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**SUBCELLULAR DISTRIBUTION OF A VOLTAGE-GATED POTASSIUM
CHANNEL: THE EFFECT OF LOCALIZATION ON CHANNEL FUNCTION**

A Dissertation Presented

by

Megan Anne Doczi

to

The Faculty of the Graduate College

of

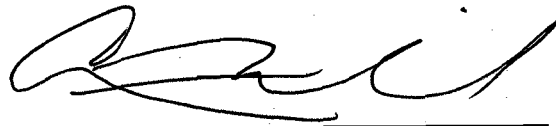
The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Anatomy and Neurobiology

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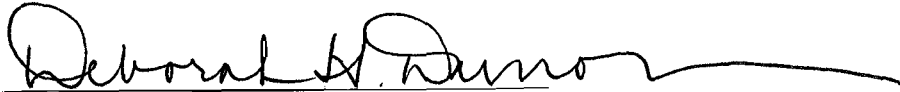
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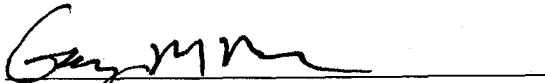


Advisor

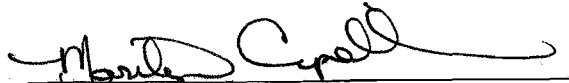
Anthony D. Morielli, Ph.D.



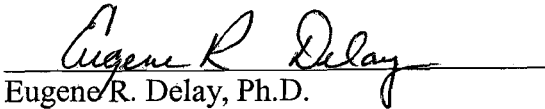
Deborah H. Damon, Ph.D.



Gary M. Mawe, Ph.D.

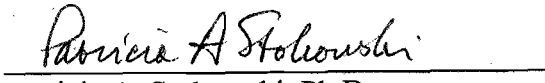


Marilyn J. Cipolla, Ph.D.



Chairperson

Eugene R. Delay, Ph.D.



**Associate Dean,
Graduate College**

Patricia A. Stokowski, Ph.D.

Date: November 11, 2009

ABSTRACT

Voltage-gated potassium channels are primary determinants of cellular excitability in the mammalian nervous system. The localization of these channels to distinct cellular compartments influences components of neuronal function, including resting membrane potential, action potential characteristics and neurotransmitter release. Thus, understanding the mechanistic basis of ion channel localization can provide fundamental insight into human physiology. The overall goal of this dissertation was to elucidate the regulatory mechanisms governing localization and function of the Kv1.3 voltage-gated potassium channel.

The sympathetic branch of the autonomic nervous system innervates many organ systems including the kidneys, heart and blood vessels and was used as a model to study endogenous Kv1.3. We found that postganglionic sympathetic neurons express Kv1.3 and that the channel exhibits a striking pattern of localization to the Golgi apparatus in the soma of these cells. Kv1.3 ionic current was also isolated from the soma of these neurons, indicating the channel is a determinant of the electrophysiological properties of sympathetic neurons. In addition, the specific inhibition of Kv1.3 with margatoxin was found to depolarize neuronal resting membrane potential, decrease the latency to action potential firing and increase nicotinic agonist-induced neurotransmitter release. Collectively, these findings demonstrate that Kv1.3 influences the function of postganglionic sympathetic neurons and led to the hypothesis that regulating channel localization may be a mechanism for modulating the activity of these cells.

In this dissertation, we propose that the observed Golgi retention of Kv1.3 may be a trafficking-dependent mechanism of channel regulation. To test this hypothesis, we used HEK293 cells as our model system. Our data show that the degree of Kv1.3 Golgi localization is inversely correlated with the amount of channel at the plasma membrane. In addition, the amplitude of Kv1.3 ionic current measured in cells with low Kv1.3 Golgi localization was significantly greater than the current measured in cells with high Kv1.3 Golgi localization. One mechanism for localizing ion channels to the Golgi apparatus involves the Class I PDZ-binding motif (X-S/T-X- Φ). Deletion of the C-terminal PDZ-binding motif of Kv1.3 decreased the intracellular Golgi localization of the channel and increased channel localization at the cell surface. Disrupting this canonical binding motif also increased the amplitude of Kv1.3 ionic current. These findings indicate that regulated subcellular distribution of the channel may be a determinant of Kv1.3 surface expression and function.

CITATIONS

Material from this dissertation has been published in the following form:

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DEDICATION

This dissertation is dedicated to my parents,

William and Christine Doczi,

for their unconditional love, support,

and patience.

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“When someone makes a decision, he is really diving into a strong current that will carry him to places he had never dreamed of when he first made the decision.”

- Paulo Coelho
The Alchemist

My life has been touched by many individuals whose guidance and support have shaped the person I am today. Although this section will attempt acknowledge them all, there is no possible way to extend my gratitude to each individual who has influenced the decisions leading up to and continuing throughout my graduate career. I would like to thank the Department of Anatomy and Neurobiology at the University of Vermont for providing an enriched learning environment that fosters scientific imagination. Thank you especially to my original mentor, Dr. Felix Eckenstein, for giving me the opportunity to immerse myself in this new culture. I would also like to acknowledge Dr. Cindy Forehand and Dr. Rae Nishi, two incredibly strong women who are ideal role models; thank you for believing in me, despite witnessing my weaknesses. Thank you also to Emily McLaughlin for her endless enthusiasm and positive presence; she is truly a breath of fresh air.

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

I. Introduction

Ion channels are integral membrane proteins that mediate rapid changes in the electrical potential across cellular membranes (Katz and Miledi, 1970). These pore forming proteins open and close in response to specific stimuli and selectively allow the passage of ions through the lipid bilayer along their electrochemical gradient (Mullins, 1975). Consequently, ion channels are major determinants of cellular excitability (Schauf and Bullock, 1979). In neurons, the complex spatial distribution of these proteins to specific subcellular compartments such as the soma, axons, dendrites and presynaptic terminals influences neuronal excitability and synaptic transmission (Chiu and Ritchie, 1981; Rasband et al., 1998). Therefore, understanding the mechanisms governing ion channel localization can provide insight into neuronal function.

This dissertation identifies the novel localization of the Kv1.3 voltage-gated potassium channel in sympathetic neurons of the autonomic nervous system. We propose that subcellular localization of Kv1.3 to the Golgi apparatus may be a mechanism of channel regulation. While trafficking-dependent mechanisms of regulation have been defined for other Kv1 family channels (Choi et al., 2005; Connors et al., 2008; Nesti et al., 2004; Watanabe et al., 2007; Zhu et al., 2003a), localization of Kv1.3 to the Golgi remains unexplored. One major mechanism for localizing ion channels to specific subcellular compartments involves protein-protein interactions (Gu et al., 2003; Kim et al., 1995; Manganas and Trimmer, 2004). The work presented here identifies a protein

interaction motif that contributes to the localization of Kv1.3 and shows that modulating this motif can alter channel function.

The consequences of altering Kv1.3 channel function span a wide range of physiological processes, from immune responsiveness to olfaction (Beeton et al., 2006; Desir, 2005; Fadool et al., 2004; Rangaraju et al., 2009; Xu et al., 2003; Xu et al., 2004). This dissertation highlights the novel expression of Kv1.3 in postganglionic sympathetic neurons and suggests that modulating channel localization in this cell type may lead to changes in sympathetic excitability. Altering the distribution of Kv1.3 ion channels in these neurons may have significant effects on regulating sympathetic effectors, defining a new physiological role for this channel. Therefore, elucidating the critical role of ion channel localization is imperative to understanding the mechanistic basis of neuronal activity.

II. Anatomy and Function of the Sympathetic Nervous System

A. The Sympathetic Nervous System

Chapter 2 of this study focuses on the expression, function and modulation of the Kv1.3 voltage-gated potassium channel, using the sympathetic division of the autonomic nervous system as a model system. The autonomic nervous system regulates homeostatic mechanisms, including gastrointestinal motility, reproduction, metabolic physiology and cardiovascular function (Bornstein et al., 2004; Ito and Scher, 1981; Jones et al., 1987). It is composed of three major subdivisions: sympathetic, parasympathetic and enteric

(Purves, 2001). In addition to providing tonic drive to target effectors, the sympathetic division of the autonomic nervous system also coordinates adaptive responses to stressful stimuli (Selye and Fortier, 1949; Purves, 2001) in an event commonly referred to as the fight or flight response. The sympathetic nervous system originates from brainstem nuclei and from the intermediolateral cell column of the spinal cord, where preganglionic sympathetic cell bodies extend from the thoracic (T1) to the upper lumbar (L2 or L3) segments (Purves, 2001). Preganglionic sympathetic neurons innervate para- and prevertebral ganglia located outside of the central nervous system (CNS) (Purves, 2001). The paravertebral ganglia are situated bilaterally along the length of the vertebral column, while prevertebral ganglia are located midline and anterior to the vertebral column (Purves, 2001).

Postganglionic sympathetic neurons (PSNs) are derived from the neural crest and contain cell bodies that reside in the para- and prevertebral ganglia (Le Douarin and Teillet, 1974). PSNs extend long, generally unmyelinated, small diameter axonal processes outward to innervate various visceral effectors as seen in Figure 1 (Glebova and Ginty, 2005; Wallin and Charkoudian, 2007). The superior cervical ganglion (SCG) is a representative paravertebral ganglion composed of PSNs and has been used to study sympathetic activity (Li and Horn, 2006; Luther and Birren, 2006). In addition to providing sympathetic innervation to the eye, major target tissues for the SCG include the lacrimal, submandibular and parotid glands (Li and Horn, 2006). The SCG also provides sympathetic innervation to blood vessels (Cassaglia et al., 2008; Li and Horn, 2006). However, the sympathetic control of blood vessels is not well understood. The distal end of a postganglionic sympathetic axon is highly branched and contains a complex network

of small membranous swellings termed varicosities (Todd, 1980). These varicosities contain mitochondria and numerous large dense-core and small dense-core neurotransmitter containing vesicles (Devine and Simpson, 1968). They are known to be sites of local neurotransmitter release that greatly increase communication between sympathetic efferents and target vessels (Devine and Simpson, 1967, 1968; Jackson and Cunnane, 2001).

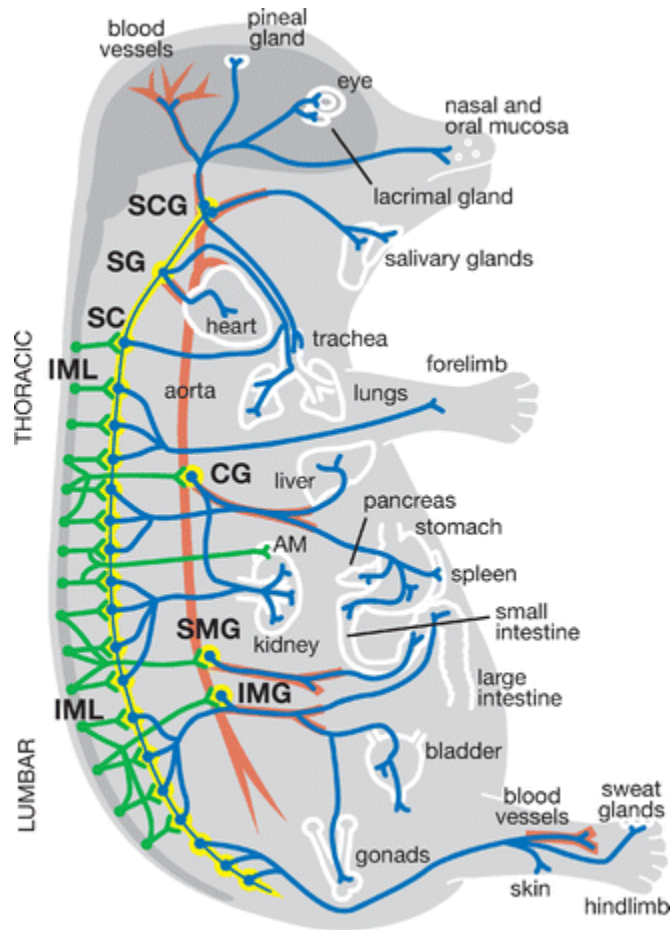


Figure 1: Anatomy of the sympathetic nervous system in a rodent model. Schematic representation of preganglionic (green) and postganglionic (blue) sympathetic innervation in a mouse. Preganglionic cell bodies are located in the intermediolateral (IML) cell column of the thoracic and lumbar spinal cord. Preganglionic fibers synapse onto postganglionic cells bodies in sympathetic ganglia (yellow). Paravertebral ganglia, such as the superior cervical ganglion (SCG), are situated bilaterally alongside the vertebral column. Postganglionic sympathetic fibers directly innervate target effectors such as the heart, kidney and blood vessels.

Glebova et al. **Growth and survival signals controlling sympathetic nervous system development.** *Annual Review of Neuroscience.* (2005) 28, pp 191-222.

B. Sympathetic Neurotransmission

Sympathetic ganglia from the adult mouse and rat are widely used as experimental models because they accurately reflect the sympathetic innervation of the vasculature in humans (Glebova and Ginty, 2005; Li and Horn, 2006). The experiments outlined in this dissertation will focus on the SCG. Phenotypic characteristics such as neuropeptide content, electrical properties and morphology of these ganglia have been previously explored, making SCG neurons a powerful model system for studying sympathetic neurons (Anderson et al., 2001a; Anderson et al., 2001b; Gibbins et al., 2000; Li and Horn, 2006; Morris et al., 1995). The experiments presented in Chapter 2 will use PSNs from primary cultures of dissociated neonatal rat SCG. Dissociated cell cultures grown *in vitro* provide the opportunity to carry out a variety of experimental techniques in a specific population of cells that can be maintained in a tightly controlled environment.

While preganglionic sympathetic neurons utilize acetylcholine (ACh) as their main neurotransmitter, postganglionic sympathetic neurons are primarily adrenergic, and utilize norepinephrine (NE) (Gibbins and Morris, 2006; Kanagy, 2005; Morales et al., 1995), as well as various neurotransmitter peptides. NE is a catecholamine neurotransmitter that is synthesized from the amino acid tyrosine in a series of enzymatic steps (Kirshner, 1957). Tyrosine is converted to dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (Kirshner, 1957). L-DOPA is then decarboxylated to form the neurotransmitter dopamine via the enzyme DOPA decarboxylase (Kirshner, 1957). In the

final step of norepinephrine biosynthesis, dopamine- β -hydroxylase oxidizes dopamine to form NE (Kirshner, 1957). The rate limiting enzyme in norepinephrine synthesis is tyrosine hydroxylase (TH) and is used to confirm the presence of postganglionic sympathetic neurons (Anderson et al., 2001b). The research presented in this dissertation utilizes TH as a marker of postganglionic neurons. Although we focus on NE release as the main measure of sympathetic activity, it is important to note that additional neurotransmitters, such as neuropeptide Y and ATP, are coreleased in conjunction with NE (Msghina et al., 1998; Wahlestedt et al., 1992). The release of NE from sympathetic nerve terminals has previously been used to measure sympathetic nerve activity and alterations in the release and reuptake of NE may provide insight into the function of these cells (Kristufek et al., 1999; Msghina et al., 1998; Socci et al., 2003; Uhrenholt and Nedergaard, 2003).

C. Electrophysiological Properties of Postganglionic Sympathetic Neurons

As described above, postganglionic sympathetic neurons communicate with target effector organs via the release of neurotransmitters such as NE (Bennett et al., 2004; McLachlan, 2007). Therefore, the function of these neurons is essential for maintaining the sympathetic regulation to various tissues, including blood vessels. ACh released from preganglionic sympathetic neurons binds to ligand-gated nicotinic receptors located in the soma of PSNs, causing membrane depolarization of the cell to reach threshold, generating an action potential which is conducted along the length of the postganglionic sympathetic axon (Muscholl et al., 1973). When the action potential reaches a

sympathetic varicosity or synaptic terminal, voltage-gated calcium channels permit entry of extracellular calcium into the cell and activate the release of neurotransmitters (Muscholl et al., 1973). Therefore, knowledge of the electrophysiological properties of PSNs is crucial to understanding the function of the sympathetic nervous system.

Postganglionic sympathetic neurons can be classified into three main categories based on their electrophysiological discharge properties upon stimulation with a depolarizing current: Phasic (Ph), tonic (T) and long afterhyperpolarizing (LAH) (Cassell et al., 1986; Jamieson et al., 2003; Keast et al., 1993; McLachlan and Meckler, 1989). Phasic neurons fire multiple bursts of action potentials transiently upon depolarization, whereas tonic neurons fire action potentials throughout the duration of a maintained depolarization (Cassell et al., 1986). LAH neurons discharge only once, due to the prolonged outward current that may last several seconds (McLachlan and Meckler, 1989). These three electrophysiological classes of sympathetic neurons are predominantly localized to different populations of ganglia (Boyd et al., 1996; Keast et al., 1993). The paravertebral chain is home to primarily Ph neurons, while T neurons and LAH neurons reside mainly in the inferior mesenteric ganglion and celiac ganglion, respectively (Jamieson et al., 2003).

As is true for all neurons, electrophysiological properties of sympathetic neurons are governed by the expression and localization of various ion channels within the cell membrane (Arnold, 2007; Choe, 2002; Kaczmarek, 2006; Lai and Jan, 2006; Roeper and Pongs, 1996). In particular, voltage-gated potassium channels (Kv) are fundamental determinants of neuronal excitability. Based upon their localization within the cell, Kv channels can have different effects on neuronal physiology and neurotransmitter release;

affecting depolarization properties if localized to the soma, or action potential propagation if distributed along the axon (Lai and Jan, 2006). This dissertation focuses on elucidating the role of the Kv1.3 ion channel in sympathetic neuronal activity, and emphasizes the importance of subcellular localization on channel function.

III. The Kv1.3 Voltage-Gated Potassium Channel

A. Kv1 Channel Structure

Voltage-gated potassium channels (Kv) are expressed throughout the mammalian nervous system, where they play a central role in regulating neuronal excitability via the maintenance of resting membrane potential, integration of synaptic input, and regulation of action potential firing (Armstrong and Hille, 1998; Catterall, 1988; Choe, 2002; Lai and Jan, 2006). These highly conserved channels are grouped into twelve families denoted Kv1 through Kv12 (Gutman et al., 2003). The Kv1 family of voltage-gated potassium channels is known as the *Shaker* family (Jan et al., 1977). First identified in *Drosophila melanogaster*, genetic mutations in the *Shaker* locus result in flies that vigorously shake their legs under anesthesia due to abnormal neuronal activity (Jan et al., 1977). The *Shaker* family consists of eight genes encoding distinct alpha subunit proteins, Kv1.1 through Kv1.8 (Gutman et al., 2005). These alpha subunits can also associate in a 1:1 stoichiometry with auxiliary beta-subunits (Kv β 1-3), which can alter channel trafficking, gating and function (Heinemann et al., 1996; Rettig et al., 1994). Functional channels are formed when four Kv1 alpha subunits assemble as homotetramers of

uniform subunits or as heterotetramers with other members of the Kv1 family (Shen and Pfaffinger, 1995). Formation of heteromultimeric channels greatly enhances functional diversity through the modulation of current conduction, as well as fine tuning channel regulation through multiple channel trafficking pathways (Choe, 2002).

Several conserved structural regions within Kv1 channels are important for regulating fundamental aspects of channel localization and function. These regions include the six transmembrane domains (Yi et al., 2001), the intracellular N-terminal tetramerization domain (Gu et al., 2003), the putative voltage sensor (Borjesson and Elinder, 2008) and the potassium ion specific pore loop (Manganas et al., 2001b; Zhu et al., 2005). Basic properties of Kv1 channel assembly, gating, selectivity and conductance are governed by these structural domains (Choe, 2002; Debanne, 2004; Dodson and Forsythe, 2004; Judge and Bever, 2006; Lai and Jan, 2006).

The work presented in this dissertation focuses on a specific member of the *Shaker* family, Kv1.3. Each Kv1.3 alpha subunit is composed of six transmembrane spanning domains, termed S1-S6 (Figure 2) (Choe, 2002). The first four transmembrane helices enable the channel to sense and respond to changes in membrane potential (Grottesi et al., 2005). In particular, the positively charged S4 segment serves as the voltage sensor and detects changes in membrane potential, rendering the channel in an open or closed state (Grottesi et al., 2005). The positive charge of the voltage sensing domain is conferred by multiple basic arginine residues in the S4 helix (Borjesson and Elinder, 2008). Upon membrane depolarization, conformational changes in the S4 domain lead to opening of the channel pore and the influx of potassium ions (Borjesson and Elinder, 2008; Grottesi et al., 2005). Between segments S5 and S6 lies the selectivity

filter, containing the signature sequence Thr-Val-Gly-Tyr-Gly that is characteristic of the pore loop (Doyle et al., 1998). Stereochemical checkpoints ensure the specificity of potassium ion flow through the narrow pathway of the pore loop (Doyle et al., 1998).

The transmembrane spanning helices of Kv1.3 are flanked by cytoplasmic termini containing protein interaction domains (Gu et al., 2003; Kosolapov et al., 2004). The intracellular N-terminal region immediately preceding the first transmembrane helix (S1) of Kv1.3 contains a stretch of approximately 130 amino acids that mediates the tetramerization of four alpha subunits to form a holochannel (Gu et al., 2003; Kosolapov et al., 2004). Accordingly, this region is referred to as the T1 tetramerization domain. In addition to promoting the proper assembly of hetero- and homotetramers (Shen and Pfaffinger, 1995), the T1 domain has also been shown to target Kv1.3 to the axonal region of neurons (Rivera et al., 2005), indicating a role for this domain in both the function and localization of Kv1 channels.

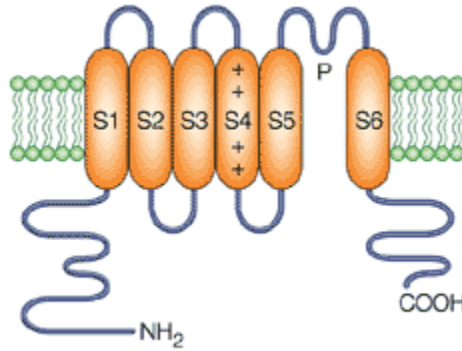


Figure 2: Voltage-gated potassium channel alpha subunit structure. The alpha subunit of Kv1 channels is composed of intracellular N- and C-termini and six transmembrane spanning domains (S1-S6). The N-terminus contains the T1 tetramerization domain, S4 (+) is the voltage sensor, and the poor loop selectivity filter (P) lies between S5 and S6.

Choe, S. **Potassium Channel Structures.** *Nature Reviews Neuroscience.* (2002) 3, pp 115-120.

