 50% acetonitrile (MeCN), 2.5% formic acid (FA) and then dried.



Peptides were then resuspended in 2.5% MeCN, 2.5% FA and loaded using a Micro-AS autosampler (Thermo Electron, Waltham, MA) onto a 100- $\mu$ m inner diameter fused silica microcapillary column prepared in-house and packed with 12 cm of reverse phase MagicC18 material (5  $\mu$ m, 200 Å; Michrom Bioresources, Inc., Auburn, CA). Using a Surveyor MS HPLC Pump Plus (Thermo Electron), peptides were eluted using a 5-35% MeCN (0.15% FA) gradient over 50 min, after a 14-min isocratic loading at 2.5% MeCN, 0.15% FA. The HPLC solvents used to achieve separation were 2.5% MeCN, 0.15% FA (solvent A) and 97.5% MeCN, 0.15% FA (solvent B). Mass spectra were acquired in an LTQ-XL linear ion trap mass spectrometer (Thermo Electron) over the entire run using 10 MS/MS scans following each survey scan. Instrument control was performed using the Xcalibur software package (version 2.0, SR2, Thermo Electron). Raw data were searched against a Kvl.2 sequence data base using Sequest software (version 27, revision 12, Thermo Electron) with no enzyme specificity and a 2-Da mass tolerance. Cysteine residues were required to have a static increase in 71 Da for acrylamide adduction. Differential modifications of 80 Da for serine, threonine, and tyrosine residues, as well as 16 Da for methionine residues, were permitted, and phospho-specific neutral loss increases to XCorr scoring were turned off. After initially identifying each phospho-tryptic peptide corresponding to serines 440 and 449, targeted MS/MS analyses on the corresponding  $m/z$  values were performed, confirming the initial results.

**Statistical Analysis:** Descriptive statistics are provided in figures as bar graphs indicating the sample mean, with *error bars* indicating S.E. Detection of statistical difference between two independent measurements was by a one-way  $t$  test. Comparison

of percent changes between pairs of independent measurements was by a two-way analysis of variance. Sample populations were considered to be significant at  $p \leq 0.05$ .

## 2.4. Results

### **cAMP enhances surface expression of Kv1.2**

Stimulation of  $\beta_2$ -adrenergic receptors with isoproterenol in *Xenopus* oocytes augments the ionic current of Kv1.2 (Huang, Morielli et al. 1994). We hypothesized that trafficking exists as a possible mechanism for this process. Flow cytometry was used to analyze changes in Kv1.2 surface levels. The basal levels of the channel were measured with cells treated with an antibody directed against an external loop of Kv1.2 (anti-Kv1.2e), as compared with a control group of cells treated with IgG. A representative histogram shift depicting increased fluorescence between the IgG control and the cells labeled with anti-Kv1.2e antibody is shown in Fig. 1A. The *inset* shows the population of intact cells selected to generate the histogram. Endogenous  $\beta_2$ -adrenergic receptors activated with the norepinephrine analog isoproterenol (10  $\mu\text{M}$ , 10 min) caused a significant increase in channel density at the cell surface ( $n = 12$ ,  $p \leq 0.001$ ) (Fig. 1B). This response was blocked by pretreatment with propranolol (10  $\mu\text{M}$ , 30 min;  $p \geq 0.1$ ), a  $\beta$ -adrenergic receptor antagonist. Thus, not only does  $\beta_2$ -adrenergic stimulation cause an increase in Kv1.2 ionic current as reported previously, it also causes a significant increase in the quantity of channels present at the surface of the cell.

Isoproterenol stimulates a signaling cascade that includes the activation of adenylate cyclase and cAMP, and therefore, we wanted to confirm that this pathway was involved. Increasing concentrations of forskolin, an activator of adenylate cyclase, significantly raised levels of Kv1.2 surface channel ( $n = 5, p \leq 0.001$ ) (Fig. 2A). A time course experiment revealed that the forskolin-induced change occurred rapidly and decayed over 1 h ( $n = 5, p \leq 0.01$ ) (Fig. 2B). Application of the cell-permeable cAMP analog chlorophenylthio-cAMP (250  $\mu\text{M}$ , 30 min) displayed a similar response to forskolin (10  $\mu\text{M}$ , 10 min), suggesting that cAMP is the main downstream effector ( $n = 12, p < 0.0001$ ) (Fig. 2C). To confirm this idea, phosphodiesterase (PDE) inhibitors were used to inhibit the degradation of cAMP, thus increasing the intracellular concentration of the second messenger. The general PDE inhibitor, isobutylmethylxanthine (100  $\mu\text{M}$ , 30 min), caused a significant increase in Kv1.2 surface levels ( $n = 18, p < 0.0001$ ) (Fig. 2D). Because isobutylmethylxanthine can inhibit both cAMP- and cGMP-specific PDEs, we decided to test a cAMP-specific PDE inhibitor. Rolipram (10  $\mu\text{M}$ , 3 min), which inhibits PDE4, also increased Kv1.2 surface levels ( $n = 6, p < 0.0001$ ). Therefore, elevating cAMP increases Kv1.2 expression on the cell surface.

### **cAMP affects Kv1.2 surface levels through PKA-independent and PKA-dependent mechanisms**

Because increasing cAMP levels enhanced the amount of Kv1.2 on the cell surface, we hypothesized that these effects were mediated by the main cAMP downstream effector, PKA. To investigate this, we treated cells with a panel of PKA

inhibitors, including myristoylated PKA inhibitor peptide (1  $\mu\text{M}$ , 10 min), KT5720 (5  $\mu\text{M}$ , 30 min), and H89 (10  $\mu\text{M}$ , 30 min). These inhibitors did not prevent either forskolin or chlorophenylthio-cAMP from increasing Kv1.2 surface levels ( $n = 12$ ,  $p \leq 0.01$ ) (Fig. 3, A-C). This suggests that cAMP can enhance surface levels independent of PKA. Interestingly, PKA does appear to have a role because application of the PKA inhibitors alone caused a significant increase in Kv1.2 surface levels ( $p \leq 0.01$ ). Therefore, we sought to confirm the existence of basal PKA activity in unstimulated cells. To that end, we inhibited adenylate cyclase with SQ22536 (500  $\mu\text{M}$ , 30 min), which resulted in a similar increase in Kv1.2 surface levels ( $n = 25$ ,  $p \leq 0.0001$ ). Furthermore, we evaluated the level of substrates phosphorylated by PKA in cell lysates with an antibody directed against a phosphorylated PKA consensus sequence (RXX(S/T)). Forskolin cause a significant increase in the amount of phosphorylated protein ( $n = 2$ ,  $p \leq 0.01$ ). Conversely, both the adenylate cyclase inhibitor, SQ22536, and the PKA inhibitor, H89, decreased phosphorylation ( $n = 3$ ,  $p \leq 0.05$ ) (Fig. 3E). This suggests that the mechanism for cAMP regulation of Kv1.2 surface levels can occur in both PKA-dependent and PKA-independent manners.

### **cAMP and PKA modulate Kv1.2 function**

We next asked whether the increased surface expression of Kv1.2 caused by forskolin or inhibitors of PKA was correlated with an increase in Kv1.2 ionic current. Forskolin induced a significant increase in Kv1.2 ionic current relative to the control;  $6.4 \pm 1.0$  nA *versus*  $3.3 \pm 1.2$  nA ( $n = 9$ ,  $p < 0.01$ ) (Fig. 4A). KT5720 also caused a similar

increase in Kv1.2 ionic current relative to the control;  $12.6 \pm 0.9$  nA *versus*  $5.8 \pm 3.5$  nA ( $n = 5$ ,  $p < 0.01$ ) (Fig. 4B). Half-activation voltage was determined from tail current activation curves fitted by a Boltzmann function. The half-activation voltage was  $-10.3 \pm 1.4$  mV in control cells and  $-11.7 \pm 3.3$  mV in forskolin-treated cells. These values are not significantly different ( $p = 0.28$ ). Therefore, the increase in ionic current caused by forskolin is not attributable to a shift in voltage dependence of Kv1.2 activation. Interestingly, KT5720 did produce a significant depolarizing shift in Kv1.2 voltage dependence ( $-6.6 \pm 0.7$  mV,  $p < 0.01$ ). However, because it is a positive shift, it cannot underlie the KT5720-induced increase in Kv1.2 steady-state ionic current. These findings indicate that the additional Kv1.2 channels in the plasma membrane upon treatment with forskolin or with PKA inhibitors are functional and thus capable of affecting membrane excitability.

**Serines 440 and 449 are phosphorylated in forskolin-treated cells, but are not responsible for the forskolin-mediated increase in surface Kv1.2**

In the course of the preceding experiments, we observed that forskolin produced a shift in the electrophoretic mobility of Kv1.2. Loss of the higher molecular mass band after treatment of cell lysates with alkaline phosphatase indicates that this shift is caused by channel phosphorylation ( $n = 2$ ) (Fig. 5A). Because forskolin caused a PKA-independent increase in Kv1.2 surface levels, we hypothesized that this shift was caused by a PKA-independent phosphorylation of Kv1.2. Moreover, the PKA inhibitor, H89 (10  $\mu$ M, 30 min) (Fig. 5B;  $n = 2$ ), had no effect on the forskolin-induced band shift.

Interestingly, this suggests that PKA is not the kinase involved and indicates that forskolin signals through a different kinase/phosphatase pathway to elicit phosphorylation of Kv1.2.

We next used mass spectrometry to identify the phosphorylation sites responsible for the shift in Kv1.2 mobility. Kv1.2 was immunoprecipitated from forskolin-stimulated HEK-K cells and then resolved by SDS-PAGE. Coomassie-stained bands of Kv1.2 were excised, diced, and digested in-gel with trypsin. Extracted peptides were subjected to liquid chromatography MS/MS and data base search analysis identified two unique phospho-tryptic peptides harboring phosphoserine 440 and phosphoserine 449 (Fig. 6A). To determine whether phosphorylation at these sites causes the mobility shift seen in Fig. 5A, we introduced the serine-to-alanine mutations S440A, S449A, and S440/449A. The forskolin-induced doublet was not observed in any of these mutant channels, confirming that these sites are critical for forskolin-induced phosphorylation of Kv1.2 (Fig. 6B).

Given that Ser-440 and Ser-449 are vital to the forskolin-induced band shift, we evaluated their roles in channel surface expression. To our surprise, these point mutations had no effect on the forskolin-mediated increase in Kv1.2 surface levels ( $n = 18, p \leq 0.01$ ) (Fig. 6C). The *inset* to Fig. 6C depicts the percent change induced by forskolin, normalized to saline control. Therefore, the forskolin-induced shift in electrophoretic mobility and the increase in surface channel levels are not causally related. Interestingly, the serine-to-alanine mutations caused a significant decrease in the unstimulated Kv1.2 surface levels ( $p \leq 0.01$ ).

### **Thr-46 is necessary for the cAMP-mediated effects on Kv1.2 surface levels**

Previous work has shown that mutation of the Kv1.2 N-terminal threonine 46 to valine (T46V) produces channels that are resistant to PKA-mediated current increases (Huang, 1994 #14). This suggests that phosphorylation of this site by PKA underlies the biophysical changes associated with current enhancement. Our findings indicate that the cAMP-mediated increase in surface Kv1.2 is PKA-independent. We therefore sought to clarify the role of Thr-46 in Kv1.2 regulation. Introduction of the T46V mutation significantly decreased Kv1.2 surface levels ( $n = 31$ ,  $p < 0.0001$ ) (Fig. 7A). Furthermore, T46V blocked the forskolin-mediated increase ( $p \geq 0.36$ ); thus, channels harboring the T46V mutation were refractory to cAMP-dependent effects on surface channel levels. To investigate the role of phosphorylation at Thr-46, we performed mass spectrometry on WT Kv1.2 derived from forskolin-treated cells. Whereas we cannot definitively conclude that Thr-46 is not phosphorylated, we did not find any peptide where Thr-46 was phosphorylated, even when we performed targeted MS/MS analysis on masses consistent with Thr-46 phospho-tryptic peptides in multiple charge states. Conversely, we did detect tryptic peptides that included Thr-46 in its unphosphorylated state. This suggests that channel modulation does not involve phosphorylation of Kv1.2 at Thr-46.

To determine whether decreased surface expression caused by the T46V mutation results from a change in Kv1.2 protein expression or stability, we evaluated protein levels by immunoblotting. The levels of T46V protein were not lower than those of WT protein (Fig. 7B), suggesting that the T46V mutation alters surface channel levels by a mechanism that does not involve changes in channel protein levels. We also note that

T46V did not alter the forskolin-induced mobility shift, consistent with the idea that the band shift is not involved in stimulus-induced changes in surface levels of Kv1.2.

### **Forskolin-mediated effects on Kv1.2 are independent of Kvβ2**

The auxiliary Kvβ2 subunit binds to Kv1.2 and can serve as a chaperone protein, guiding newly synthesized channels to the cell surface (Shi, Nakahira et al. 1996). This interaction requires an intact T1 domain within Kv1.2, and mutation of Thr-46, which lies within the T1 domain of Kv1.2, disrupts Kvβ2 binding (Gu, Jan et al. 2003). Our finding that the T46V mutation also blocks forskolin effects on surface channels suggests a role for Kvβ2 in this process. We hypothesized that the effects observed with the T46V mutation result from loss of Kvβ2 binding. To test this we first verified that the T46V mutation blocks interaction of Kv1.2 with Kvβ2. The Kvβ2 subunit does not co-immunoprecipitate with Kv1.2 T46V but does interact with WT Kv1.2 (Fig. 8A). We next examined the effect of Kvβ2 on the forskolin-induced increase in surface Kv1.2. The absence of Kvβ2 significantly decreased Kv1.2 surface levels in unstimulated cells; however, it did not prevent the forskolin-induced increase ( $n = 34, p < 0.0001$ ) (Fig. 8B). Because the Kvβ2 subunit has no apparent role in the cAMP-mediated effects on Kv1.2 surface levels, we conclude that the T46V mutation exerts its effects on Kv1.2 through a mechanism independent of Kvβ2.



### **cAMP modulates Kv1.2 trafficking at the plasma membrane**

Elevation of Kv1.2 surface levels can be achieved by increased outward trafficking or by decreased inward trafficking of channels. Possibly, cAMP exerts its effects by regulating trafficking of newly synthesized channels from the Golgi apparatus to the plasma membrane. To test this idea, brefeldin A (BFA) was used to compromise the integrity of the Golgi apparatus (Yoo, Fang et al. 2005). Although BFA treatment (5  $\mu\text{M}$ , 1 h) disrupted the Golgi apparatus (Fig. 9A), it had no effect on the ability of forskolin to increase surface levels of Kv1.2 ( $n = 18$ ,  $p < 0.0001$ ) (Fig. 9B). This indicates that the cAMP-mediated increase in Kv1.2 surface levels does not involve changes in Golgi trafficking of the channel.

Alternatively, cAMP may decrease inward trafficking of Kv1.2 channels from the cell surface. Endocytosis is an important mechanism for the regulation of Kv1.2 (Nesti, Everill et al. 2004). We hypothesized that cAMP inhibits the steady-state endocytotic trafficking of Kv1.2, thereby increasing surface channel levels. Dynamin affects endocytotic trafficking in multiple ways (Herskovits, Burgess et al. 1993) and has a key role in Kv1.2 endocytosis (Nesti, Everill et al. 2004). We therefore targeted this protein to block Kv1.2 inward trafficking. Overexpression of a dominant negative form of dynamin, dynamin K44A, completely blocked the forskolin-mediated increase in Kv1.2 surface levels ( $n = 12$ ,  $p \geq 0.19$ ) (Fig. 9C). To further explore this idea, we targeted cortactin, another protein with a central role in Kv1.2 trafficking (Hattan, Nesti et al. 2002). We have shown previously that depletion of cortactin with siRNA blocks Kv1.2 inward trafficking (Williams, Markey et al. 2007). Cortactin knockdown completely inhibited the

ability of forskolin to modulate surface levels of Kv1.2 ( $n = 8, p \geq 0.08$ ) (Fig. 9, D and E). Thus, both cortactin and dynamin are essential for cAMP-mediated effects on Kv1.2 surface levels. We therefore conclude that cAMP exerts its effects by modulating the inward trafficking of Kv1.2.

## 2.5. Discussion

Altered cAMP levels can result in changes in neuronal plasticity (Chen, Suntres et al. 2007), hormonal secretion (Yang, Fransson et al. 2004), and gene expression (Hosoda, Feussner et al. 1994); diverse feedback loops ensure a regulated balance of cAMP (Bauman, Soughayer et al. 2006). In this study, we have shown dual mechanisms for cAMP modulation of Kv1.2 trafficking. First, we demonstrated that elevation of cAMP caused a significant increase in Kv1.2 surface levels and that this effect is independent of PKA. Next, we showed that decreasing cAMP also caused a significant increase in Kv1.2 surface levels, but that this effect is PKA-dependent. Thus, basal cAMP levels form a homeostatic set point for Kv1.2 surface expression. We propose that through its effects on Kv1.2 function, cAMP homeostasis may act as a buffer for cellular excitability (Fig. 10).

Previous studies in *Xenopus* oocytes show that application of PKA alters the biophysical properties of Kv1.2 to increase the ionic current (Huang, Morielli et al. 1994). Here we showed in HEK293 cells that inhibiting PKA increases Kv1.2 at the cell surface (Fig. 3) and that this increase corresponds to an increase in ionic current (Fig. 4). Thus, it appears that in *Xenopus* oocytes PKA increases Kv1.2 activity, whereas in HEK293 cells PKA decreases its activity. Therefore, depending on the cellular environment, PKA can

affect Kv1.2 function by directly impacting the channel's biophysical properties or by affecting channel trafficking. Indeed, these mechanisms are not necessarily mutually exclusive and may act in concert with PKA-independent effects of cAMP to precisely modulate Kv1.2 function.

Although PKA does have a role in Kv1.2 trafficking, the effect of forskolin on Kv1.2 is independent of PKA. Despite this, we found that mutation of a potential PKA phosphorylation site (T46V) completely eliminates the effect of forskolin on Kv1.2 surface levels. This result may be explained by a phosphorylation-independent role for Thr-46. Minor *et al.* (Minor, Lin et al. 2000) have shown that Thr-46 forms a hydrogen bond involved in maintaining T1 domain structure and that mutation of Thr-46 disrupts the T1 domain. This prevents interaction of the isolated T1 domain of Kv1.2 with the Kv $\beta$ 2 subunit (Gu, Jan et al. 2003). These findings added a new facet to previous research that shows a chaperone-like role for Kv $\beta$ 2 in promoting cell surface expression (Shi, Nakahira et al. 1996) and axonal targeting of Kv1.2 (Campomanes, Carroll et al. 2002). We confirmed that the entire  $\alpha$ -subunit harboring the T46V mutation cannot bind Kv $\beta$ 2 (Fig. 8). This is consistent with our finding that Kv1.2 T46V had significantly reduced surface expression relative to the wild-type channel. However, the mechanism by which T46V blocks the forskolin-induced trafficking of the channel (Fig. 7) does not involve the loss of Kv $\beta$ 2 binding (Fig. 8). Therefore, correct positioning of the T1 domain appears to be required for multiple but distinct types of channel trafficking, including Kv $\beta$ 2-mediated and, as we have reported here, Kv $\beta$ 2-independent and cAMP-dependent trafficking of Kv1.2.

We found no evidence for forskolin-induced phosphorylation of Thr-46; however, forskolin does induce phosphorylation at other sites. Biochemical and mass spectrometry studies suggest that forskolin induces a PKA-independent phosphorylation of Kv1.2 at Ser-440 and Ser-449 (Fig. 6). We found that these sites are critical for Kv1.2 surface expression, but surprisingly they do not appear to be involved in the forskolin-induced increase in surface channel. Nevertheless, phosphorylation at one or both of these sites may be necessary for the channel to reach the cell surface or to prevent channel degradation. In fact, Ser-440 seems to be critical for protein stability, as the S440A mutation visibly decreases protein levels (Fig. 6B). Furthermore, we observed a notable change in the pattern of higher molecular mass Kv1.2 bands in the S449A mutant channel. This may represent altered channel glycosylation, indicating disruption of normal channel maturation. Therefore, these sites are important for channel surface expression, but do not appear to play a direct role in the acute response to forskolin.

Further evidence that altered biosynthetic trafficking is not involved comes from our finding that disruption of the Golgi apparatus with brefeldin A has no effect on the cAMP-dependent, PKA-independent increase in Kv1.2 surface levels. Instead, this effect appears to involve modulation of constitutive channel endocytosis. Previous reports have shown that Kv1.2 surface expression is modulated by endocytosis of the channel protein. This process is dependent on both dynamin (Nesti, Everill et al. 2004) and cortactin (Williams, Markey et al. 2007). We showed that disruption of either dynamin or cortactin blocks the forskolin-induced increase in surface levels (Fig. 9). We conclude that forskolin modulates Kv1.2 surface levels by modulating the constitutive endocytosis of

the channel. Therefore, both positive and negative regulation of Kv1.2 surface expression are governed by modulated channel endocytosis. In this way, Kv1.2 serves to link membrane excitability with a complex array of trafficking signals.

## **2.6. Acknowledgements**

We thank Megan A. Doczi, Lee D. Stirling, Brandon Field, and Jim Kilcoyne for their input during preparation of the manuscript. This work was supported in part by National Institutes of Health Grant 5 P20 RR016435-07 from the Center of Biomedical Research Excellence Program of the National Center of Research Resources, by the Vermont Cancer Center of the Vermont Genetics Network through National Institutes of Health Grant P20 RR16462 from the Institutional Development Award Networks of Biomedical Research Excellence Program of the National Center for Research Resources (to B. A. B.), and by National Institutes of Health Grant R0NS050623 (to A. D. M.).

## **2.7. Abbreviations**

PKA, protein kinase A; BFA, brefeldin A, GFP, green fluorescent protein; WT, wild type; FA, formic acid; HPLC, high pressure liquid chromatography; HEK, human embryonic kidney; MS/MS, tandem mass spectrometry; PDE, phosphodiesterase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; siRNA, small interfering RNA.

## 2.8. Figure Legends

**Figure 1: Kv1.2 surface levels increase in response to isoproterenol.** (A) Histograms represent the distribution of cells labeled with IgG (gray) and the distribution of cells in which surface Kv1.2 was labeled with a fluorescent antibody (black). The inset shows a representative density plot of the cell population used to obtain the histograms. (B) Mean Kv1.2 surface levels in cells treated with 10 $\mu$ M isoproterenol (ISO) are significantly higher than in control (CTR) cells. Pretreatment with 10  $\mu$ M propranolol (PRO) blocks the effect of isoproterenol. (\*\*,  $p < 0.01$  versus control).

**Figure 2: Stimuli that increase cAMP enhance Kv1.2 surface levels.** (A) Kv1.2 surface levels are shown as a function of forskolin concentration. Data points at each concentration were taken 10 min after the application of forskolin. (B) Kv1.2 surface levels are shown as a function of time after the application of 10  $\mu$ M forskolin. (C) Mean Kv1.2 surface levels are shown after treatment with 10  $\mu$ M forskolin (*FSK*) for 10 min or 250  $\mu$ M CPT-cAMP for 30 min, revealing that such treatments significantly increase surface Kv1.2 relative to the control (*CTR*). (D and E) The PDE inhibitors isobutylmethylxanthine (*IBMX*; 100 $\mu$ M) and rolipram (*ROL*; 10 $\mu$ M) significantly increased the surface levels of Kv1.2. (\*\*,  $p < 0.01$  versus control).

