Novel Structural and Physiological Functions of High Conductance K+ Channels of the Slo Family

Gonzalo Budelli
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Novel Structural and Physiological Functions of High Conductance K⁺ Channels of the Slo Family

by

Gonzalo Budelli

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2013

St. Louis, Missouri
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Abstract of the Dissertation

Novel Structural and Physiological Functions of High Conductance K⁺ Channels of the Slo Family

by

Gonzalo Budelli

Doctor of Philosophy in Neurosciences

Washington University in Saint Louis, 2013

Professor Lawrence Salkoff, Chair

The SLO family channels are high conductance K⁺ channels that are gated both by voltage and intracellular ions. Structurally they resemble voltage gated channels but have additional large conserved intracellular domains appended on the C-terminal that allow them to be gated by different intracellular ions. Two members of this unique family of K⁺ channels are Slo1 (BK) which is activated by Ca²⁺, and Slo2.2 (Slack) which is activated by sodium. Both channels are widely expressed in the brain and other tissues in many species from C. elegans to humans.

The large conductance Ca²⁺- activated K⁺ channel (Slo1 or BK for Big conductance K⁺ channel) is widely distributed and controls many different physiological processes including cellular excitability, neurotransmitter release, muscle contraction, hair cell function, insulin release, and blood pressure. Defects in Slo1 channels have been associated with hypertension, autism and mental retardation, obesity, asthma, epilepsy, and cerebellar ataxia. Slo1 channels are activated by Ca²⁺, voltage, and Mg²⁺ through different allosteric pathways providing a model system to study allosteric coupling and pathways in channel gating and protein function. The structure of Slo1 has two functional domains, a “Core” consisting of seven transmembrane elements (S0-S6) which assemble to form a voltage sensing domain which allosterically confers voltage sensitivity.
to the pore gate domain, and a “Tail” that forms a large intracellular gating ring thought to confer 
Ca$^{2+}$ and Mg$^{2+}$ sensitivity through different transduction pathways from gating ring to Core. The 
large modular Slo1 channel is known to undergo many complex allosteric interactions during 
channel gating, some within subdomains of the Core itself, some within the massive Tail, and 
some between Tail and Core. Because of its great size and complexity it has not been possible to 
understand all these allosteric structural changes nor dissect the contributions of the different 
transduction pathways to channel gating. A new and valuable tool for answering these questions 
would be the ability to express the voltage-sensitive Core alone, free of the influence of the large 
and complex Tail. This would allow the determination of the baseline gating properties of the 
isolated Core, which would permit experiments such as adding the transduction pathways back 
one at a time and in combination, to reveal the functions of each. Unfortunately, it has not 
previously been possible to express the Core without the gating ring. However, we have been 
able to develop novel constructs of the Core without the gating ring that I have been able to 
express and analyze using the *Xenopus* oocyte heterologous expression system. I will show that 
currents from these constructs are from heterologously expressed gating ring-less channels and 
not from possible endogenously expressed channels. This allows determination for the first time, 
of the baseline properties of the Slo1 Core without passive or active allosteric input from the 
gating ring. The studies I performed show that the baseline properties of the isolated Core differ 
considerably from the properties of the intact Slo1 channel in the isolation of Ca$^{2+}$. This shows 
that the gating ring imparts passive properties and interactions with the core, even in the absence 
of Ca$^{2+}$. Thus, removing the gating ring reduces single-channel conductance $\sim$30%, removes all 
Ca$^{2+}$- and Mg$^{2+}$-sensitivity, greatly reduces single channel mean open channel duration and burst 
duration; and right-shifts the G/V relation. Knowing these baseline properties of the Core then
provides us with a novel tool and a guide for understanding the allosteric basis for gating in Slo1 channels. Such knowledge may facilitate the development of agents to restore normal function in genetic syndromes where Slo1 channels are involved. Also, this more complete understanding of how these complicated channels function could be important for understanding other channels that are activated by more than one factor (as TRP channels) or for other proteins which undergo complicated allosteric structural changes.