B. Physiological Significance of Kv1 Channels

Voltage-gated potassium channels play a major role in regulating cellular excitability through the delicate control of potassium ion flow across the plasma membrane (Yi et al., 2001). These channels open and close in response to changes in membrane potential (Borjesson and Elinder, 2008) and facilitate events such as neuronal action potential propagation. Therefore, perturbations in Kv1 channel expression and localization could have significant consequences on neuronal function. Through the study of mutant channels and transgenic gene-targeted deletions, the biological importance of

Kv1 family channels has been well characterized (Brew et al., 2007; Lopantsev et al., 2003; Tschritter et al., 2006).

Point mutations in the Kv1.1 potassium channel gene (KCNA1) have been linked to a rare autosomal dominant disorder in humans. Known as episodic ataxia type 1 (EA1), this channelopathy is characterized by intermittent episodes of unsteady gait, uncoordinated movements, and involuntary muscle twitches and is frequently associated with unprovoked epileptic seizures (Manganas et al., 2001a; Smart et al., 1998). Kv1.1-null mice recapitulate these symptoms and also demonstrate increased brain growth and megencephaly (Persson et al., 2005; Petersson et al., 2003), predominantly in the hippocampus and ventral cortex (Almgren et al., 2007; Persson et al., 2007). Additionally, targeted deletion of KCNA1 in mice results in the hyperexcitability of CA3 pyramidal cells, providing further evidence for high seizure susceptibility in these animals (Lopantsev et al., 2003). Kv1.1 alpha subunits commonly co-assemble with Kv1.2 to form heteromultimeric tetramers in neurons (Brew et al., 2007; Wang et al., 1994). These two Kv1 subtypes are often localized to juxtaparanodal regions in myelinated axons, where they contribute to regulating action potential propagation (Brew et al., 2007; Lai and Jan, 2006; Rasband and Shrager, 2000). Deletion of the gene that encodes for the Kv1.2 alpha subunit (KCNA2) also leads to spontaneous generalized seizures in mice (Brew et al., 2007), underscoring the importance of Kv1 channels in the maintenance of neuronal excitability.

This dissertation focuses on Kv1.3, a *Shaker* family potassium channel closely related to Kv1.1 and Kv1.2. Although this channel exhibits limited expression in the central nervous system, high levels of Kv1.3 have been detected in brain regions such as

the dentate gyrus of the hippocampus, the hypothalamus and the olfactory bulb (Fadool and Levitan, 1998; Grosse et al., 2000; Veh et al., 1995). Also, Kv1.3 accounts for the largest proportion of outward current from olfactory bulb mitral cells (Fadool et al., 2004). Gene-targeted deletion of this channel results in neurons with modified action potential characteristics; less current is required to elicit the same spike frequencies observed in wild-type cells (Fadool et al., 2004). In addition, behavioral assessments revealed that Kv1.3-null mice have a lower threshold for odor detection that is accompanied by increased odor discrimination, suggesting a link between neuronal excitability and behavior (Fadool et al., 2004). Conversely, recent studies indicate that a single-nucleotide polymorphism in the human Kv1.3 gene (KCNA3) results in a gain of function; accordingly, carriers of this polymorphism show impairments in olfactory function (Guthoff et al., 2009).

Kv1.3 has also been highlighted as an important component of other physiological processes. Phenotypic examination of Kv1.3 deficient mice (Kv1.3^{-/-}) indicates that the channel is a determinant of body weight and energy homeostasis (Xu et al., 2003). In comparison to control littermates, Kv1.3^{-/-} mice display decreased body weight, increased metabolic rate and are resistant to diet-induced obesity (Xu et al., 2004). Kv1.3 has also been found to contribute to the regulation of peripheral insulin sensitivity and glucose metabolism (Xu et al., 2004). Inhibiting Kv1.3 has been shown to decrease inflammatory cytokines, thereby increasing insulin sensitivity (Desir, 2005; Xu et al., 2004). In addition, a polymorphism in the promoter region of human KCNA3 that alters transcriptional activity is associated with impaired glucose tolerance and reduced insulin sensitivity (Tschritter et al., 2006). These findings also provide rationale to explore

Kv1.3 as a potential therapeutic target for metabolic diseases such as diabetes mellitus (Desir, 2005; Esler et al., 2001).

Homotetrameric Kv1.3 channels are the major voltage-gated potassium channels expressed in T-lymphocytes, key mediators of autoimmune disease (Beeton et al., 2006; Panyi, 2005; Panyi et al., 2006; Panyi et al., 2004a). In these cells, the channel plays a central role in regulating resting membrane potential, Ca^{2+} signaling cascades, and antigen-dependent activation and proliferation (Lin et al., 1993). The expression of Kv1.3 has been shown to dramatically increase when CD4^+ and CD8^+ T-lymphocytes are activated and inhibition of the channel suppresses downstream signaling cascades that lead to cell proliferation and cytokine production (Beeton et al., 2006). This research has placed Kv1.3 at the center of emerging therapeutic targets for T-cell mediated autoimmune diseases (Beeton et al., 2006).

C. Kv1 Channel Pharmacology

Because a plethora of diverse ion channels exist within cellular membranes, isolating current from voltage-gated potassium channels is a challenging task. However, pharmacological reagents that specifically block Kv channels have been used to resolve current from distinct channels (Garcia-Calvo et al., 1993; Kirpekar et al., 1977; MacKinnon and Yellen, 1990; Vacher et al., 2007). In addition, these pharmacological tools have been used to decipher the physiological role of differential channel localization (Panyi et al., 2006). Two main molecular pharmacological agents are capable of blocking or modulating Kv channels: organic compounds and venom-derived peptide toxins

(Grissmer et al., 1994; Gutman et al., 2005; Judge and Bever, 2006; Wei et al., 2005). These powerful probes have been used to investigate various Kv channel currents in a variety of cell types.

Most potassium channels possess the ability to pass ammonium ions, however the quaternary derivative, tetraethylammonium chloride (TEA), acts as an open channel blocker of Kv channels (Cena et al., 1985; Crouzy et al., 2001; Heginbotham and MacKinnon, 1992; MacKinnon and Yellen, 1990). While the effect of intracellular TEA is quite uniform in blocking Kv channels, the extracellular application of TEA is variable; Kv1 channels demonstrate one of the lowest sensitivities to this organic compound, allowing the use of TEA as a tool to resolve currents produced by these channels (MacKinnon and Yellen, 1990).

Another organic compound, 4-aminopyridine (4-AP), blocks a variety of Kv channels, having the greatest binding affinity for delayed rectifying channels of the Kv1 family (Judge and Bever, 2006; Kirpekar et al., 1977; Msghina et al., 1998; Przywara et al., 1992; Thorneloe et al., 2001). This compound is known as an open channel blocker because upon application, the drug enters the pore region of an open channel, and becomes trapped within the intracellular face of the channel pore (Judge and Bever, 2006). Researchers report that the addition of 4-AP to rabbit carotid arteries preloaded with [³H]-norepinephrine greatly enhances the stimulation-evoked overflow of NE, suggesting that voltage-gated potassium channel blockade increases neurotransmitter release from sympathetic neurons (Uhrenholt and Nedergaard, 2003). Studies of ocular hypertension in rabbits indicate that the topical application of 4-AP can elevate intraocular pressure (IOP) (Socci et al., 2003). Increases in IOP are accompanied by

increases in aqueous levels of NE (Socci et al., 2003). In isolated rabbit iris-ciliary body preparations, 4-AP causes a significant increase in field-stimulated NE release from sympathetic nerves (Socci et al., 2003). These experiments suggest that Kv channels in sympathetic nerves are important for regulating neurotransmitter release and provide a rationale for isolating specific Kv1 family currents from postganglionic sympathetic neurons.

Venom-derived peptide toxins are small, compact globular proteins that are highly potent Kv channel inhibitors selective for specific classes of ion channels (Judge and Bever, 2006). At low nanomolar concentrations, the snake-derived peptide toxin, dendrotoxin (DTX), can specifically inhibit Kv1.1 and Kv1.2 channels (Dodson et al., 2003; Gasparini et al., 1998; Haghdoost et al., 2006; Harvey, 2001; Judge and Bever, 2006). Similarly, the scorpion-derived peptide toxin, margatoxin (MgTx), can specifically inhibit Kv1.3 at low concentrations (Bednarek et al., 1994; Garcia-Calvo et al., 1993; Helms et al., 1997; Judge and Bever, 2006; Shah et al., 2003). While it is possible for some venom-derived toxins to incorporate into the lipid bilayer and modulate the voltage sensing region of potassium channels (Swartz and MacKinnon, 1995), DTX and MgTX exert their channel blockade in a different way. These toxins bind to the extracellular mouth of the pore region, blocking the flow of potassium ions through the channel (Judge and Bever, 2006). The experiments outlined in this dissertation use both DTX and MgTX as pharmacological tools to specifically isolate endogenous Kv1.1/Kv1.2 and Kv1.3 currents, respectively. While venom-derived toxins used at specific concentrations are useful for blocking Kv1 currents, strong sequence homology of these channels may preclude toxin specificity in some instances. Therefore it is

important to note that additional molecular approaches such as RNA interference or transgenic knockouts may be needed to fully validate the scientific findings resulting from toxin blockade.

D. Kv1.3 Regulation

Voltage-gated potassium channels are subject to multiple mechanisms of regulation (Figure 3). Transcriptional regulation of Kv channels influences membrane excitability by up- or downregulating the amount of available channel protein within a given cell (Guthoff et al., 2009; Tschritter et al., 2006). Functional diversity is also regulated at various checkpoints within the biosynthetic pathway. At the level of translation, assembly of homo- or heterotetramers occurs in the ER and the ratio of subunit expression influences channel regulation (Kerr et al., 2001; Vicente et al., 2008). Primary amino acid motifs within the channel protein itself commonly control export from and retention in the ER (Li et al., 2000; Ma and Jan, 2002). Trafficking through the Golgi apparatus also serves as a locus for regulation; N-linked glycosylation of Kv1 family channels in the Golgi is required for efficient plasma membrane expression (Khanna et al., 2001; Zhu et al., 2001; Zhu et al., 2003). Once in the plasma membrane, select ion channels undergo local trafficking events such as endocytosis and recycling (Nesti et al., 2004), shuttling them away from and back into the cell membrane. Many of these regulatory events are mediated by direct chemical modification of the protein, known as post-translational modification.

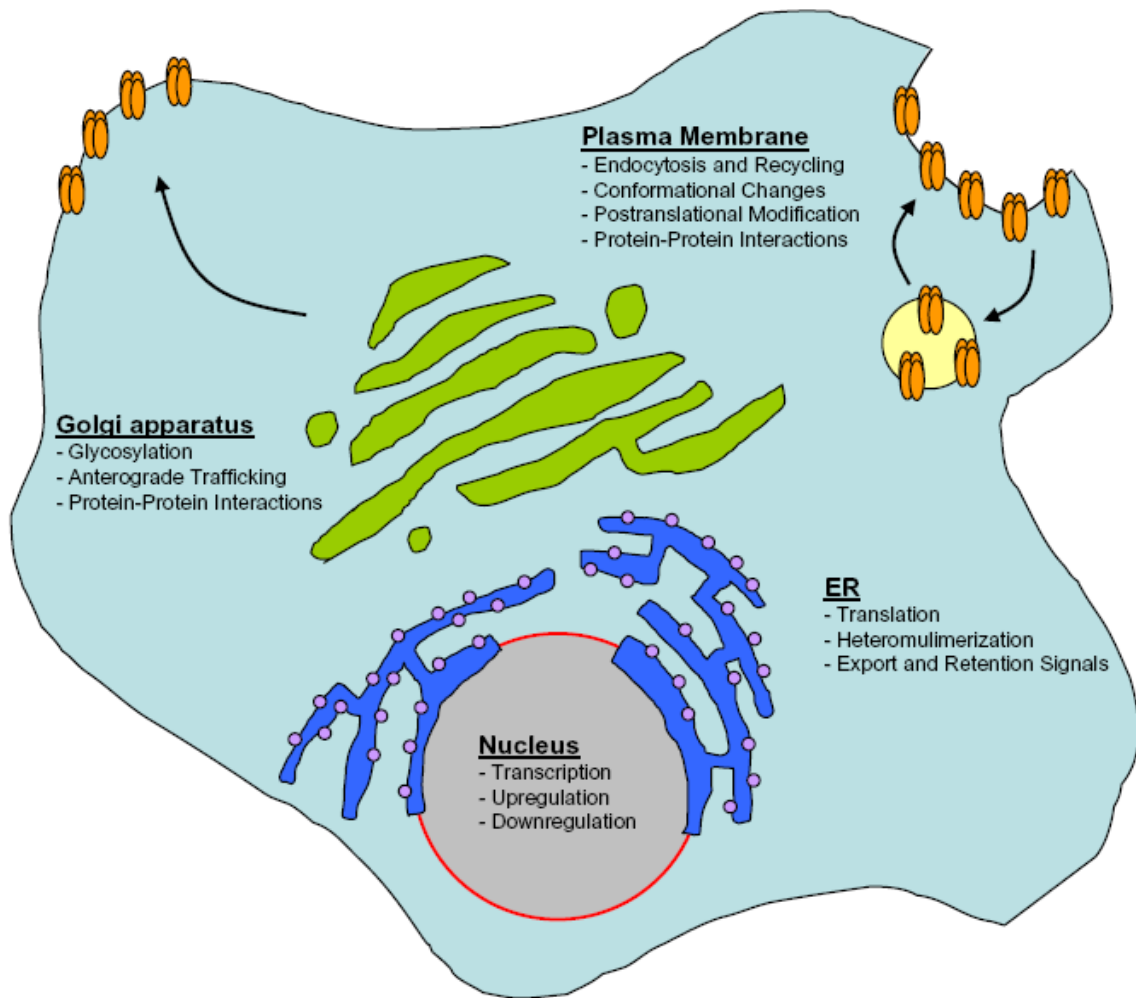


Figure 3: Voltage-gated potassium channel regulation. Kv1 channels are subject to multiple regulatory mechanisms, which occur in various cellular compartments such as the nucleus, endoplasmic reticulum (ER), Golgi apparatus, and plasma membrane.

Post-translational modification events play a key role in regulating the function of many voltage-gated ion channels (Catterall, 1988). Early studies reported that Kv1.3 current was functionally regulated via manipulation of protein kinase A (PKA) and protein kinase C (PKC) (Cai and Douglass, 1993). These findings, coupled with evidence that Kv1.3 can be directly phosphorylated at specific sites, led to speculation that serine/threonine phosphorylation was the major mechanism of Kv1.3 regulation in cell types as diverse as T-lymphocytes and *Xenopus* oocytes (Attali et al., 1992; Payet and Dupuis, 1992). Studies involving other Kv1 channels also demonstrate that PKA and PKC alter single channel properties and current conductance, providing further evidence that serine/threonine phosphorylation plays a key role in Kv1 channel regulation (Huang et al., 1994; Tsai et al., 1997).

In addition to extensive studies on serine/threonine phosphorylation of ion channels, the direct phosphorylation of tyrosine residues has also emerged as a key mechanism of channel regulation (Huang et al., 1993). In 1993, Huang et al found that the activity of Kv1.2 is modulated by tyrosine phosphorylation; m1 muscarinic acetylcholine receptor activation induced tyrosine phosphorylation of specific residues led to a suppression of Kv1.2 ionic current, without altering channel kinetics (Huang et al., 1993). Later studies determined that the mechanism of Kv1.2 current suppression was a physical removal of the channel from the plasma membrane known as endocytosis (Nesti et al., 2004). These findings were the first to link tyrosine phosphorylation dependent signaling to cellular excitability via the trafficking-dependent regulation of a voltage-gated potassium channel.

Following this seminal discovery, tyrosine phosphorylation of other ion channels has become an area of considerable interest (Holmes et al., 1996; MacFarlane and Sontheimer, 2000; Wang, 1999). Kv1.3 also contains multiple tyrosine residues that lie within potential consensus sites for tyrosine kinase-mediated phosphorylation (Figure 4). In 1996, Holmes et al published the first report of tyrosine phosphorylation modulating the functional properties of Kv1.3. This study showed that pharmacological inhibition of tyrosine phosphatase activity via pervanadate application, increased tyrosine phosphorylation of Kv1.3 in a dose-dependent manner (Holmes et al., 1996). This increase in Kv1.3 tyrosine phosphorylation was also accompanied by a concomitant suppression of Kv1.3 ionic current that was eliminated by introducing a single point mutation (Y449F) (Holmes et al., 1996). These data provided the first evidence that direct phosphorylation of Kv1.3 is associated with functional suppression of ionic current (Holmes et al., 1996).

Since the initial discovery that phosphorylation suppresses Kv1.3 current, both cellular and receptor tyrosine kinases have been found to alter the functional properties of Kv1.3 (Colley et al., 2004; Fadool et al., 1997; Marks and Fadool, 2007). Coexpression of Kv1.3 with the constitutively active nonreceptor tyrosine kinase, v-Src, suppresses channel current (Fadool et al., 1997). In addition, brain derived neurotrophic factor (BDNF)-induced activation of neurotrophin receptor tyrosine kinase B (TrkB) results in putative phosphorylation of multiple tyrosine residues on both the N- and C-terminus of Kv1.3 and is accompanied by suppression of current magnitude in rat olfactory bulb neurons (Colley et al., 2004). Similarly, insulin-induced neuromodulation of Kv1.3 occurs through insulin receptor (IR) kinase activity at specific tyrosine residues (Marks

and Fadool, 2007). While several physiological stimuli suppress Kv1.3 ionic current in a tyrosine phosphorylation dependent manner (Colley et al., 2004; Fadool et al., 1997; Marks and Fadool, 2007), the exact mechanism of functional suppression remains largely unexplored.

Major advancements in understanding the function and regulation of Kv1.3 were achieved by studying mice with a gene-targeted deletion of the channel (Biju et al., 2008; Fadool et al., 2004; Tucker et al., 2008). Along with post-translational modifications, protein-protein interactions have also been found to regulate Kv1.3 function (Fadool et al., 2004; Marks and Fadool, 2007). The expression of various modulatory kinases and scaffolding proteins normally associated with the channel was greatly altered in Kv1.3 null mice (Fadool et al., 2004). In addition to both the cellular kinase, Src, and the receptor tyrosine kinase, TrkB, Kv1.3-null mice also exhibited upregulation of the synaptic adaptor protein, post-synaptic density 95 (PSD-95) (Fadool et al., 2004). A recent study demonstrated that PSD-95 clusters Kv1.3 channels in specific membrane microdomains and significantly suppresses current magnitude of these channels via an activity-dependent mechanism (Marks and Fadool, 2007). These findings indicate a role for adaptor proteins in modulating the localization and function of voltage-gated potassium channels (Marks and Fadool, 2007).

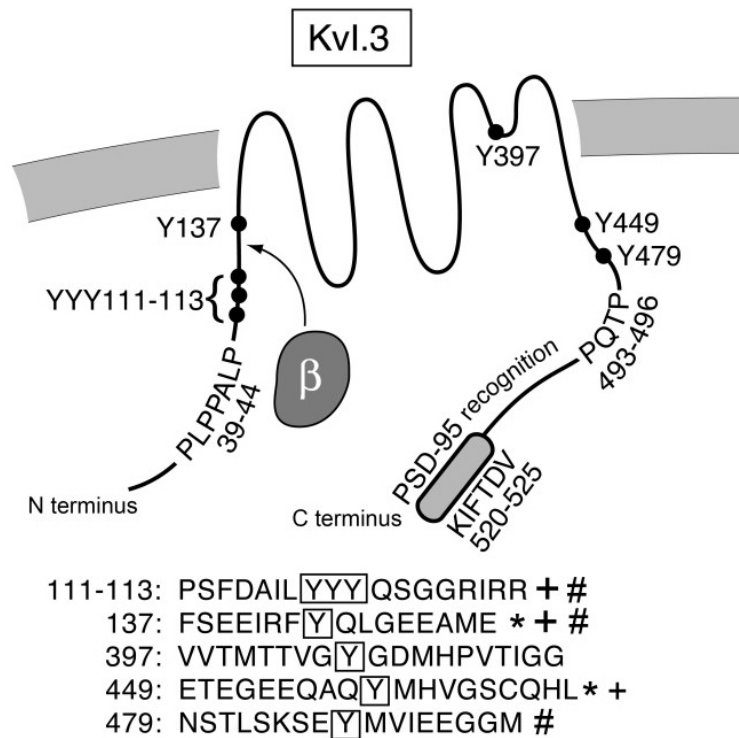


Figure 4: The primary structure of Kv1.3 contains multiple regulatory motifs. The cytoplasmic N- and C-termini, as well as the poor loop region of Kv1.3, contain multiple tyrosine residues (●) that lie within consensus sequences for phosphorylation by Src kinase (*), TrkB (#), and insulin receptor kinase (+). In addition, Kv1.3 contains two proline rich domains (PXXP) which may bind SH3 domain containing proteins. The extreme C-terminus of Kv1.3 contains a consensus sequence (X-(S/T)-X-φ) known to bind PDZ domain containing proteins, such as the canonical PDZ protein, PSD-95.

Colley et al. **Brain-derived neurotrophic factor modulation of Kv1.3 channel is disregulated by adapter proteins Grb10 and nShc.** *BMC Neuroscience.* (2009) 10:8.

IV. PDZ Domain Interactions

A. Structural Basis of PDZ Domain Recognition

Similar to many other ion channels, Kv1.3 contains a consensus sequence for the binding of PDZ domain containing proteins (Kim et al., 1995). Often arranged in tandem arrays or in conjunction with other interaction domains, PDZ domains are protein-protein recognition modules that can assemble large molecular complexes and participate in diverse signaling assemblies (Feng and Zhang, 2009; Harris and Lim, 2001; Kim and Sheng, 2004). These domains were originally discovered as homologous regions in diverse signaling proteins (Cho et al., 1992; Woods and Bryant, 1993). The name PDZ is an acronym derived from the first three proteins in which these domains were found: postsynaptic density-95 (PSD-95), discs-large (DLG) and zona-occludens-1 (ZO-1) (Harris and Lim, 2001; Jemth and Gianni, 2007). As depicted in Figure 5, PDZ domains are composed of approximately 90 amino acid residues, folded into six beta sheets (β A- β F) and two alpha helices (α A, α B), forming an extended groove between strand β B and helix α B (Harris and Lim, 2001; Jemth and Gianni, 2007). This groove recognizes specific PDZ-binding motifs, also referred to as PDZ ligands, found at the extreme carboxyl-terminus of transmembrane receptors and ion channels (Harrison, 1996). A highly conserved four residue sequence in the carboxylate-binding loop of the extended groove, known as the signature Gly-Leu-Gly-Phe (GLGF) motif, forms hydrogen bonds with the PDZ ligand (Morais Cabral et al., 1996). The extended groove binds the

consensus sequence of the PDZ ligand with varying affinity based on preferential residue specificity (Harris and Lim, 2001).