**Figure 3. Effects of cAMP and PKA on Kv1.2.** Treatment with the PKA inhibitors

1 $\mu$ M myristoylated PKA inhibitor peptide (*mPKI*; A), 10 $\mu$ M H89 (B), or 5 $\mu$ M KT5720 (*KT*; C) caused a significant elevation of Kv1.2 surface levels. These inhibitors did not block the increase of surface Kv1.2 caused by the addition of 250  $\mu$ M chlorophenylthio-cAMP (A) or 10  $\mu$ M forskolin (*FSK*; B and C). (D) The adenylate cyclase inhibitor SQ22536 (*SQ*; 500 $\mu$ M) also significantly increased Kv1.2 surface levels. (E) An immunoblot of control (*CTR*) cell lysates or lysates from cells treated with forskolin, SQ22536, or H89 was probed with an antibody directed against a phosphorylated PKA consensus sequence (*left*). Equal loading was confirmed by probing with anti-GAPDH antibody. Phosphorylated RXX(S/T) substrate band densities were normalized to GAPDH, averaged over at least two experiments, and are depicted as bar graphs (*right*). \*\*,  $p < 0.01$  and \*,  $p < 0.05$  relative to control; ^,  $p < 0.01$  relative to inhibitor alone.

**Figure 4. Forskolin and KT5720 increase Kv1.2 ionic current.** (A) Kv1.2 ionic current measured in the absence (*left*) or presence (*right*) of forskolin. Currents were evoked by voltage steps from -70 to +50 mV in 10-mV increments and are normalized to the steady-state current at +50 mV in control cells. Current traces for each condition represent the average of nine cells. (B) Kv1.2 ionic current measured in the absence (*left*) or presence (*right*) of KT5720. Currents were evoked by voltage steps from -70 to +50mV in 10-mV increments and are normalized to the steady-state current at +50 mV in control cells. Current traces for each condition represent the average of five cells.

**Figure 5. Forskolin-induced, PKA-independent phosphorylation of Kv1.2. (A)**

Immunoblots of lysates from HEK-K cells treated with forskolin (*FSK*) reveal a forskolin-induced shift in Kv1.2 electrophoretic mobility relative to untreated control cells. This mobility shift was diminished when HEK-K cell lysates were incubated with active alkaline phosphatase (*AP*), but not when they were incubated with inactive alkaline phosphatase. Blots were probed with anti-Kv1.2 monoclonal antibody. (B) Immunoblots of lysates from control (*CTR*) or forskolin-treated HEK-K cells reveal that pretreatment with the PKA inhibitor, H89, did not alter the forskolin-induced band shift. Blots were probed with anti-Kv1.2 monoclonal antibody. Equal loading was confirmed with anti-GAPDH antibody.

**Figure 6. Forskolin-mediated increase in surface Kv1.2 is not dependent on**

**phosphorylation at Ser-440 or Ser-449. (A)** Low energy collision-induced dissociation MS/MS spectra indicate phosphorylation on serine 440 (*left*) and serine 449 (*right*). (B) HEK-M cells were transiently transfected with Kv $\beta$ 2, GFP, and either WTKv1.2 or mutant versions of Kv1.2. The electrophoretic mobility of Kv1.2 was analyzed with anti-Kv1.2 monoclonal antibody in control cells and in cells treated with forskolin. GAPDH was used as a loading control. Note that the forskolin-induced doublet is lost in all three mutant Kv1.2  $\alpha$ -subunits. (C) Surface levels of WT Kv1.2 and mutant Kv1.2 channels were evaluated in the presence or absence of forskolin. (\*\*,  $p < 0.01$ ). The *inset* depicts the percent change induced by forskolin normalized to control. In contrast, all mutations



caused a significant reduction in the Kv1.2 surface levels in unstimulated cells. ( $\wedge$ ,  $p < 0.0001$ ).

**Figure 7. T46V prevents the cAMP-mediated increase in Kv1.2 surface levels. (A)**

HEK-M cells were transiently transfected with Kv $\beta$ 2, GFP, and either WT Kv1.2 or Kv1.2 T46V. The T46V mutation significantly decreased Kv1.2 surface channel levels in unstimulated cells. ( $\wedge$ ,  $p < 0.0001$ ). Forskolin caused a significant increase in WT Kv1.2 surface levels (\*\*,  $p < 0.0001$ ); however, the T46V mutation blocked the forskolin-induced increase in surface Kv1.2 ( $p < 0.36$ ). (B) The immunoblot shows that the T46V mutation did not decrease the amount of Kv1.2 detected by anti-Kv1.2 monoclonal antibody. Equal loading was confirmed with anti-GAPDH antibody. Note that forskolin causes a mobility shift of both the WT and T46V channels.

**Figure 8. Forskolin modulation of Kv1.2 does not require Kv $\beta$ 2. (A)**

Immunoprecipitation (*IP*) of Kv1.2 from lysates of HEK-M cells transfected with a combination of WTKv1.2, Kv1.2 T46V, and Kv $\beta$ 2 as indicated revealed that WT Kv1.2, but not Kv1.2T46V, interacts with Kv $\beta$ 2. Lysates confirm expression of both proteins. (B) HEK-M cells were transfected with combinations of WTKv1.2 (*K*) and Kv $\beta$ 2 ( $\beta$ ) as indicated. Surface levels of Kv1.2 in the presence or absence of Kv $\beta$ 2 indicate that Kv $\beta$ 2 is required for efficient expression of surface Kv1.2. ( $\wedge$ ,  $p < 0.0001$ ). In the absence of Kv $\beta$ 2, forskolin (*FSK*) produces a significant increase in

Kv1.2 surface levels. (\*\*,  $p < 0.0001$ ). The *inset* shows the percent change in Kv1.2 surface levels evoked by forskolin in the presence or absence of Kv $\beta$ 2.

**Figure 9. cAMP mediates trafficking of Kv1.2 at the plasma membrane. (A)**

Treatment with BFA (*right*) disrupted the Golgi apparatus relative to cells treated with carrier solution (MeOH; *left*). The Golgi was labeled with anti-GM130 antibody (*green*) and the actin cytoskeleton was labeled with phalloidin (*red*). *Scale bars*=20 $\mu$ m. (B)

Treatment with BFA caused a significant decrease in Kv1.2 surface levels. ( $\wedge$ ,  $p < 0.0001$ ).

In contrast, BFA pretreatment did not block the ability of forskolin (*FSK*) to evoke a significant increase in Kv1.2 surface levels. (\*\*,  $p < 0.0001$ ).

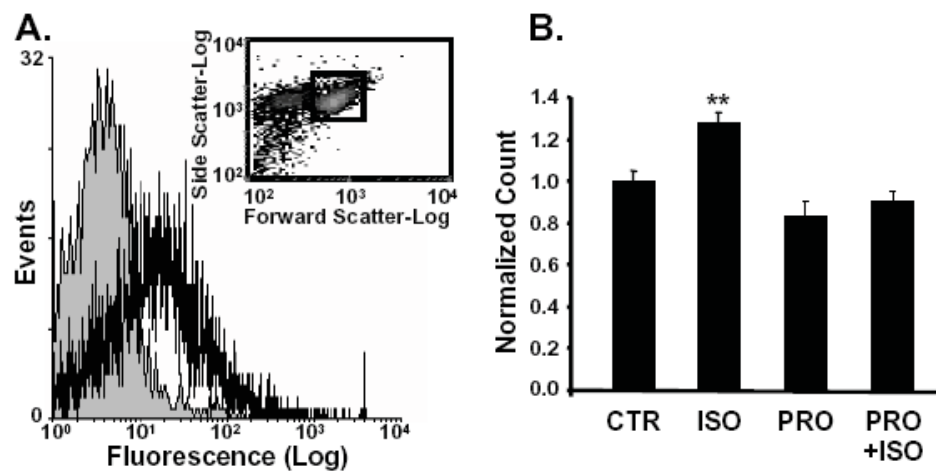
(C) Transfection of HEK-K cells with dominant negative dynamin (*DYNK44A*) blocked the forskolin-induced surface channel increase relative to the control. ( $p > 0.19$ ).

(D) Depletion of cortactin blocked forskolin modulation of Kv1.2 surface levels. Transfection of cells with non-targeting siRNA (*NT*) had no effect on the ability of forskolin to increase Kv1.2 surface levels. (\*\*,  $p < 0.001$ ). In contrast, transfection with siRNA targeted against cortactin (*CORT*)

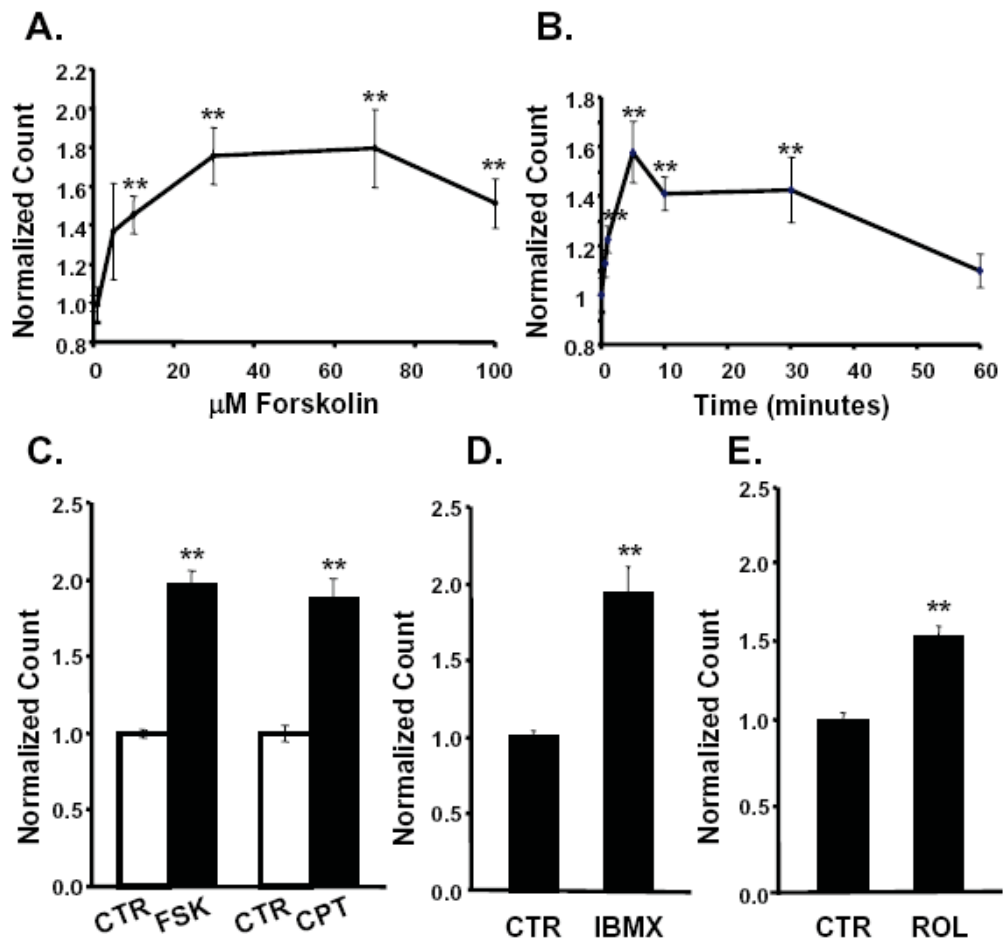
completely blocked the forskolin-induced increase in Kv1.2 surface levels ( $p > 0.08$ ).

(E) Immunoblots confirm cortactin depletion by siRNA. Immunoblots of lysates from HEK-K cells transfected with either non-targeting siRNA or siRNA directed against cortactin were probed with anti-cortactin and anti-GAPDH antibodies. The bar graph depicts density of the anti-cortactin immunopositive band normalized to GAPDH from three independent experiments.

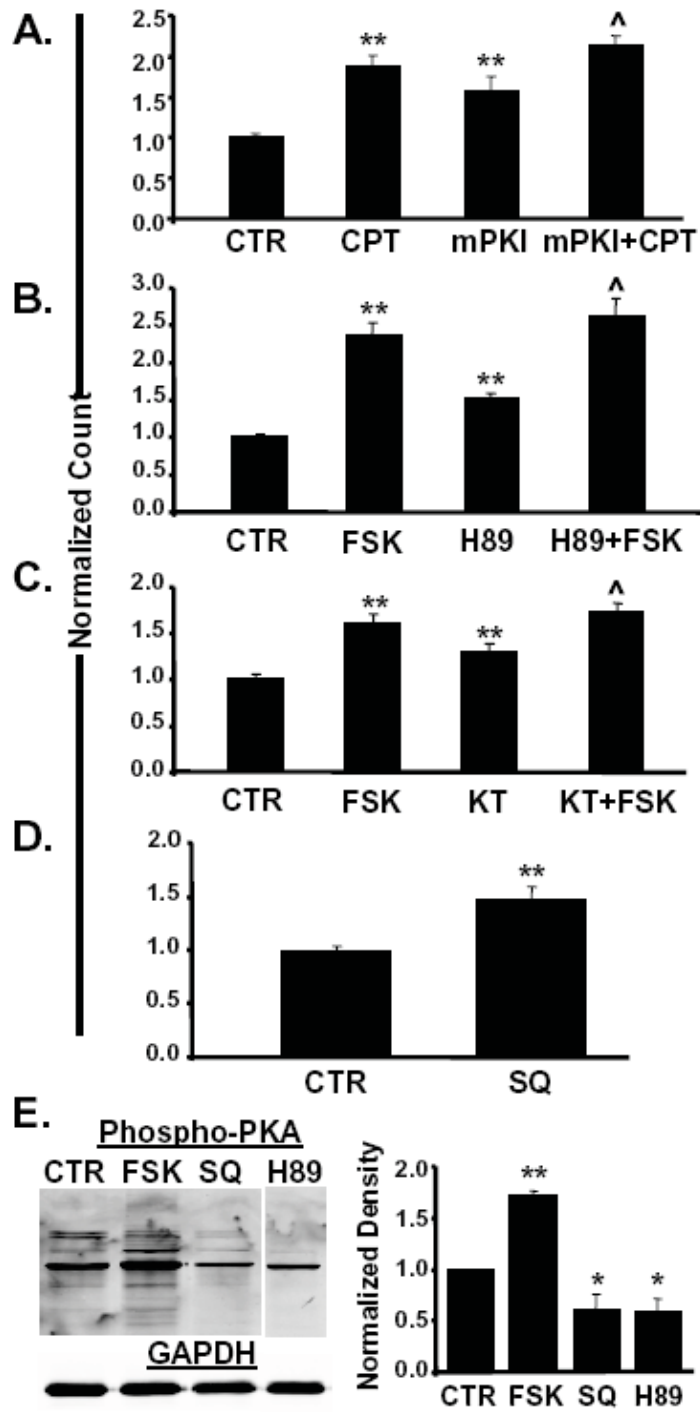
**Figure 10. Proposed model for Kv1.2 regulation by cAMP.** A continuous gradient of cAMP is proposed to produce a discontinuous modulation of Kv1.2 surface expression, resulting in a narrow window of increased electrical excitability in the cell.



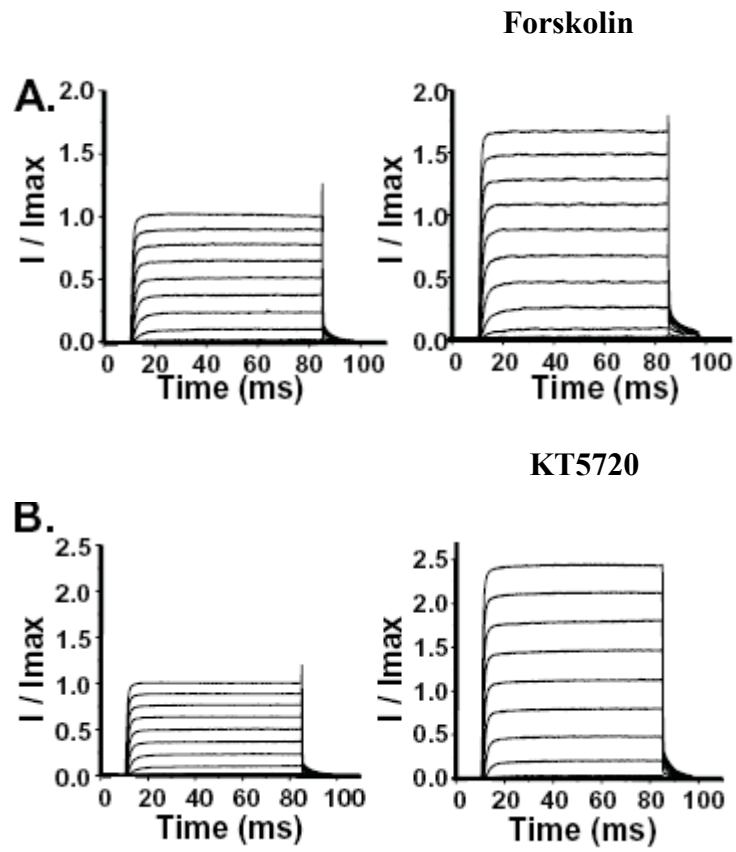
**Figure 1:** Kv1.2 surface levels increase in response to isoproterenol.



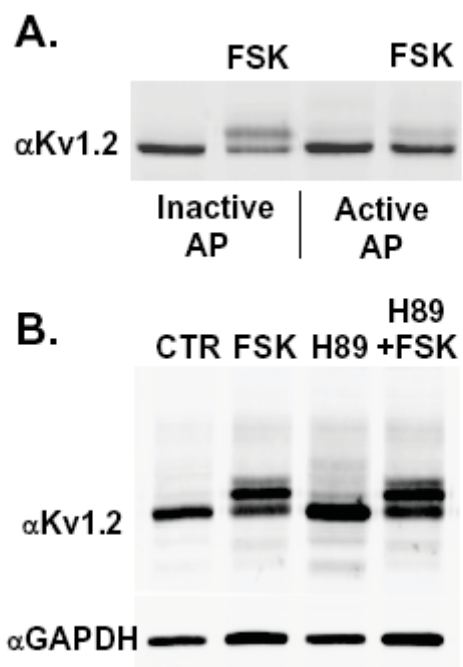
**Figure 2:** Stimuli that increase cAMP enhance Kv1.2 surface levels.