The goal of the second project was to reveal the physiological relevance of Slo2 (Slack and Slick) Na⁺-dependent K⁺ channels. The discovery of high conductance Na⁺-dependent K⁺ channels in heart and brain presented a conundrum, the sodium concentrations needed to activate these channels in inside-out patches far exceeded the intracellular concentration of Na⁺ under normal physiological conditions. Thus, it was proposed that Na⁺-dependent K⁺ channels were an emergency conductance only activated under very special conditions such as during hypoxia or ischemia where the Na⁺ levels increase inside the cell. However, other reports indicated that these channels could be active under normal physiological conditions. Also, there is evidence of these channels being widely expressed all over the mammalian brain. I present data here showing that one of the largest components of the delayed outward current that is active under physiological conditions in many mammalian neurons, such as medium spiny neurons of the striatum and tufted-mitral cells of the olfactory bulb, is expressed by Na⁺-activated K⁺ channels and has previously gone unnoticed. Previous studies of K⁺ currents in mammalian neurons may have overlooked this large outward component because the sodium channel blocker tetrodotoxin (TTX) is typically used in studies of K⁺ channels. However, we found that TTX also eliminated this Na⁺-dependent delayed outward component in rat neurons as a secondary consequence. Unexpectedly, we found that the activity of persistent inward sodium current is highly effective
at activating this large Na\(^+\)-dependent (TTX sensitive) delayed outward current. Using siRNA
techniques, I identified the Slo2.2 channel as a carrier of this delayed outward current. These
findings have far reaching implications for many aspects of cellular and systems neuroscience, as
well as clinical neurology and pharmacology.

The final part of this dissertation involves the study of the effect of divalent cations on Slo2.2
channels. The activating effect of virtually all divalent cations on Slo1 is well documented, but
the effect of divalent cations on Slo2.2 channels is largely unstudied. In exploring this question, I
was surprised to observe that all of the divalent ions that activate Slo1 channels have the opposite
effect on Slo2.2 channels; they reduce channel activity. After making this observation I turned
my attention towards understanding the mechanism of blocking. I considered two hypotheses: 1)
Divalent ions blocked the pore of Slo2.2 channels, and 2) Divalent ions functioned at a site away
from the pore and either competed with sodium ion binding or produced allosteric changes
leading to channel inhibition. My results indicate that the effect of divalent ions on Slo2.2 is not
by blocking the pore. I also showed that the blocking effect of divalent cations on Slo2.2
channels is conserved in the orthologous channel from *Drosophila* which has been cloned in our
lab. In addition, I show that the *Drosophila* Slo2 channel is sodium dependent, unlike the Slo2
channel in another invertebrate, *C. elegans*, which lacks sodium sensitivity and is instead,
activated by Ca\(^{2+}\). Finally, by site-directed mutagenesis, we have tentatively located the site of
interaction of divalent cations with the Slo2.2 channel. In the conclusion to this section, I
discuss the possible physiological relevance of my findings to a proposed mechanism of action
of Slo2 channels.
**Acknowledgments**

First of all, I want to say thank you to Valentina for being always there. Your advice, support and help were essential for me to achieve this. Thank you also for making me happy and for being a wonderful mom of our beautiful Antonia.

I want to thank Larry (Lorenzo) for being a great mentor and friend. Thank you for having your door open for me whenever I needed and for being so enthusiastic about science and my work. I also want to thank all the members of Salkoff’s lab that I have had the luck of working with. In special to Celia (I’ll miss you), Alice (I don’t know what I am going to do without you), Aguan (you will be always a reference), Berry, Jonathan, Pato and Travis. I want to especially thank Qi, for his dedication and for putting up with me.