PDZ domain recognition specificity is dictated by the primary amino acid sequence of the C-terminal PDZ-binding motif that binds in the extended groove (Songyang et al., 1997). Consensus binding sequences are divided into three main categories, defined as Class I (X-(S/T)-X- ϕ -COOH), Class II (X- ϕ -X- ϕ -COOH) and Class III (X-X-C-COOH) (Songyang et al., 1997). With the exception of internal β -hairpin folds, as seen in the binding of neuronal nitric oxide synthase (nNOS) to syntrophin (Christopherson et al., 1999), virtually all PDZ domains recognize terminal carboxyl motifs (Christopherson et al., 1999; Harris and Lim, 2001; Hillier et al., 1999; Trejo, 2005). Even in the case of this internal PDZ motif recognition, the same energetic requirements observed in C-terminal binding are maintained because the tertiary structure of the internal loop mimics a chain terminus (Gee et al., 1998). The nomenclature of these terminal residues is well established; the C-terminal amino acid is termed P₀, while upstream N-terminal residues are termed P₋₁, P₋₂, P₋₃, etc (Harris and Lim, 2001). Studies suggest that residues in the P₀ and P₋₂ position are most important for proper PDZ domain recognition and serve as critical docking sites in the binding groove (Jelen et al., 2003; Sheng and Sala, 2001).

Other residues in the PDZ binding motif may confer specificity and serve to fine tune the PDZ interaction (Harris and Lim, 2001). The amino acid residue located at the P₋₃ position also makes contact with the PDZ domain, and has been shown to participate in hydrogen bonding of the ligand with the groove (Sheng and Sala, 2001). Ionotropic

glutamate receptors (NMDA), voltage-gated sodium channels, and some *Shaker* potassium channels contain a glutamate residue at the P₃ position that may be involved in mediating the selectivity of PDZ domain protein interactions (Songyang et al., 1997). In the case of Kv1.3, P₃ is occupied by phenylalanine, a nonpolar hydrophobic residue containing a benzyl side chain. It is the only member of the *Shaker* family that does not contain either a glutamate or leucine residue at this position (Kim et al., 1995) and therefore may be recognized by PDZ domain proteins distinct from other Kv1 channels. Moreover, the localization of Kv1.3 may be regulated by specific PDZ interactions that are distinct from other Kv1 family channels.

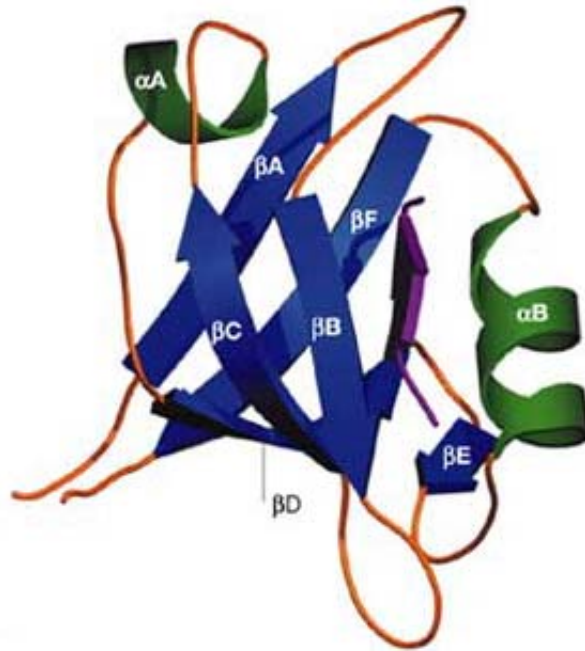


Figure 5: The tertiary structure of PDZ domains. PDZ domains are composed of approximately 90 amino acids forming six beta strands (βA - βF ; blue) and two alpha helices (αA , αB ; green). Between beta strand B and alpha helix B lies the extended groove, which binds the extreme C-terminal PDZ ligand (purple) of transmembrane receptors and ion channels.

Kim et al. **PDZ domain proteins of synapses.** *Nature Reviews Neuroscience.* (2004) 5, pp 771-781.

B. Role of PDZ Interactions in Protein Localization

The localization of proteins to distinct cellular compartments strongly influences cellular function. Perhaps the most fundamental demonstration of the role PDZ domain containing proteins play in compartmental localization is exemplified by the process of

polarized signaling. For example, the plasma membrane of epithelial cells is divided into apical and basolateral surfaces, which are separated by tight junctions (Kim, 1997; Mostov et al., 2003). Maintenance of this cell polarity is crucial for normal cellular function and is facilitated by the asymmetrical distribution of receptors, transporters and ion channels between these membranes (Kim, 1997; Mostov et al., 2003; Moyer et al., 1999). PDZ domains are essential components in determining this cellular polarity; they have been shown to specifically localize proteins to both the apical and basolateral membranes (Fanning and Anderson, 1999).

Neurons also have a highly polarized cellular structure, most often composed of somatic, dendritic and axonal compartments (Dotti et al., 1988). Several studies have shown that PDZ domain interactions are integral for anchoring proteins at specific subcellular sites (Harris and Lim, 2001; Kim et al., 1995). These molecular scaffolds help maintain the specific protein localization required for neuronal signaling (Kim et al., 1995; Srivastava et al., 1998). Spatial restriction of Kv1 channels to the axon initial segment of neurons is mediated by a macromolecular protein complex involving post-synaptic density 93 (PSD-93) (Ogawa et al., 2008). In addition, PSD-95 has been shown to cluster Kv1 channels in heterologous cells (Kim et al., 1995; Marks and Fadool, 2007), as well as differentially recruit these channels into neuronal lipid rafts (Wong and Schlichter, 2004), providing further evidence for the role of PDZ domains in protein localization.

Altering interactions with the C-terminal PDZ binding motif of ion channels leads to significant changes in their localization and function (Haggie et al., 2004; Nehring et al., 2000; Roche et al., 2001). For example, association of an inward rectifier potassium

channel, Kir2.3, with PSD-95 functionally suppresses the activity of the channel by reducing its single channel conductance (Nehring et al., 2000). Deleting the PDZ binding motif of the NR2B subunit of the ionotropic glutamate receptor, N-methyl-D-aspartate (NMDA), results in channel internalization from the plasma membrane (Roche et al., 2001). Furthermore, removing the PDZ binding motif of the Cystic Fibrosis transmembrane conductance regulator (CFTR) disrupts the normal intracellular trafficking of the channel, underscoring the importance of this motif in protein localization and function (Haggie et al., 2004).

Minimal evidence exists for defining the role of the Kv1.3 C-terminal PDZ-binding motif in regulating the localization and function of this channel. Early studies have demonstrated that similar to other Kv1 channels, the C-terminal tail of Kv1.3 associates with PSD-95 (Kim et al., 1995). Subsequent analyses of Kv1.3-null mice have shown that deletion of the channel alters the expression of various scaffolding proteins, including PSD-95 (Fadool et al., 2004). These findings are consistent with previous observations of the reciprocal regulation of Kv1.3 (Holmes et al., 1997). While Src kinase has been shown to suppress Kv1.3 current, the overexpression of Kv1.3 downregulates Src (Holmes et al., 1997). A recent study identified the functional ramifications of coexpressing Kv1.3 and PSD-95 in a heterologous system (Marks and Fadool, 2007). Co-immunoprecipitation of Kv1.3 and PSD-95 is dependent on the presence of specific C-terminal residues; phosphorylation-independent point mutations in the PDZ-binding motif of Kv1.3 disrupted the channel/adaptor complex (Marks and Fadool, 2007). While molecular interaction of the Kv1.3 C-terminal PDZ binding motif with PSD-95 is responsible for channel clustering in the plasma membrane, the PSD-95

mediated decrease in the activity-dependent peak current magnitude of Kv1.3 relies on domain interactions beyond canonical PDZ binding (Marks and Fadool, 2007). The work presented in this dissertation is the first to identify a role for the PDZ-binding motif in Golgi localization of Kv1.3 and aims to elucidate the functional consequences of abolishing this protein interaction motif.

C. PDZ Interactions and the Golgi Apparatus

Intracellular localization and trafficking are distinct properties that have been shown to extensively influence ion channel function (Gu et al., 2003; Nesti et al., 2004). PDZ domains have been shown to contribute to the localization of proteins to intracellular organelles, such as the Golgi apparatus (Gentzsch et al., 2003; Guggino, 2004; He et al., 2004). Several ion channels are known to localize to the Golgi apparatus (Cheng et al., 2002; Gentzsch et al., 2003; Zhu et al., 2001). Analysis of the intracellular distribution of other Kv1 family channels reveals that Kv1.4 accumulates in the Golgi, whereas Kv1.1 and Kv1.2 are primarily found in the ER (Zhu et al., 2001, 2003a). The CFTR associates with a Golgi resident protein presumed to tether CFTR to this organelle and potentially regulate channel transit to the plasma membrane (Cheng et al., 2002; Guggino, 2004). Another chloride channel, ClC-3B, also localizes to the Golgi apparatus and has been found to associate with the same Golgi resident proteins as CFTR (Gentzsch et al., 2003).

Biosynthetic trafficking is an important mechanism for determining the level of mature protein within a cell and can be regulated at multiple stages (Ma and Jan, 2002;

Ma et al., 2001). Although many motifs exist for modulating endoplasmic reticulum (ER) retention and export (Zarei et al., 2004; Zhu et al., 2003a), the Golgi apparatus also appears to serve as a checkpoint for anterograde protein trafficking (Cheng et al., 2002; Gentzsch et al., 2003; He et al., 2002). Glycosylation in the Golgi apparatus is a major determinant of protein trafficking to the plasma membrane (He et al., 2002; Watanabe et al., 2007; Zhu et al., 2001). For example, a glycosylation-deficient mutant of the beta 1 adrenergic receptor (β 1AR) leads to functional protein production, but decreased receptor surface expression (He et al., 2002). Analysis of the intracellular distribution of Kv1 family channels reveals that Kv1.4 accumulates in the Golgi and illustrates a role for trans-Golgi glycosylation in the surface expression of Kv1 channels (Watanabe et al., 2007; Zhu et al., 2001).

In addition, protein-protein interactions in the Golgi apparatus also regulate biosynthetic trafficking (Cheng et al., 2002; Gentzsch et al., 2003; He et al., 2004). For example, CFTR channels interact with the Golgi-associated protein, CFTR-associated ligand (CAL) (Cheng et al., 2002). Overexpression of CAL significantly reduces CFTR surface expression and ionic current by potentially sequestering the channel to the Golgi, an interaction mediated by the C-terminal PDZ-binding motif of the channel (Cheng et al., 2002). Since the discovery of CAL, this PDZ domain protein has been found to interact with other ion channels in the Golgi apparatus (Gentzsch et al., 2003). The ClC-3B chloride channel is present primarily in the Golgi, where it also associates with CAL, alternatively known as Golgi-associated PDZ and coiled-coil motif containing protein (GOPC) (Gentzsch et al., 2003). Finally, the carboxyl terminus of β 1AR, which contains a PDZ-binding motif, also associates with CAL (He et al., 2004). Overexpression of CAL

results in an intracellular accumulation of β 1AR, which is accompanied by a concurrent decrease in cell surface receptor (He et al., 2004). This dissertation identifies the Kv1.3 voltage gated potassium channel as being enriched in the Golgi apparatus and explores the role of the PDZ-binding motif in this subcellular localization.

V. Summary

The investigations associated with this project have focused on the function and regulation of the Kv1.3 voltage-gated potassium channel. Chapter 2 presents novel evidence indicating that Kv1.3 is a determinant of postganglionic sympathetic neuronal function. Inhibition of the channel alters resting membrane potential, latency to action potential firing, and neurotransmitter release in these cells. Before Chapter 2 was published in the American Journal of Physiology, Kv1 mRNAs were known to be expressed within sympathetic ganglia (Dixon and McKinnon, 1996), but little was understood about the location or function of specific Kv1 channels in these neurons. In this chapter, we also show that Kv1.3 exhibits a striking pattern of localization to a discrete intracellular compartment, overlapping with a marker for the Golgi apparatus. Not only did this research establish the importance of Kv1.3 in sympathetic neuronal function, it also unlocked the possibility that Golgi retention of Kv1.3 may play a role in regulating the functional properties of these neurons.

The remainder of this dissertation describes the use of a heterologous expression system to focus on the intracellular Golgi localization of Kv1.3 as a potential mechanism for channel regulation. This research quantitatively identifies the inverse relationship between Kv1.3 Golgi localization and the amount of channel in the plasma membrane.

The extreme C-terminal region of Kv1.3 contains a PDZ-binding motif which is known to participate in protein-protein interactions that aid channel localization to various cellular compartments (Kim et al., 1995). We show that removal of this PDZ-binding motif decreases Kv1.3 Golgi localization and increases Kv1.3 ionic current, suggesting that Golgi retention may be a mechanism for regulating the amount of functional channel in the plasma membrane. Collectively, the work in this dissertation identifies Kv1.3 as an important determinant of sympathetic neuronal function and provides insight into the regulatory checkpoints of the channel that may serve as targets for Kv1.3-mediated drug therapy in human disease.

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**CHAPTER 2: KV1.3 CHANNELS IN POSTGANGLIONIC SYMPATHETIC
NEURONS: EXPRESSION, FUNCTION, AND MODULATION**

Megan A. Doczi¹, Anthony D. Morielli^{2*}, Deborah H. Damon^{2*}

¹Department of Anatomy and Neurobiology

²Department of Pharmacology

University of Vermont College of Medicine

Burlington, VT 05401

* These authors contributed equally.

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Corresponding Author: Anthony D. Morielli
Department of Pharmacology
University of Vermont College of Medicine
89 Beaumont Avenue
Burlington, VT 05405
Phone: 802-656-4500
Fax: 802-656-4523
Email: Anthony.Morielli@uvm.edu

Abstract

Kv1.3 channels are known to modulate many aspects of neuronal function. We tested the hypothesis that Kv1.3 modulates the function of postganglionic sympathetic neurons. RT PCR, immunoblot and immunohistochemical analyses indicated that Kv1.3 channels were expressed in these neurons. Immunohistochemical analyses indicated that Kv1.3 protein was localized to neuronal cell bodies, processes, and nerve fibers at sympathetic neurovascular junctions. Margatoxin (MgTX), a specific inhibitor of Kv1.3, was used to assess the function of the channel. Electrophysiological analyses indicated that MgTX significantly reduced outward currents ($p < 0.05$; $n = 18$ (control) and 15 (MgTX)), depolarized resting membrane potential, and decreased the latency to action potential firing ($p < 0.05$; $n = 11$ (control) and 13 (MgTX)). The primary physiological input to postganglionic sympathetic neurons is acetylcholine, which activates nicotinic and muscarinic acetylcholine receptors. MgTX modulated nicotinic acetylcholine receptor agonist-induced norepinephrine release ($p < 0.05$; $n \geq 6$), and MgTX-sensitive current was suppressed upon activation of muscarinic acetylcholine receptors with bethanechol ($p < 0.05$; $n = 12$). These data indicate that Kv1.3 affects the function of postganglionic sympathetic neurons, which suggests that Kv1.3 influences sympathetic control of cardiovascular function. Our data also indicate that modulation of Kv1.3 is likely to affect sympathetic control of cardiovascular function.

Introduction

The sympathetic nervous system is a major determinant of cardiovascular function and is implicated in cardiovascular disease (Carlson et al., 2000; Dakak et al.,

1995; Dhital et al., 1988; Esler et al., 2006; Fingerle et al., 1991; Guyenet, 2006; Karoon et al., 1995; Kolo et al., 2004; Lee et al., 1987; Luo et al., 2003; Sauzeau et al., 2006; Schrier and Abraham, 1999). Many effects of the sympathetic nervous system on cardiovascular function are mediated via neurotransmitters released from postganglionic sympathetic neurons innervating blood vessels. The mechanisms governing neurotransmitter release at sympathetic neurovascular junctions are not completely understood.

Kv1 family potassium channels consist of eight genes encoding distinct alpha subunit proteins, Kv1.1 through Kv1.8, and are expressed throughout the nervous system (Dixon and McKinnon, 1996; Guan et al., 2006; Gutman et al., 2003; Hatton et al., 2001; Ichikawa and Sugimoto, 2003; Malin and Nerbonne, 2001; Nashmi et al., 2000; Popratiloff et al., 2003; Szulczyk et al., 2006; Tsaor et al., 1992; Veh et al., 1995; Wang et al., 1994). Functional Kv1 channels are formed when four Kv1 alpha subunits assemble as homotetramers or as heterotetramers with other members of the Kv1 family. These channels affect a range of neuronal functions (Dodson et al., 2002; Fadool et al., 2004; Glazebrook et al., 2002; Haghdoost et al., 2006; Kupper et al., 2002; McFarlane and Pollock, 2000; McKay et al., 2005; Mo et al., 2002), including spike frequency adaptation (McKay et al., 2005; Mo et al., 2002) and the regulation of cellular excitability in response to synaptic input (Glazebrook et al., 2002). Postganglionic sympathetic neurons express Kv1 channels (Dixon and McKinnon, 1996; Malin and Nerbonne, 2001; Szulczyk et al., 2006) and inhibition of these channels has been reported to modulate neurotransmitter release from these neurons (Ikushima et al., 1981; Jackson et al., 2001;

Uhrenholt and Nedergaard, 2003). However, the mechanisms involved in Kv1 channel modulation of neurotransmitter release in sympathetic neurons are largely unexplored.

Kv1.3 channels play a key role in a wide range of physiological phenomena. Kv1.3 is required for the activation of T-lymphocytes, and is thus a determinant of immune function (Beeton et al., 2006). Inhibition of Kv1.3 facilitates translocation of the insulin sensitive glucose transporter, GLUT4, to the plasma membrane of adipocytes (Li et al., 2006) and skeletal muscle (Xu et al., 2004), and is thus a determinant of glucose homeostasis. Kv1.3 has also been shown to contribute to body weight regulation and energy homeostasis, processes that are regulated by the sympathetic nervous system (Xu et al., 2003). In neurons, Kv1.3 has been reported to modulate action potential firing (8, 26).

Here we evaluate the role of Kv1.3 in postganglionic sympathetic neuron function. We demonstrate that this channel is present and functional in postganglionic sympathetic neurons. Kv1.3 was detected throughout the neurons, in both soma and processes. It contributed to outward currents recorded from the soma and was a determinant of resting membrane potential and neurotransmitter release. These studies show that Kv1.3 channels are important determinants of postganglionic sympathetic neuronal function and have important implications for understanding the effects of the sympathetic nervous system on cardiovascular function.

Materials and Methods

Animals. The use of animals in the present studies was in accordance with the National Institutes of Health guidelines for the humane care and use of animals in research and

was approved by the Institutional Animal Care and Use Committee of the University of Vermont. Neonatal Sprague Dawley rats were used to obtain superior cervical sympathetic ganglia (SCG). Adult postpartum female Sprague Dawley rats were used to obtain SCG and tail arteries. The postpartum females used in the present studies were the mothers of the neonatal rats and were used to minimize the number of animals.

Neuronal Culture. Postganglionic sympathetic neurons were isolated from the SCG of neonatal (3 - 4 days) Sprague Dawley rats (males and females). Ganglia were dissociated for 10 minutes at 37°C in a collagenase/hyaluronidase solution (10 mg/ml bovine serum albumin, 4 mg/ml collagenase, 1 mg/ml hyaluronidase in Dulbecco's phosphate buffered saline) and then for 10 minutes in trypsin (3 mg/ml added to trypsin-EDTA). Dissociated cells were resuspended in neuronal growth medium (DMEM/F12 supplemented with 10% NuSerum (BD Biosciences), 5% fetal bovine serum (Invitrogen) and penicillin/streptomycin), supplemented with NGF (BD Biosciences; 50 ng/ml), and applied to collagen-coated tissue culture dishes. The cells were allowed to attach overnight in a humidified 5% CO₂ environment maintained at 37°C. Non-neuronal cells were then growth arrested with mitomycin C (Sigma; 10 µg/ml for 1 hour). These cultures of neurons will be subsequently referred to as dissociated neurons.

RT-PCR. RNA was isolated with RNAeasy mini kits from Qiagen. Equal amounts of RNA were reverse transcribed (RetroScript, Ambion) and equal amounts of cDNA amplified (Amplitaq Gold, Applied Biosystems). PCR primers and annealing temperatures are indicated in Table 1. PCR products were electrophoresed on 1.2% agarose gels containing ethidium bromide and visualized with UV light. All PCR reactions included (-) RT and (-) template controls. Amplified PCR products were

sequenced by the University of Vermont DNA facility to confirm the identity of the DNA.

Western Analyses. Tissues and cells were lysed and homogenized in enhanced RIPA buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA 0.25% deoxycholate 1% NP40, 10% glycerol, 1% protease inhibitor cocktail (Sigma), 1 mM dithiothreitol, 0.1% sodium dodecyl sulfate; pH 8.0). Samples were diluted with equal volumes of electrophoresis running buffer, boiled for 5 minutes and electrophoresed on 4 – 20% gradient acrylamide gels. Samples were then transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 0.05% Tween and 3% nonfat dry milk (30 minutes at room temperature) and then incubated overnight at 4 C in PBS-Tween containing 3% nonfat dry milk, and primary antibody (0.2 µg/ml anti-Kv1.3 (NeuroMab) and 1 µg/ml anti-Tyrosine Hydroxylase (TH; Sigma)). The Kv1.3 mouse monoclonal antibody, IgG2a isotype, was raised against synthetic peptide amino acids 485-506 of rat Kv1.3 (clone L23/27; Lot # 413-5RR-07). The TH mouse monoclonal antibody recognizes the N-terminal epitope between amino acids 40-152 of rodent and human TH (Lot # 016K4857). Unbound primary antibody was removed with three 5 minute washes (PBS-Tween). The membranes were then incubated in PBS-Tween containing 3% nonfat dry milk and horseradish peroxidase-conjugated secondary antibodies (1:3000; Biorad) for one hour at room temperature. The horseradish peroxidase was detected with enhanced chemiluminescence (Pierce) and documented on autoradiographic film.