**Figure 3.** Effects of cAMP and PKA on Kv1.2.

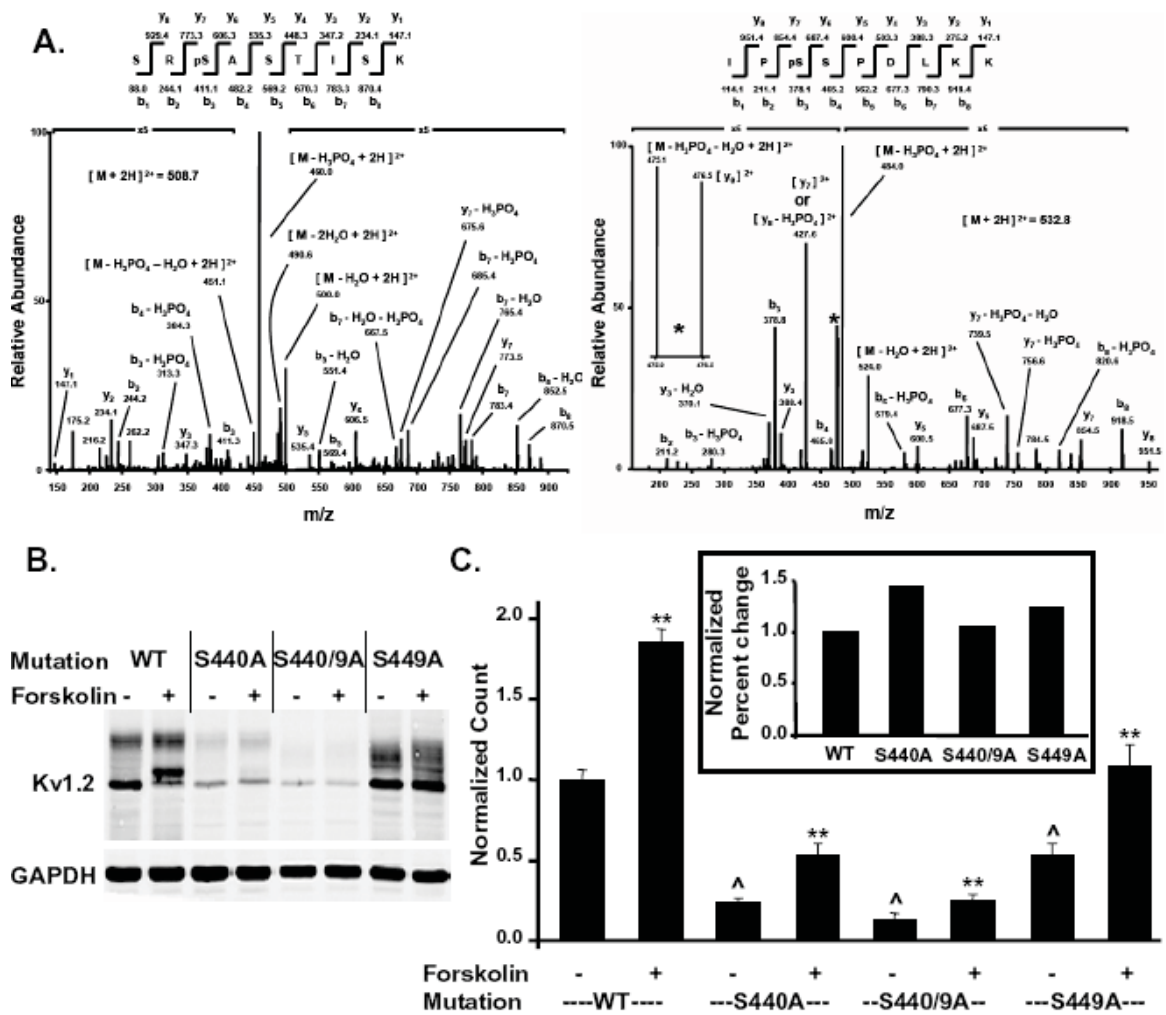


**Figure 4.** Forskolin and KT5720 increase Kv1.2 ionic current.

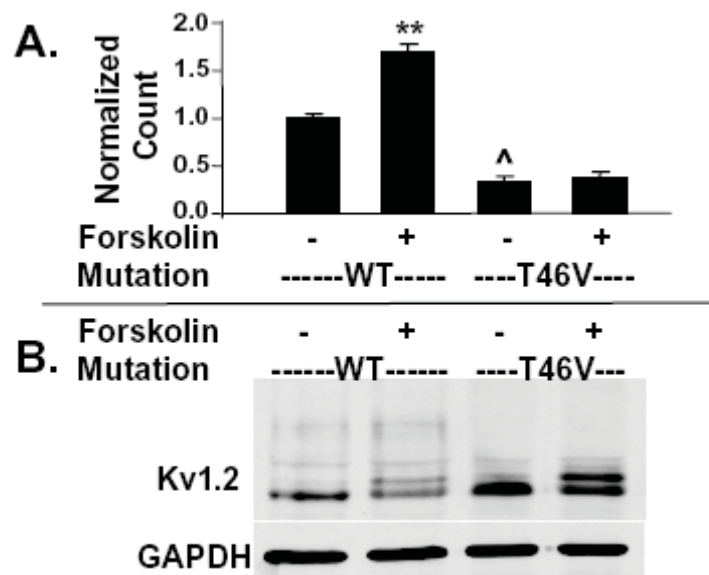


**Figure 5.** Forskolin-induced, PKA-independent phosphorylation of Kv1.2.

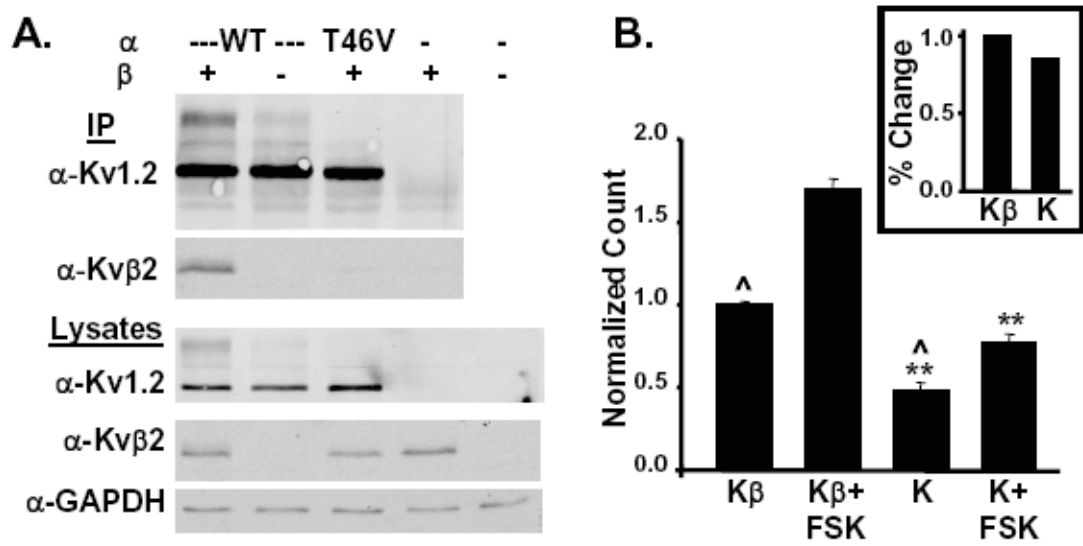




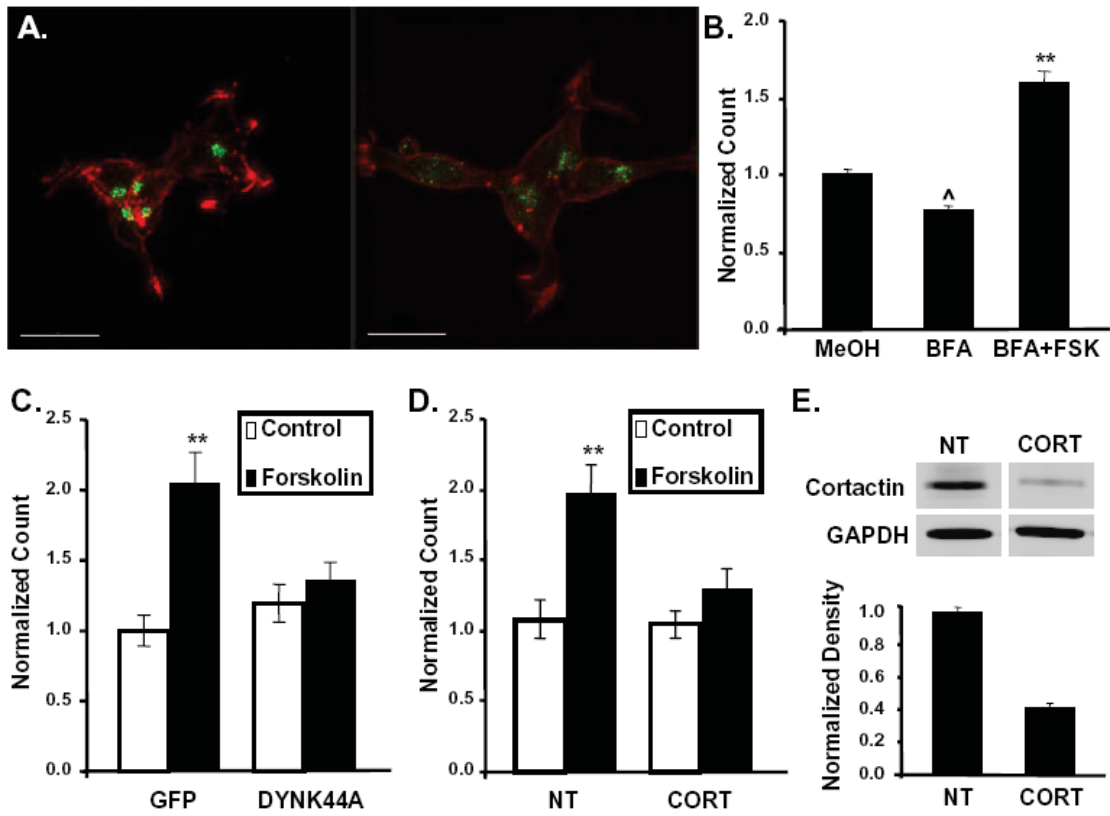
**Figure 6.** Forskolin-mediated increase in surface Kv1.2 is not dependent on phosphorylation at Ser-440 or Ser-449.



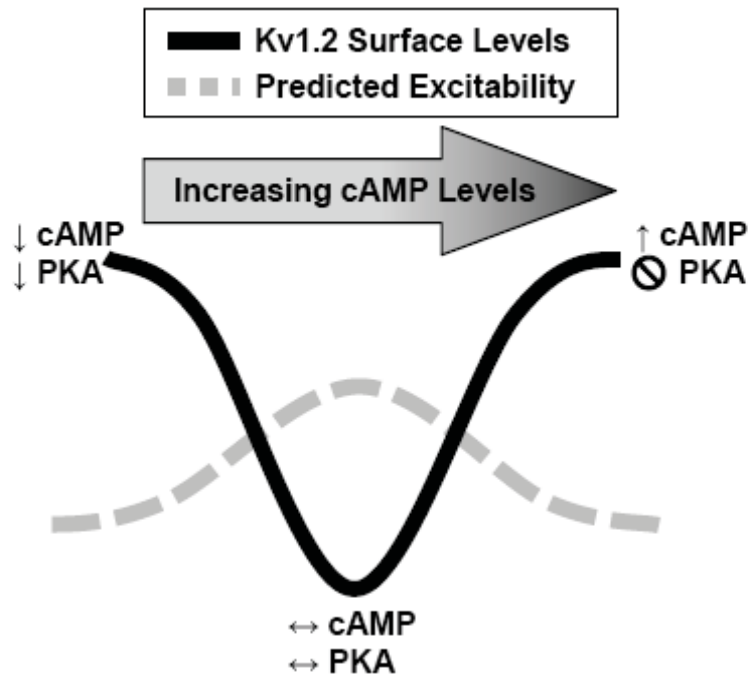
**Figure 7.** T46V prevents the cAMP-mediated increase in Kv1.2 surface levels.



**Figure 8.** Forskolin modulation of Kv1.2 does not require Kv $\beta$ 2.



**Figure 9.** cAMP mediates trafficking of Kv1.2 at the plasma membrane.



**Figure 10.** Proposed model for Kv1.2 regulation by cAMP.

**CHAPTER 3: A ROLE FOR THE PROTEIN ARGININE  
METHYLTRANSFERASE, PRMT8: REGULATION OF VOLTAGE-GATED  
POTASSIUM CHANNEL TRAFFICKING**

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Running Head: PRMT8 regulation of Kv1.2 trafficking

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### 3.1. Abstract

Protein arginine methyltransferases (PRMTs) are emerging as an important new class of regulatory proteins. Biological functions of several PRMT family members have been described, however the role of other PRMT isoforms is not well understood. In this study, we identify a role for PRMT8 in protein trafficking. Expression of PRMT8 in HEK293 cells caused an increase in the surface levels of the voltage-gated potassium channel, Kv1.2. Endocytosis and recycling are involved in maintaining the homeostasis of Kv1.2 surface expression, however, PRMT8 did not affect these processes. Instead, we found that PRMT8 modulates biosynthetic trafficking of Kv1.2 by facilitating ER exit. The PRMT8-mediated increase in Kv channel surface expression was greater in a mutant channel that preferentially localizes to the ER (Kv1.2N207Q) compared to Kv1.2WT channels. In contrast, the PRMT8-mediated change in surface expression was less pronounced in a closely related channel preferentially localized to the Golgi apparatus (Kv1.3). Moreover, PRMT8 promoted surface expression of the high mannose glycosylated form of Kv1.2, characteristic of immature, ER-localized channels. PRMT8 also enhanced Kv1.2 total protein levels, most likely by decreasing the amount of channel protein available for ER-associated degradation (ERAD). Collectively, these findings identify a novel role for the protein arginine methyltransferase, PRMT8, as a regulator of biosynthetic protein trafficking.

### 3.2. Introduction

Protein arginine methyltransferases (PRMTs) are a family of enzymes that catalyze the addition of methyl groups to arginine side chains (Bedford 2007). There are currently at least ten known human PRMTs, all of which have a conserved binding domain for S-adenosylmethionine, the methyl donor (Lee and Stallcup 2009). PRMT isoforms diverge, however, in their substrate specificity, binding partners and localization (Pahlich, Zakaryan et al. 2006). PRMT8 is a recently identified methyltransferase primarily localized to neurons in the hippocampus and cerebellum (Lee, Sayegh et al. 2005; Taneda, Miyata et al. 2007). PRMT8 is unique amongst its family members not only because of its characteristic neuronal expression, but because it harbors an N-terminal myristoylation site that can localize it to the plasma membrane (Sayegh, Webb et al. 2007). Despite what is known about its specific localization, the biological role of PRMT8 is poorly understood.

Collectively, physiological consequences of PRMT family proteins include transcriptional regulation, RNA processing, altered signal transduction and DNA repair (Bedford and Richard 2005). These PRMT-mediated effects are generally a result of arginine methylation-induced changes in substrate function. Arginine methylation is a covalent modification that results in a bulkier side group, altered hydrogen bonding and increased hydrophobicity (Boisvert, Chenard et al. 2005). Interestingly, there are examples of methylation-independent functions of PRMTs (Pahlich, Zakaryan et al.



2008; Perreault, Gascon et al. 2009). These depend on direct protein binding to specific domains within PRMTs.

A recent proteomic study in Jurkat cells identified a number of PRMT8 binding partners, offering critical insights to the potential function of this protein (Pahlich, Zakaryan et al. 2008). PRMT1, which is highly homologous to PRMT8, was among the proteins identified. These two methyltransferases can form dimers and it has been suggested that PRMT8 may localize PRMT1 activity to the plasma membrane (Lee, Sayegh et al. 2005). The RNA-binding protein, Ewing's sarcoma (EWS) also interacts with PRMT8 and interestingly, this physical association is independent of the methylation state of EWS (Pahlich, Zakaryan et al. 2008). In addition, SH3-domain containing proteins, such as the tyrosine kinase FYN, can bind proline rich domains within the N-terminus of PRMT8 (Sayegh, Webb et al. 2007). PRMT8 also interacts with actin (Pahlich, Zakaryan et al. 2008). Therefore, in addition to its methyltransferase activity, PRMT8 may act as a scaffold linking its binding partners with actin dependent processes.

Actin dynamics and tyrosine phosphorylation converge to regulate the voltage-gated potassium channel Kv1.2. Tyrosine phosphorylation of Kv1.2 evokes channel endocytosis from the plasma membrane, a process that requires direct interaction of Kv1.2 with the actin regulating proteins RhoA and cortactin (Nesti, Everill et al. 2004; Williams, Markey et al. 2007; Stirling, Williams et al. 2009). Since Kv1.2 is expressed in the same brain regions as PRMT8 (Sheng, Tsaur et al. 1994), we utilized it as a model for evaluating the role of PRMT8 in protein trafficking. Here, we show that PRMT8

regulates Kv1.2 surface levels by facilitating exit of the channel from the endoplasmic reticulum. These findings identify regulation of protein trafficking as a new role for the protein arginine methyltransferase, PRMT8.

### 3.3. Results

#### **PRMT8 increases the surface expression of Kv1.2**

To investigate the potential role of PRMT8 in protein trafficking, we evaluated its effects on the voltage-gated potassium channel Kv1.2. Trafficking of this ion channel is a major mechanism for its regulation. Using flow cytometry, we assessed the levels of Kv1.2 at the cell surface in HEK-K cells transiently transfected with pRK5-PRMT8 or with pRK5 vector alone. Surface Kv1.2 levels in cells expressing PRMT8 were significantly greater relative to control cells (Figure 1A). This result suggests that PRMT8 may be involved in the regulation of Kv1.2 trafficking.

PRMT8 is highly homologous to PRMT1. PRMT8 and PRMT1 have similar substrate specificities, suggesting that PRMT1 may also modulate Kv1.2. We found that surface expression of Kv1.2 was unaffected by PRMT1, despite being significantly increased by PRMT8 in the same experiment (Figure 1B). Equal expression of PRMT1 and PRMT8 was confirmed by immunoblot analysis of lysates from cells transfected with PRMT1-myc or PRMT8-myc (Figure 1C). Thus, despite the similarities between these two methyltransferases, the increase in Kv1.2 surface levels is specific to PRMT8.

The highly specialized N-terminus of PRMT8, not present in PRMT1, contains a myristoylation motif. Myristoylation of PRMT8 not only confers plasma membrane

localization (Lee, Sayegh et al. 2005), but also may affect its enzymatic activity and scaffolding functions (Sayegh, Webb et al. 2007). To test the role of PRMT8 myristoylation in modulating Kv1.2, we used a mutant form which lacks the myristoylation site (PRMT8G2A). Expression of PRMT8G2A did not increase Kv1.2 surface levels while the non-mutant PRMT8 caused a significant increase in the same experiment (Figure 1D). Immunoblot analysis confirmed equal expression of both proteins (Figure 1E). Myristoylation of PRMT8 therefore appears to be an important determinant of its ability to regulate Kv1.2 trafficking.

### **PRMT8 does not affect plasma membrane trafficking of Kv1.2**

Surface Kv1.2 levels are maintained by a balance of constitutive endocytosis and recycling at the plasma membrane (Stirling, Williams et al. 2009). Previous studies have shown that inhibiting steady-state channel internalization causes an increase in channel surface levels (Stirling, Williams et al. 2009). We therefore hypothesized that PRMT8 may increase Kv1.2 surface levels by affecting channel trafficking at the cell surface. Channel endocytosis was determined by measuring internalization of a fluorescently labeled Kv1.2-e antibody applied to live HEK-K cells. Non-internalized antibody was removed by acid wash (Stirling, Williams et al. 2009). The total remaining fluorescence represents channels internalized from the cell surface (Nesti, Everill et al. 2004; Stirling, Williams et al. 2009). Channel internalization was not blocked in cells expressing PRMT8 (Figure 2A). In the same experiment, PRMT8 caused a significant

increase in surface channel levels (Figure 2A). This suggests that PRMT8 does not elevate Kv1.2 surface levels by inhibiting steady-state channel endocytosis.

Internalized channels can be recycled back to the cell surface or targeted to degradation pathways (Morrison, Moore et al. 1996; Letierrier, Bonnard et al. 2004), thus determining the lifetime of surface Kv1.2 channel protein. Although PRMT8 did not block channel internalization, it may limit transfer of internalized channel to a degradation pathway. This would increase surface Kv1.2 levels by increasing the amount of internalized channel available to recycle back to the plasma membrane. To determine whether PRMT8 affects surface channel degradation, surface Kv1.2 was pulse-labeled with biotin and loss of biotinylated channel protein was analyzed over time. The relative loss of surface Kv1.2 at time points up to 10 hours was not affected by expression of PRMT8 (Figure 2B). Collectively, these findings indicate that PRMT8 does not affect Kv1.2 surface levels by influencing steady-state trafficking or degradation of plasma membrane localized Kv1.2.

### **PRMT8 promotes exit of Kv1.2 from the Endoplasmic Reticulum**

Since PRMT8 does not affect Kv1.2 trafficking at the plasma membrane, we asked if it increases Kv1.2 surface expression by altering biosynthetic trafficking of the channel. Kv1.2 glycosylation has been shown to affect biosynthetic trafficking and channel surface expression. During its biosynthesis, Kv1.2 undergoes multiple stages of glycosylation. The addition of a high mannose sugar occurs in the endoplasmic reticulum (ER). In the Golgi apparatus, mannose sugars are trimmed and replaced by the complex,

branched glycosylation characteristic of mature channel (Nagaya and Papazian 1997). These forms of glycosylated Kv1.2 are indicated by their distinct electrophoretic mobilities (Manganas and Trimmer 2000). The high mannose glycosylated form of Kv1.2 characteristic of channel localized to the ER runs as at an apparent molecular weight of approximately 65KDa (Manganas and Trimmer 2000). The mature glycosylated form runs at higher apparent molecular weights, ranging from 80-86KDa (Manganas and Trimmer 2000; Watanabe, Zhu et al. 2007; Connors, Ballif et al. 2008). Because Kv1.2 electrophoretic mobility indicates the type of channel glycosylation, it can be used to evaluate the channel's progression through the biosynthetic pathway. We evaluated the glycosylation pattern of Kv1.2 channels at the cell surface in PRMT8 expressing cells. Surface proteins were biotinylated, collected onto Neutravidin beads, and the glycosylation pattern of biotinylated Kv1.2 was assessed by immunoblot. As expected, expression of PRMT8 increased the amount of biotinylated Kv1.2, reflecting an increase in channel surface expression (Figure 3A). Interestingly, in cells expressing PRMT8, the high mannose form of Kv1.2 appeared to be considerably more pronounced at the cell surface (Figure 3A, left). This was confirmed by determining the ratio of signal density of the high mannose to the mature glycosylated surface channel (Figure 3B). PRMT8 also increased the total amount of Kv1.2 present in the cell (Figure 3A, right); analysis of eight independent experiments showed a significant  $128 \pm 32$  percent increase ( $p < 0.01$ ). To determine whether the altered glycosylation pattern in PRMT8 expressing cells was due to this increase in total protein levels, we transfected increasing amounts of plasmid encoding Kv1.2 and measured the ratio of surface high mannose to surface mature

glycosylated channel. Both surface (Figure 3A, left) and total (Figure 3A, right) Kv1.2 levels increased as a function of transfected plasmid concentration. In contrast, the ratio of surface high mannose to surface mature glycosylated channel was unaffected by increasing Kv1.2 protein levels (Figure 3B). Thus, PRMT8 selectively alters the type of glycosylated Kv1.2 that reaches the cell surface, suggesting a role for PRMT8 in the biosynthetic trafficking of Kv1.2.

The increased surface expression of the high mannose form of Kv1.2 could be explained by a direct effect of PRMT8 on the process of channel glycosylation. Alternatively, PRMT8 may promote high mannose glycosylated Kv1.2 surface expression by facilitating the transport of ER-localized Kv1.2 directly to the cell surface. To test both of these ideas, we utilized a mutant form of Kv1.2, Kv1.2N207Q. This mutant channel lacks the only glycosylation site within the channel and is also preferentially localized to the ER (Watanabe, Zhu et al. 2007). Consistent with previous studies, surface expression of Kv1.2N207Q was significantly lower than Kv1.2WT (Figure 4A, left). If PRMT8 affects Kv1.2 surface expression by directly altering channel glycosylation, the non-glycosylated Kv1.2N207Q channels should be resistant to this effect. In contrast, if PRMT8 acts by promoting exit of Kv1.2 from the ER, the ER-retained Kv1.2N207Q channels should therefore exhibit a greater PRMT8 mediated increase in surface expression relative to Kv1.2WT channels. Strikingly, not only did PRMT8 increase the surface levels of Kv1.2N207Q channels, the increase was significantly greater than that elicited with Kv1.2WT channels (Figure 4A). Importantly, this enhanced PRMT8 effect is not due to a preferential increase of Kv1.2N207Q total

protein levels; PRMT8 increased the total protein levels of Kv1.2N207Q, however, it did so to the same extent as for Kv1.2WT (Figure 4B). Thus, PRMT8 does not directly affect channel surface levels by changing channel glycosylation or by changing protein expression levels. Instead, these findings suggest a model whereby PRMT8 elevates surface channel levels by facilitating exit of channel from the ER. According to this model, the effect of PRMT8 is directly related to the degree of ER retention of channel protein. To further test this idea, we evaluated the effect of PRMT8 on Kv1.3, a channel related to Kv1.2, but that is preferentially localized to the Golgi apparatus (Doczi, Morielli et al. 2008). As predicted, the PRMT8-mediated increase in surface Kv1.3 channel levels was significantly decreased relative to Kv1.2 (Figure 4C).

### **3.4. Discussion**

Utilizing the well-studied trafficking mechanisms of a voltage gated potassium channel, we discovered a new role for the protein arginine methyltransferase, PRMT8 as a regulator of channel exit from the ER. Protein export from the ER is a regulated process that diverts misfolded proteins to a degradation pathway and regulates the biosynthetic trafficking of correctly assembled proteins. ER export signals are encoded within the primary sequence of some potassium channels and can determine channel surface density in neurons (Ma, Zerangue et al. 2001). This process can be influenced by protein:protein interactions. For example, the PDZ-domain protein SAP97 regulates the ER exit of Kv1.4 (Tiffany, Manganas et al. 2000). These interactions can be modulated by post-translational modification (Scott, Blanpied et al. 2001; Xia, Hornby et al. 2001; Scott,

Blanpied et al. 2003), lending an added level of control to the exit of proteins from the ER. This study adds PRMT8, a protein with both scaffolding abilities and methyltransferase activity, to the regulatory mechanisms governing this process.

PRMT8 significantly increased surface expression of Kv1.2 (Figure 1A). The first indication that PRMT8 regulates ER trafficking was the augmented appearance at the cell surface of the high mannose glycosylated form of Kv1.2 relative to the mature glycosylated form (Figure 3). Most Kv1.2 at the cell surface is the mature glycosylated form, and the high mannose form of the channel is primarily localized to the ER (Manganas and Trimmer 2000). Importantly, expressing increasing amounts of Kv1.2 caused an increase in surface expression of both glycosylated forms, but had no effect on their relative level of expression at the cell surface (Figure 3). PRMT8, in contrast, increased the relative surface expression of the high mannose to the mature glycosylated form of the channel (Figure 3). This can be explained by a PRMT8-mediated enhancement of Kv1.2 trafficking out of the ER, in part through a pathway that bypasses the Golgi. By circumventing the glycosylation trimming and branching processes that occur in the Golgi, some Kv1.2 trafficked to the cell surface would be unchanged from the high mannose glycosylated form. Alternatively, the mechanism for the increased appearance of the high mannose form of Kv1.2 at the cell surface could involve PRMT8-mediated block of the trimming and branching processes. We show that PRMT8 likely does not act by directly affecting glycosylation though, since it is able to alter surface levels of a non-glycosylated mutant of Kv1.2 (Kv1.2N207Q; Figure 4A). It is important to note that Kv1.2N207Q, which completely lacks glycosylation, is not equivalent to the



immature, high mannose glycosylated form of the channel. Instead, we utilized Kv1.2N207Q as a tool to evaluate the direct effects of PRMT8 on channel glycosylation.

The idea that PRMT8 modulates ER exit is supported by experiments utilizing channels with different degrees of ER retention. Kv1.2N207Q and the high mannose glycosylated form of Kv1.2 are similar in that they are both retained in the ER. Thus, if PRMT8 is promoting ER exit of the channel, we hypothesized that it would alter Kv1.2N207Q surface levels to a much greater extent than Kv1.2WT. Indeed, Kv1.2N207Q shows a greater PRMT8-mediated change in channel surface levels compared to Kv1.2WT (Figure 4A). In contrast, Kv1.3, a channel with reduced ER localization relative to Kv1.2WT was less sensitive to PRMT8 (Figure 4C).