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Last I want to thank my parents (for everything), to my sister (for being there and for trusting in me) and to all my friends. I want to particularly thank my friends Juju, Fefa and Maru that were with me during this long process (I’ll see you soon!).
Chapter 1

Introduction
Ion channels are essential to electrical activity in all cells and all electrical signaling in the brain. Understanding ion channel function is crucial for comprehending the physiology of excitable cells in health and disease. Also, there is a great interest in studying ion channels because they are targets of toxins and numerous pharmacological drugs. Ion channels are transmembrane proteins which form pores that allow ions to cross the lipid bilayer. Some channels are constitutively open (usually called leak channels) but most channels are open only part of the time under certain given conditions. When an ion channel opens, thousands or even millions of ions cross the membrane following their electrochemical gradients. Ion channels are expressed in every cell and perform essential physiological functions. First, ion channels work in combination with pumps and carriers to transport water and ions to regulate cellular volume. Second, ion channels are essential players to determine the cell membrane potential. In excitable cells the coordinated opening and closing of different ion channels produce fast changes in the electrical potential difference across the membrane that can be autoregenerated and propagated long distances. Third, ions coming into the cell can be second messengers, the most studied second messenger is calcium which can activate various enzymes that modulate metabolic pathways or trigger different processes as neurotransmitter release in neurons, or contraction in muscle cells.

Different properties of ion channels have been used to classify them. The most relevant three are: (1) Selectivity, some channels are very selective to one ion over the others. Selectivity to Na\(^+\), K\(^+\), Ca\(^{2+}\) or Cl\(^-\) ions are often used to classify ion channels. (2) The stimulus that gates the channel; the most important factors to classify channels are: membrane voltage, ligand and mechanical force. Gated channels open and close stochastically but their open probability is dramatically changed by some of these factors. There are channels that respond only to voltage or only to a ligand but, there are others that respond to more than one factor, including the
channels that are the focus of this dissertation. (3) Structure, the number of transmembrane segments or the number of pore domains have also been used to classify channels. Channels have also been grouped in different classes based on sequence homology in addition to their physiological properties.

This dissertation is focused on the family of ion channels which are selective to potassium ion. Potassium channels constitute a large and highly diverse family of ion channels which is far more extensive than that of sodium and calcium channels combined. This may be because, after the depolarizing influence of sodium or calcium channels, what happens next with regard to voltage is largely due to the action of the different potassium channels in the cell. Figure 1 illustrates a variety of active membrane potential changes seen in a variety of cell types, and many of the differences illustrated are the consequence of the different types of potassium channels in the cells. Thus, depending on their properties, potassium channels may control the amplitude, duration, frequency and shape of action potentials. Potassium channels may also control the intrinsic properties of cell excitability, and have as large an influence in controlling the threshold of active responses, as sodium or calcium channels. As will be further discussed, potassium channels of the Slo family, can also play an essential role as a feedback mechanism for calcium or sodium entry, and can thus, appropriately tune the excitability of a cell to changing conditions.

The main focus of this dissertation is the study of the functional properties and the physiological roles of a subfamily of potassium channels called the Slo family (Salkoff et al., 2006). In particular my studies focus on the Slo1 and Slo2.2 channels. Slo1 is one of the most studied K⁺ channels, their high conductance and their dual modulation by voltage and calcium have attracted biophysicists since it was first observed. Even though Slo1 channels have been
extensively studied, due to their complexity there are many aspects of Slo1 channels that are still not well understood. I am fortunate to be able to present here a unique result that I believe will open up a new perspective on how these channels function and help us to better understand how the calcium- and the voltage-dependent domains interact. I also worked on the less studied sodium-dependent Slo2.2 channel, I will present results showing that, contrary to what many proposed, Slo2.2 is activated by sodium currents under physiological conditions. Finally, I will show that many divalent cations have opposite effects on Slo1 and Slo2.2; although intracellular divalent cations activate Slo1, I will show that they all inhibit Slo2.2.

The following sections will introduce the Slo family of potassium channels with regard to their discovery and history, their structure, their function and their involvement in physiology and disease.