Immunofluorescence. Cultures of postganglionic sympathetic neurons were rinsed in PBS and fixed in 4% formaldehyde in PBS for 12 min. SCG and tail arteries were dissected from adult postpartum female rats and fixed in 4% formaldehyde in PBS for 1

or 2 hours, respectively. Ganglia were immersed in 30% sucrose in PBS overnight and frozen in Tissue-Tek O.C.T. compound (Electron Microscopy Sciences) and sliced to a thickness of 30 μm at -20°C . SCG sections and dissociated neurons were permeabilized with 0.2% Triton X-100 in PBS for 5 min and subsequently rinsed in PBS. Tail arteries were permeabilized with acetone for 5 min and rinsed in PBS. Cultures, SCG sections, and whole mount tail arteries were blocked for 1 hour in 3% goat serum, 0.1% fish skin gelatin in PBS. A rabbit polyclonal TH antibody (0.16 $\mu\text{g}/\text{ml}$; Chemicon), raised against denatured rat TH (Lot # 0512016843), was used to identify neurons. A rabbit polyclonal GM130 antibody (0.7 $\mu\text{g}/\text{mL}$; Calbiochem), raised against recombinant protein containing amino acids 371-990 of human GM130 (Lot # D00004465), was used to identify the Golgi apparatus. The same monoclonal Kv1.3 antibody (0.84 $\mu\text{g}/\text{mL}$; NeuroMab) used for western analyses was also used to identify Kv1.3. Cells were incubated in primary antibodies overnight at 4°C , followed by three 5 min washes in PBS. Alexa Fluor Goat anti-mouse (cultures 568 nm; SCG and tail arteries 647 nm) and goat anti-rabbit (488 nm) secondary antibodies (4 $\mu\text{g}/\text{ml}$; Invitrogen) were applied for 1 hour at room temperature and all samples were mounted using ProLong Gold antifade reagent (Invitrogen). All images were taken using the Olympus IX70 microscope and DeltaVision Restoration Imaging System (Applied Precision, LLC) and background subtracted with an IgG2a isotype control (R&D Systems).

Neuronal transfection. Neurons were transfected using the Helios Gene Gun (BioRad) with pEGFP or pEGFP-Kv1.3. pEGFP coated bullets were a generous gift from Dr. Victor May (University of Vermont, Department of Anatomy and Neurobiology). pEGFP-C1-Kv1.3 was a generous gift from Dr. Jürgen Kupper ((Kupper, 1998); Max

Planck Institute of Biochemistry, Martinsried, Germany). Cells were studied 48 hrs after transfection.

Electrophysiology. Electrophysiological recordings were performed at room temperature, utilizing the whole cell patch clamp technique. Data acquisition and analysis were obtained using the Axopatch 200B (Axon Instruments) patch clamp amplifier and pCLAMP 9.2 (Axon Instruments) software. Electrodes were pulled in two stages from thin wall filament glass capillary tubing (Warner Instruments) and fire polished to a resistance ranging from 1 – 2 M Ω . Voltage clamp recording solutions were as follows (in mM): external (bath) solution 100 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgCl₂, 23 glucose, 5 Na HEPES, 0.001 tetrodotoxin (TTX), 10 tetraethylammonium (TEA), pH 7.4; internal (pipette) solution: 120 KCl, 3.69 CaCl₂, 0.094 MgCl₂, 5 BAPTA, 5 EDTA, 5 Na HEPES, 5 glucose, pH 7.2. Pharmacological agents were applied at the following concentrations: 1 nM Margatoxin (MgTX; Alomone), 100 nM α -Dendrotoxin (DTX; Research Biochemicals International) and 100 μ M Bethanechol (BeCh; Sigma). Cells were held at -60 mV, followed by a 20 ms hyperpolarization to -90 mV, and stepped from -70 mV to +50 mV in 10 mV increments. Leak currents (P/8) were subtracted from all traces. Averaged MgTX traces were subtracted from averaged control traces to obtain the resolved Kv1.3 current. Current clamp recording solutions were the same as listed above, except that TTX and TEA were omitted. Resting membrane potential was monitored for 100 ms, followed by 400ms current injections of the following magnitudes (in pA): -500, 500, 1000, 1500, 2000, and 2500. Latency to action potential firing was defined as the time between the start of the current injection and the peak of the action potential. Action

potential width was measured at half the action potential amplitude as previously described (Guan et al., 2007).

Norepinephrine release. Norepinephrine (NE) release was assessed using tritiated norepinephrine purchased from Amersham. These assays were performed using HEPES-buffered Krebs solution [122 mM NaCl, 3 mM KCl, 0.4 mM MgSO₄ · H₂O, 1.2 mM KH₂PO₄, 10 mM Glucose, 20 mM HEPES, 1.3 mM CaCl₂ · 2H₂O, 1 mM Ascorbic Acid, 10 μM Pargyline, pH 7.4]. Cells were preincubated at 37°C with 100 nM tritiated norepinephrine for 30 minutes. The cells were then washed (6 x 5 minutes) and stimulated with a nicotinic agonist, dimethylphenylpiperazinium (DMPP; 30 μM; Sigma). The remaining cell associated NE was then extracted with acidified ethanol. NE in all samples was collected and analyzed using a Beckman LS6000IC Liquid Scintillation Counter (Beckman Instruments). Stimulated release was calculated using the following equation: (stimulated cpm – background cpm) / (total cpm available for release).

Statistics. Data are presented as means ± standard errors. Unpaired Student's t-tests assuming unequal variances were used to determine statistical differences. Differences were considered significant if $p \leq 0.05$.

Results

To begin to assess the function of Kv1.3 channels in postganglionic sympathetic neurons, we first characterized the expression and subcellular localization of these channels. Kv1.3 mRNA and protein were detected in dissociated neurons (d), and in intact neonatal (n) and adult (a) ganglia (figure 1A). Potassium channel effects on

neuronal physiology are strongly influenced by their location within the cell, therefore we used immunofluorescence microscopy to examine the subcellular localization of Kv1.3 protein (figure 1B and 1C). Neurons were identified with antibodies directed against tyrosine hydroxylase. In dissociated neurons and freshly isolated tissues, Kv1.3 was detected in both the soma and processes. In the soma, Kv1.3 exhibited a striking pattern of localization to a discrete intracellular compartment that overlaps with the GM130 Golgi apparatus marker in dissociated neurons (figure 2). All dissociated neurons observed expressed Kv1.3 and exhibited this intracellular localization pattern.

Because the striking intracellular localization detected with anti-Kv1.3 is not typical for a membrane-associated ion channel, we used GFP-Kv1.3 as an alternate approach to assess the subcellular localization of Kv1.3. The distribution of overexpressed GFP-Kv1.3 overlapped with that detected by Kv1.3 immunofluorescence (figure 3). The distribution of overexpressed GFP differed considerably from GFP-Kv1.3 and Kv1.3 immunofluorescence (figure 3). Intracellular compartmentalization of Kv1.3 was also observed in sympathetic neurons in adult ganglia (figure 1C).

We next determined the function of Kv1.3 in postganglionic sympathetic neurons. We used whole cell patch clamp electrophysiology in the absence and presence of margatoxin (MgTX), a specific inhibitor of Kv1.3 (Garcia-Calvo et al., 1993). To resolve Kv1.3 currents, all measurements were recorded in the presence of 1 μ M TTX and 10 mM TEA to block currents generated by sodium and non-Kv1 family potassium channels. Steady state currents measured in untransfected dissociated postganglionic sympathetic neurons were significantly suppressed by MgTX (1 nM; $n = 15$) relative to control ($n = 18$; $p < 0.02$) (figure 4A). This indicates that endogenous Kv1.3 channels

contribute to outward current recorded from the soma of postganglionic sympathetic neurons. To confirm that MgTX inhibited Kv1.3, steady state currents were also measured in dissociated postganglionic sympathetic neurons expressing GFP-Kv1.3. Outward currents in transfected cells were markedly increased, indicating that GFP-Kv1.3 was functional in these cells. MgTX (1 nM) elicited an 86% decrease in this current (figure 4B; $n = 3$; $p < 0.002$). Activation curves were generated from tail currents and fit to a Boltzmann function. These curves are shown in figure 4C for both endogenous and GFP-Kv1.3. Half activation voltages ($V_{1/2}$) determined from these curves were -22.8 mV for endogenous MgTX-sensitive current and -27.2 mV for GFP-Kv1.3, values consistent with previously published reports (Vicente et al., 2008a).

At concentrations higher than that used in the present studies, MgTX has been reported to inhibit Kv1.6 (Garcia-Calvo et al., 1993). To evaluate the contribution of Kv1.6 to the MgTX-sensitive current measured in figure 4A, we measured steady state ionic currents in dissociated postganglionic sympathetic neurons in the presence of α -dendrotoxin (DTX), an inhibitor of Kv1.6, as well as Kv1.1 and Kv1.2 channels (Harvey, 2001). DTX (100 nM) had no effect on steady state currents in these cells ($p > 0.05$; $n \geq 10$) (figure 4D). Steady state outward currents recorded in HEK 293 cells stably transfected with Kv1.2 were significantly suppressed in the presence of DTX (100 nM; $n = 4$; $p < 0.05$; data not shown), confirming the effectiveness of this inhibitor. These findings support our conclusion that the MgTX-sensitive current shown in figure 4A is attributable to Kv1.3.

Current clamp electrophysiology was used to elucidate the physiological role of Kv1.3 current in dissociated postganglionic sympathetic neurons. Resting membrane

potential measured in the presence of MgTX ($-45.7 \text{ mV} \pm 2.1$; $n = 13$) was significantly depolarized relative to control ($-51.9 \text{ mV} \pm 2.3$; $n = 11$; $p < 0.05$) (figure 5B). In addition, the latency to action potential firing in the presence of MgTX ($26.9 \text{ ms} \pm 1.3$; $n = 13$) was significantly less than control ($39.9 \text{ ms} \pm 5.4$; $n = 11$; $p < 0.05$) (figure 5C). MgTX did not alter the width of the action potential measured at half peak amplitude (figure 5D).

Acetylcholine (ACh) is the preganglionic neurotransmitter for postganglionic sympathetic neurons. ACh activates both nicotinic (nAChR) and muscarinic (mAChR) acetylcholine receptors in these neurons (Lechner et al., 2003). Activation of mAChRs is known to suppress ionic current of Kv1 family channels (Huang et al., 1993). We found that bethanechol (BeCh; $100 \text{ } \mu\text{M}$), a mAChR selective agonist, significantly decreased outward current measured in dissociated neonatal sympathetic neurons (figure 6A; $n = 12$; $p < 0.05$). Inhibition of Kv1.3 with MgTX abrogated the effect of BeCh, suggesting that activation of mAChRs suppresses Kv1.3 current in these cells (figure 6B; $n = 12$; $p > 0.05$). In HEK cells lacking muscarinic receptors, but transfected with GFP-Kv1.3, BeCh did not decrease Kv1.3 current at any voltage (data not shown; $n = 8$; $p > 0.05$). These data indicate that BeCh did not directly affect Kv1.3.

The ultimate function of postganglionic neurons is to release neurotransmitter, thereby affecting the function of sympathetic targets. Therefore, we assessed the effect of Kv1.3 on NE release from these neurons. Acetylcholine is the physiological stimulus for neurotransmitter release from these neurons. This effect is primarily mediated by activation of nAChRs. Therefore, we used DMPP, a nAChR selective agonist to stimulate neurotransmitter release. Application of $30 \text{ } \mu\text{M}$ DMPP stimulated NE release

from dissociated neonatal neurons. Inhibition of Kv1.3 with MgTX (1 nM) increased NE release from these cells (figure 7; n = 6; p < 0.05).

Discussion

The present studies provide novel evidence that Kv1.3 determines the function of postganglionic sympathetic neurons. Expression analyses indicated that Kv1.3 is present in these neurons. Electrophysiological analyses indicated that this channel contributes to outward current and the maintenance of resting membrane potential. Pharmacological inhibition of Kv1.3 increased neurotransmitter release. In addition, we demonstrated that this channel was modulated by preganglionic mechanisms. Postganglionic sympathetic neurons are important determinants of cardiovascular function. Thus, our studies strongly suggest that Kv1.3 channels and their modulation are important determinants of sympathetic control of cardiovascular function.

The present studies are the first to demonstrate that Kv1.3 protein is expressed in postganglionic sympathetic neurons. RT-PCR, immunoblot, and immunohistochemical analyses indicate that Kv1.3 was in the soma. Kv1.3 was concentrated in an intracellular compartment (figures 1, 2 and 3). Similar localization of Kv1.3 has been reported in other cells and tissues (Guan et al., 2006; O'Connell and Tamkun, 2005). Figure 2 indicates that this compartment is the Golgi apparatus. Kv1.3 was also detected in the processes of dissociated neurons as well as in nerve fibers on the adventitial surface of freshly isolated tail arteries. These data suggest that Kv1.3 channels are likely to affect the function of postganglionic sympathetic neurons in general and in particular, to affect sympathetic control of vascular function.

Functional analyses of Kv1.3 indicate that this channel is a determinant of the electrophysiological properties of postganglionic sympathetic neurons. MgTX, a specific inhibitor of Kv1.3 (Garcia-Calvo et al., 1993), decreased outward currents measured in these cells (figure 4A). In contrast, DTX, an inhibitor of Kv1.6, Kv1.1 and Kv1.2 (Harvey, 2001), had no effect on outward currents in these neurons (figure 4D). This indicates that MgTX is specific for Kv1.3, and that Kv1.1, Kv1.2 and Kv1.6 do not significantly contribute to the outward current measured in these neurons. Action potential analyses presented in figure 5 indicate that Kv1.3 contributes to the maintenance of resting membrane potential. MgTX significantly decreased the latency to action potential firing, consistent with the depolarization of resting membrane potential. This is the first demonstration that Kv1.3 affects the function of postganglionic sympathetic neurons.

Our immunohistochemical and functional analyses indicate that Kv1.3 is localized to both the plasma membrane and Golgi apparatus in postganglionic sympathetic neurons. We detected Kv1.3 current in non-transfected cells indicating surface localization of the channel (figure 4A). In non-transfected cells, surface expression of Kv1.3 was below the level of detection of our immunohistochemical analyses (figure 1B). In neurons that were transfected with GFP-Kv1.3, surface expression was easily detectable (figure 3). In both non-transfected and transfected neurons, our immunohistochemical analyses detected a fraction of Kv1.3 localized to the Golgi apparatus (figures 2, 3). Cellular localization is an important determinant of ion channel function (Lai and Jan, 2006). Our data thus suggest that Golgi localization or retention may be a determinant of surface expression and function of Kv1.3.

The primary preganglionic input to these neurons is ACh. It is well known that activation of mAChRs modulates KCNQ potassium channels in postganglionic sympathetic neurons and that this modulation affects neurotransmitter release (Lechner et al., 2003). In addition, previous studies indicate that activation of mAChRs modulates the function of Kv1 channels (Huang et al., 1993). We show that activation of mAChRs suppresses Kv1.3 current in postganglionic sympathetic neurons (figure 6). This data demonstrates a novel effect of mAChR activation, a novel mechanism of Kv1.3 modulation, and suggests a new mechanism by which mAChR activation modulates membrane excitability.

In addition to activating mAChRs, release of ACh from preganglionic neurons activates nAChRs. Nicotinic AChR activation is the primary mechanism for generating action potentials and eliciting neurotransmitter release from postganglionic sympathetic neurons. We assessed the effects of Kv1.3 on nicotinic receptor-induced NE release. Our data indicate that inhibition of Kv1.3 enhanced NE release (figure 7). This suggests that Kv1.3 is acting to suppress membrane excitability and thereby inhibit neurotransmitter release. These findings are consistent with previous reports demonstrating that inhibition of Kv1 family channels enhances neurotransmitter release. The work of Jackson et al. (Jackson et al., 2001) and Uhrenholt et al. (Uhrenholt and Nedergaard, 2003) indicate that inhibition of Kv1 channels enhances NE release at sympathetic neurovascular junctions. Our findings that Kv1.3 channels are present in processes innervating arteries and that MgTX enhances NE release, suggest that these channels play a role in modulating sympathetic neurovascular transmission.

Our data clearly indicate that Kv1.3 channels influence the function of postganglionic sympathetic neurons derived from the SCG of the rat, a representative paravertebral sympathetic ganglion. The postganglionic sympathetic neurons in this ganglion innervate many targets including blood vessels (Li and Horn, 2006). Kv1.3 was expressed in all of the neurons that were studied, suggesting that Kv1.3 affects sympathetic regulation of all SCG targets. The studies of Dixon and McKinnon (Dixon and McKinnon, 1996) indicate that Kv1.3 is also expressed in prevertebral ganglia suggesting a general role for Kv1.3 in postganglionic sympathetic neuronal function. Our finding that Kv1.3 is expressed in the fibers innervating the tail artery (figure 1C) strongly suggests that Kv1.3 affects neurotransmitter release at sympathetic neurovascular junctions. This would suggest that Kv1.3 is a determinant of sympathetic control of blood flow and blood pressure.

Perspectives and Significance

Compelling evidence suggests that the sympathetic nervous system contributes to the development and/or maintenance of cardiovascular disease (Carlson et al., 2000; Dakak et al., 1995; Dhital et al., 1988; Esler et al., 2006; Fingerle et al., 1991; Guyenet, 2006; Karoon et al., 1995; Kolo et al., 2004; Lee et al., 1987; Luo et al., 2003; Sauzeau et al., 2006; Schrier and Abraham, 1999; Small et al., 2002). Sympathetic activity is increased in hypertensive animals and humans, and sympathoinhibition decreases blood pressure (Dhital et al., 1988; Guyenet, 2006; Karoon et al., 1995; Kolo et al., 2004; Lee et al., 1987; Sauzeau et al., 2006). Hypertension is also a complication of diabetes and obesity. Elevated sympathetic activity is thought to contribute to these forms of

hypertension (Carlson et al., 2000; Esler et al., 2006). Sympathetic activity is increased in many patients with heart failure, and this increased activity contributes to the pathology of this disease (Schrier and Abraham, 1999; Small et al., 2002). The present studies suggest that Kv1.3 channels in postganglionic sympathetic neurons are determinants of sympathetic activity and therefore are potential therapeutic targets for the prevention and treatment of cardiovascular disease.

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Figure Legends

Figure 1: Kv1.3 channels are present in postganglionic sympathetic neurons. A) RT-PCR (top; n = 2) shows expression of mRNA for Kv1.3 in neonatal dissociated neurons (d), neonatal sympathetic superior cervical ganglia (SCG) (n) and adult sympathetic SCG (a). PCR reactions included minus reverse transcriptase (-RT) and minus template (-T) controls. Kv1.3 and tyrosine hydroxylase (TH) immunoblots (bottom; n = 2) show corresponding protein expression. TH was used as a marker of postganglionic sympathetic neurons. Approximate molecular weight is noted with arrows. B) Immunolocalization of Kv1.3 (top) and TH (bottom) in the soma (left) and processes (right) of dissociated postganglionic sympathetic neurons *in vitro* (n = 2). Scale bar = 10 μ m. C) Immunolocalization of Kv1.3 (top) and TH (bottom) in neuronal soma of intact adult sympathetic SCG (left) and of processes innervating adult rat tail arteries (right) (n = 2). SCG scale bar = 10 μ m; tail artery scale bar = 30 μ m.

Figure 2: Kv1.3 overlaps with a marker for the Golgi Apparatus in postganglionic sympathetic neurons. Immunoreactivity of endogenous Kv1.3 (red) overlaps with the Golgi marker, GM130 (green), in the soma of dissociated postganglionic sympathetic neurons. The far right panel depicts the overlap between these two antibodies (merge; n = 3). Scale bar = 10 μ m.

Figure 3: Localization of transfected GFP-Kv1.3 versus endogenous Kv1.3 in postganglionic sympathetic neurons. GFP-Kv1.3 (top left) and immunoreactive Kv1.3 (top right) overlap in postganglionic sympathetic neurons (n = 3). Neurons transfected with GFP only (bottom left) do not overlap with immunoreactive Kv1.3 (bottom right) (n = 3). Scale bar = 10 μ m.

Figure 4: Voltage clamp analyses of Kv1.3 in postganglionic sympathetic neurons.

A) Ionic current measured in untransfected dissociated sympathetic neurons in the absence (control; n = 18) and presence of 1 nM Margatoxin (MgTX; n = 15). Current traces for each condition represent the average of unpaired measurements made in multiple cells. * indicates MgTX was significantly different from control ($p \leq 0.05$; unpaired t-test). Resolved Kv1.3 current was obtained by subtracting MgTX from control. B) Ionic current measured in dissociated sympathetic neurons transfected with GFP-Kv1.3 in the absence (control; n = 3) and presence of 1 nM MgTX (n = 3). Current traces for each condition represent the average of unpaired measurements made in multiple cells. * indicates MgTX was significantly different from control ($p \leq 0.02$; unpaired t-test). Resolved GFP-Kv1.3 current was obtained by subtracting MgTX from control. C) Activation curves were plotted from the tail current of endogenous Kv1.3 and GFP-Kv1.3 and fit to a Boltzmann equation. D) Ionic current measured in dissociated sympathetic neurons in the absence (control; n = 10) and presence of 100 nM DTX (n = 18; $p > 0.05$).

Figure 5: Current clamp analyses of Kv1.3 in postganglionic sympathetic neurons.

A) Representative traces of phasic action potentials recorded from the soma of dissociated postganglionic sympathetic neurons in the absence (control; black line) and presence (MgTX; red line) of 1 nM MgTX. B) The resting membrane potential, C) the latency to action potential firing, and D) the action potential width as measured at half the action potential amplitude in the absence (open bars; n = 11) and presence (closed bars; n = 13) of 1 nM MgTX. * indicates MgTX was significantly different from control ($p < 0.05$; unpaired t-test).

Figure 6: Preganglionic modulation of Kv1.3 in postganglionic sympathetic neurons.

A) Steady state current-voltage curves for postganglionic sympathetic neurons in the absence (open symbols) and presence (closed symbols) of muscarinic acetylcholine receptor activation using 100 μ M Bethanechol (BeCh). B) Steady state current-voltage curves for postganglionic sympathetic neurons treated with 1nM MgTX in the absence (open symbols) and presence (closed symbols) of 100 μ M BeCh. * indicates BeCh was significantly different from control (n = 12; $p < 0.05$; unpaired t-test).

Figure 7: Kv1.3 affects norepinephrine release from postganglionic sympathetic neurons.

Tritiated norepinephrine release from neuronal cultures in response to a 30 μ M dimethylphenylpiperazinium (DMPP) treatment in the absence (open bar) and presence (closed bar) of MgTX (1 nM, 10 min). * indicates MgTX was significantly different from control ($p < 0.05$; unpaired t-test).