The PRMT8-induced increase in total Kv1.2 protein levels (Figure 3) is also consistent with PRMT8 as a regulator of ER exit. ER-associated degradation (ERAD) is a quality control mechanism thought to target misfolded or misassembled proteins for degradation. However, it is also involved in the regulation of normal proteins retained in the ER, as shown for the cystic fibrosis transmembrane conductance regulator (Zhang, Nijbroek et al. 2001), and for the voltage-gated Shaker (Khanna, Lee et al. 2004) and Kv1.5 channels (Kato, Ogura et al. 2005). PRMT8 could increase channel surface levels by directly blocking ERAD, which would increase total protein levels, making more channel available for trafficking to the cell surface. Alternatively, PRMT8 could affect ER exit directly, increasing trafficking of channel to the surface. In this case, the increase in total protein levels would result from decreased availability of channel for ERAD. Our data distinguish between ERAD block and ERAD evasion. PRMT8 increased surface

levels of the ER-retained mutant Kv1.2N207Q to a far greater extent than Kv1.2WT, but that was not mimicked by a parallel increase in total Kv1.2N207Q protein levels (Figure 4A,B). Thus, the increase in total protein does not appear to drive increased surface channel expression. Instead, PRMT8 likely increases both channel surface levels and total protein levels by directly promoting ER exit and thereby indirectly preventing ER-associated degradation.

Myristoylation of PRMT8 localizes it to the plasma membrane, which may in turn affect its function (Lee, Sayegh et al. 2005). We show that the effect on Kv1.2 requires an intact PRMT8 N-terminal myristoylation site (Figure 1C). Since Kv1.2 is a membrane protein, it seems logical that myristoylation-induced targeting of PRMT8 to the plasma membrane would influence its effects on the channel. For example, PRMT8 localized to the cell surface may promote trafficking of Kv1.2 from ER in regions where the ER is directly adjacent to the plasma membrane. Whether this depends on the arginine methyltransferase activity of PRMT8, its scaffolding function, or both, remains to be determined. It is equally plausible that plasma membrane localization of PRMT8 is not the critical factor. Myristoylation has also been hypothesized to affect PRMT8 activity directly. The N-terminus of PRMT8 can act as an autoinhibitory domain and myristoylation is thought to relieve this autoinhibition, thereby increasing methyltransferase activity (Sayegh, Webb et al. 2007). Additionally, myristoylation is thought to induce a conformational change, revealing proline rich domains in the N-terminus and affecting PRMT8 activity as a scaffold (Sayegh, Webb et al. 2007). Recent findings indicate that this scaffolding function may be independent of the

methyltransferase function of PRMT8 (Pahlich, Zakaryan et al. 2008). Thus, mutation of the myristoylation site may alter Kv1.2 trafficking by directly affecting PRMT8 methyltransferase or scaffolding functions within the ER. Given the multiple roles that myristoylation has on PRMT8 function, the precise mechanisms by which PRMT8 modulates ER exit of Kv1.2 awaits further study.

To date, most studies on PRMTs suggest that they function as part of a broad network of proteins that govern gene regulation (Bedford and Clarke 2009). Interestingly, recent reports indicate that this can involve PRMT mediated regulation of protein trafficking; PRMT1, the methyltransferase with the highest homology to PRMT8, can methylate RNA helicase A, altering its nuclear import (Smith, Schurter et al. 2004). In addition to its more immediate effects on gene regulation, PRMT1 modulates insulin receptor trafficking through methylation of a heterogeneous nuclear ribonucleoprotein, hnRNPQ (Iwasaki 2008). Our findings identify PRMT8 as a regulator of protein biosynthetic trafficking. This is consistent with the accepted role of PRMTs in regulating protein expression, but also adds to the emerging paradigm of PRMTs as regulators of protein trafficking.

We report that PRMT8 promotes the ER exit of the voltage gated potassium channel Kv1.2. This represents the first biological role for this protein arginine methyltransferase. PRMT8 was originally identified by homology to other methyltransferases, and indeed, *in vitro* assays show that PRMT8 is a functional methyltransferase (Lee, Sayegh et al. 2005). Thus, arginine methylation may play an important, but unexplored role in the process of regulated ER export. Additionally,

PRMT8 is primarily localized to brain neurons (Taneda, Miyata et al. 2007). One intriguing possibility is that PRMT8 integrates signals at the neuronal cell surface with local biosynthetic protein trafficking.

### 3.5. Materials and Methods

**Materials:** Antibody directed against the first extracellular loop of Kv1.2 (Kv1.2e) was developed with assistance from Biosource International (Camarillo, CA). The following antibodies were used: Kv1.2 monoclonal (Kv1.2m) from UC Davis (Davis, CA), GAPDH monoclonal from Millipore (Billerica, MA), Myc monoclonal from Invitrogen (Carlsbad, CA), Flag-M2 monoclonal ( $\alpha$ -Flag) from Sigma (St. Louis, MO). NHS-SS-biotin (Cat# 21331) and the High Capacity Neutravidin Agarose Resin (Cat#29204) were from Thermo Scientific (Rockford, IL).

**Tissue Culture:** Two stable cell lines were used: human embryonic kidney 293 cells stably expressing M1 muscarinic acetylcholine receptors and Kv $\beta$ 2 (HEK- $\beta$ ), and HEK- $\beta$  cell line also stably expressing Kv1.2 (HEK-K). Cells were cultured as previously reported (Connors, Ballif et al. 2008).

**DNA Constructs and Transfection:** Kv1.2 and Kv1.3-Flag were all expressed with the pRK5 mammalian expression vector. GFP-PRMT8, GFP-PRMT1 and GFP-PRMT8G>A (here referred to as PRMT8G2A) constructs were generous gifts from Dr. Mark Bedford (The University of Texas, M.D. Anderson Cancer Center). PRMT8, PRMT1 and PRMT8G2A were subcloned from pEGFP into pRK5 and myc vectors. Primers were purchased from Operon Biotechnologies (Huntsville, AL). Kv1.2N207Q was generated

by mutagenesis using the Stratagene QuikChange Site-Directed Mutagenesis kit (La Jolla, CA). Cells were transiently transfected with various constructs and pEGFP using the TransIT-293 liposomal transfection reagent (Mirus; Madison, WI).

**Flow Cytometry:** Surface Kv1.2 levels were measured by flow cytometry as described (Connors, Ballif et al. 2008). Channel internalization was measured using the Kv1.2e antibody labeled with the Zenon Alexa Fluor 488 Rabbit IgG labeling kit (Invitrogen, Cat#Z25302) as previously described (Stirling, Williams et al. 2009). Surface Kv1.3 was labeled with  $\alpha$ -Flag that detected a Flag epitope inserted between AA 222 and AA 223 in the first extracellular loop of the channel. This epitope does not disrupt channel trafficking or function (unpublished data). Background labeling was detected with isotype specific IgG (R&D Systems; Minneapolis, MN). Secondary labeling was with SPRD conjugated antibody (Southern Biotech; Birmingham, AL).

**Biotinylation and Immunoblot:** Cells were washed with room temperature HBSS (Invitrogen Cat#14025) then incubated with 25mM NHS-SS-biotin in HBSS or in HBSS alone for 30 minutes, followed by washing with quench solution (HBSS/Tris buffer pH 7.4). For time course experiments, cells were returned to warm serum-free culture media and incubated at 37 degrees for indicated time points prior to lysis. For other experiments cells were immediately lysed in RIPA buffer (50mM Tris, 150mM NaCl, 11mM EDTA, 0.25% deoxycholate, 1% NP40, 10% glycerol, 1mM NaF, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM Na<sub>4</sub>BAPTA, protease inhibitors (Sigma Cat#P8340), pH 8.0). Lysates were cleared by sequential centrifugation at 20,000  $\times$  g for 4 min and 95,000  $\times$  g for 10 min. Biotinylated proteins were collected by rocking with Neutravidin beads, eluted and separated by SDS-

PAGE. Western blot detection of Kv1.2 was with  $\alpha$ Kv1.2m. Blots were imaged and quantified with the Odyssey Infrared Imaging System (Li-Cor; Lincoln, NE).

**Statistical Analysis:** Descriptive statistics are provided in figures as bar graphs indicating the sample mean with error bars indicating the standard error of the mean.

Detection of statistical difference was by one-way t-test, and considered to be significant at  $p \leq 0.05$ .

### **3.6. Acknowledgements**

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### **3.7. Abbreviations**

GFP, green fluorescent protein; WT, wild type; HEK, human embryonic kidney; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBSS, hank's balanced salt solution

### 3.8. Figure Legends

**Figure 1. Expression of PRMT8 increases surface levels of Kv1.2.** (A) Kv1.2 at the cell surface, determined by flow cytometry, was significantly greater in HEK-K cells expressing PRMT8 relative to vector transfected control (n=110; \*\*p<0.0001). (B) Expression of PRMT1 did not change surface levels of Kv1.2 (p=0.15), however, PRMT8 had a significant effect in the same experiment (n=24; \*\*p<0.0001). (D) Expression of a mutant form of PRMT8 lacking the N-terminal myristoylation site, PRMT8G2A, did not increase surface Kv1.2 levels, but did elicit a small but significant decrease relative to control (n=27; ^p=0.03). PRMT8 caused a significant increase in the same experiment (\*\*p<0.0001). (C,E) To confirm PRMT protein expression, lysates from HEK-K cells expressing either GFP as a control (Ctr), GFP and myc-tagged PRMT8 (P8), GFP and myc-tagged PRMT1 (P1;C) or GFP and myc-tagged PRMT8(G2A) (G2A; E) were analyzed by immunoblot probed with  $\alpha$ -myc and  $\alpha$ -GFP antibodies.

**Figure 2. PRMT8 effects on steady-state trafficking of Kv1.2 at the plasma membrane.** (A) Steady-state internalization of Kv1.2, determined by flow cytometry, is not changed by PRMT8 expression (n=6; p=0.11). In the same experiment, PRMT8 significantly increased surface levels of Kv1.2 relative to control (n=4; \*\*p<0.0001). (B) Steady-state loss of surface Kv1.2 is unaffected by expression of PRMT8. Graph represents densitometry measurements of surface biotinylated Kv1.2 resolved by immunoblot in PRMT8 expressing or control cells. Values are normalized to the t=0 time

point and are not significantly different between PRMT8 and control at each time point (n=2, p>0.05).

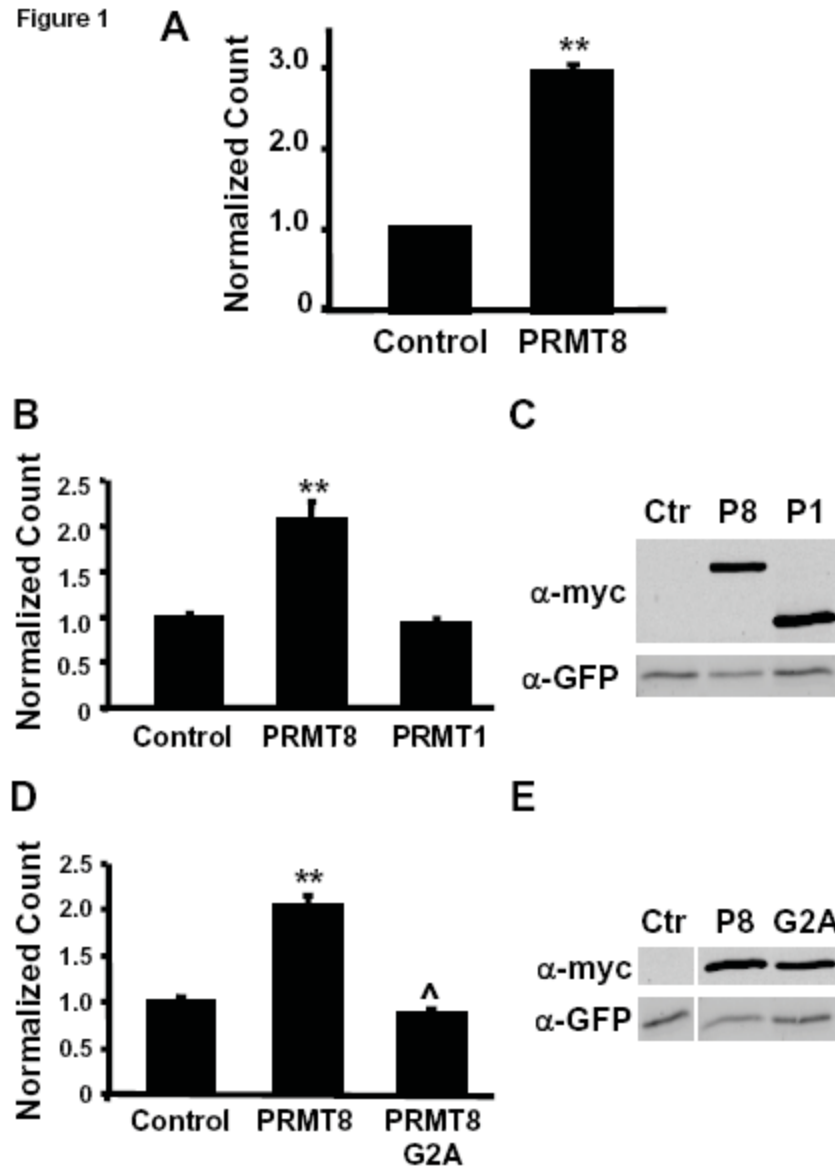
**Figure 3. PRMT8 affects the biosynthetic trafficking of Kv1.2.** (A, left)  $\alpha$ -Kv1.2m immunoblot of biotinylated surface Kv1.2 in HEK- $\beta$  cells transfected with increasing concentrations of pRK5-Kv1.2 (0.25 $\mu$ g = K; 0.5 $\mu$ g = K+; 1 $\mu$ g = K++) or with 0.25 $\mu$ g pRK5-Kv1.2 + PRMT8 (KP). The absence of GAPDH signal confirms selective biotinylation of surface proteins. Graph represents quantification of Kv1.2 signal intensity. (A, right) Immunoblot of the pre-pulldown lysate (input) and graph representing the total amount of Kv1.2 protein for each condition, using GAPDH as a loading control. (B) Ratios of the densitometry measurements of the biotinylated high mannose Kv1.2 to the biotinylated mature glycosylated Kv1.2 reveal that increasing amounts of total Kv1.2 protein (K+ and K++) proportionally increased the mature and immature forms of Kv1.2 at the cell surface compared to K (p>0.1). PRMT8, however, significantly increases the amount of high mannose Kv1.2 relative to the mature glycosylated form at the plasma membrane (n=2; \*\*p<0.01).

**Figure 4: PRMT8 mediates channel exit from the ER.** HEK- $\beta$  cells were transfected with combinations of Kv1.2WT, Kv1.2N207Q and PRMT8 as indicated. (A) The surface levels of the Kv1.2N207Q mutant channel were significantly less than Kv1.2WT (left; n=17; \*\*p<0.0001). PRMT8 significantly increased the surface levels of both Kv1.2WT and Kv1.2N207Q (\*\*p<0.0001 vs. Kv1.2WT; ^ p<0.0001 vs. Kv1.2N207Q). However,



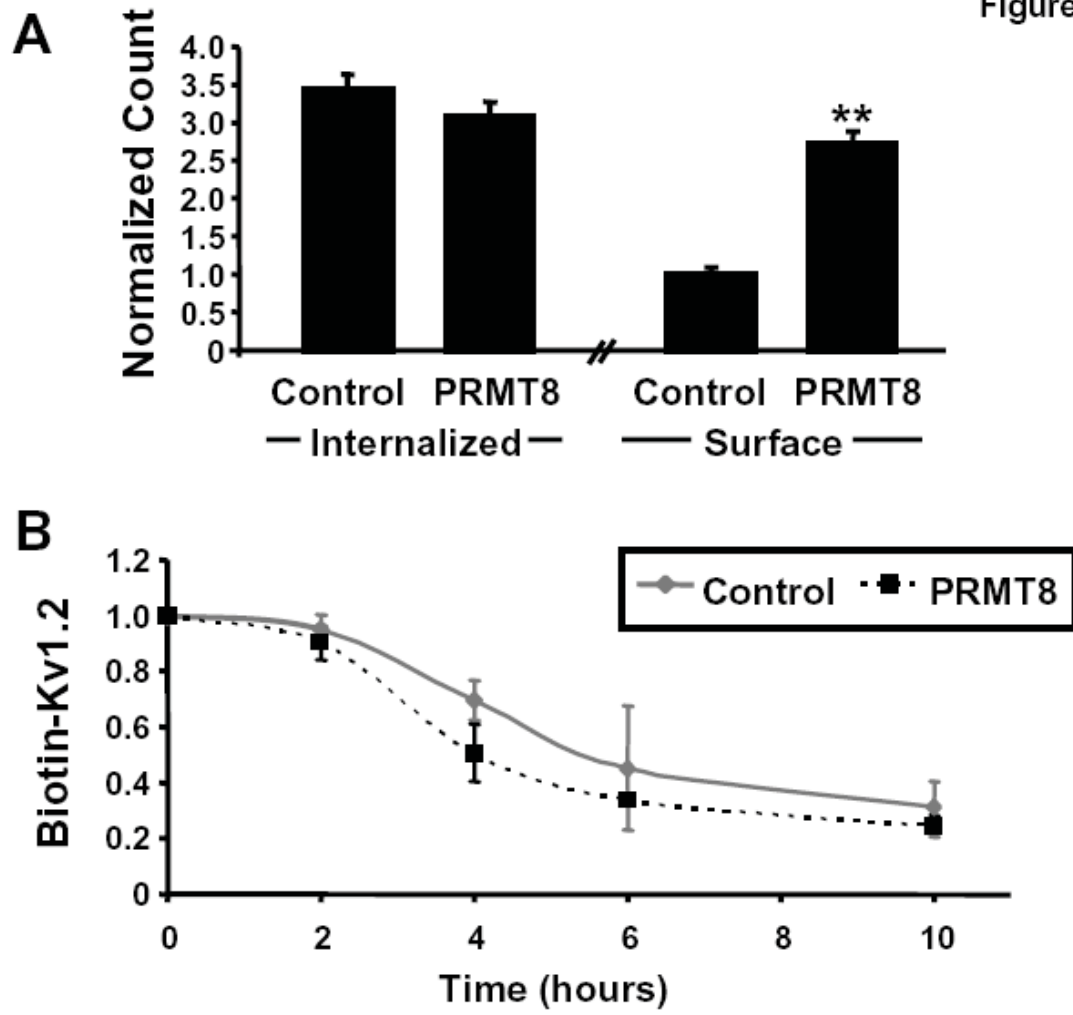
the PRMT8-mediated increase is significantly greater for Kv1.2N207Q as compared to Kv1.2WT channels (right; n=18; \*\*p<0.0001). (B) PRMT8 (P) caused an increase in both Kv1.2WT (K) and Kv1.2N207Q (N) total protein levels, as detected by immunoblot (left). HEK- $\beta$  cells ( $\beta$ ) were used as a control. Graph depicts the percent change in quantified Kv1.2 signal caused by PRMT8, revealing no difference between Kv1.2WT and Kv1.2N207Q (n=4; p=0.3). (C) Percent increase in Kv1.3 surface levels mediated by PRMT8 was significantly smaller relative to Kv1.2 (n=8; \*p<0.018).

Figure 1

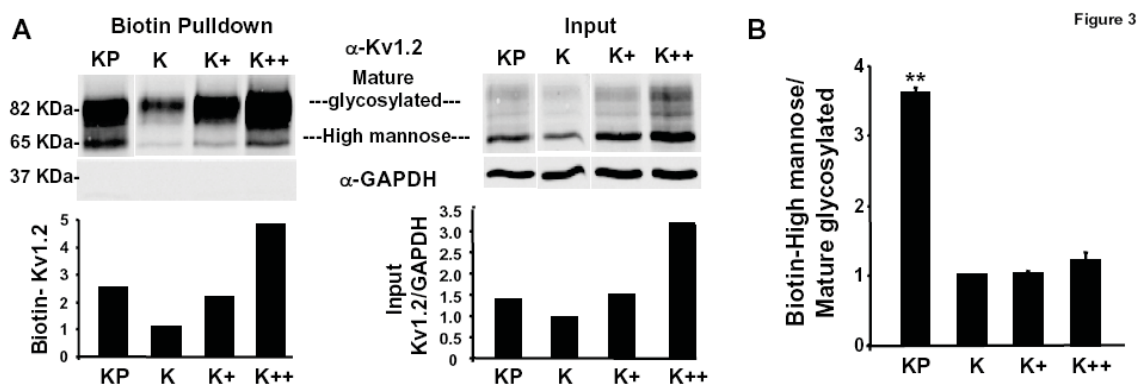


**Figure 1.** Expression of PRMT8 increases surface levels of Kv1.2

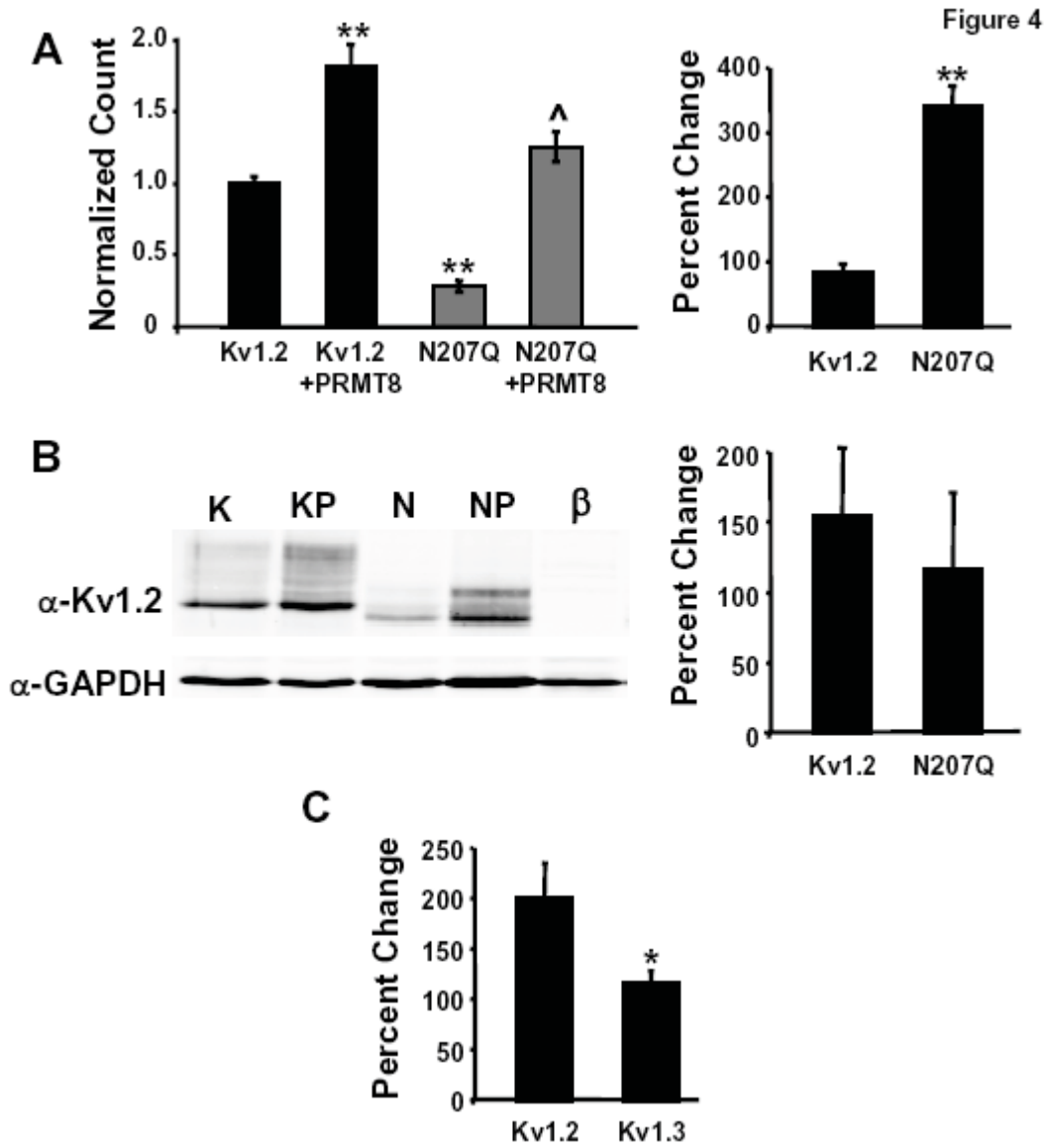
Figure 2



**Figure 2.** PRMT8 effects on steady-state trafficking of Kv1.2 at the plasma membrane



**Figure 3.** PRMT8 affects the biosynthetic trafficking of Kv1.2



**Figure 4: PRMT8 mediates channel exit from the ER**

## CHAPTER 4: COMPREHENSIVE DISCUSSION

As selective pores within the plasma membrane, ion channels regulate ion gradients that are critical for cellular function. Research focusing on the regulatory mechanisms governing ion channels can provide valuable insight into the understanding of human physiology. Therefore, the main goal of this dissertation was to elucidate mechanisms involved in the regulation of the voltage-gated ion channel, Kv1.2. Channel trafficking was identified as a major component in the positive regulation of Kv1.2. Positive trafficking, which yields a net increase of channel protein at the cell surface, was shown to involve cAMP/PKA modulation of channel steady-state endocytosis and PRMT8-regulated Kv1.2 exit from the Endoplasmic Reticulum. Therefore, this research describes regulated trafficking of Kv1.2 occurring both before and after the channel reaches the cell surface and also adds the cAMP/PKA pathway and the protein arginine methyltransferase, PRMT8, to the signaling network governing Kv1.2 trafficking.

The second chapter of this dissertation explores mechanisms by which the cAMP/PKA pathway regulates Kv1.2. Previous work described the involvement of this pathway in the regulation of the Kv1.2 ionic current (Huang, Morielli et al. 1994), however, the role of cAMP/PKA in Kv1.2 trafficking was unknown. The hypothesis that the cAMP/PKA pathway would positively influence Kv1.2 trafficking was based on the directionality of the PKA-mediated increase of the Kv1.2 ionic current (Huang, Morielli et al. 1994). Interestingly, we found that either increasing or decreasing intracellular cAMP enhanced the amount of Kv1.2 on the cell surface, suggesting that basal cAMP levels maintain homeostasis of surface Kv1.2 (Chapter 2, Figures 1-3). While endocytosis

had been previously shown to negatively regulate the channel (Nesti, Everill et al. 2004), this was the first demonstration of channel trafficking governing the positive regulation of the Kv1.2 ionic current.

In this study, we defined roles for both PKA-dependent and PKA-independent pathways in the modulation of Kv1.2 surface expression. Inhibition of PKA or adenylate cyclase caused an increase in surface Kv1.2 levels and we concluded that basal activity of the cAMP/PKA pathway is involved in regulating basal levels of surface Kv1.2.

Investigation of the cAMP-dependent, PKA-independent effects on Kv1.2 revealed a requirement for dynamin and cortactin, proteins involved in regulating Kv1.2 endocytosis (Chapter 2, Figure 9). Thus, we concluded that the mechanisms by which both cAMP-mediated pathways regulate Kv1.2 trafficking involve modulation of constitutive channel endocytosis. The process of steady-state channel internalization has been shown to occur by a cholesterol-dependent mechanism and includes channel endocytosis, vesicle sorting and recycling back to the cell surface (Morrison, Moore et al. 1996; Leterrier, Bonnard et al. 2004; Stirling, Williams et al. 2009). Each step of constitutive channel endocytosis relies on specific proteins; for example, ROCK, a downstream effector of RhoA, has been implicated in the recycling segment (Stirling, Williams et al. 2009). Further research is needed to identify which of these constitutive endocytosis pathways is affected by altering cAMP levels. To investigate this, in addition to testing the involvement of RhoA, temperature block experiments can be utilized to block the sorting and recycling portions of constitutive channel endocytosis. By doing so, specific targets for the cAMP/PKA pathway in steady-state channel internalization can be identified.

By using a reductionist approach and model cell system, we were able to delineate the rather complex cAMP/PKA involvement in Kv1.2 regulation. This research significantly impacted the interpretation of more recent studies in the lab focused on *in vivo* Kv1.2 regulation in the cerebellum. Kv1.2 has previously been shown to localize to the pinceau, the pre-synaptic area of cerebellar basket cells that surround the initial axon segment of Purkinje cells (Sheng, Tsaur et al. 1994). This synapse is especially important because Purkinje cell axons form the main output of the cerebellar cortex. Inhibiting Kv1.2 function here has been shown to increase inhibitory postsynaptic currents recorded from Purkinje cells, therefore decreasing Purkinje cell excitability and cerebellar cortex output (Southan and Robertson 1998). Given the important role of Kv1.2 at this anatomical location, we predict that regulation of Kv1.2 function here would ultimately modulate all output of the cerebellar cortex. Interestingly, synaptic activity at the pinceau is regulated by cAMP/PKA pathways (Yung, Leung et al. 2001; Yoshida, Hashimoto et al. 2002; Lee, Chen et al. 2005). Feedback from Purkinje cells involves the release of both secretin and cannabinoids which act on their respective receptors in the basket cells. Secretin receptors can activate adenylate cyclase through  $G_{\alpha s}$  (Vilardaga, Ciccarelli et al. 1994), while cannabinoid receptors CB1 and CB2 inhibit adenylate cyclase through  $G_{\alpha i}$  (Felder, Joyce et al. 1995). Preliminary studies from the Morielli Lab, show that modulation of both of these pathways affects the surface expression of Kv1.2 in cerebellar slices (Michael Williams, unpublished data). Furthermore, there seems to be both PKA-dependent and PKA-independent pathways regulating Kv1.2 in the cerebellum. These results parallel the findings in HEK293 cells, presented in this



dissertation. Thus, the work within this dissertation provides a foundation for future *in vivo* experimental design and interpretation of results.

Within Chapter Two, new phosphorylation sites in the C-terminus of Kv1.2 are identified (Figure 6). Phosphorylation at S440 and S449 was confirmed by mass spectrometry and shown to have a critical role in the basal surface levels of Kv1.2. These sites were not, however, involved in the forskolin-mediated increase in surface Kv1.2 levels. Interestingly, based on western blot analysis of channels mutated at either S440 or S449, we were able to predict the role for phosphorylation at these sites. The S440A mutation decreased the amount of total channel protein as compared to Kv1.2WT, suggesting that phosphorylation of S440 is critical for channel stability. Kv1.2S449A, on the other hand, caused a dramatic change in the glycosylation pattern of the channel relative to Kv1.2WT, indicating that phosphorylation of S449 is involved in channel biosynthesis. Research from the Trimmer lab has since shown, by quantitative mass spectrometry, that phosphorylation of S440 is only detected in the mature glycosylated form of Kv1.2, which is mostly localized to the cell surface (Yang, Vacher et al. 2007). This supports the hypothesis that phosphorylation of this site is involved in channel stability once the channel has reached the plasma membrane. Yang et al. also demonstrate that S449 phosphorylation is not restricted to the mature, glycosylated form of the channel, thus implying that phosphorylation at this site can occur during the biosynthetic progression of the channel. Further research is needed to fully determine the role for phosphorylation of these C-terminal serines in Kv1.2 function.

After the publication of Chapter Two, we sought to identify the kinase(s) responsible for the phosphorylation of S440 and S449. As an initial assay, we evaluated the effect of kinase inhibitors on Kv1.2 surface levels and the forskolin-induced molecular weight shift of Kv1.2 previously shown require phosphorylation at S440 or S449. Some of these data are presented in Appendix A, specifically I show the involvement of MAP Kinase, Casein Kinase and AMP Kinase in Kv1.2 regulation. Not presented in this dissertation are data showing that inhibition of other kinases including, CaM Kinase II, PKC, Cyclin Dependent Kinase, PKG and PI3 Kinase did not result in a block of the forskolin-mediated band shift. Recently published work from the Cole lab identified PKA as a kinase that can phosphorylate S449 (Johnson, El-Yazbi et al. 2009). Since Chapter Two explores the involvement of PKA in Kv1.2 phosphorylation and trafficking, our conclusions can now incorporate this new information. We showed that PKA inhibition did not alter the band shift produced by forskolin (Figure 5) and proposed that forskolin signals through a different kinase/phosphatase pathway to elicit phosphorylation of Kv1.2. We now conclude that although PKA phosphorylates Kv1.2 directly, PKA phosphorylation is not necessary for the forskolin-mediated Kv1.2 band shift. This is an important distinction and implies that using the band shift to investigate kinases that directly phosphorylate Kv1.2 may not be the best assay since it may depend on two potential phosphorylation sites. The forskolin-induced band shift may, in fact, involve Kv1.2 phosphorylation by PKA, however another kinase may be able to substitute when PKA is inhibited. Importantly, the kinase that is involved in phosphorylation at S440 has not been identified. To clarify the contribution of various

kinases in Kv1.2 phosphorylation and regulation, it will be necessary to use techniques that are more direct than the forskolin-induced band shift, including mass spectrometry, different combinations of kinase inhibitors and phospho-specific antibodies.

The findings by Johnson et. al. also allow a re-evaluation of our conclusions on the involvement of PKA and S449 phosphorylation in Kv1.2 trafficking. We show that S449 is necessary for normal, basal Kv1.2 surface levels, as the basal surface levels of the Kv1.2S449A mutant channel were significantly lower than Kv1.2WT. If this mutation prevents phosphorylation of S449 to cause these decreased surface levels, then inhibiting the kinase responsible for the phosphorylation should yield the same decreased surface expression as Kv1.2S449A. Furthermore, since PKA was identified as a kinase responsible for S449 phosphorylation, PKA inhibition should decrease surface Kv1.2 levels through this mechanism. However, we show that inhibiting PKA increases the surface expression of the channel (Chapter 2, Figure 3). These seemingly conflicting data can be explained in a few ways. First, PKA inhibition could have effects other than inhibiting phosphorylation at S449. These potential effects include altering the function of proteins that interact with the channel or altering the phosphorylation of other sites within Kv1.2 to cause an increase in channel surface expression. Second, channels targeted to the plasma membrane by acute PKA inhibition may already be phosphorylated at S449, either by PKA before it was inhibited or by another kinase. A more targeted analysis of the involvement of PKA phosphorylation in basal trafficking of Kv1.2 awaits further study.

Chapter three of this dissertation details the role of the protein arginine methyltransferase, PRMT8, in regulating the trafficking of Kv1.2. This research highlights a distinct aspect of the positive trafficking of Kv1.2. That is, not trafficking of the channel once it has reached the plasma membrane, but trafficking of Kv1.2 from the endoplasmic reticulum to the cell surface (Chapter 3, Figure 2-4). Our extensive knowledge of Kv1.2 trafficking, specifically the channel's glycosylation patterns as a readout of its biosynthetic progression, made Kv1.2 an excellent model protein to investigate the role of PRMT8 in protein trafficking.

Interestingly, our data indicate that PRMT8 may serve a general trafficking role for a variety of ER-localized proteins. We show that surface levels of Kv1.3, a channel closely related to Kv1.2, were increased by PRMT8, albeit to a significantly lesser extent (Chapter 3, Figure 4). Trafficking of other ER-localized proteins may also be affected by PRMT8. Implications for PRMT8-mediated ER exit in the brain are particularly interesting due to the high compartmentalization of protein synthesis machinery in dendrites. ER outposts can be localized to some dendritic spines, including those from hippocampal and purkinje neurons (Villa, Sharp et al. 1992; Pierce, van Leyen et al. 2000; Toresson and Grant 2005). PRMT8-mediated ER exit could be important for neuronal processes such as local protein synthesis. PRMT8 may facilitate the trafficking of locally synthesized proteins to the plasma membrane. Considerable experimental evidence suggests that synapse-specific protein synthesis is involved in long-term memory (Martin, Barad et al. 2000; Sutton and Schuman 2006). PRMT8 may, thus, have

the capacity to regulate learning and memory-linked processes, including long-term potentiation and long-term depression.

Learning and memory require long-term changes and interestingly, the data presented in this dissertation may provide a mechanism by which those lasting modifications may occur. Currently, methylation is thought to be an irreversible post-translational modification. If methylation is involved the PRMT8-mediated ER exit, then this could indicate a mechanism for long-term regulation of ER exit. Experimental evidence for the irreversibility of methylation, however, is sparse and may simply reflect our limited understanding of this signaling pathway. In fact, the first arginine demethylase, JMJD6, was recently identified (Chang, Chen et al. 2007), and may be the first member of an entire family of demethylases that await discovery. Nevertheless, depending on when and where demethylases are expressed, methylation has the potential to be at least a semi-permanent modification. Thus, besides defining the first biological role for PRMT8, this work presents exciting possibilities for future experiments based on elucidating the involvement of arginine methylation in learning and memory.

In conclusion, I began these studies with the goal of elucidating positive trafficking pathways involved in the regulation of Kv1.2. I uncovered specific roles for the cAMP/PKA pathway, direct channel phosphorylation, and PRMT8 in regulating the trafficking of this channel. The work presented here defines multiple checkpoints for positive channel trafficking, including regulation both before and after the channel has reached the plasma membrane. This research defines positive trafficking as an essential mechanism for the regulation of this voltage gated ion channel.

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## APPENDICES

Appendix A consists of data that are related to the second chapter of this dissertation. This work includes analysis of different stimuli that alter Kv1.2 surface levels and is presented here as an initial attempt at defining other pathways that may modulate Kv1.2 trafficking. The involvement of calcium, actin and microtubule disrupting drugs, kinase inhibitors, and EPAC/Rap in the cAMP-mediated increase of Kv1.2 surface levels are all explored here. Furthermore, we reveal the potential involvement of AMP Kinase as the enzyme responsible for the phosphorylation of the C-terminal serines identified in Chapter 2. Lastly, both H<sub>2</sub>O<sub>2</sub> and a mutant form of Kv1.2 unable to be ubiquitinated are investigated in relation to their involvement in Kv1.2 regulation.

In Appendix B, research conducted as part of revisions for the paper “Pattern specific mono-ubiquitylation of Kv1.2 determines the polarity of its trafficking at the plasma membrane” is presented. These data are included as part of the scope of my research and to provide the foundation for future experiments.

Extensive analysis of a Kv1.2 mutant, R100K, is presented in Appendix C. The preliminary nature of this study prevented it from being included as a chapter, however it will hopefully be followed up and submitted. This mutation profoundly alters Kv1.2 surface levels and ionic current, suggesting that it is essential for proper channel function. The altered glycosylation patterning and beta subunit binding suggests a modified progression through the biosynthesis pathway. Interestingly, this channel can function at

the cell surface but is differentially affected by various stimuli that cause channel endocytosis. This mutation has the potential to clarify channel specificity for one endocytosis pathway over another.

Finally, data related to Chapter 3 of this dissertation are presented in Appendix D. The information and conclusions from this work will help elucidate other mechanisms of channel regulation, especially those involving PRMT8. Data in this appendix include the effect of general inhibition of arginine methylation on Kv1.2 surface levels, experiments relating to the involvement of the beta subunit in the PRMT8-mediated regulation of Kv1.2, effects of lysosomal and proteasomal inhibition on Kv1.2 surface levels, and the association of Kv1.2 with the heat shock protein HSP70. Collectively, this work may be of interest to the Morielli lab and others studying Kv1.2 regulation.

## **Appendix A: Data relating to Chapter Two**

### **A.1. Background**

During the course of collecting data for the cAMP/PKA paper, I investigated a number of pathways that seemed likely to be involved in the regulation of Kv1.2. These initial experiments define multiple signaling networks that alter Kv1.2 regulation. This work is included here as it identifies potential projects and is relevant to future studies.

### **A.2. Results and Discussion**

#### Effect of altered extracellular calcium on Kv1.2 surface levels

In Chapter 2 we showed that decreased adenylate cyclase activity caused an increase in Kv1.2 surface levels as measured by flow cytometry (Connors, Ballif et al. 2008). The activity of some adenylate cyclase isoforms is controlled by calcium (Willoughby and Cooper 2007). Therefore, we asked if altering the calcium concentration would also change levels of Kv1.2 at the cell surface. Interestingly, lowering the amount of extracellular calcium increased the amount of Kv1.2 at the cell surface (Figure A1). These data show the involvement of calcium in Kv1.2 regulation and suggests that pathways that alter the calcium concentration may also affect this channel.

#### The cytoskeleton plays an integral role in regulating surface Kv1.2 levels

The actin cytoskeleton has been shown to play a role in regulating the amount of Kv1.2 present at the cell surface (Nesti, Everill et al. 2004). Inhibiting actin filament

formation with latrunculin A, a toxin that binds monomeric actin, has been shown to decrease surface Kv1.2 levels (Nesti, Everill et al. 2004). Alternatively, Cytochalasin B, which caps the plus end of actin filaments, causes a significant increase in Kv1.2 surface levels (Figure A2-A). This interesting disconnect may be a valuable insight to the actin-based mechanisms of Kv1.2 trafficking. Additionally, Forskolin (10uM, 10min) was not able to elicit a significant change in surface Kv1.2 levels with Cytochalasin B as compared to control.

Cholchicine, a blocker of microtubule polymerization, has a negative effect on the surface levels of Kv1.2, suggesting for the first time that microtubules are involved in the trafficking of this channel (Figure A2-B). Inhibiting microtubule formation does not however, alter the forskolin-induced increase in Kv1.2 surface levels.

#### Casein Kinase and MAP Kinase are involved in regulating the basal levels of surface Kv1.2

During the investigation of kinases that may regulate Kv1.2, inhibition of casein kinase and MAP kinase proved to have significant effects on Kv1.2 surface levels. A3, a general casein kinase inhibitor, increased the amount of Kv1.2 on the cell surface (Figure A3-A). This drug, however, is non-selective and does have some effect on inhibiting PKA at high concentrations. Blocking PKA leads to increased surface levels (Chapter 2) and could be the mechanism by which A3 exerts its effect. Thus, the involvement of casein kinase in Kv1.2 regulation warrants further investigation.



The MAP Kinase inhibitor, SB203580, caused a significant increase in surface Kv1.2 levels (Figure A3-B). Thus this kinase may be involved in regulating the basal levels of Kv1.2. MAP Kinase has been shown to regulate Heat shock proteins (Helmbrecht, Zeise et al. 2000) and this is particularly interesting due to the interaction between Kv1.2 and Hsp70 (Appendix D).

#### EPAC and RAP effects on Kv1.2 surface levels

Efforts to explore downstream effectors of the cAMP pathway, other than PKA, led to an investigation of the proteins EPAC and Rap. cAMP binds and activates EPAC, which is a guanine nucleotide exchange factor for Rap. Activation of EPAC with the agonist, 8-pCPT-2-O-Me-cAMP, significantly increases surface Kv1.2 levels (Figure A4-A). These results were highly variable between experiments.

Transient transfection of wild-type (WT) and dominant negative (DN) Rap also increased surface Kv1.2 levels, while constitutively active (CA) Rap had no effect (Figure A4-B). This result is opposite of what we would have expected if the mechanism for the EPAC-effect involved EPAC activation of Rap. Despite the effect of WT and DN Rap on basal Kv1.2 surface levels, treatment with Forskolin (10 $\mu$ M, 10min) produced an increase comparable to control (Figure A4-C). CA Rap, on the other hand, significantly increased the effect of forskolin on Kv1.2 surface levels. Collectively, these data suggest a role for EPAC and Rap in the regulation of Kv1.2, however a more extensive analysis is required.

## Effects of Oxidative Stress on the regulation of Kv1.2

AMP Kinase (AMPK) is thought of as an energy sensor as it is activated by changes in the AMP:ATP ratio. Since Kv1.2 is an oxygen sensitive channel and oxidative stress can activate AMPK, we tested the possibility that this kinase was involved in the regulation of Kv1.2. Specifically; we explored the role of AMPK in the forskolin-mediated effects on Kv1.2, including the electrophoretic shift and increased surface levels (Chapter 2). Pre-treatment with Compound C, a selective and reversible inhibitor of AMPK, completely prevented the band shift induced by forskolin (Figure A4-A). This is particularly intriguing as it suggests that AMPK may be involved in the direct phosphorylation of Kv1.2 at either S440 or S449. Flow cytometric analysis of Kv1.2 surface levels reveals that application of Compound C significantly increases the basal levels of Kv1.2 (Figure A5-B). Remarkably, the forskolin-mediated increase in surface levels is not seen in the presence of Compound C (Figure A5-B).

Since oxidative stress is known to stimulate AMPK, we used hydrogen peroxide ( $H_2O_2$ ) to test the effects of AMPK activation. At high concentrations of  $H_2O_2$  we observed a distinct shift in the electrophoretic mobility of Kv1.2 (Figure A6-A). This suggests that  $H_2O_2$  may activate AMPK to cause a band shift similar to forskolin and is in line with the data in Figure A4, where inhibiting AMPK prevents this shift. Various concentrations of  $H_2O_2$  are all able to cause a significant increase in the amount of Kv1.2 at the cell surface (Figure A6-B). Although this result is paradoxical to the result seen with Compound C, as they act in similar directions, not opposing, it is consistent with the data in Chapter 2, however, as a homeostatic balance of Kv1.2 levels is maintained by a

single pathway. It is important to note that H<sub>2</sub>O<sub>2</sub> has many other effects, including inhibition of protein tyrosine phosphatases. Thus, the mechanism behind the H<sub>2</sub>O<sub>2</sub>-induced modulation of Kv1.2, including the requirement for AMP Kinase, warrants further investigation.

Intriguingly, the polyphenol Resveratrol, a component of red wine, has been shown to be a potent activator of AMPK (Dasgupta and Milbrandt 2007). Resveratrol has vasodilatory effects and it has been suggested that regulation of Kv channels is involved (Novakovic, Bukarica et al. 2006). The mechanism for the Resveratrol-mediated vasodilation could possibly involve AMPK mediated phosphorylation and trafficking of Kv1.2. The involvement of AMPK in the regulation of Kv1.2 is an exciting area of research that I hope will be continued.

#### Kv1.2 Ubiquitination and Phosphorylation

There has been considerable work in the Morielli Lab on ubiquitination of Kv1.2, including evidence that specific patterning of channel ubiquitination alters channel trafficking. There are six potential ubiquitin sites on the C-terminus of the channel. These lysines are very close to the phosphorylation sites identified on the C-terminus of Kv1.2, S440 and S449. Phosphorylation at S440 or S449 can cause a mobility shift of Kv1.2. I hypothesized that these post-translational modifications may be dependent on one another. To test this I used a mutant version of Kv1.2 that is unable to be ubiquitinated (Total K→R) and evaluated the effect of forskolin via western blot. Interestingly, the Total K→R mutant channel can no longer form a doublet when treated

with forskolin (Figure A7). This may be due to an altered conformation of the C-terminus, thus preventing accessibility of the kinase for phosphorylation. Nevertheless, the effect of post-translational modifications on the conformation and ultimately greater regulation of Kv1.2 is an attractive area of research.