Tables

Table 1: Forward and reverse PCR primer sequences

mRNA	Primer Sequences	Annealing Temp (°C)
Kv1.3	5'-GTA CTT CGA CCC GCT CCG CAA TGA -3' 5'-GGG CAA GCA AAG AAT CGC ACC AG -3'	59

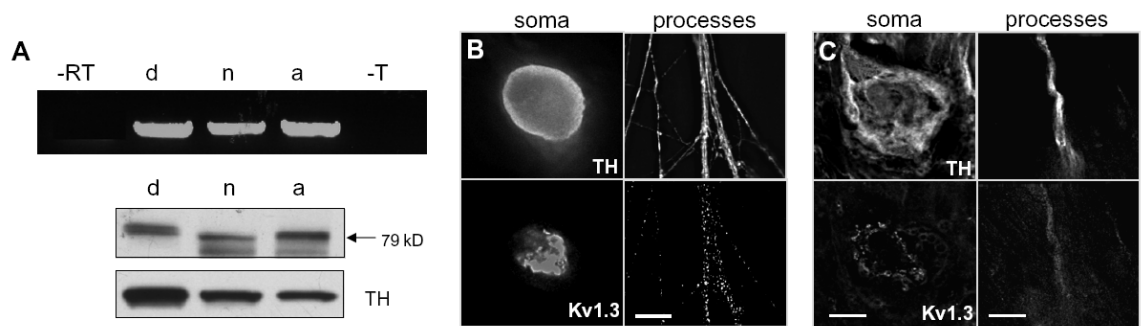


Figure 1: Kv1.3 channels are present in postganglionic sympathetic neurons.

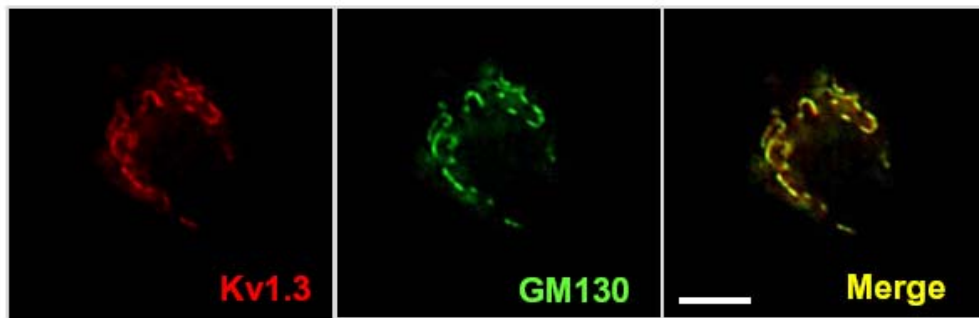


Figure 2: Kv1.3 overlaps with a marker for the Golgi Apparatus in postganglionic sympathetic neurons.

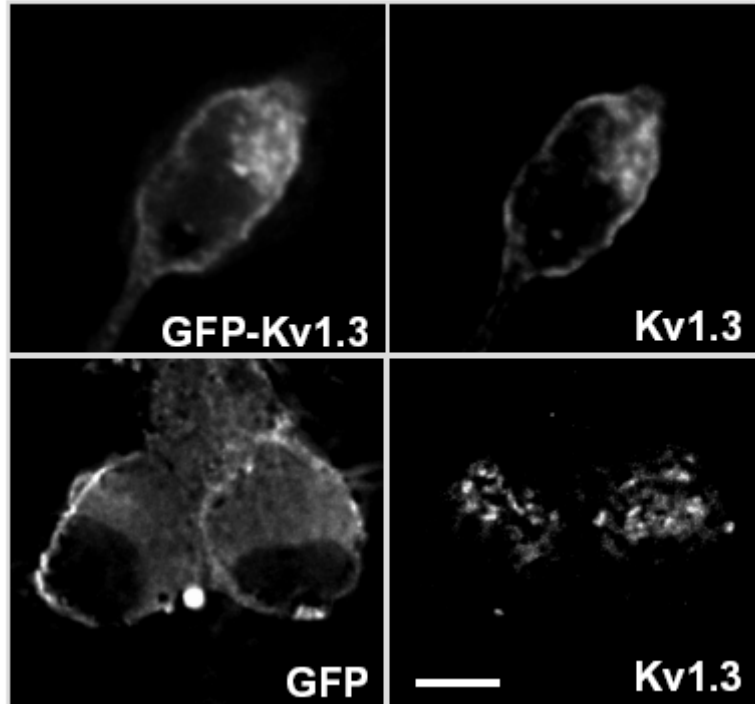


Figure 3: Localization of transfected GFP-Kv1.3 versus endogenous Kv1.3 in postganglionic sympathetic neurons.

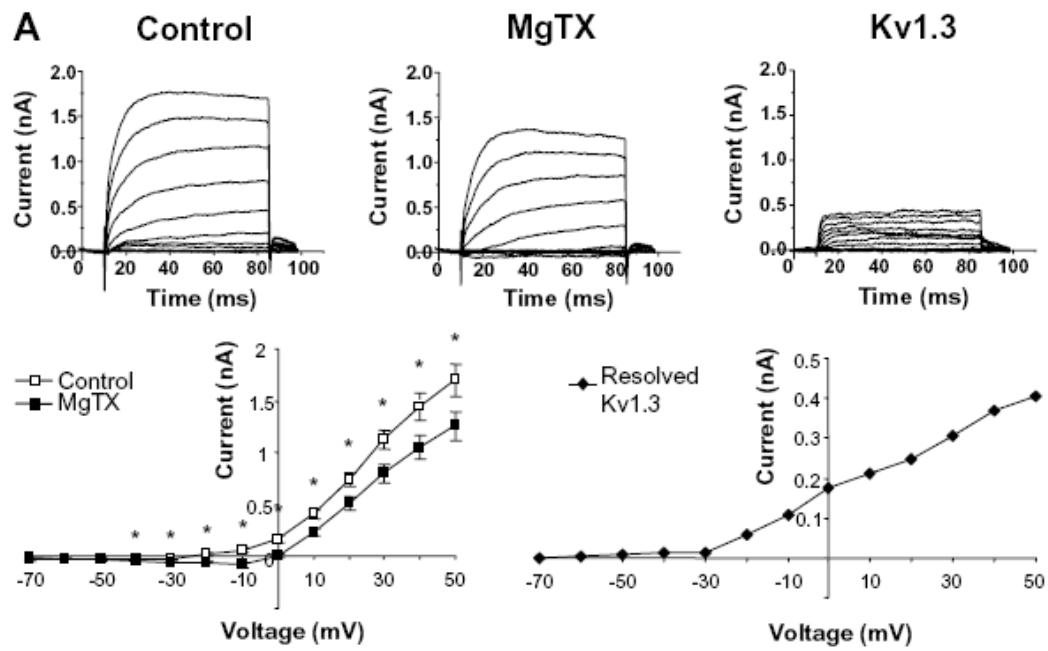


Figure 4A: Voltage clamp analyses of Kv1.3 in postganglionic sympathetic neurons.

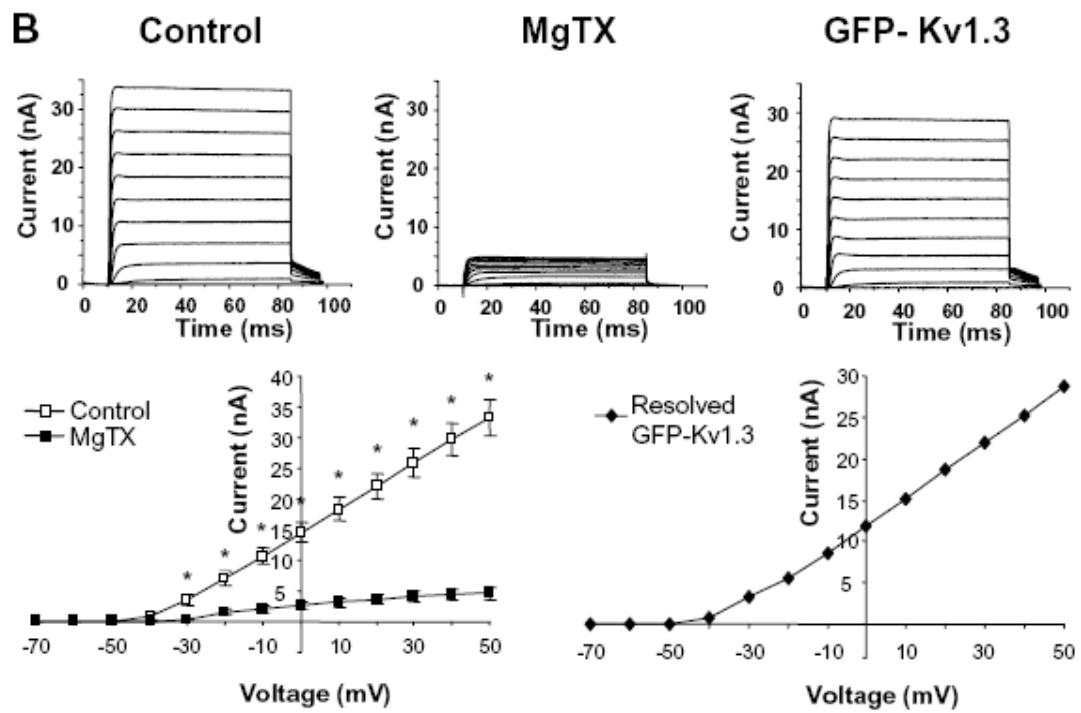


Figure 4B: Voltage clamp analysis of Kv1.3 in postganglionic sympathetic neurons.

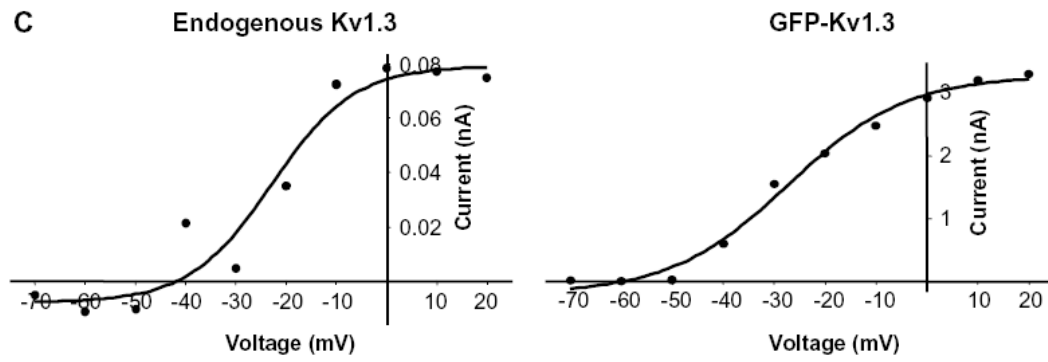


Figure 4C: Voltage clamp analysis of Kv1.3 in postganglionic sympathetic neurons.

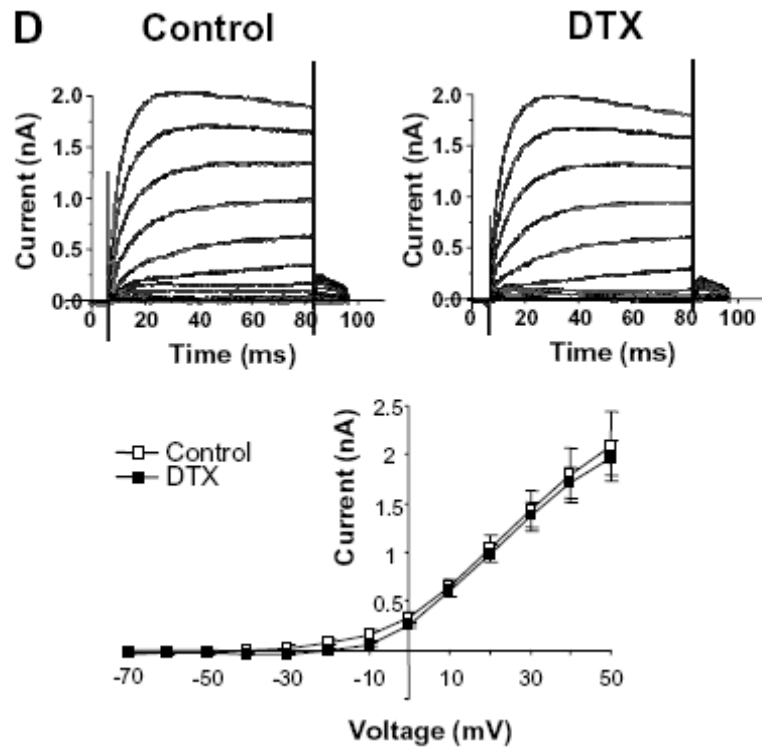


Figure 4D: Voltage clamp analysis of Kv1.3 in postganglionic sympathetic neurons.

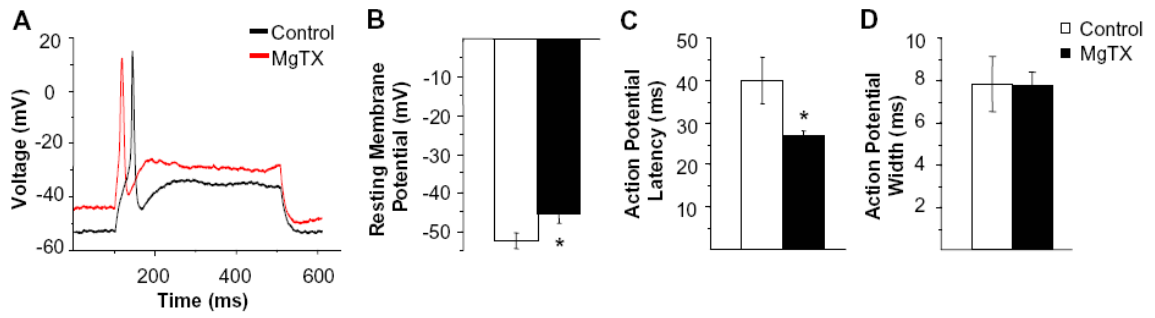


Figure 5: Current clamp analyses of Kv1.3 in postganglionic sympathetic neurons.

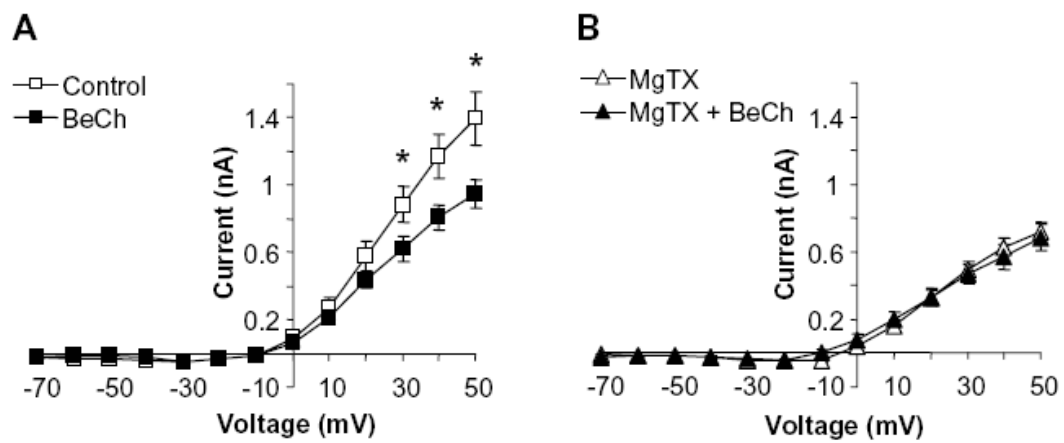


Figure 6: Preganglionic modulation of Kv1.3 in postganglionic sympathetic neurons.

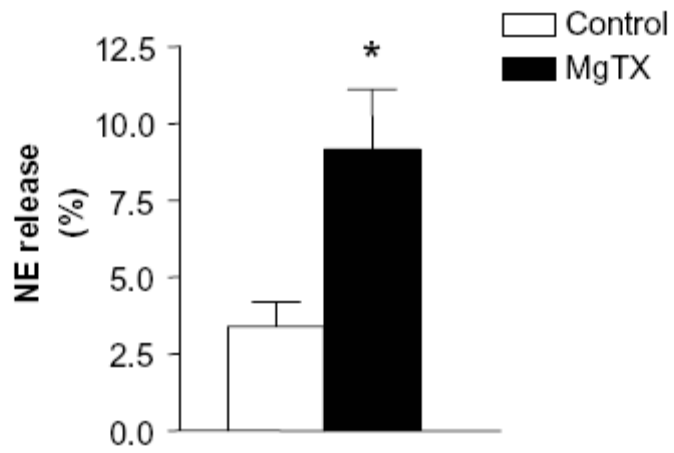


Figure 7: Kv1.3 affects norepinephrine release from postganglionic sympathetic neurons.

**CHAPTER 3: PDZ-BINDING MOTIF MEDIATED GOLGI ENRICHMENT OF
KV1.3 CONTRIBUTES TO CHANNEL LOCALIZATION AND FUNCTION**

Megan A. Doczi¹, Deborah H. Damon^{2*}, Anthony D. Morielli^{2*}

¹Department of Anatomy and Neurobiology

²Department of Pharmacology

University of Vermont College of Medicine

Burlington, VT 05401

* These authors contributed equally.

Running Head: Golgi Localization of Kv1.3

Corresponding Author: Anthony D. Morielli
Department of Pharmacology
University of Vermont College of Medicine
89 Beaumont Avenue
Burlington, VT 05405
Phone: 802-656-4500
Fax: 802-656-4523
Email: Anthony.Morielli@uvm.edu

Abstract

The voltage-gated potassium channel, Kv1.3, plays an important role in regulating membrane excitability in diverse cell types ranging from neurons to T-lymphocytes. Subcellular localization of ion channels is a key mechanism for determining their function. We observed that Kv1.3 is localized primarily to the Golgi apparatus and the plasma membrane. The degree of Kv1.3 Golgi localization is inversely correlated with the amount of channel at the plasma membrane. The amplitude of Kv1.3 ionic current measured in cells with low Kv1.3 Golgi localization was significantly greater than the current measured in cells with high Kv1.3 Golgi localization. We therefore hypothesized that Golgi localization is a determinant of Kv1.3 function. One mechanism for localizing ion channels to the Golgi apparatus involves the Class I PDZ-binding motif (X-S/T-X-Φ). The C-terminal region of Kv1.3 contains a canonical PDZ-binding motif. Deletion of this motif decreases Kv1.3 Golgi localization and increases channel localization at the cell surface. Deletion of this region also increased the amplitude of Kv1.3 ionic current. We propose that Golgi retention serves as a checkpoint for regulating the surface expression and function of Kv1.3.

Introduction

The Kv1.3 voltage-gated potassium channel is a key determinant of function in many cell types. Kv1.3 plays a central role in regulating resting membrane potential, Ca²⁺ signaling cascades, and antigen-dependent activation and proliferation in T-lymphocytes, key mediators of autoimmune disease (Beeton et al., 2006; Panyi, 2005; Panyi et al., 2004b). In adipocytes and skeletal muscle, Kv1.3 influences glucose

transport, (Desir, 2005; Li et al., 2006; Li et al., 2007) and studies in Kv1.3 deficient mice indicate that the channel is a determinant of body weight and energy homeostasis (Fadool et al., 2004; Tucker et al., 2008; Xu et al., 2003). Several studies also demonstrate that Kv1.3 influences neuronal resting membrane potential, action potential characteristics, neurotransmission, and synaptic refinement (Biju et al., 2008; Doczi et al., 2008; Kupper et al., 2002), further underscoring the physiological significance of Kv1.3 in multiple cellular processes.

The localization of ion channels to distinct cellular compartments can influence cellular function. For example, activity-regulated local translation of Kv1.1 in hippocampal dendrites contributes to channel surface expression, potentially modulating local cellular excitability (Raab-Graham et al., 2006). Modulated endocytosis of Kv1.2 facilitates both positive and negative plasma membrane trafficking of the channel in HEK293 cells, in turn altering Kv1.2 activity at the cell surface (Connors et al., 2008; Nesti et al., 2004). Plasma membrane expression of the Cystic Fibrosis transmembrane conductance regulator (CFTR), a chloride channel that is enriched in the Golgi apparatus, is potentially regulated via channel transit from the Golgi to the plasma membrane (Cheng et al., 2002; Cheng et al., 2004; Guggino, 2004; Moyer et al., 2000). Previous work from our laboratory indicates that in postganglionic sympathetic neurons, Kv1.3 also exhibits localization to the Golgi apparatus (Doczi et al., 2008). In this study, we propose that Kv1.3 Golgi localization is a mechanism for regulating channel activity at the cell surface, in turn influencing cellular function.

One mechanism for localizing ion channels to the Golgi apparatus involves the Class I PDZ-binding motif (X-S/T-X-Φ). This motif is found at the extreme carboxy-

terminus of proteins and serves as a ligand for the binding of postsynaptic density-95/discs-large/zona-occludens-1 (PDZ) domain containing proteins (Hung and Sheng, 2002). CFTR channels contain a PDZ-binding motif that associates with the Golgi localized PDZ domain protein, CFTR associated ligand (CAL), potentially tethering the channel to this subcellular compartment (Cheng et al., 2002; Cheng et al., 2004; Guggino, 2004; Moyer et al., 2000). The ClC-3B chloride channel is a PDZ-binding isoform of the ClC family that also exhibits intracellular localization to the Golgi apparatus (Gentzsch et al., 2003). Similarly, ClC-3B transit from the Golgi to the plasma membrane is dependent upon interactions with PDZ domain proteins. Potassium channels also contain PDZ-binding motifs and interactions of these motifs with PDZ proteins play a role in channel localization. For example, the PDZ domain containing scaffold protein, Postsynaptic density-93 (PSD-93), induces clustering of Kv1 channels to the axon initial segment in neurons (Ogawa et al., 2008). Altering associations with the C-terminal PDZ-binding motif of potassium ion channels can lead to significant changes in channel function (Cheng et al., 2002; Cheng et al., 2004; Guggino, 2004; Mery et al., 2002; Swiatecka-Urban et al., 2002). Coexpression of an inward rectifier potassium channel, Kir2.3, with the synaptic adaptor protein, post synaptic density 95 (PSD-95), functionally suppresses the activity of the channel by reducing its single channel conductance (Nehring et al., 2000).

The C-terminus of Kv1.3 contains a PDZ-binding motif which is known to associate with PDZ domain containing proteins (Kim et al., 1995). A recent study demonstrates that PSD-95 clusters Kv1.3 channels into specific membrane microdomains (Marks and Fadool, 2007). In addition, PSD-95 also suppresses the current magnitude of

Kv1.3 channels via an activity-dependent mechanism (Marks and Fadool, 2007). Thus, PDZ interactions play an integral role in the localization and function of Kv1.3.

In the present study, we test the hypothesis that the PDZ-binding motif of Kv1.3 plays a role in regulating the intracellular Golgi localization and electrophysiological function of the channel. We show that Golgi localization of WT Kv1.3 is inversely correlated with surface expression of the channel. Disrupting the C-terminal PDZ-binding motif of Kv1.3 significantly decreased Golgi localization of the channel. In addition, this mutation also caused an increase in Kv1.3 ionic current. These findings suggest that the PDZ-binding motif dependent localization of Kv1.3 to the Golgi apparatus is a mechanism for regulating the level of functional channel at the cell surface.