### A.3. Figure Legends

**Figure A1:** Low calcium increases surface Kv1.2 levels. Flow cytometric analysis reveals that reducing extracellular calcium from 1.8mM to 100nM causes a significant increase in the amount of Kv1.2 at the cell surface (n=6; \*\* pval<0.01 vs. control).

**Figure A2:** Disrupting the cytoskeleton alters basal Kv1.2 surface levels. (A) Application of the actin inhibitor Cytochalasin B (Cyto B; 10 $\mu$ M for 10min) caused a significant increase in the surface levels of Kv1.2 (\*\*pval<0.001 vs. control). Forskolin (10 $\mu$ M, 10min) caused a significant increase in surface Kv1.2 levels in the absence of the inhibitor (\*\*pval<0.001 vs. control), but this effect was not seen when cells were pretreated with Cytochalasin B (n=5). (B) The microtubule inhibitor Colchicine (20 $\mu$ M, 60min) caused a significant decrease in the amount of Kv1.2 detected at the cell surface (n=6, \*\*pval<0.01 vs. control). The forskolin-mediated effect on Kv1.2 was seen in both the presence and absence of Colchicine (\*\*pval<0.01 vs. control, ^pvals<0.01 vs. colchicine).

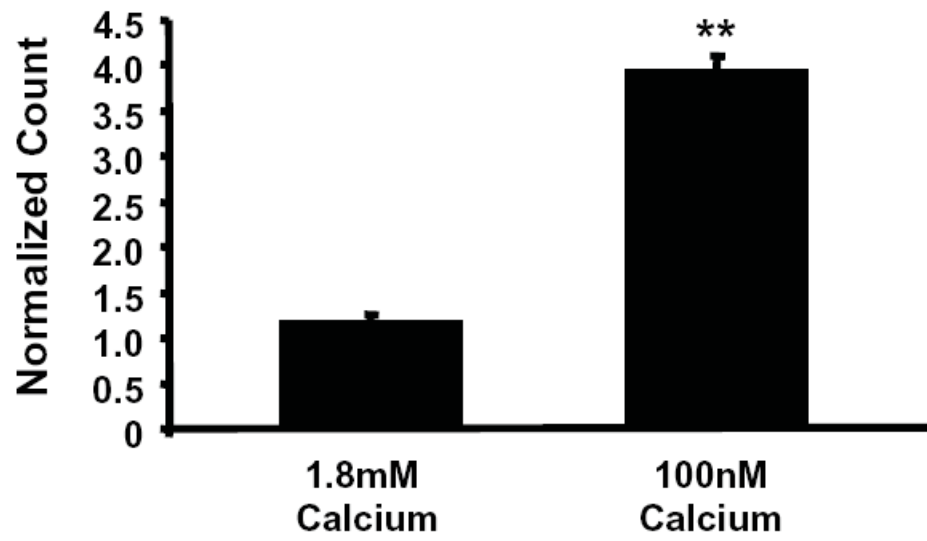
**Figure A3:** Inhibitors of Casein Kinase and MAP Kinase increase the basal surface levels of Kv1.2. Both the casein kinase inhibitor, A3, (A; 50 $\mu$ M or 100 $\mu$ M, 20 min) and the MAP Kinase inhibitor, SB203580 (B; 10 $\mu$ M, 30 min) significantly increase the Kv1.2 surface levels. (n=6, \*pval<0.05 vs. control, \*\*pval<0.01 vs. control)

**Figure A4:** The cAMP downstream effectors EPAC and Rap significantly affect the basal levels of Kv1.2 at the cell surface. (A) Increasing concentrations of the EPAC agonist, 8-pCPT-2-O-Me-cAMP, caused a significant increase in the surface levels of Kv1.2 (10min, n=6, \*pval<0.05 vs. control, \*\*pval<0.01 vs. control) (B) Transient transfection of WT and dominant negative (DN) Rap caused Kv1.2 surface levels to significantly increase (n=18, \*pval<0.05 vs. control). (C) Forskolin (10 $\mu$ M, 10min) consistently caused a significant increase in surface Kv1.2 levels. Transient transfection of constitutively active (CA) Rap caused the percent increase with Forskolin to be significantly higher than control (\*\*pval<0.01 vs. control)

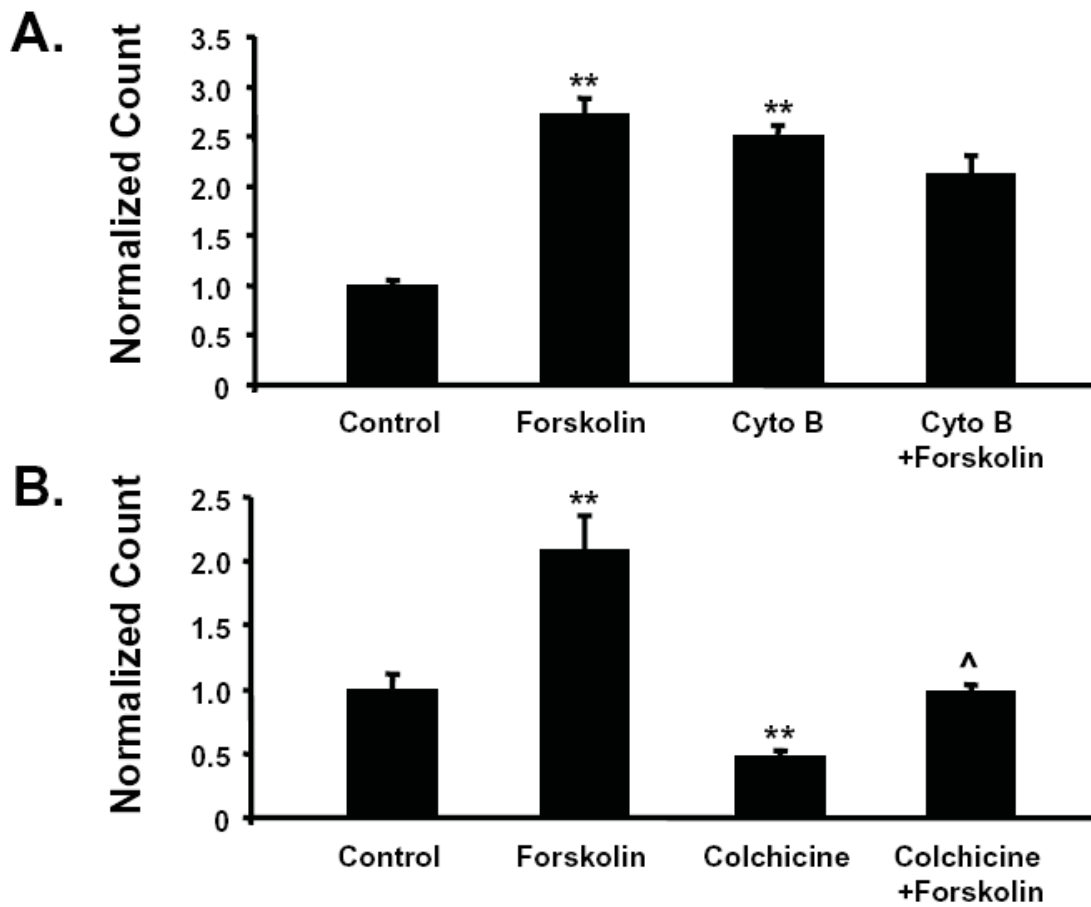
**Figure A5:** Effects of AMP Kinase inhibition on Kv1.2 regulation. (A) Pretreatment with Compound C (C; 20 $\mu$ M, 30min), an inhibitor of AMP Kinase, prevents the shift in Kv1.2 electrophoretic mobility caused by forskolin (F; 10 $\mu$ M, 10min) treatment. GAPDH shows equal loading of the lanes. (n=2) (B) Both Forskolin and Compound C caused a significant increase in surface Kv1.2 levels (n=6; \*\*pval<0.01 vs. control), however pretreatment with Compound C prevented the forskolin-mediated increase in Kv1.2 surface levels (pval=0.108).

**Figure A6:** Effects of H<sub>2</sub>O<sub>2</sub> on Kv1.2. (A) Forskolin (F; 10μM, 10min) is known to cause a phosphorylation-dependent shift in the electrophoretic mobility of Kv1.2 as compared to Control (C). Increasing concentrations of H<sub>2</sub>O<sub>2</sub> were applied to cells for 10 minutes and then cell lysates were analyzed by western blot. 1mM H<sub>2</sub>O<sub>2</sub> caused a shift of Kv1.2 similar to that seen with forskolin. GAPDH was used as a loading control. (B) All concentrations of H<sub>2</sub>O<sub>2</sub> significantly increased surface Kv1.2 levels as determined by flow cytometry (n=6, \*pval<0.05 vs. control, \*\*pval<0.01 vs. control).

**Figure A7:** Kv1.2 mutated to prevent ubiquitination does not form a doublet when treated with forskolin. Kv1.2 with lysines in the N and C-termini mutated to arginine does not form a band shift when treated with forskolin (F; 10μM, 10 min) as compared to control. GAPDH was used as a loading control.

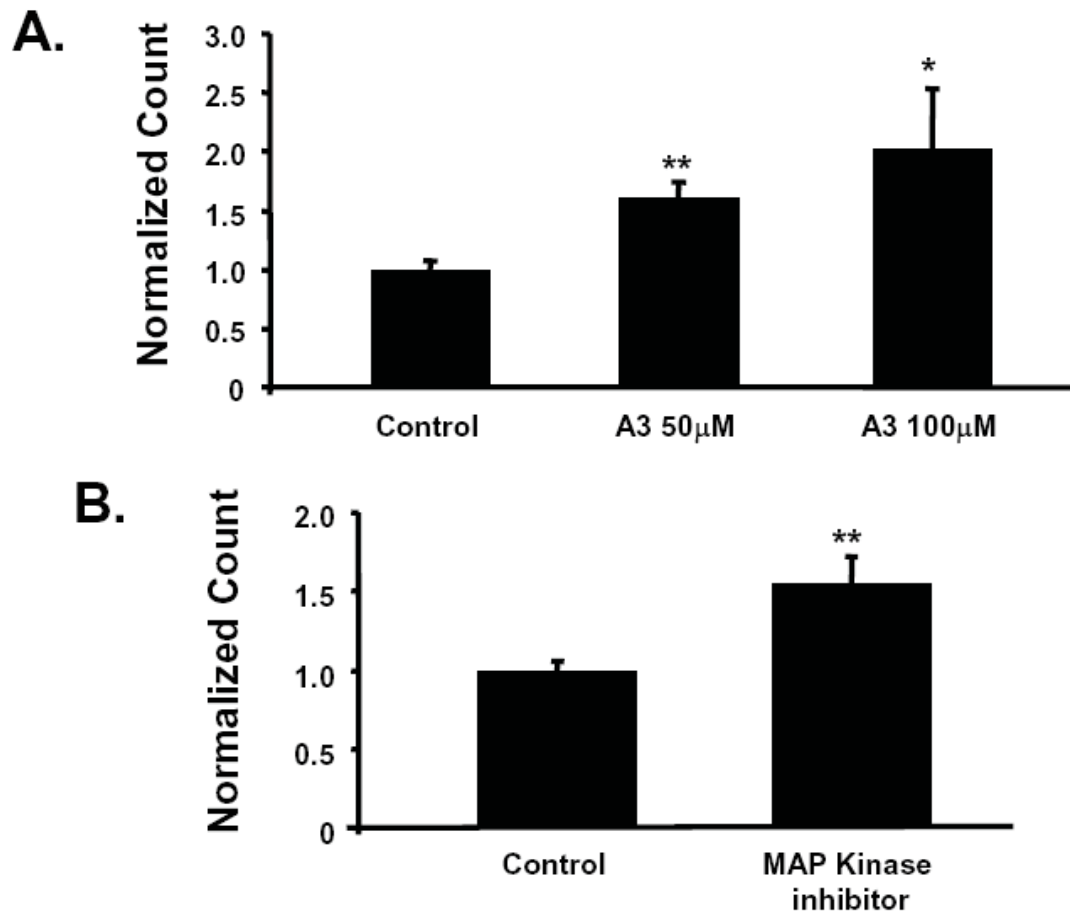


**Figure A1:** Low calcium increases surface Kv1.2 levels

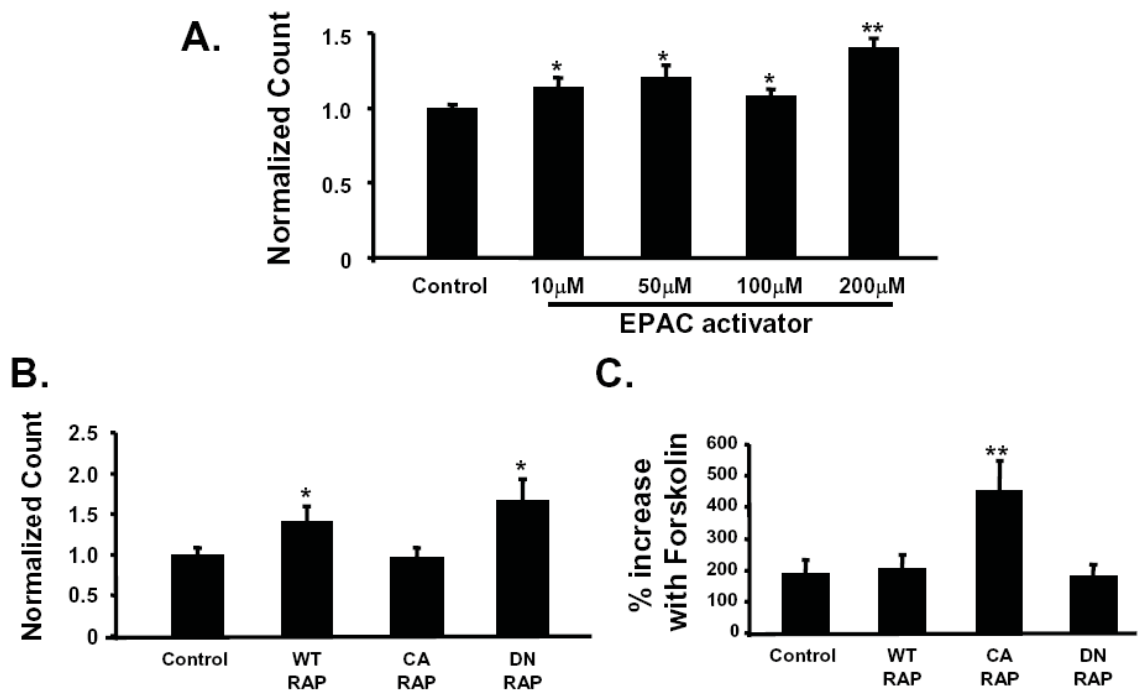


**Figure A2:** Disrupting the cytoskeleton alters basal Kv1.2 surface levels



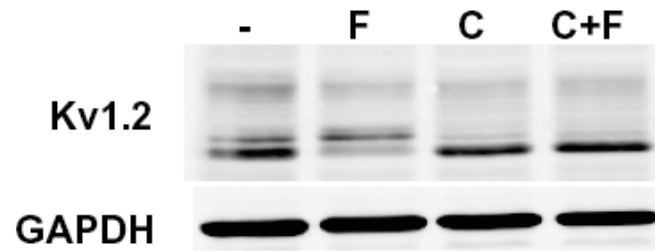


**Figure A3:** Inhibitors of Casein Kinase and MAP Kinase increase the basal surface levels of Kv1.2

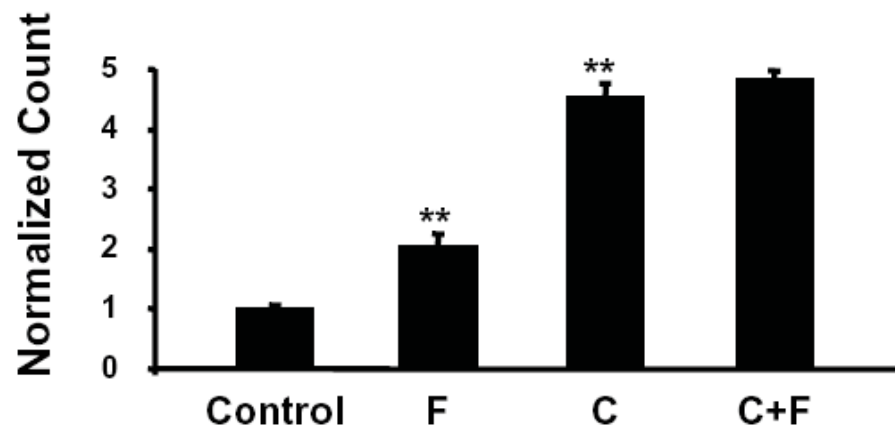


**Figure A4:** The cAMP downstream effectors EPAC and Rap significantly affect the basal levels of Kv1.2 at the cell surface

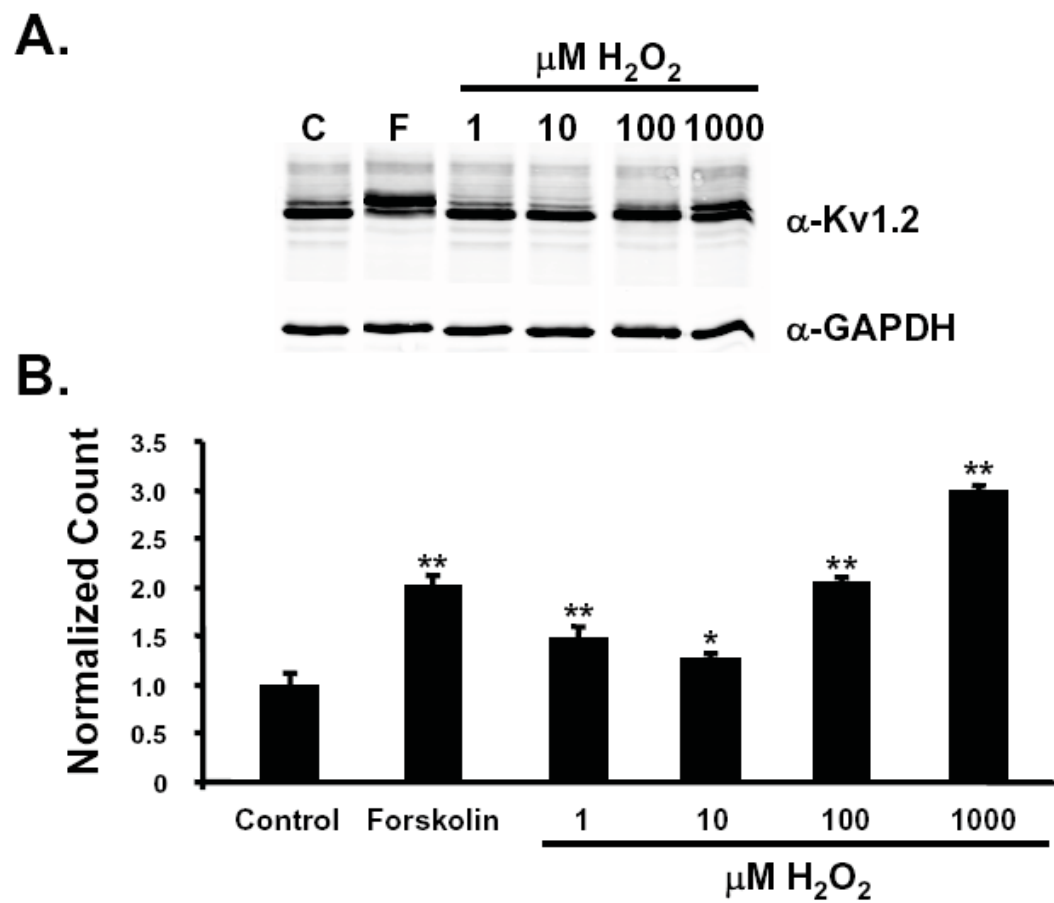
**A.**



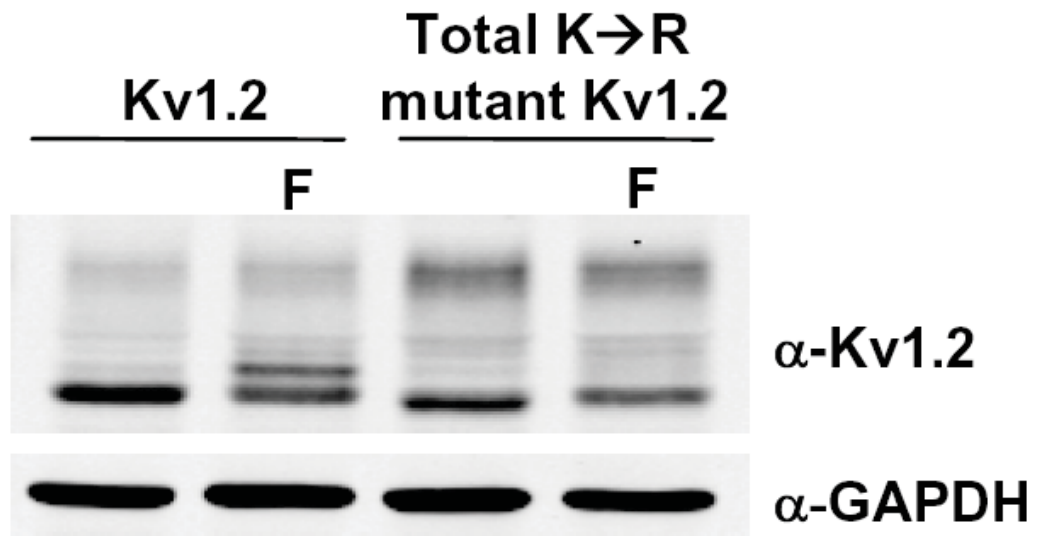
**B.**



**Figure A5:** Effects of AMP Kinase inhibition on Kv1.2 regulation



**Figure A6:** Effects of  $\text{H}_2\text{O}_2$  on Kv1.2



**Figure A7:** Kv1.2 mutated to prevent ubiquitination does not form a doublet when treated with forskolin

**Appendix B: Data relating to the paper “Pattern specific mono-ubiquitylation of Kv1.2 determines the polarity of its trafficking at the plasma membrane”**

**B.1. Background**

Protein ubiquitination is a post-translational modification that can be a signal for protein degradation. In the paper in preparation, entitled, “Pattern specific mono-ubiquitination of Kv1.2 determines the polarity of its trafficking at the plasma membrane” we describe the role of ubiquitination in the regulation of Kv1.2. This includes the involvement of region-specific mono-ubiquitination of the Kv1.2 alpha subunit by the E3 ubiquitin ligase c-Cbl. In this appendix, I provide a critical figure to the paper that shows the interaction of Kv1.2 and Cbl in the brain. The paper as a whole proposes that mono-ubiquitination of Kv1.2 can cause both positive and negative regulation of the channel.

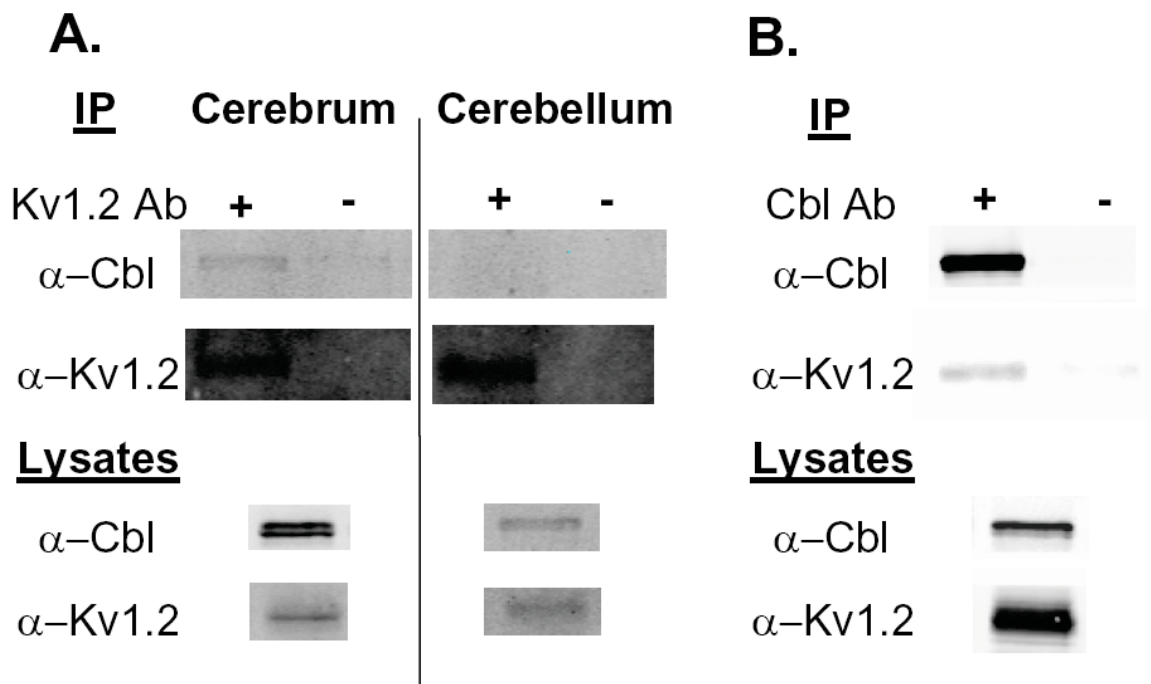
**B.2. Results and Discussion**

If Kv1.2 is ubiquitinated, it would interact with an E3 ubiquitin ligase that would transfer the ubiquitin moiety. We hypothesized that this interaction might be strong enough to detect by immunoprecipitation (IP). First, Kv1.2 was immunoprecipitated from either adult male rat cerebrum or adult male rat cerebellum. Cbl could be detected in the cerebrum IP, but not the cerebellum (Figure B1-A). It is possible that the channel interacts with this protein differently in various parts of the

brain. Both Cbl and Kv1.2 were detected in the lysates. The reverse-IP was performed with a polyclonal Cbl antibody in adult male rat cerebrum (Figure B1-B). The interaction between the two proteins is seen here as well. These data support the other data included in the paper describing the involvement of mono-ubiquitylation in Kv1.2 trafficking.

### **B.3. Figure Legend**

**Figure B1:** Kv1.2 and Cbl interact in the brain. (A) Immunoprecipitation (IP) of Kv1.2 from male rat cerebrum reveal that the channel interacts with Cbl. Kv1.2 was immunoprecipitated with the in-house polyclonal Kv1.2 antibody and Cbl was probed for with the Cbl-m antibody from BD Biosciences. To confirm IP of Kv1.2, the Kv1.2m antibody was used. Interestingly, IP of Kv1.2 from rat cerebellum did not show an interaction with Cbl. Enhanced RIPA was used to solubilize the tissue and lysates confirm presence of both proteins. (B) The reverse IP also showed an interaction between Cbl and Kv1.2 in the cerebrum. Cbl was immunoprecipitated with the polyclonal Cbl antibody from Santa Cruz and Kv1.2 was visualized with the Kv1.2m antibody. In the condition where the Cbl-p antibody was not added to the lysates, there was no detectible Cbl with the Cbl-m antibody, or Kv1.2 with the Kv1.2 antibody. Presence of both proteins is confirmed in the lysates.



**Figure B1:** Kv1.2 and Cbl interact in the brain



## **Appendix C: Data relating to R100K, a Kv1.2 mutant**

### **C.1. Background**

Voltage gated potassium channels are comprised of tetramers of Kv alpha subunits. Cytoplasmic assembly of the holotetramer is conferred by the T1 domain within each alpha subunit. The T1 domain has a polar interface that contains amino acids that make contact with the T1 domains of adjacent alpha subunits. R100 is a non-paired amino acid positioned at the top of the T1 domain in close proximity to Y132, a tyrosine known to be involved with channel endocytosis. We investigated the effects of mutation of this amino acid on channel regulation.

Negative regulation of Kv1.2 ionic current is evoked by activation of pathways that elicit tyrosine phosphorylation of the channel protein. One such pathway involves M1 muscarinic receptor-induced phosphorylation of Kv1.2 leading to endocytosis of the channel and thus suppression of Kv1.2 ionic current (Nesti, Everill et al. 2004). Kv1.2 endocytosis occurs through both clathrin-dependent and cholesterol-dependent endocytosis (Stirling, Williams et al. 2009). Kv1.2 suppression and endocytosis elicited by M1 muscarinic receptors appears to involve a clathrin-dependent pathway, while that elicited by serum occurs in a cholesterol-dependent manner. Here, we present evidence indicating that R100 is involved in regulating the type of endocytosis the channel undergoes.

## C.2. Results and Discussion

### R100K mutation produces a functional channel

R100 is situated at the top of the T1 domain as shown in Figure C1-A. The inset shows a top-down view depicting R100 from each of the four alpha subunits. Channels harboring a mutation of R100 to lysine (R100K) were functional and were detected at the plasma membrane by flow cytometry. Kv1.2R100K protein levels at the cell surface were, however, significantly decreased as compared to the WT channel (Figure C1-B). Similarly, the ionic current recorded from cells expressing the mutant channel was significantly decreased compared to the WT channel (Figure C1-C).

### The R100K mutation does not prevent the channel's interaction with the Kvβ2 subunit

Immunoprecipitation of Kv1.2 from HEK-B cells transfected with either Kv1.2WT or Kv1.2R100K revealed that the R100K mutation does not block binding to Kvβ2 (Figure C2-A). Interestingly, the R100K mutation seems to increase the interaction with the beta subunit (Figure C2-B). The increase is not statistically significant, most likely due to the small sample size for this experiment. We hypothesized that the Kv1.2R100K low surface levels were possibly due to the altered Kvβ2 binding. Figure C2-C shows that the levels of Kv1.2R100K at the cell surface are susceptible to the absence of Kvβ2 similarly to the Kv1.2WT. Thus, the low Kv1.2R100K surface levels are not due to changes in beta subunit binding.

### Channel glycosylation is altered by the introduction of a mutation at R100

During the Kv $\beta$ 2 experiments we noted that the patterning of Kv1.2R100K on the western blot was different than Kv1.2WT. Decreased total protein levels could possibly be responsible for the lower surface levels and decreased ionic current of Kv1.2R100K. Analysis of total protein levels between Kv1.2R100K and Kv1.2WT did not reveal any difference between the two (Figure C3-C). Interestingly, there was a significant difference in the relative amounts of the upper, mature glycosylated form of the channel (Figures C3-A&B). This suggests that Kv1.2R100 is possibly required for the biosynthetic maturation processing of the channel.

### Kv1.2R100K channels differ from Kv1.2WT in the response to specific stimuli that modulate channel endocytosis

Strikingly, Kv1.2R100K channels were far less sensitive to M1 mAChR induced endocytosis, but retained their ability to undergo serum induced endocytosis. Activation of M1 muscarinic acetylcholine receptors with carbachol causes a 50% suppression of Kv1.2WT, however this effect is reduced to a 20% suppression in Kv1.2R100K channels (Figure C4-A). Similar results were obtained with the protein tyrosine phosphatase inhibitor, pervanadate (Figure C4-B). In contrast, the negative suppression induced by serum is not affected by the R100K mutation (Figure C4-C).

The altered responsiveness of Kv1.2R100K to various stimuli may be explained by the multiple pathways of Kv1.2 endocytosis. Kv1.2R100K is significantly altered in

the response to carbachol and pervanadate as compared to Kv1.2WT. These pathways elicit channel endocytosis through a clathrin-mediated pathway. Alternatively, serum-induced endocytosis involves a cholesterol-dependent pathway, and this is unaffected by the R100K mutation. These data suggest that R100 is essential to clathrin-dependent endocytosis and that R100 modulates the response of Kv1.2 to stimuli that alter channel trafficking.

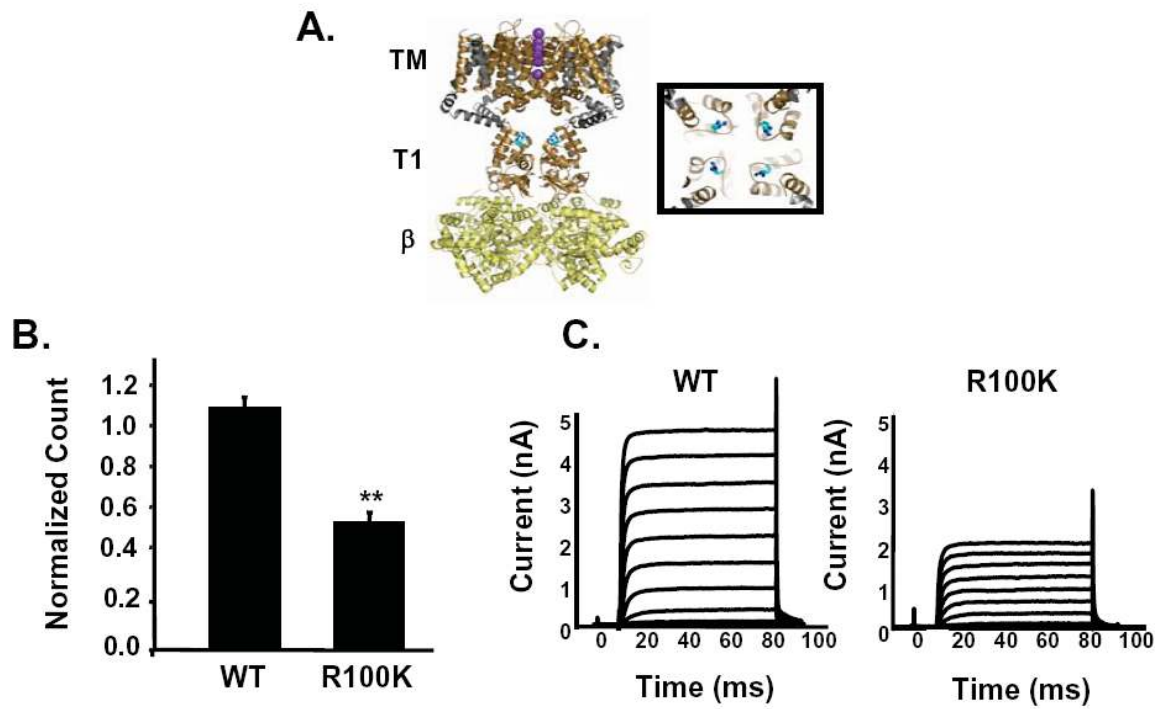
### C.3. Figure Legends

**Figure C1.** Introduction of a mutation at R100 (R100K) produces functional channel. (A) Crystal structure of Kv1.2 highlighting R100. Side view of holotetramer with R100 (indicated in aqua) depicted on all four alpha subunits. Inset shows a top down view. (TM: Transmembrane domain; T1: T1 domain consisting of amino acids 33-138;  $\beta$ : Beta subunits) The structure was adapted from the following reference: (Long, Campbell et al. 2005). (B) Flow cytometric analysis of unstimulated HEK-B cells transiently transfected with either Kv1.2WT or Kv1.2R100K. The R100K mutation causes a 47% decrease in the amount of channel at the cell surface (\*\* $p < 0.0001$  vs. control). (C) Steady state ionic current for transiently transfected Kv1.2WT and Kv1.2R100K in HEK-B cells. Current traces for each condition represent the average of measurements made in at least 7 cells. The R100K mutation causes an average of a 57% decrease in the amount of outward current, compared to WT channel.

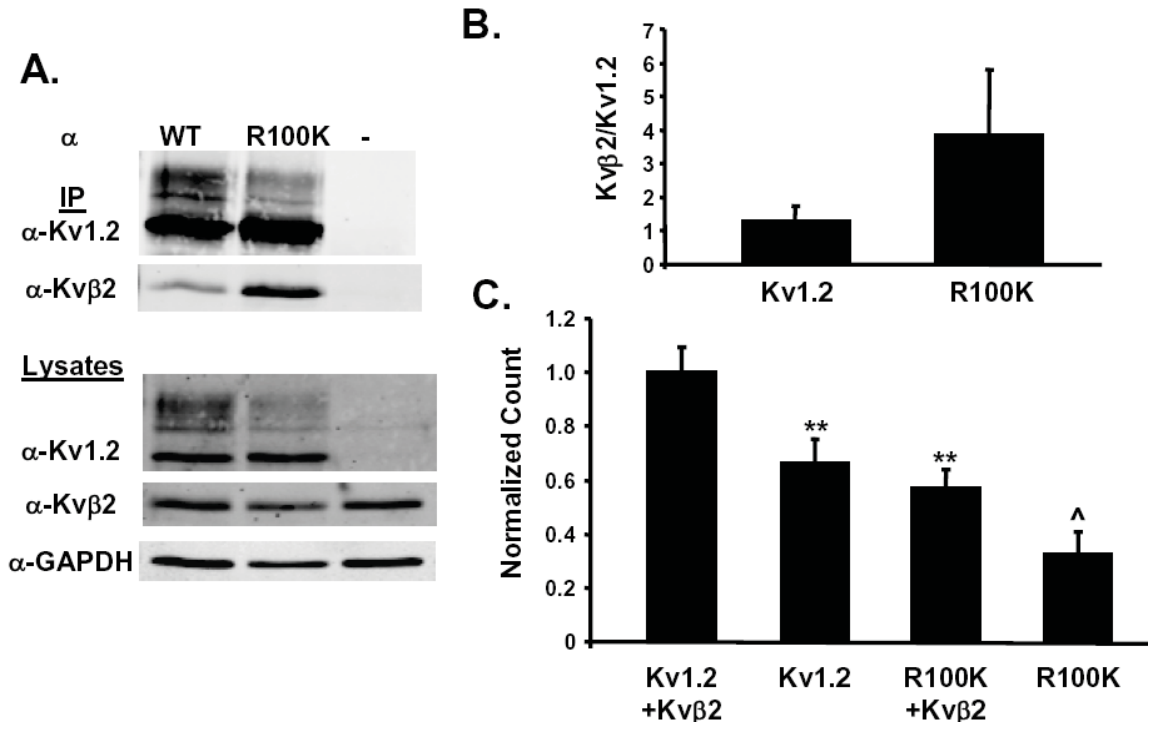
**Figure C2:** Kv1.2R100K may have altered Kv $\beta$ 2 binding; however this is not responsible for the decreased surface channel levels. (A) Immunoprecipitation of Kv1.2 from lysates of HEK-B cells transfected with either Kv1.2WT or Kv1.2R100K as indicated, reveal that both channels interact with Kv $\beta$ 2. Lysates confirm expression of both proteins. (B) Graph of the relative amount of Kv $\beta$ 2 co-immunoprecipitated with Kv1.2WT or mutant Kv1.2. The increase of Kv $\beta$ 2 binding to Kv1.2R100K as compared to Kv1.2WT is not statistically significant (pval=0.112), however this may be due to a low sample size (n=4). (C) Surface levels of Kv1.2WT or Kv1.2R100K in the presence or absence of Kv $\beta$ 2 indicate that Kv $\beta$ 2 is not responsible for the decreased surface levels of Kv1.2R100K. Kv $\beta$ 2 is required for efficient basal expression of surface Kv1.2WT and Kv1.2R100K. (\*\*p<0.01 vs. control or ^ vs. Kv1.2R100K).

**Figure C3:** The R100K mutation diminishes the upper, mature glycosylation bands. (A) Western blot analysis of lysates from cells transfected with either Kv1.2WT or Kv1.2R100K reveals that this mutation reduces the intensity of the upper bands, while keeping total protein levels the same. Kv1.2 was detected with the Kv1.2m antibody and GAPDH was used as a loading control. (B) Analysis of the intensity of the upper, glycosylation bands relative to the total Kv1.2 signal shows that Kv1.2R100K displays a significantly lower amount of glycosylation (\*\*pval<0.0001 vs. control). (C) Total protein levels of Kv1.2WT and Kv1.2R100K are not statistically different (pval=0.46; n=3).

**Figure C4:** Mutation of R100 selectively alters channel suppression. (A) Carbachol (CCH, 10 $\mu$ M, 10min) elicits a 47% decrease in Kv1.2WT surface levels, however carbachol stimulation only decreases surface levels of Kv1.2R100K by 16% (n=18, pval=0.18). The R100K mutation blocks carbachol-induced suppression by 65%. (B) Pervanadate (Per, 32 $\mu$ M, 10min) elicits a 54% decrease in Kv1.2WT surface levels, however Kv1.2R100K surface levels are decrease by only 20% in cells stimulated with pervanadate (n=12, pval=0.14). Thus, the R100K mutation decreases pervanadate-induced channel suppression by 64%. (C) Serum (10%, 10min) elicits a 56% decrease in Kv1.2WT surface levels. Serum stimulation also decreases Kv1.2R100K surface levels by 51% (\*\*pval<0.0001 vs. control, ^pval<0.0001 vs. Kv1.2R100K).

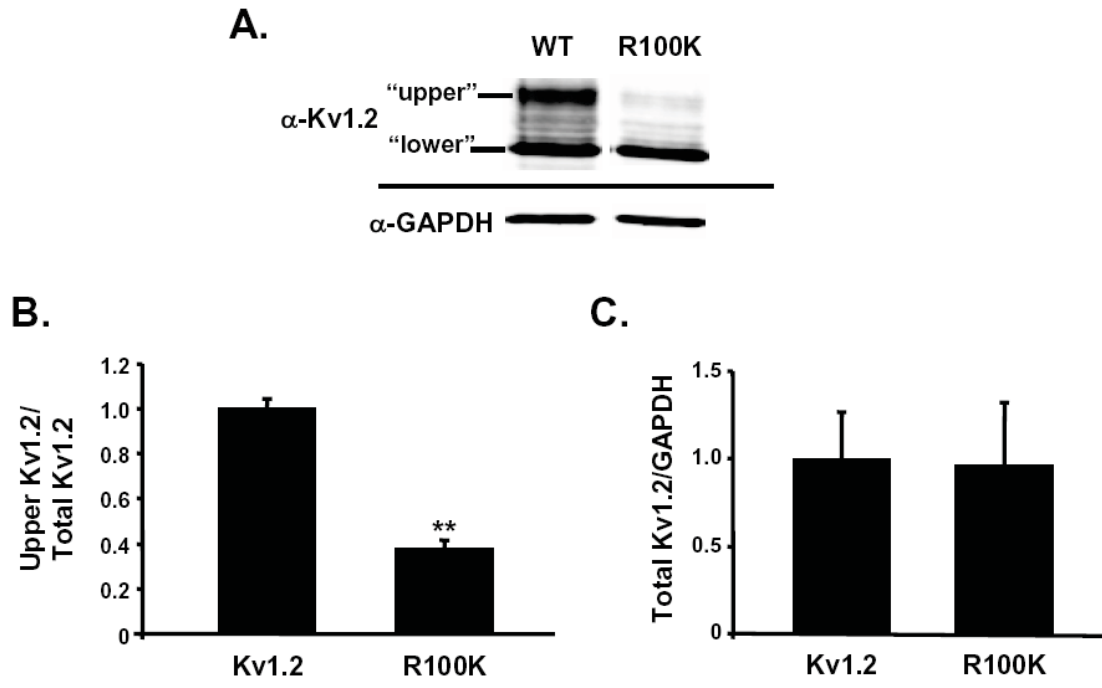


**Figure C1.** Introduction of a mutation at R100 (R100K) produces functional channel

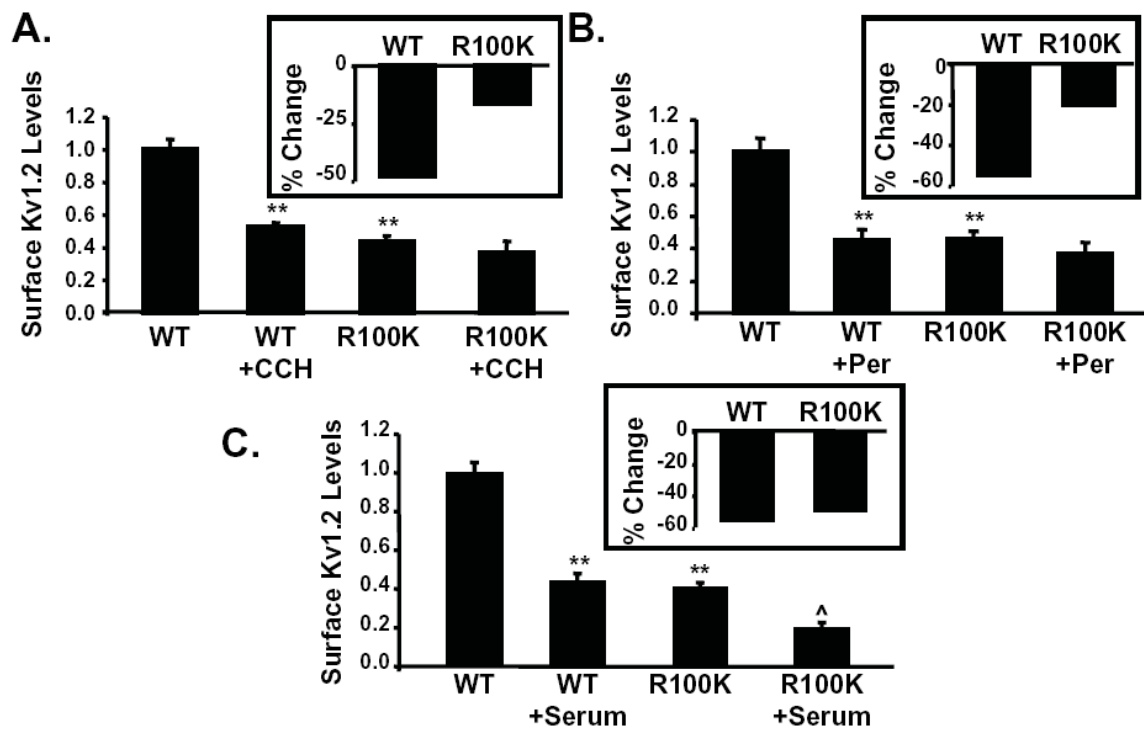


**Figure C2:** Kv1.2R100K may have altered Kv $\beta$ 2 binding; however this is not responsible for the decreased surface channel levels





**Figure C3:** The R100K mutation diminishes the upper, mature glycosylation bands



**Figure C4:** Mutation of R100 selectively alters channel suppression

## **Appendix D: Data relating to Chapter Three**

### **D.1. Background**

The research presented here was collected while investigating the mechanism by which PRMT8 increases Kv1.2 surface levels (Chapter 3). Similar to Appendix A, these findings may provide the basis for future studies.

### **D.2. Results and Discussion**

#### Kv1.2 surface levels are enhanced by inhibiting cellular arginine methylation

We tested the effect of a general arginine methylation inhibitor on Kv1.2 surface levels. Treatment with adenosine dialdehyde (AdOx, 20 $\mu$ M, 12hours) significantly decreased the amount of methylated proteins detected by an antibody against asymmetrically dimethylated arginine sidechains (Figure D1-A). This analysis confirms the efficacy of AdOx. Figure D1-B shows an AdOx-induced increase in the amount of Kv1.2 at the cell surface as compared to control. AdOx is a broad protein arginine methylation inhibitor, however, and these results are subject to only a general interpretation. Possibly the increase we see in Kv1.2 surface levels is the net effect of inhibiting methylation of a variety of proteins involved in the channel's regulation. Nevertheless, these data point to a role for arginine methylation and protein arginine methyltransferases in the regulation of Kv1.2. These data will become more relevant as specific inhibitors of the various PRMT isoforms become available.