Materials and Methods

Cell Culture and Transient Transfection- Clonal human embryonic kidney 293 (HEK293) cells were maintained in DMEM/F-12 (Invitrogen) supplemented with 10% fetal bovine serum, 10 U/ml penicillin and streptomycin, and 2 mM L-glutamine. Cells were transiently transfected with 3.0ug of GFP-Kv1.3/-Flag or GFP-Kv1.3- Δ TDV/-Flag using the Lipofectamine LTX transfection reagent (Invitrogen). Confluent transfected cultures were plated to a low density (25,000 cells/cm²) onto poly-D-lysine-coated tissue culture plates (Corning Glass Works) and subsequently placed in serum-free medium overnight for experimentation the following day.

Mutagenesis- All mutagenesis reactions were performed using the Quickchange Lightning Site-Directed Mutagenesis Kit (Stratagene), following the manufacturer's protocol. The GFP-Kv1.3- Δ TDV truncation was created by inserting two premature stop

codons into the wild-type channel using the following primer sequence and its reverse complement: 5'-GTCAACATCATCAAAAAGATATTCTGATGAACTGATGTCTAATAGGGATCCACC-3'. Similar to previous reports (Panyi et al., 2003; Panyi et al., 2004a), the Flag epitope was created by inserting the amino acid sequence, DYKDDDDK, into the first extracellular loop of GFP-Kv1.3/- Δ TDV and incorporating D222 as the first aspartic acid residue in the Flag epitope using the following primer sequence and its reverse complement: 5'-TCTCCGTCGCAGGACTACAAGGACGACGACGACAA GGTGTTTGAGGCTGCC-3'.

Immunofluorescence– To detect Kv1.3 on the cell surface, transiently transfected HEK293 cells were live labeled with anti-Flag M2 antibody (1 μ g/mL; Sigma) at 37°C for 15 min. Cells were then rinsed 3 x 5 min in PBS, fixed in 4% formaldehyde in PBS for 20 min, and subsequently permeabilized with acetone for 5 min and rinsed in PBS. Cells were then blocked for 20 min at 37°C in 3% goat serum, 0.1% fish skin gelatin in PBS. Cells were incubated 20 min at 37°C in a rabbit polyclonal GM130 antibody (0.7 μ g/mL; Calbiochem), raised against recombinant protein containing amino acids 371-990 of human GM130 (Lot # D00004465), to identify the Golgi apparatus and subsequently rinsed 3 x 5 min in PBS. Alexa Fluor goat anti-mouse (647 nm) and goat anti-rabbit (568 nm) secondary antibodies (4.0 μ g/ml; Invitrogen) were applied for 20 min at 37°C and all samples were mounted using ProLong Gold antifade reagent (Invitrogen). All images were taken using the Olympus IX70 microscope and DeltaVision Restoration Imaging System (Applied Precision, LLC). Golgi Localization was quantified as follows: Golgi region of interest (ROI) was defined using the GM130 marker, and the amount of GFP-

Kv1.3 signal intensity present in the Golgi ROI was expressed as a percent of the total GFP-Kv1.3 signal for the entire cell ((GFP-Kv1.3 in Golgi region / Total GFP-Kv1.3) X 100). Surface expression was quantified as follows: the intensity of the live labeled anti-Flag M2 antibody was normalized to the total GFP-Kv1.3-Flag intensity of the cell (surface Kv1.3/ total Kv1.3).

Immunoblot Analysis - 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 Na₃VO₄, 1 mM Na₄ BAPTA, 1mM 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 x g for 5 min, and the resulting supernatant was combined with sample buffer and separated by SDS-PAGE. Western blotting detection of Kv1.3 was done with anti-Kv1.3 monoclonal antibody (0.42ug/mL; NeuroMab). A monoclonal anti-GAPDH antibody (1.0ug/mL; Millipore) was used to control for loading efficiency. Blots were imaged and quantified with the Odyssey infrared imaging system (Li-Cor Biosciences).

Electrophysiology-Electrophysiological recordings were performed at room temperature, utilizing the whole cell patch clamp technique. Data acquisition and analysis were obtained using the Axopatch 200B (Axon Instruments) patch clamp amplifier and pCLAMP 9.2 (Axon Instruments) software. Electrodes were pulled in two stages from thin wall filament glass capillary tubing (Warner Instruments) and fire polished to a resistance ranging from 1 – 2 MΩ. Voltage clamp recording solutions were as follows (in mM): external (bath) solution 100 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgCl₂, 23 glucose,

5 Na HEPES, pH 7.4; internal (pipette) solution: 120 KCl, 3.69 CaCl₂, 0.094 MgCl₂, 5 BAPTA, 5 EDTA, 5 Na HEPES, 5 glucose, pH 7.2. Cells were held at -60 mV, followed by a 20 ms hyperpolarization to -90 mV, and stepped from -70 mV to +50 mV in 10 mV increments. Leak currents (P/8) were subtracted from all traces.

MBP Pulldown— pMAL-Kv1.3-C-term, pMAL-Kv1.3 Δ TDV-C-term, and the empty pMAL vector were transformed into BL21 Gold bacterial cells (Stratagene). Cultures were grown in LB supplemented with Glucose/Ampicillin and protein expression was induced by adding 0.3mM IPTG for 2 hours. Cells were harvested at 4000x g for 10 min and resuspended in Column Buffer (20mM Tris-HCl, 200mM NaCl, 1mM EDTA, 1mM sodium azide, 1mM DTT). Cells were lysed and the protein extracts (MBP-Kv1.3, MBP- Δ TDV or MBP) were coated onto Amylose resin (New England Biolabs). HEK293 cells transfected with pGW1-CMV-myc-PSD-95 were lysed in RIPA and precleared with MBP coated Amylose resin by rocking for 30 min at 4°C. Precleared myc-PSD-95 lysates were then rocked with either MBP-Kv1.3, MBP- Δ TDV or MBP coated Amylose resin for 30 min at 4 °C, washed 3x in RIPA, needle aspirated, combined with sample buffer and separated by SDS-PAGE. Western blotting detection of myc-PSD-95 was done with an anti-Myc monoclonal antibody (0.1 ug/mL; Invitrogen) and Kv1.3 was detected using an anti-Kv1.3 monoclonal antibody (0.42 ug/mL; NeuroMab). Blots were imaged and quantified with the Odyssey infrared imaging system (Li-Cor Biosciences).

Statistical Analysis - Data are presented as means \pm standard errors. As noted, either nonparametric Man-Whitney U tests, unpaired Student's t-tests assuming unequal

variances, or one-way ANOVA with post hoc Bonferroni test were used to determine statistical differences. Differences were considered significant if $p \leq 0.01$.

Results

Golgi localization of Kv1.3- Previous studies have shown that Kv1.3 is localized to the Golgi apparatus in postganglionic sympathetic neurons (Doczi et al., 2008). The present study explores the role of Kv1.3 Golgi localization on channel function using HEK293 cells as a model system. To determine the degree of Kv1.3 concentration in the Golgi apparatus, cells were transfected with GFP-Kv1.3, processed for immunofluorescence and labeled with the Golgi apparatus marker, anti-GM130, to delineate the Golgi region. For each cell, GFP-Kv1.3 intensity was measured in two regions of interest: one encompassing the entire cell (total Kv1.3) and one in the Golgi region (Golgi Kv1.3). Golgi localization (%) was calculated as $((\text{Golgi Kv1.3} / \text{total Kv1.3}) \times 100)$. Figure 1A illustrates that Kv1.3 has a distribution in the Golgi compartment ranging from 8.4 % to 50 %, with a mean Golgi localization of $28.5 \% \pm 3.1$ ($n = 20$). The photomicrographs in Fig 1B were selected to depict GFP-Kv1.3 transfected cells exhibiting Golgi localization in both the upper range above the mean (46.0%; top) and the lower range below the mean (9.6%; bottom).

The Golgi distribution of Kv1.3 is distinct from that observed with a closely related channel, Kv1.2 (Fig. 1A). The mean Golgi localization of GFP-Kv1.2 ($12.6 \% \pm 0.85$; $n = 20$) was significantly less than mean Golgi localization of GFP-Kv1.3 ($28.5 \% \pm 3.1$; $n = 20$; $p < 0.0001$). A representative photomicrograph depicting the intracellular distribution of Kv1.2 is shown in Figure 1C. These data suggest that the high Golgi

enrichment of GFP-Kv1.3 is a property of the channel itself, and is not a general property of Kv1 channels expressed in HEK293 cells.

Kv1.3 surface expression is inversely related to Golgi localization of the channel-

In addition to enrichment in the Golgi apparatus, Kv1.3 is also present in the plasma membrane (Doczi et al., 2008). Because anterograde trafficking of proteins to the plasma membrane can be regulated at the level of the Golgi apparatus, we hypothesized that Kv1.3 Golgi localization may be a determinant of Kv1.3 surface expression. To measure the amount of GFP-Kv1.3 localized to the plasma membrane, we introduced a FLAG epitope (DYKDDDDK) into the extracellular loop between S1 and S2 transmembrane helices (GFP-Kv1.3-Flag). Electrophysiological studies shown in Figure 2 confirm no significant difference between B) the outward current or C) the half activation voltage of GFP-Kv1.3 (open symbols) and GFP-Kv1.3-Flag (closed symbols).

To determine if Golgi localization of Kv1.3 influences its surface expression, we quantified each parameter within the same cell. Fluorescence microscopy was used to measure total Kv1.3 protein and immunofluorescence analysis was used to measure Golgi localized Kv1.3 and surface Kv1.3. Surface Kv1.3 was labeled by applying anti-Flag M2 antibody to live cells. The representative photomicrographs shown in Figure 2B illustrate a cell transfected with GFP-Kv1.3-Flag (green) and live labeled with anti-Flag-M2 (blue) to detect the extracellular Kv1.3-Flag epitope. The restriction of Flag signal to the plasma membrane, and the absence of Flag signal in the Golgi apparatus, confirmed the anti-Flag M2 antibody had access only to channel at the cell surface. To quantify Kv1.3 surface expression, the intensity of the live labeled anti-Flag M2 antibody was normalized to the

total GFP-Kv1.3-Flag intensity of the cell (surface Kv1.3/ total Kv1.3). Linear regression analysis revealed that Kv1.3 surface expression was inversely correlated with Golgi localization of the channel (R-squared = 0.56; n = 82) (Fig 2D). These data indicate that cells with increased Kv1.3 Golgi localization exhibit decreased expression of the channel at the cell surface, suggesting the Golgi apparatus may serve as an internal reservoir for the channel.

To facilitate analysis, cells were binned into two categories based on their distribution relative to the median percent Golgi localization. Cells are described as having high or low Kv1.3 Golgi localization if the percent channel Golgi localization falls above or below the median, respectively. Figure 2E depicts the surface expression of GFP-Kv1.3-Flag channels exhibiting high or low Golgi localization. The median Golgi localization for GFP-Kv1.3-Flag transfected cells was 15.9%. Figure 2E demonstrates the normalized surface expression (Kv1.3 surface/Kv1.3 total) of GFP-Kv1.3-Flag in the low Golgi localized population (n = 41; mean = 2.2 ± 0.12) was significantly higher than the surface expression of the high Golgi localized Kv1.3 population (n = 41; mean = 1.0 ± 0.10 ; $p < 0.01$) (Fig 2E).

Golgi localization and Kv1.3 ionic current- We next asked if the increase in surface expression of Kv1.3 is a determinant of channel function. Since Kv1.3 Golgi localization was inversely correlated with surface expression, we tested the hypothesis that a similar relationship exists between Kv1.3 Golgi localization and Kv1.3 ionic current. Because electrophysiological recordings were performed in live cells, we visually categorized GFP-Kv1.3 transfected cells as having either high or low Golgi

localization before each recording. To confirm that visual categorization parallels the previous immunofluorescence data (Fig 2E), we conducted a blind assay to assign previously captured photomicrographs of GFP-Kv1.3-Flag transfected cells into high and low Golgi localized bins. The results of that visual categorization were then compared to the quantitative categorization. Visual categorization correctly binned 79.3% of cells into their respective high Golgi localized (above the median) and low Golgi localized (below the median) category. No high Golgi cells were incorrectly identified as low Golgi. Therefore any inaccurate visual categorization would only underestimate the significance of the data presented.

Steady-state ionic current was measured from cells visually categorized as having high (Fig 3A) or low (Fig 3B) channel localization to the Golgi apparatus. The traces depicted in Figure 3 represent the average outward current recorded from cells in each category. Steady-state current voltage relationships were plotted for cells exhibiting both high (closed symbols) and low (open symbols) Kv1.3 Golgi localization (Fig 3C). The outward current from cells categorized as having high Kv1.3 Golgi localization ($18.2 \text{ nA} \pm 2.1$; $n = 15$) was significantly lower than cells categorized as having low Kv1.3 Golgi localization ($26.0 \text{ nA} \pm 2.4$; $n = 12$; $p < 0.05$). There was no significant difference in the voltage dependence of high and low Golgi localized Kv1.3 channels (Fig 3D), indicating that the increase in outward current was not due to a decreased voltage dependence of channel activation. These data suggest that Kv1.3 ionic current may depend on Golgi localization of the channel.

Disrupting the C-terminal PDZ binding motif of Kv1.3 decreases Golgi localization of the channel- The C-terminal region of Kv1.3 contains a PDZ- binding motif which is known to associate with PDZ domain containing proteins, aiding in localization and function of the channel to various cellular compartments (Marks and Fadool, 2007). To determine the importance of this motif in Kv1.3 Golgi localization, we created a truncated form of the channel lacking the last three amino acids (Δ TDV). Equal amounts of GFP-Kv1.3-WT (WT) and GFP-Kv1.3- Δ TDV (Δ TDV) were separately transfected into HEK293 cells and immunoblot analyses indicate no significant difference in total channel protein (Fig 4A). Both WT and Δ TDV bands were detected at the expected molecular mass of 100-kDa. Equal loading was confirmed by normalizing both WT and Δ TDV band densities to glyceraldehyde-3 phosphate dehydrogenase (GAPDH). There was no significant difference in the corresponding mean densitometry between WT (n = 8; 1.0 ± 0.7) and Δ TDV (n = 8; 0.92 ± 0.14 ; p = 0.29).

To confirm that the Δ TDV truncation successfully disrupted the PDZ binding motif of Kv1.3 and abolished the binding of PDZ domain containing proteins, we performed a standard pulldown assay using PSD-95, a canonical PDZ domain containing protein known to bind to Kv1.3 (Kim et al., 1995; Marks and Fadool, 2007). The pMAL-Kv1.3-C-term construct was used to express Maltose Binding Protein (MBP) fused to the C-terminal region of Kv1.3. In addition to MBP-Kv1.3-C-term (WT), two other fusion proteins were created: MBP-Kv1.3- Δ TDV (Δ TDV) and a MBP-only (MBP) control. MBP, WT, and Δ TDV coated beads were incubated with HEK293 cell lysates containing myc-PSD95. Figure 4B illustrates that PSD95 binds to the C-terminus of Kv1.3-WT and that binding is abolished with the Δ TDV truncation.

To address the hypothesis that the PDZ binding motif of Kv1.3 contributes to Golgi localization of the channel, HEK293 cells were transfected with GFP-Kv1.3- Δ TDV (Δ TDV) and processed for immunofluorescence. To quantify Golgi localization of Δ TDV, we used the Golgi apparatus marker, anti-GM130. Figure 4D illustrates that Golgi localization of Δ TDV ($11.12\% \pm 1.4$; $n = 20$) was significantly less than WT ($28.5\% \pm 3.0$; $n = 20$; $p < 0.0001$). In fact, all Δ TDV data points fell below the WT mean (Fig 4D). The representative photomicrograph in Fig 4C depicts the intracellular localization of Kv1.3- Δ TDV (9.8%). These data show that removal of the PDZ binding motif of Kv1.3 decreases Golgi localization of the channel and suggest that this motif may be involved in the Golgi-mediated regulation of the channel.

Disrupting the C-terminal PDZ binding motif of Kv1.3 increases outward functional current and plasma membrane expression of the channel- We next tested the hypothesis that the PDZ binding motif of Kv1.3 contributes not only to Golgi localization, but also surface expression and Kv1.3 channel function. Steady-state outward current was measured from GFP-Kv1.3-Flag-WT transfected cells exhibiting both high ($15.5 \text{ mV} \pm 0.78$; $n = 15$) and low ($9.2 \text{ mV} \pm 0.77$; $n = 15$) channel Golgi localization (Figure 5A and B). Recordings from both high and low Golgi localized GFP-Kv1.3-Flag-WT demonstrate increased outward current in the low Golgi localized cells. This finding parallels the previous electrophysiological data for GFP-Kv1.3 (Fig 3) and indicates that introducing the Flag epitope does not alter the significant difference in current between the high and low Kv1.3 Golgi localized cells. Since we found that the Δ TDV truncation decreased Golgi localization of Kv1.3, we hypothesized that deleting this PDZ binding

motif would more closely represent the current conducted from the low Golgi localized Kv1.3 channels and in turn increase outward functional current. Steady-state outward current measured from GFP-Kv1.3-Flag- Δ TDV ($16.1 \text{ mV} \pm 1.0$; $n = 27$) transfected HEK293 cells indicate that the channel is functional and that disrupting the PDZ binding motif significantly increases Kv1.3 outward current (Fig 5C). The corresponding current–voltage relationships (Fig 5D) for high and low Golgi localized GFP-Kv1.3-Flag-WT and GFP-Kv1.3-Flag- Δ TDV (Δ TDV) demonstrate that outward current recorded from the Δ TDV channels is significantly higher than high Golgi localized WT Kv1.3. There is no significant difference between outward current of Δ TDV and the low Golgi localized WT Kv1.3 channel. Activation curves for high and low Golgi localized WT and Δ TDV were plotted from tail currents and fit to a Boltzmann equation to determine the half activation voltages ($V_{1/2}$) for each channel (Fig. 5E). No significant difference was observed in the $V_{1/2}$ for WT ($-20.7 \text{ mV} \pm 1.4$; $n = 15$) and Δ TDV (-21.2 ± 0.94 ; $n = 27$; $p > 0.05$) channels, indicating that the increase in Δ TDV current is not due to alterations in voltage dependence.

To test the hypothesis that the observed increase in Kv1.3 current is due to increased channels at the cell surface, we performed an immunofluorescence assay to measure both Golgi localization and surface expression of GFP-Kv1.3-Flag- Δ TDV transfected HEK293 cells. Similar to the non Flag construct, the Golgi localization of GFP-Kv1.3-Flag- Δ TDV ($11.6\% \pm 0.57$; $n = 59$) was significantly less than GFP-Kv1.3-Flag-WT ($21.7\% \pm 1.6$; $n = 82$; $p < 0.0001$). For the purpose of comparison, the GFP-Kv1.3-Flag-WT surface expression data from Figure 2E is shown. As described earlier, the WT population was binned into two categories, high and low, based on median Golgi

localization (16.0%). A one-way ANOVA followed by post hoc Bonferroni test for multiple comparisons revealed the normalized surface expression of GFP-Kv1.3-Flag- Δ TDV (n = 59; mean = 1.6 ± 0.11) was significantly higher than surface expression of high Golgi GFP-Kv1.3-Flag-WT (n = 41; mean = 1.0 ± 0.10 ; $p < 0.0001$) and lower than low Golgi GFP-Kv1.3-Flag-WT (n = 41; mean = 2.2 ± 0.12 ; $p > 0.05$), suggesting that the Δ TDV truncation decreases Golgi localization of Kv1.3, while increasing surface expression of the channel. Coupled with the increase in ionic current, these data also suggest the PDZ binding-motif disruption-mediated increase in Kv1.3 plasma membrane expression may involve a novel mechanism for anterograde Golgi trafficking of the channel.

Discussion

Subcellular localization and trafficking are distinct properties that have been shown to extensively influence ion channel function (Lai and Jan, 2006). In this study, we have shown that Kv1.3 is enriched in the Golgi apparatus and that the degree of this enrichment is inversely correlated with the amount of channel in the plasma membrane. Removal of the C-terminal PDZ-binding motif of Kv1.3 decreases Golgi localization and increases channel at the cell surface. In turn, this decrease in Golgi localization is accompanied by an increase in Kv1.3 ionic current. We propose that retention of Kv1.3 in the Golgi apparatus is a mechanism for regulating the amount of functional Kv1.3 channels at the cell surface (Fig 6).

Biosynthetic trafficking is an important mechanism for determining the level of mature protein within a cell and can be regulated at multiple stages. Although several motifs exist for modulating the endoplasmic reticulum (ER) retention and export of potassium channels (Zarei et al., 2004; Zhu et al., 2005; Zhu et al., 2003a, b), the Golgi apparatus may also serve as a checkpoint for anterograde protein trafficking (Cheng et al., 2002; Gentsch et al., 2003; He et al., 2002). We show that disrupting the PDZ-binding motif of Kv1.3 significantly decreases the amount of Kv1.3 channel in the Golgi apparatus (Fig 4), suggesting that PDZ-mediated interactions may contribute to the regulated biosynthetic trafficking of this channel. Disrupting the PDZ-binding motif of Kv1.3 also increases the amount of channel at the cell surface and the amount of Kv1.3 ionic current (Fig 5), supporting the idea that Golgi retention serves as a checkpoint for regulating Kv1.3 channel activity. The Δ TDV mutation increased surface expression of Kv1.3 by 60.0%, and increased Kv1.3 ionic current by 75.0%. These increases could result from a separate mechanism for regulating Kv1.3 channel at the plasma membrane. This is consistent with previous studies demonstrating that posttranslational modification of Kv1.3 can lead to alterations in Kv1.3 ionic current. For example, phosphorylation of Kv1.3 by cellular and receptor tyrosine kinases has been shown to induce current suppression of the channel (Bowby et al., 1997; Colley et al., 2004; Fadool et al., 1997; Holmes et al., 1996; Marks and Fadool, 2007; Tucker and Fadool, 2002). In addition, protein interactions have also been shown to modulate Kv1.3 activity. Coexpression of the synaptic adaptor protein, PSD-95, significantly suppresses Kv1.3 peak current magnitude in a use-dependent fashion (Marks and Fadool, 2007). Therefore, separate

mechanisms of Kv1.3 channel modulation at the plasma membrane may alter the function of the channel without affecting surface expression.

The notion that Kv1.3 Golgi localization serves as a checkpoint for regulating Kv1.3 function is also consistent with our observation that Kv1.3 Golgi localization is variable in HEK293 cells (Fig 1). The reason for this cell to cell variability is currently not known, however it could result from differences in steady-state signaling profiles between cells, resulting in altered effects on PDZ interactions. Many class I PDZ-binding motifs (X-S/T-X-Φ) are susceptible to posttranslational modifications that may alter the interaction of these motifs with PDZ domain containing proteins (Arnold and Clapham, 1999; Ikenoue et al., 2008; Lin et al., 2006; Matsuda et al., 1999). For example, phosphorylation of these motifs via specific serine/threonine kinases has been shown to perturb protein-protein interactions. Phosphorylation of AMPA receptor GluR2 subunits by PKC can reduce the receptor binding affinity for GRIP, a synaptic PDZ domain-containing protein (Matsuda et al., 1999). Similarly, casein kinase II can phosphorylate the NR2B subunit of NMDA receptors and disrupt the interaction of NR2B with both PSD-95 and SAP102, in turn decreasing surface expression of this receptor (Lin et al., 2006). Alternatively, acetylation of a lysine residue present in the PDZ-binding motif of PTEN has been shown to play a role in mediating PDZ domain interactions (Ikenoue et al., 2008). Therefore, posttranslational modification of the Kv1.3 PDZ-binding motif may alter protein-protein interactions and contribute to the observed variability of Kv1.3 Golgi localization in HEK293 cells.

Other Kv1 family channels also contain similar PDZ-binding motifs, although the mechanisms of channel regulation differ considerably (Kim et al., 1995). We found that

Kv1.2, which also contains a C-terminal PDZ-binding motif, did not display the same degree of Golgi localization as Kv1.3 (Fig 1). These results are consistent with reports indicating that subcellular distribution of Kv1.2 is mainly in the endoplasmic reticulum (ER) and highlight a fundamental difference between two closely related channels of the same family (Shi and Trimmer, 1999). These differences in intracellular distribution may reflect differences in the various trafficking pathways involved in channel regulation. For instance, cyclic AMP-mediated increases in plasma membrane Kv1.2 have been shown to be independent of Golgi trafficking (Connors et al., 2008); Modulated endocytosis at the plasma membrane is a major determinant of Kv1.2 activity at the cell surface (Nesti et al., 2004). In contrast to Kv1.2, our data suggest that the Golgi apparatus may function as a reservoir for Kv1.3 channel regulation. Overall, our findings suggest an additional, trafficking dependent mechanism for regulating Kv1.3 function in the cell, which exists in parallel with other mechanisms of channel regulation.

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Figure Legends

Figure 1: Intracellular distribution of Kv1.3. A) Vertical scatter plot depicting the mean Golgi localization (%) and distribution of both GFP-Kv1.3 (n = 20; mean = 28.5% \pm 3.1) and GFP-Kv1.2 (n = 20; mean = 12.6 % \pm 0.85) transfected HEK293 cells. GFP-Kv1.2 Golgi localization is significantly decreased compared to GFP-Kv1.3 (p <0.0001, Mann-Whitney test) B) Representative photomicrographs of HEK293 cells transfected with GFP-Kv1.3 (green) demonstrate a differential intracellular distribution of the channel. Each photomicrograph is an example of a cell in the range above (46.0%) and below (9.6%) the mean GFP-Kv1.3 Golgi localization. B) Representative photomicrograph of an HEK293 cell transfected with GFP-Kv1.2 (green) demonstrates a decreased percentage of this channel (13.0%) detected in the Golgi region. The GM130 marker (red) was used to define the Golgi region. Scale bar = 10 μ m.

Figure 2: Kv1.3 surface expression is inversely related to Golgi localization of the channel. A) Steady-state current-voltage relationships for GFP-Kv1.3 (open symbols) and GFP-Kv1.3-Flag (closed symbols) indicate no difference in channel function due to the presence of the extracellular Flag epitope. B) Activation curves plotted from the tail current of GFP-Kv1.3 (open symbols) and GFP-Kv1.3-Flag (closed symbols) indicate no difference in voltage sensitivity due to the presence of the extracellular Flag epitope. C) Representative photomicrograph of an HEK293 cell transfected with GFP-Kv1.3-Flag (green) and live labeled with anti-Flag-M2 (blue) to detect the extracellular Kv1.3-Flag

epitope, and thus Kv1.3 at the cell surface. After fixation, cells were labeled with anti-GM130 (red) to define the Golgi region and to subsequently calculate Golgi localization (GFP-Kv1.3 in Golgi region / Total GFP-Kv1.3). D) Scatter plot depicting the relationship between Kv1.3 surface expression and Golgi localization for GFP-Kv1.3-Flag. Linear regression analysis indicates that Kv1.3 surface expression is inversely correlated with Golgi localization of the channel ($n = 82$; $R\text{-squared} = 0.56$). E) Normalized surface expression of GFP-Kv1.3-Flag transfected HEK293 cells. Kv1.3 was binned into categories based on Golgi localization above (high; black bar) or below (low; white bar) the mean value (21.7%). Surface expression was calculated (anti-Flag-M2 / total GFP-Kv1.3-Flag) and both the high and low Golgi values were normalized to the mean value for cells exhibiting high GFP-Kv1.3-Flag Golgi localization. Surface expression of the low Golgi localized GFP-Kv1.3 population ($n = 54$; $\text{mean} = 2.2 \pm 0.12$) is significantly higher than surface expression of the high Golgi localized GFP-Kv1.3 population ($n = 28$; $\text{mean} = 1.0 \pm 0.10$; $p < 0.0001$; unpaired t-test). Scale bar = 10 μm .

Figure 3: Kv1.3 ionic current is inversely related to Golgi localization of the channel. Ionic current evoked by stepped voltages from -70 to + 50 mV in GFP-Kv1.3 transfected HEK293 visually categorized as having A) high or B) low Golgi localization. Visual categorization was confirmed by blind assessment of Golgi localization, yielding zero false low Golgi localized channels. Current traces are averages of currents from multiple cells ($n \geq 12$). C) Steady-state current-voltage relationships for GFP-Kv1.3 in cells categorized as having high (closed symbols) or low (open symbols) Golgi localization. Ionic current was significantly greater in cells categorized as having low

Kv1.3 Golgi localization. (* $P \leq 0.05$; unpaired t-test). D) Activation curves plotted from the tail current indicate no difference in channel voltage sensitivity in cells categorized as having high (closed symbols) and low (closed symbols) Kv1.3 Golgi localization.

Figure 4: Disrupting the C-terminal PDZ binding motif of Kv1.3 decreases Golgi localization of the channel. A) Representative immunoblots and corresponding normalized immunodensitometry demonstrating no significant difference in protein expression of GFP-Kv1.3-WT (WT; mean = 1.0 ± 0.08) and GFP-Kv1.3- Δ TDV (Δ TDV; mean = 0.92 ± 0.14) in cells transfected with equal amounts of plasmid DNA (n = 8; p > 0.05). GAPDH indicates equal loading between lanes and was used to normalize Kv1.3 band intensities. B) Immunoblot of eluant from an *in vitro* maltose binding protein (MBP) pulldown assay of lysates from HEK 293 cells transiently transfected with myc-PSD-95. Protein was collected with beads coated with either an MBP only control, MBP fused to the C-terminus of Kv1.3-WT (WT) or Kv1.3- Δ TDV (Δ TDV). Blots were probed with anti-Myc antibody (top) or anti-Kv1.3 C-term antibody (bottom). Removal of the PDZ binding motif of Kv1.3 abolishes the channels ability to pull down the canonical PDZ domain protein, PSD-95. C) Representative photomicrograph of an HEK293 cell transfected with GFP-Kv1.3- Δ TDV (green) and labeled with the Golgi apparatus marker, GM130 (red; n = 20). D) Vertical scatter plot depicting the Golgi localization (%) and distribution of GFP-Kv1.3 (WT) and GFP-Kv1.3- Δ TDV (Δ TDV) expressed in HEK293 cells. For the purpose of comparison, the GFP-Kv1.3 (WT) data from Figure 1A is shown. The Δ TDV truncation (n = 20; mean = 11.1 ± 1.39) significantly decreased Kv1.3

Golgi localization compared to WT channel (n = 20; mean = 28.5% ± 3.1; Mann-Whitney test; p < .0001). Scale bar = 10µm

Figure 5: Disrupting the C-terminal PDZ binding motif of Kv1.3 increases ionic current and plasma membrane expression of the channel. Ionic current measured in HEK 293 cells transfected with GFP-Kv1.3-Flag-WT and visually categorized as having A) high or B) low Kv1.3 Golgi localization. C) Ionic current measured in HEK 293 cells transfected with GFP-Kv1.3-Flag-ΔTDV (ΔTDV). Current traces for each condition are the average of currents from multiple cells (n ≥ 15). D) Steady-state current-voltage relationships for GFP-Kv1.3-Flag-WT exhibiting high (open diamonds; n = 15; mean = 9.2 mV ± 0.8) and low (open triangles; n = 15; mean = 15.5 mV ± 0.8) Golgi localization, as well as GFP-Kv1.3-Flag-ΔTDV (closed squares; n = 27; mean = 16.1 mV ± 1.0) demonstrate a significant difference between WT high Golgi and ΔTDV (P ≤ 0.02; unpaired t-test). No difference in ionic current was detected between WT low Golgi and ΔTDV. E) Activation curves plotted from the tail current indicate no difference in channel voltage sensitivity in cells expressing GFP-Kv1.3-Flag-WT exhibiting high (open squares) and low (open triangles) Golgi localization and GFP-Kv1.3-Flag-ΔTDV (closed squares). F) GFP-Kv1.3-Flag surface expression in cells categorized as having high (n = 28; mean = 1.0 ± 0.10) or low (n = 54; mean = 2.2 ± 0.12) Golgi localization, compared to surface expression of GFP-Kv1.3-ΔTDV (ΔTDV; n = 59; mean = 1.6 ± 0.11). ΔTDV surface expression is significantly higher than surface expression of the WT high Golgi localized population (p < 0.0001).

Figure 6: Proposed model for Kv1.3 regulation. As Golgi localization of Kv1.3 increases, both surface expression and ionic current decrease, a trend potentially mediated by the C-terminal PDZ-binding motif of the channel. Conversely, as Kv1.3 Golgi localization decreases, both surface expression and ionic current increase, suggesting increased channel activity at the cell surface in response to disrupting the PDZ-binding motif.

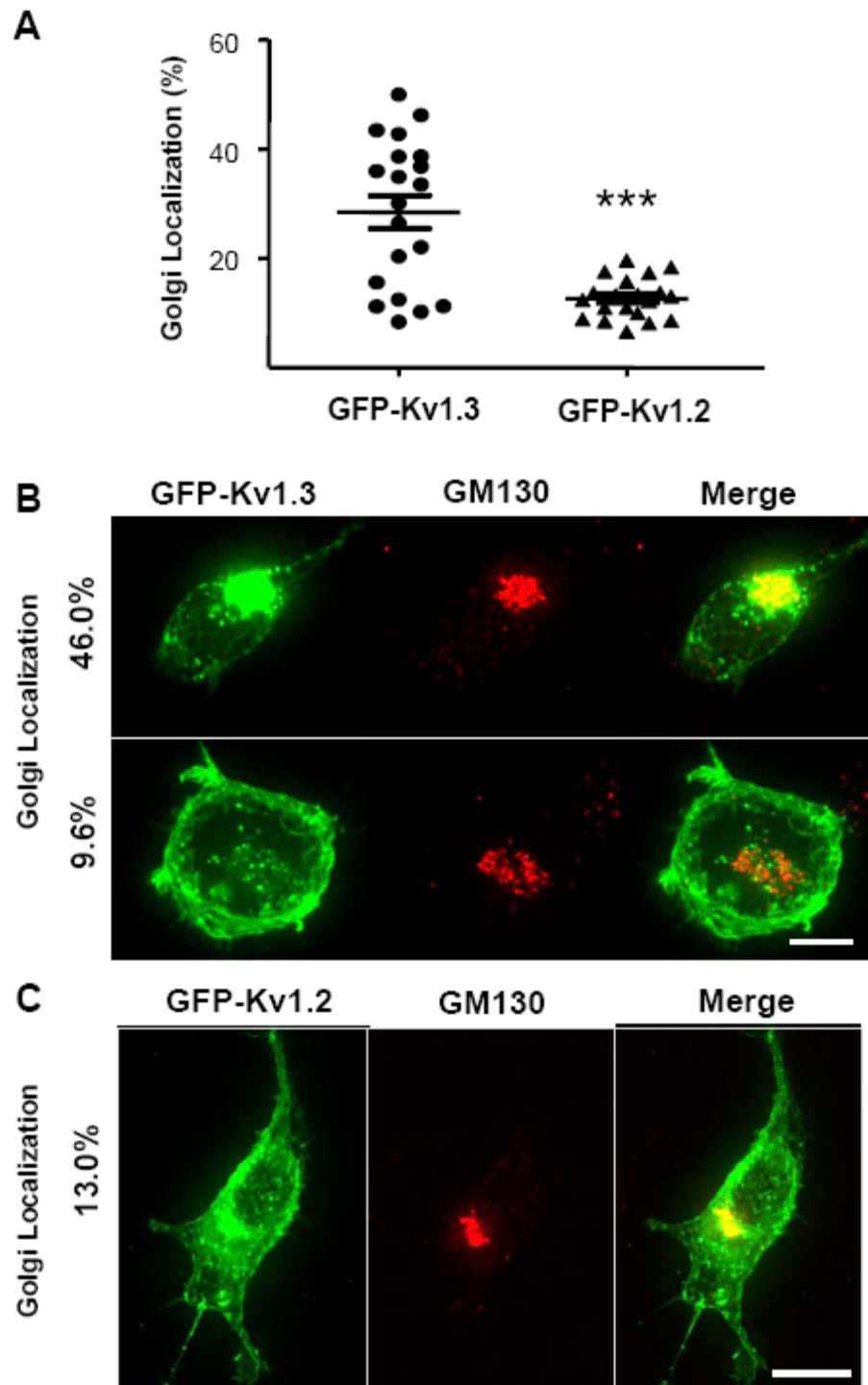


Figure 1: Intracellular distribution of Kv1.3.

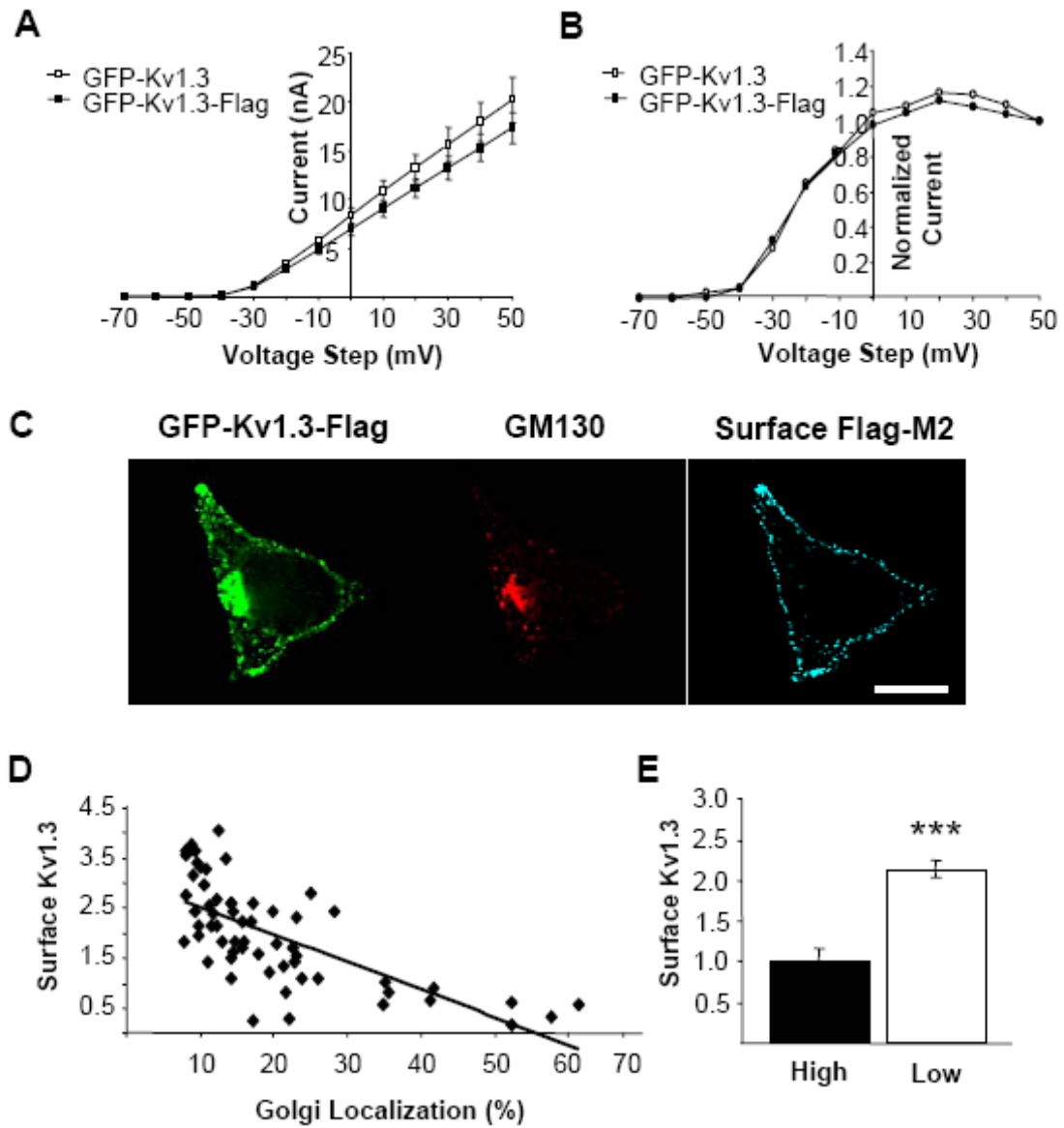


Figure 2: Kv1.3 surface expression is inversely related to Golgi localization of the channel.

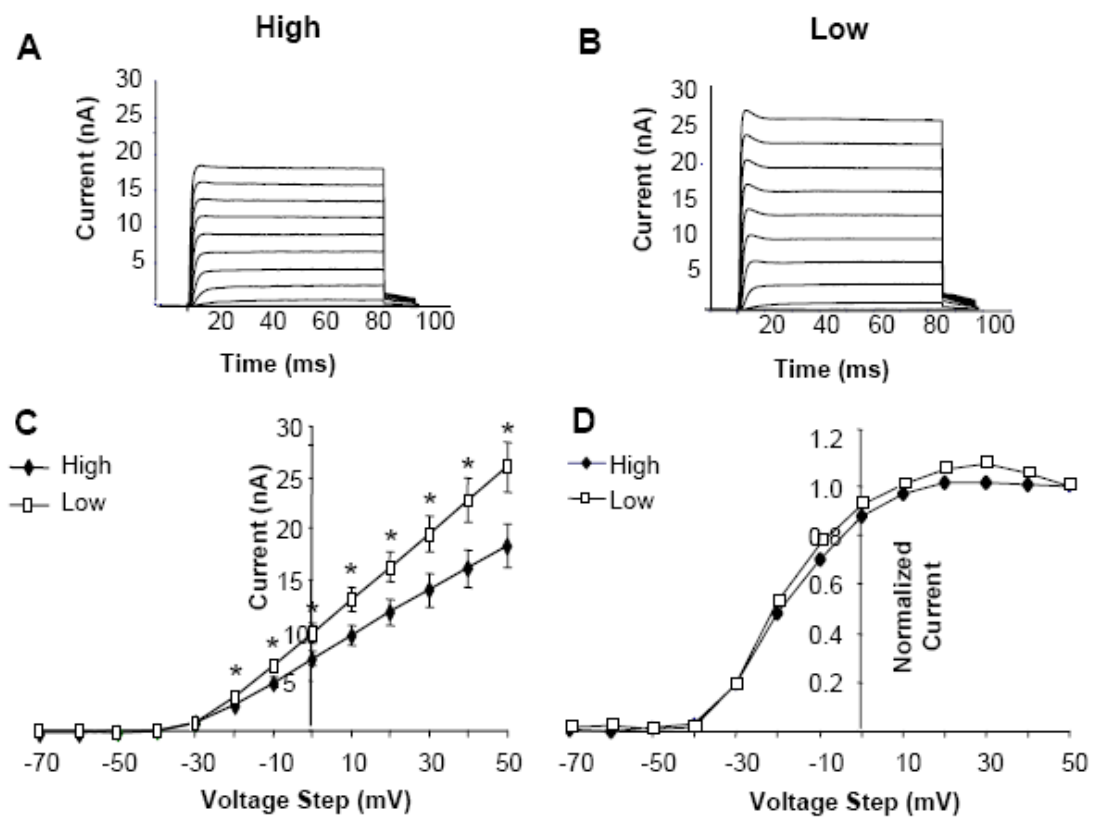


Figure 3: Kv1.3 ionic current is inversely related to Golgi localization of the channel.

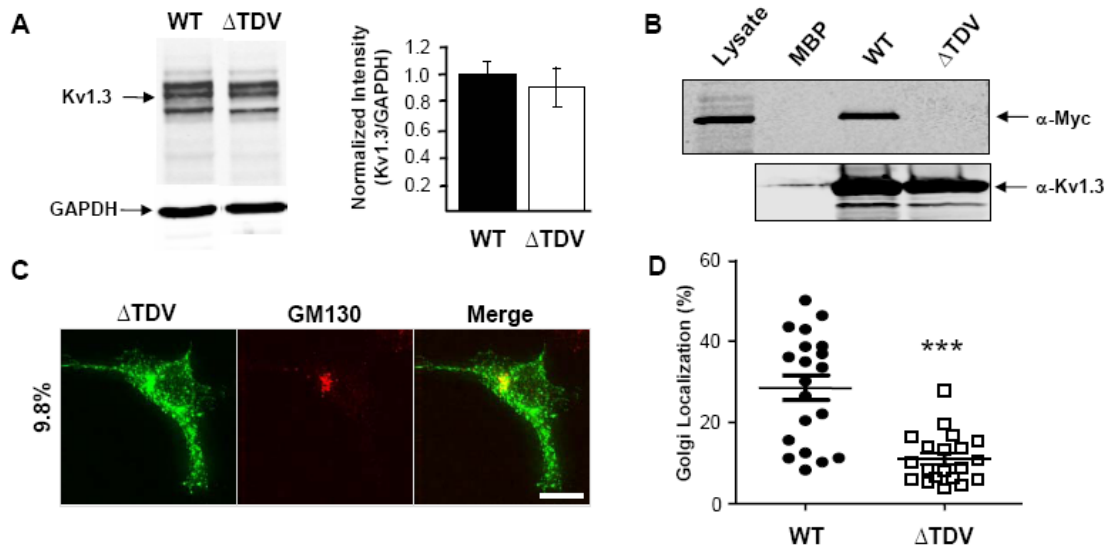


Figure 4: Disrupting the C-terminal PDZ binding motif of Kv1.3 decreases Golgi localization of the channel.