## The involvement of Kv $\beta$ 2 in the PRMT8-mediated effects on Kv1.2

In Chapter 3, we show that PRMT8 alters the biosynthetic trafficking of Kv1.2 from the ER to the plasma membrane to cause an increase in Kv1.2 surface expression. One example of this is the ER-retained Kv1.2N207Q channel, which is trafficked to the surface by PRMT8 to a significantly greater extent than Kv1.2WT (Figure 4A). It has been suggested that the auxiliary subunit, Kv $\beta$ 2, promotes biosynthetic processing of Kv1.2 as expression of the beta subunit has been shown to increase Kv1.2 surface expression in some cells (Manganas and Trimmer 2000). We hypothesized that if the absence of the beta subunit retards Kv1.2 biosynthetic processing and retains it in the ER, then the effect of PRMT8 should be enhanced. PRMT8 was not only able to increase the surface expression of Kv1.2 in the absence of Kv $\beta$ 2, but its effect trends toward being significantly greater than when Kv $\beta$ 2 is present (Figure D2-A). If repeating this experiment yields a statistically significant increase in the PRMT8 effect on ER-retained Kv1.2 minus Kv $\beta$ 2, the conclusions proposed in Chapter 3 will be considerably strengthened.

Interestingly, PRMT8 and Kv $\beta$ 2 produce similar increases in surface Kv1.2 levels (Figure D2-B). Expression of both proteins further enhances Kv1.2 surface levels. Western blot analysis reveals that PRMT8 and Kv $\beta$ 2 have also have similar effects on the glycosylation patterning of Kv1.2 (Figure D2-C). Expression of either protein increases the presence of the mature glycosylated Kv1.2 on a western blot, and the effect of both proteins is additive (Figure D2-C,D). This raises the interesting possibility that brain-specific methyltransferase, PRMT8 may be substituting for Kv $\beta$ 2. Of particular interest is

finding that in Kv $\beta$ 2/Kv $\beta$ 1 knockout mice, Kv1.2 trafficking to the surface of cerebellar basket cells is unchanged (Connor, McCormack et al. 2005). Further research is needed to investigate whether PRMT8 has a role in Kv1.2 regulation in the cerebellum.

#### Effects of inhibiting lysosomal pH on Kv1.2 surface levels

We hypothesized that a mechanism for the PRMT8-mediated increase in surface Kv1.2 was inhibiting degradation of the channel. Lysosomal degradation can be reduced with the drug Chloroquine, as it disrupts lysosomal (and endosomal) pH. We tested the effects of this drug on Kv1.2 surface levels and found that Chloroquine significantly increased surface Kv1.2 levels (Figure D3). Preventing channel destruction, and generally interrupting endosomal trafficking, possibly increases the amount of channel available to recycle back to the plasma membrane. At higher concentrations, Chloroquine dramatically reduced the PRMT8-mediated increase in surface channel levels. This indicates that the mechanisms involved in the PRMT8 effects on Kv1.2 require intact lysosomes and/or endosomes and point towards a role for protein trafficking. Further testing is required to pinpoint the cause of the chloroquine effects on the PRMT8-induced increase in Kv1.2 surface levels.

#### Effects of proteasomal inhibition on Kv1.2 regulation

Proteins can be degraded by a proteasomal pathway which involves protein ubiquitination. Kv1.2 is thought to be regulated by ubiquitination. Therefore, we tested the effects of Mg132, an inhibitor of the proteasome, on Kv1.2 surface levels. As seen in

Figure D4, Mg132 significantly increased surface Kv1.2 levels. Furthermore, the PRMT8-mediated increase in surface Kv1.2 levels was not observed in the presence of Mg132. This suggests that part of the PRMT8 mechanism may involve preventing or circumventing proteasomal degradation of the channel.

Our biotinylation experiments show evidence that PRMT8 promotes the surface expression of an immature version of Kv1.2 (Chapter 3, Figure 3). Thus, we tested what form of Kv1.2 reached the cell surface after application of Mg132. Clearly, Mg132 (M) increases the amount of surface, biotinylated channel as compared to control (C; Figure D6). Proteasomal inhibition seems to promote only the fully mature glycosylated channel to the cell surface, as compared to PRMT8 (P).

#### Kv1.2 interacts with Heat shock protein 70

In our search for a mechanism for the PRMT8 mediated increase in surface Kv1.2 levels, we came across an interaction between Kv1.2 and Hsp70. PRMT8 and Hsp70 have been shown to interact (Pahlich, Zakaryan et al. 2008) and Hsp70 was identified in a preliminary mass spectrometry search of proteins that co-IP with Kv1.2. To confirm the mass spectrometry identification, we immunoprecipitated Kv1.2 and probed for Hsp70 with a polyclonal Hsp70 antibody (Figure D6).

This interaction is the basis for exceptionally interesting research projects as the implications for the co-IP of this ion channel and heat shock protein are wide ranging. First, it is possible that Hsp70 may be tied to the channels oxygen-sensing properties or the oxidoreductase activity of Kv $\beta$ 2. Also, both Hsp70 and Kv1.2 are implicated in

regulating REM sleep (Wada, Sei et al. 2006; Douglas, Vyazovskiy et al. 2007). The discovery of this protein:protein interaction may additionally lead to future insights about the channel's biosynthesis pathway as Hsp70 is involved with the biosynthetic progression of many proteins (Zhang, Nijbroek et al. 2001; Ficker, Dennis et al. 2005; Zwang, Hoffert et al. 2009).

We tested various conditions to see if we could enhance the interaction between Kv1.2 and Hsp70. Transfection of PRMT8, treatment with forskolin (10 $\mu$ M, 10min) and transfection of a non-glycosylated form of Kv1.2 (N207Q) all seemed to increase the Kv1.2:Hsp70 interaction. The initial mass spectrometry data was collected from forskolin treated cells, and thus we predicted that forskolin treatment may increase the co-IP. We also tested PRMT8 because of its known interaction with Hsp70. Lastly, we tested the N207Q mutant because it is thought to be retained in the ER. Hsp70 is involved with ER-associated degradation (ERAD) and N207Q is potentially held in the ER by an increased interaction with proteins with a known role in ERAD. The conclusion that these stimuli increase the Kv1.2:Hsp70 interaction is extremely preliminary must be repeated. If verified, a regulated interaction between Hsp70 and Kv1.2 would certainly provide critical insights into the regulation of the channel.

### **D.3. Figure Legends**

**Figure D1:** Inhibiting methylation increases Kv1.2 surface levels. (A) Western blot of lysates pretreated with or without AdOx (20 $\mu$ M, 12hours) was analyzed by densitometry. The asymmetric dimethyl arginine (ASYM24) antibody signal was evaluated and

GAPDH was used as a loading control. (n=4; \*\*p<0.01 vs. control) (B) Flow cytometric analysis of Kv1.2 surface levels reveals that pretreating cells with AdOx (20µM, 12 hours) significantly increases the amount of Kv1.2 detected at the cell surface (n=18, \*\*p<0.01 vs. control).

**Figure D2:** Involvement of Kvβ2 in PRMT8-mediated effects on Kv1.2. (A) PRMT8 significantly elevated surface Kv1.2 levels both the presence and absence of Kvβ2. The magnitude of this effect trended towards statistical significance (pval=0.08), however repeating this experiment is necessary. (B) Both Kvβ2 and PRMT8 (P8) significantly increase Kv1.2 surface levels (n=10; \*\*p<0.001). The degree by which Kvβ2 or PRMT8 increase Kv1.2 surface levels is not statistically different (p=0.31) (C) Co-transfection of Kvβ2 (β) or PRMT8 (P) cause a similar increase in the glycosylated (upper) form of Kv1.2 as compared to control (C). (B) Graphical analysis of the ratio of the upper Kv1.2 bands to the total Kv1.2 signal is represented. Kv1.2 was detected with the Kv1.2m antibody.

**Figure D3:** Disrupting endosomal and lysosomal pH increases basal surface Kv1.2 levels and alters the PRMT8-mediated increase. Chloroquine (C; 10, 50 or 100µM, 30min) treatment causes a significant increase of levels of Kv1.2 at the cell surface. (n=9, \*\*p<0.01 vs. control). PRMT8 (P) also causes a significant increase of basal Kv1.2 levels, however this increase is absent in the presence of 50µM Chloroquine and

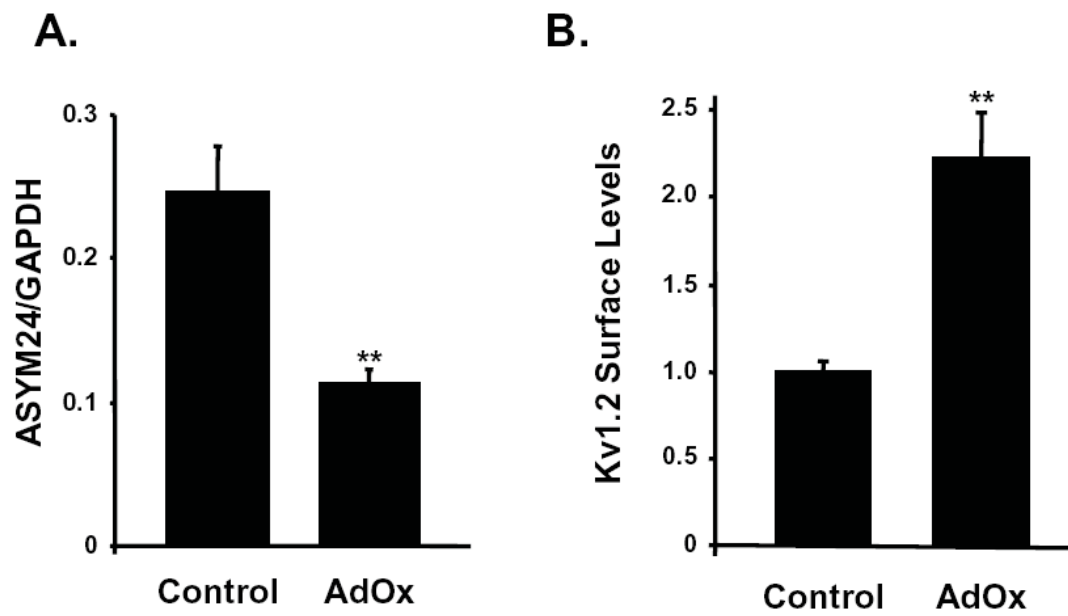


decreased, but still significant, with the application of 100 $\mu$ M Chloroquine ( $p > 0.01$  vs. Chloroquine without PRMT8).

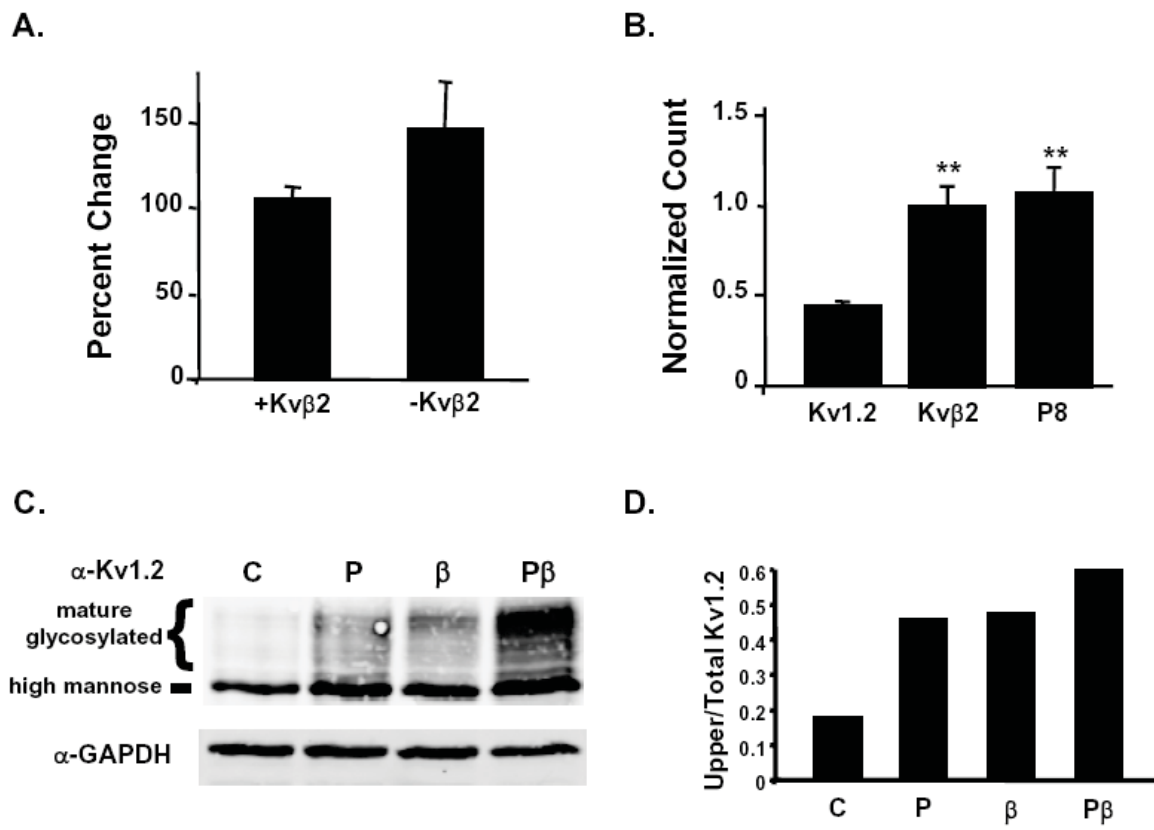
**Figure D4:** Inhibiting the proteasome increases basal surface Kv1.2 levels and prevents and further increase caused by PRMT8. Application of the proteasome inhibitor Mg132 (M; 10 $\mu$ M, 2 hours) significantly increases Kv1.2 surface levels (\*\* $p < 0.01$  vs. control). The PRMT8 (P) and Mg132 effects are not additive, as there is no significant difference between the Mg132 and PRMT8+Mg132 (PM; n=6).

**Figure D5:** Inhibiting the proteasome increases surface Kv1.2 levels by promoting the expression of the fully glycosylated form of Kv1.2. Biotin pulldown reveals that Mg132 and PRMT8 promote the surface expression of Kv1.2 differently. As compared to control (C), transfection of PRMT8 (P), Mg132 (M) or PRMT8 and Mg132 (PM) show an increase in the amount of channel detected on the cell surface. GAPDH and No Biotin (NB) were analyzed as controls. (Ladder=L).

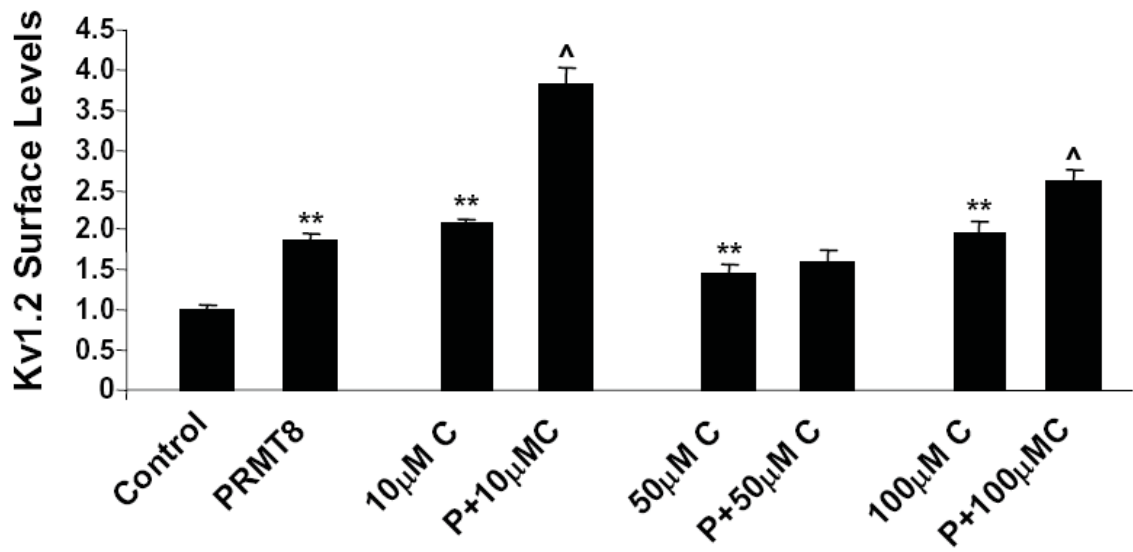
**Figure D6:** Kv1.2 and Hsp70 interact. Kv1.2 was immunoprecipitated from HEK-B cells (B) with the Kv1.2m antibody and the western blot was probed with an Hsp70 polyclonal antibody from Cell Signaling. Controls include HEK-B cells with no transfected Kv1.2 and IP with IgG. This preliminary findings suggest that transfection of PRMT8 (P), treatment with Forskolin (F; 10 $\mu$ M, 10min) or the N207Q mutation (N) may increase the interaction between Hsp70 and Kv1.2. (N=4 for K and P, N=1 for others).



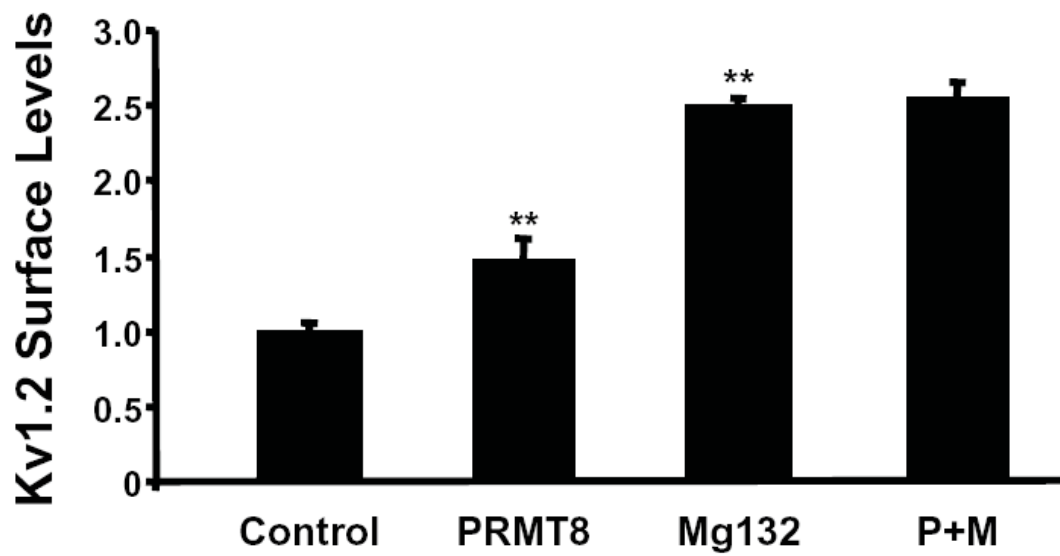
**Figure D1:** Inhibiting methylation increases Kv1.2 surface levels



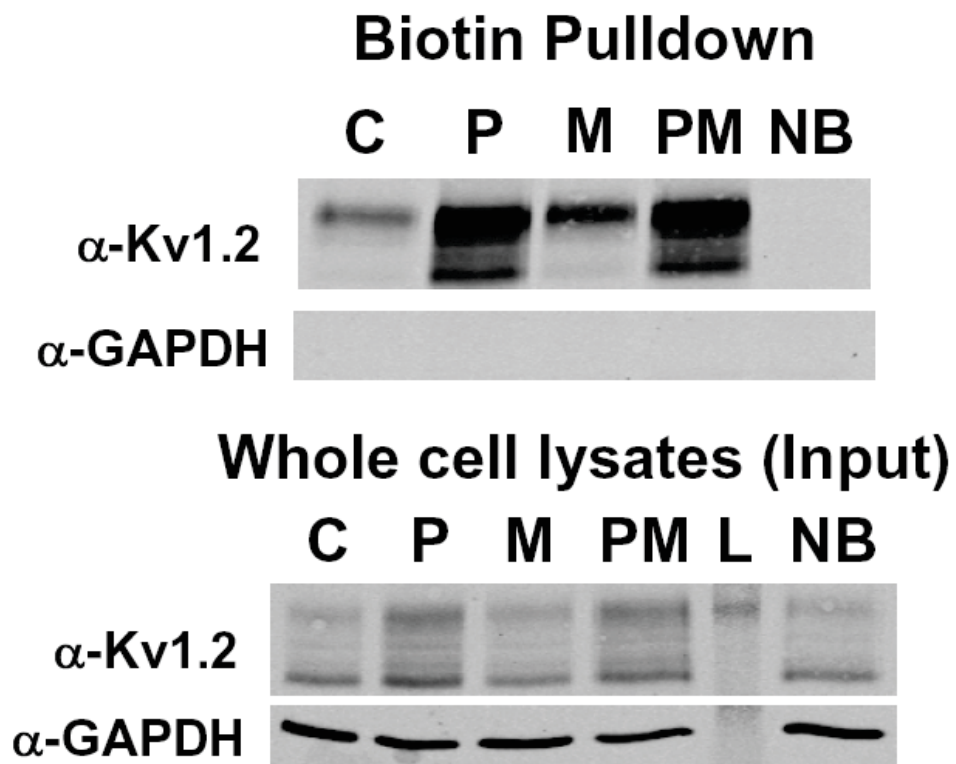
**Figure D2:** Involvement of Kvβ2 in PRMT8-mediated effects on Kv1.2



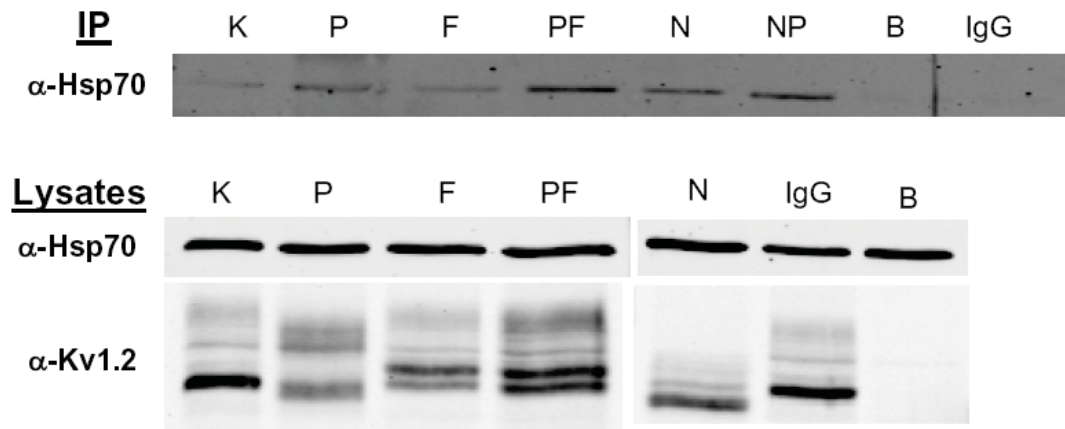
**Figure D3:** Disrupting endosomal and lysosomal pH increases basal surface Kv1.2 levels and alters the PRMT8-mediated increase



**Figure D4:** Inhibiting the proteasome increases basal surface Kv1.2 levels and prevents and further increase caused by PRMT8



**Figure D5:** Inhibiting the proteasome increases surface Kv1.2 levels by promoting the expression of the fully glycosylated form of Kv1.2



**Figure D6:** Kv1.2 and Hsp70 interact