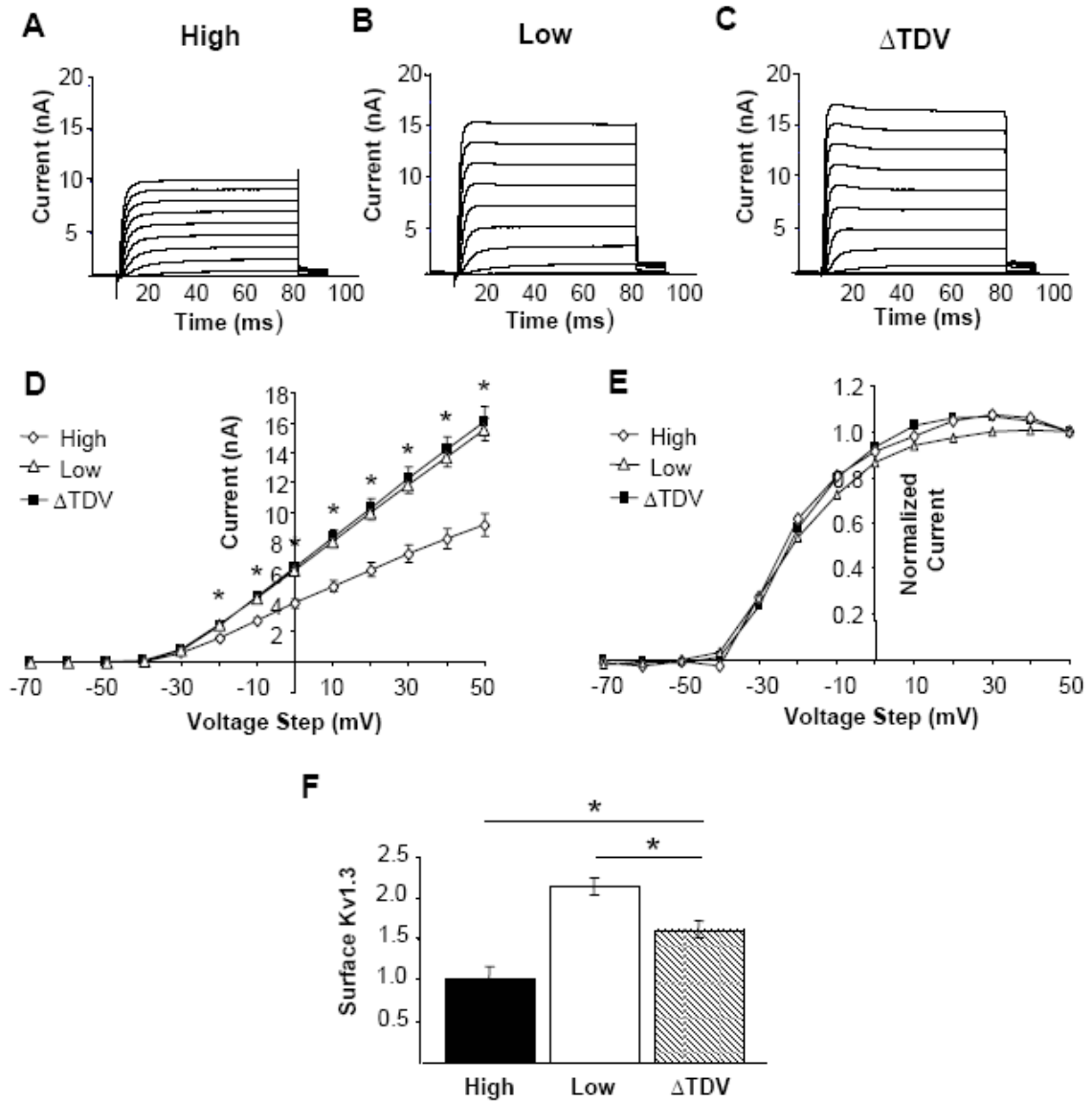


Figure 5: Disrupting the C-terminal PDZ binding motif of Kv1.3 increases ionic current and plasma membrane expression of the channel.

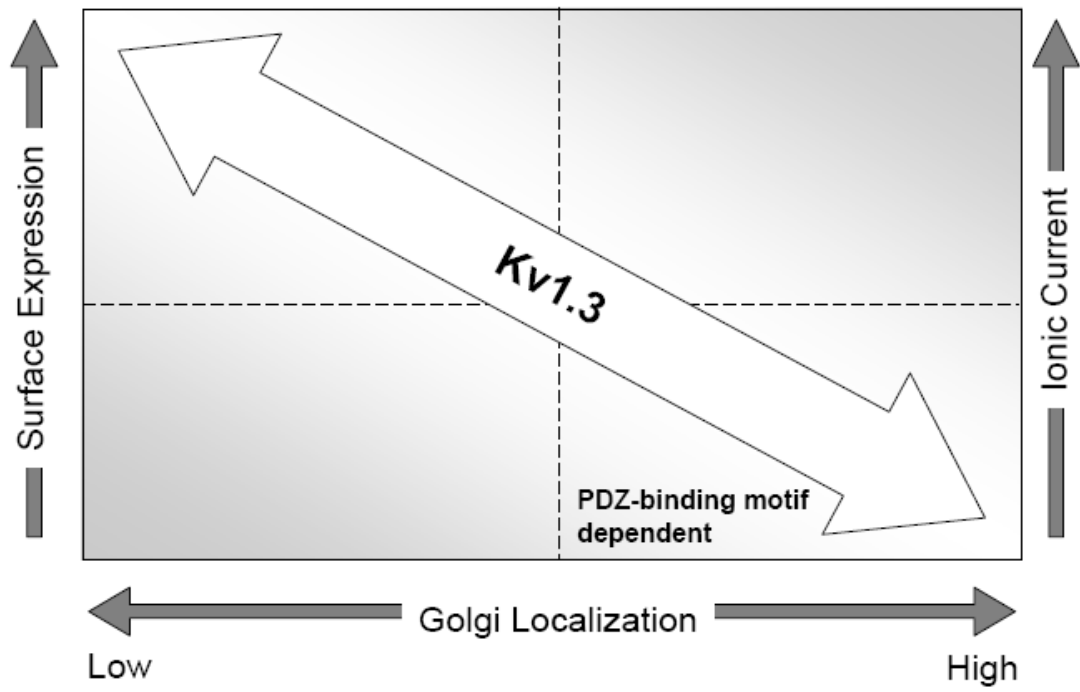


Figure 6: Proposed model for Kv1.3 regulation.

CHAPTER 4: COMPREHENSIVE DISCUSSION

Ion channels are key determinants of excitability in multiple cell types, including neurons (Schauf and Bullock, 1979). The localization of these ion channels to distinct cellular compartments greatly influences various components of neuronal function such as resting membrane potential, action potential characteristics and neurotransmission (Chiu and Ritchie, 1981; Lai and Jan, 2006; Rasband et al., 1998). Thus, understanding the regulatory mechanisms governing ion channel localization can provide fundamental insight into human physiology. The main goal of this dissertation was to elucidate the localization, function, and regulation of the voltage-gated potassium channel, Kv1.3. The experiments outlined in Chapter 2 of this study reveal the novel role of Kv1.3 as a determinant of sympathetic neuronal activity. In addition to plasma membrane expression, Kv1.3 also exhibited intracellular localization to the Golgi apparatus in these neurons. Using a model cell system, the data presented in Chapter 3 reveal a role for the PDZ-binding motif in mediating this Golgi localization of Kv1.3. Collectively, this research emphasizes how channel localization can influence the functional importance of Kv1.3 and suggests a new mechanism of channel regulation.

In Chapter 2, we used neurons from the sympathetic branch of the autonomic nervous system as a model to study endogenous Kv1.3. Previous research showed that Kv1 channel mRNA was present in superior cervical ganglia (Dixon and McKinnon 1996). In addition, previous reports demonstrated that using 4-aminopyridine to block Kv1 family channels in postganglionic sympathetic neurons increased neurotransmitter release (Ikushima et al., 1981; Uhrenholt and Nedergaard, 2003). However, the specific

ion channels involved remained largely unexplored. We found that sympathetic neurons express Kv1.3 protein and that the channel is localized to both the soma and processes (Chapter 2, Figure 1). Kv1.3 ionic current was also pharmacologically isolated from the soma, indicating that the channel is a determinant of the electrophysiological properties of these cells (Chapter 2, Figure 4). Extensive work in T lymphocytes has identified Kv1.3 as a major determinant in the regulation of resting membrane potential (Deutsch and Chen, 1993; Koo et al., 1997; Lin et al., 1993; Mello de Queiroz et al., 2008). Through mechanisms involving membrane depolarization and the subsequent attenuation of Ca^{+2} influx, selective Kv1.3 blockade suppresses the signaling cascades that lead to T lymphocyte activation and proliferation (Koo et al., 1997; Lin et al., 1993). In this study, we show that Kv1.3 also influences the resting membrane potential and function of postganglionic sympathetic neurons; inhibition of the channel depolarizes resting membrane potential, decreases the latency to action potential firing, and increases nicotinic agonist-induced neurotransmitter release (Chapter 2, Figures 5 and 7).

The research presented in Chapter 2 is the first demonstration that Kv1.3 contributes to sympathetic neuronal function and provides evidence that regulating Kv1.3 conductance can modulate neuronal excitability. Muscarinic acetylcholine receptor (mAChR) activation has previously been shown to suppress other K^{+} channels, namely Kv1.2 (Huang et al., 1993; Lechner et al., 2003). Initial studies exploring Kv1.2 regulation found that mAChR activation led to suppression of the channel's ionic current (Huang et al., 1993). The primary preganglionic input to postganglionic neurons is acetylcholine (ACh) and our studies present novel evidence that Kv1.3 current can also be modulated through mAChR activation (Chapter 2, Figure 6). Although tyrosine

phosphorylation-induced endocytosis has been defined as the mechanism for Kv1.2 current suppression (Huang et al., 1993), and Kv1.3 ionic current is also known to be modulated via tyrosine phosphorylation (Holmes et al., 1996) , no direct evidence exists to link mAChR activation to Kv1.3 trafficking events. Further studies may aim to elucidate the mechanism of stimulus-induced Kv1.3 current suppression to determine whether this phenomenon is dependent upon altered channel biophysics, potentially resulting from conformational changes within the channel itself, or due to negative trafficking of the channel away from the plasma membrane.

The unusual observation that Kv1.3 exhibits localization to a discrete intracellular compartment (Chapter 2, Figures 2 and 3) led to the hypothesis that retention of this channel in the Golgi apparatus may constitute a trafficking-dependent mechanism of regulation. While it is known that Kv1.3 undergoes tyrosine phosphorylation-induced current suppression at the plasma membrane (Holmes et al., 1996), no evidence exists linking decreased current to trafficking events such as endocytosis. Therefore, exploring the intracellular localization of Kv1.3 may provide evidence for an additional, trafficking-dependent mechanism of channel regulation which may operate concurrently with the biophysical regulation observed in other studies. Although similar intracellular localization of Kv1.3 has been previously reported in other cell types such as the HEK293 model cell system (Vicente et al., 2008a) and pyramidal neurons (Guan et al., 2006), the work outlined in Chapter 3 is the first to quantify this Kv1.3 Golgi localization. In addition, this study proposes a novel mechanism of channel regulation that is PDZ-binding motif dependent. The third chapter of this dissertation is dedicated to elucidating the role of Kv1.3 Golgi localization in channel function at the cell surface.

We show that Kv1.3 channels expressed in HEK293 cells localize to the Golgi apparatus with a high degree of variability (Chapter 3, Figure 1). Interestingly, the degree of this Golgi localization is inversely correlated with surface expression of the channel, suggesting the Golgi apparatus may serve as a checkpoint for the regulated trafficking of Kv1.3 to the plasma membrane (Chapter 3, Figure 2).

Previous work indicates that other ion channels are also localized to the Golgi apparatus (Cheng et al., 2002; Gentzsch et al., 2003). CFTR exhibits intracellular accumulation in the Golgi where it associates with a Golgi resident PDZ domain protein presumed to tether CFTR to this organelle and potentially regulate the channels transit to the plasma membrane (Cheng et al., 2002). Another chloride channel, ClC-3B, also localizes to the Golgi and has been found to associate with the same Golgi resident PDZ domain proteins as CFTR (Gentzsch et al., 2003). These findings, coupled with the knowledge that PDZ interactions play a major role in the localization and function of many proteins (Sheng and Sala, 2001), led us to hypothesize that Golgi localization of Kv1.3 may depend on protein associations with the C-terminal PDZ-binding motif of the channel. Interestingly, disrupting the PDZ-binding motif of Kv1.3 eliminated the striking intracellular localization of the channel to the Golgi apparatus, without altering the total amount of Kv1.3 protein within the cell (Chapter 3, Figure 4). This decrease in Golgi localization was accompanied by an increase in Kv1.3 ionic current (Chapter 3, Figure 5), indicating a potential role for the PDZ-binding motif in such processes as the regulation of Golgi retention-mediated channel trafficking to the plasma membrane or stabilization of functional channels already at the cell surface. Subsequent analysis also reveals an

increase in the number of channels at the cell surface (Chapter 3, Figure 5), providing evidence for a trafficking dependent mechanism of Kv1.3 channel regulation.

One possible mechanism for regulating Kv1.3 Golgi localization may involve protein-protein interactions. In a native HEK23 cell system, endogenous proteins may interact with the C-terminal PDZ-binding motif of Kv1.3 to retain this channel in the Golgi. Deleting this motif may disrupt these protein interactions, leading to increased channels at the cell surface and increased Kv1.3 ionic current. Further investigation may identify associations with specific PDZ domain proteins and reveal a role for these interactions in the anterograde trafficking pathways of the channel. While other members of the Kv1 family also contain C-terminal PDZ binding-motifs, their specific primary amino acid sequence varies (Kim et al., 1995). Previous studies have defined a role for the amino acids upstream from the final C-terminal residue, termed P₀, in the recognition and binding of PDZ domain containing proteins (Jelen et al., 2003; Sheng and Sala, 2001). Other members of the Kv1 family contain either a glutamate or leucine residue at the upstream P₋₃ position, which may be involved in mediating the selectivity of PDZ interactions (Kim et al., 1995; Songyang et al., 1997). However, the P₋₃ position of Kv1.3 is occupied by phenylalanine, a nonpolar hydrophobic residue containing a large benzyl side chain. Therefore, the distinct Golgi localization of Kv1.3 may be mediated by a PDZ domain protein interaction whose specificity is dependent on this residue. The differences in channel localization within the same family can be addressed with future experiments including mutational analysis of specific amino acids, as well as constructing chimeric channels exchanging C-terminal domains. Alternatively, similar PDZ domain proteins may bind to the C-terminal sequence of Kv1 family channels; however, variability at

other amino acid positions outside the PDZ-binding motif may alter interactions between the binding of accessory proteins of a larger complex.

Our reports are consistent with recent work indicating that the PDZ domain protein, PSD-95, suppresses the peak current magnitude of Kv1.3 over the course of repeated stimulation (Marks and Fadool, 2007). However, Marks et al also report that a phosphorylation-independent mutation of critical residues in the PDZ-binding motif of Kv1.3 abolishes this PSD-95 induced activity-dependent current suppression. However, no change in outward current between wild-type Kv1.3 and this PDZ mutation was reported (Marks and Fadool, 2007). Because our study employs a truncated form of Kv1.3 that lacks the phosphorylation site in the PDZ-binding motif, further investigation may reveal a role for post translational modification of this motif in the modulation of channel function at the plasma membrane.

The data presented in this dissertation indicate that disrupting the PDZ-binding motif of Kv1.3 abolishes the binding of the channel to the canonical PDZ domain protein, PSD-95, and suggest that disrupting this motif may also interfere with the binding of other PDZ domain containing proteins involved in channel regulation. Additionally, we demonstrate that disrupting the PDZ-binding domain of Kv1.3 also decreases the Golgi localization of the channel. Although specific PDZ protein interactions in the Golgi have yet to be identified for Kv1.3, future directions may be aimed at pinpointing associated PDZ proteins responsible for regulating trafficking of the channel at the level of this organelle. We speculate that disrupting the PDZ-binding motif of Kv1.3 would disrupt the association of the channel with these Golgi resident PDZ proteins, in turn disrupting the Golgi regulation of Kv1.3.

Anterograde trafficking of Kv1.3 from the Golgi apparatus to the cell surface may be a regulated process, introducing the possibility that localization of Kv1.3 to this organelle may serve as a reservoir of readily releasable channel. Posttranslational modification of PDZ binding-motifs has been shown to mediate their interaction with PDZ domain containing proteins (Chung et al., 2004; Matsuda et al., 1999). Therefore, serine/threonine phosphorylation of the Kv1.3 PDZ binding motif may be a mechanism for altering protein-protein interactions, in turn regulating the affinity of the channel for Golgi resident PDZ domain proteins. Decreased binding of Kv1.3 to Golgi associated PDZ proteins may lead to the regulated release of the channel from this compartment, increasing the amount of channel available for plasma membrane targeting. Similar to the phosphorylation-mediated disruption of protein interactions, the PDZ-binding motif deletion described in this dissertation may also eliminate the binding of Kv1.3 to specific Golgi resident PDZ proteins.

Neurons contain compartmentalized structures such as the soma, axons, dendrites and presynaptic terminals (Dotti et al., 1988), components not present in HEK293 cells. The later portion of this study uses HEK293 cells to explore the relationship between intracellular Golgi localization of Kv1.3 and surface expression of the channel, however, these cells can only be used to study channel trafficking to and from a noncompartmentalized plasma membrane. A major determinant of neuronal function is the localization and distribution of ion channels along neuronal processes (Chiu and Ritchie, 1981; Lai and Jan, 2006; Rasband et al., 1998). Therefore, it is important not only to consider the regulation of plasma membrane expression of Kv1.3, but also how the spatial distribution of this channel can be modulated. Chapter 1 demonstrates that

endogenous Kv1.3 in postganglionic sympathetic neurons is enriched in the Golgi apparatus. Depending on the plasma membrane targeting of Kv1.3, regulated release of this channel from the Golgi may have differential effects on cellular function. Increased Kv1.3 in the somal plasma membrane compartment may lead to a hyperpolarization of resting membrane potential. Alternatively, an increase in the number of channels targeted to the axonal region may result in a faster rate of action potential repolarization. Ultimately, the trafficking of Kv1.3 from the Golgi apparatus to various plasma membrane compartments may decrease neuronal firing, shorten action potential duration and attenuate neurotransmitter release from these cells. Because altering the distribution of Kv1.3 in the neuronal membrane can have such widespread effects on neuronal function, it is important to gain a broader understanding of the regulation of these channels.

The experiments presented in this dissertation have several limitations. Although HEK293 cells are a commonly used heterologous expression system for studying the biophysical properties and trafficking pathways of cloned ion channels (Connors et al., 2008; Marks and Fadool, 2007; Nesti et al., 2004; Vicente et al., 2008b), observations made in this cell line require further investigation in more physiologically relevant systems. It is important to note that the conclusions in Chapter 3 of this study are based on data collected from exogenous ion channel expression in HEK293 cells. Although this work has established a foundation for understanding the effect of localization on Kv1.3 function, future directions include manipulating endogenous Kv1.3 in primary neuronal cell culture. *In vitro* neuronal manipulations, such as disrupting Kv1.3 protein-protein interactions or RNA interference of endogenous channels, may provide insight into how

these channels and their associations affect neuronal function is a more physiologically relevant system. Furthermore, the present studies measure [H^3]-norepinephrine release from exogenously loaded neurons in cell culture in response to nAChR stimulation in the presence of a Kv1.3 inhibitor. The dissociated neurons used in this method have been subject to multiple enzymatic treatments which have altered them from their native state and extracellular environment. The dissociation processes strips the neurons of their axons and dendrites, therefore any neurite outgrowth in culture may have physiological characteristics different from *in vivo* preparations. Furthermore, experimental manipulation of exogenously loading these cultured cells with [H^3]-norepinephrine may have altered the physiological stores of endogenous norepinephrine, potentially contributing to experimental error. While [H^3]-norepinephrine release in this study is measured as a percentage of total radiotracer loading, it is important to note that the amount of exogenously introduced neurotransmitter may reflect more than the endogenous norepinephrine stores, thereby creating a larger pool from which an amplified release may be observed. Alternatively, this technique may be limited by cell to cell variability in the rate and efficiency of neurotransmitter uptake, attenuating the observed norepinephrine release in response to nAChR activation.

Because sympathetic neurons communicate with target tissues such as blood vessels via the release of this neurotransmitter (Bennett et al., 2004; McLachlan, 2007), measuring the change in circulating plasma norepinephrine in response to Kv1.3 inhibition in the intact animal can provide valuable information regarding the role these channels play in overall sympathetic function without introducing artificial neurotransmitters. In addition, determining the direct effects of Kv1.3 blockade on

arterial diameter and blood flow can provide greater insight into the role these channels play *in vivo*. Furthermore, this study does not incorporate pharmacological agents to disrupt Kv1.3 trafficking. Therefore, the complimentary approach of using protein transport or microtubule assembly inhibitors for studying the effect of localization on ion channel function would further aid in elucidating the mechanisms involved in Kv1.3 regulation.

An additional limitation of this study is the use of the venom-derived toxin, MgTX, to inhibit Kv1.3. While the specificity of channel inhibition is dependent on toxin concentration, discrepancies exist in defining this optimal range. An alternative approach to inhibiting endogenous Kv1.3 with MgTX is incorporating an antisense strategy such as RNA interference to eliminate Kv1.3 protein production within cultured cells. In terms of future studies, MgTX application *in vivo* may not be an effective approach due to the instability of peptide application in a living animal, as well as the inability to specifically administer the peptide to select regions. For this reason, directed gene knockdown may provide a more thorough understanding of the effects of chronically eliminating Kv1.3.

In summary, the work presented in this dissertation is the first to identify the Kv1.3 voltage-gated potassium channel as a key determinant of sympathetic neuronal activity. Initial studies sought to characterize the expression and function of Kv1.3 in these neurons and in the process, revealed a novel pattern intracellular localization to the Golgi apparatus. This work has identified a role for the PDZ-binding motif in mediating Golgi localization, surface expression and function of Kv1.3, leading us to propose that the regulated subcellular distribution of the channel is a determinant of Kv1.3 function.

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