Figure 1. Examples of electrical activity in excitable cells. Many characteristics of amplitude, duration, frequency and shape of action potentials depend on the different types of potassium channels present in these cells. Modified from Hille, 2nd edition.
K⁺ Channels and the Slo family

K⁺ channels are expressed in every studied cell and play important physiological roles in both excitable and non-exitable cells. The resting potential in almost all cells, both excitable and non-exitable, is determined by a particular class of potassium channels called “leak” potassium channels that are open at the resting potential. This makes the resting potential of most cells close to the K⁺ equilibrium potential. Thus, K⁺ channels are key players in determining the passive properties of most cell membranes, and as will be discussed, they also play a major role in shaping the active properties of excitable cells. In neurons and muscle cells they play crucial roles in action potential repolarization, regulation of neurotransmitter release and regulating the level of cell excitability. Genetic analysis has shown that many diseases are linked to mutations in K⁺ channels, including: long QT syndrome, BFNC (benign familial neonatal convulsions), and various neuromuscular disorders.

After the development of the voltage clamp and the patch clamp techniques, electrophysiological studies showed the great abundance and diversity of K⁺ channel currents expressed in different cells. Sequencing of many genomes made clear that K⁺ channels are the most abundant type compared with sodium, calcium or chloride channels. A large and conserved extended gene family of potassium channels is present in all metazoan (animal) genomes, both vertebrate, and invertebrate. In C.elegans there are more than 70 genes encoding K⁺ channels and the number is even greater in humans.

Figure 2 shows a schematic of structurally fundamental K⁺ channel types, and suggests their possible evolution. The basic domain included in all K⁺ channels is two transmembrane segments (2TM) connected by an intramembrane pore forming loop (Figure 2), all potassium channels need four pore domains to make a channel; thus, most K⁺ channels are tetramers.
Indeed, most K$^+$ channels are homotetramers consisting of four identical subunits. The simplest K$^+$ channels consist of four identical 2TM subunits (Figure 2), and channels of this type are present in both prokaryotes and eukaryotes. A tandem gene duplication of this basic 2TM structure is believed to have given rise to the two pore domain channels which have four transmembrane segments per subunit (4TM channels or 2P). Channels which use 4TM subunits are dimers and not tetramers as are most K$^+$ channels. Both 2TM and 2P-channels are controlled (gated) in many ways, but lack a specific region of their structure to sense voltage. Thus, none of them has intrinsic voltage-gating. Voltage-gated K$^+$ channels apparently evolved when this basic 2TM motif structure became associated with a four transmembrane segment domain which contained positive charges responding to voltage changes across the membrane. This resulted in the K$^+$ channel subfamily composed of six transmembrane (6TM) segments. Voltage-gated (6TM) channels express currents with different properties (Figure 3) which have been used to classify them in two main groups, rapidly-inactivating (A-type currents) and the slowly-inactivating (delayed rectifier) currents. These voltage gated K$^+$ channels are key players in shaping action potentials which can be markedly different depending on the channel composition of the cell.

The Slo family is a small family of voltage-dependent K$^+$ channels that acquired the ability of being activated by intracellular ions and a host of other factors (Figure 4). It is plausible to think that during evolution a voltage-dependent K$^+$ channel acquired a long C-terminal cytoplasmic domain (“Tail”) which bound intracellular ions and other physiological factors and via allosteric interactions transduced the effect to the voltage-dependent “Core” of the channel.
Figure 2. Evolution of the K⁺ channel family. Proposed evolution of K⁺ channels from a primitive 2TM domain channel. A duplication of this gene will lead to the 4TM (two pore domain) family. The ancient 2TM channel would be associated to a voltage sensor domain to form the 6TM voltage dependent channels. It is possible that a voltage dependent channel acquired a long intracellular domain to create the Slo family.
Figure 3. Channels encoded by Shaker, Shal, Shab and Shaw express currents that cover a wide range of biophysical properties. Outward currents recorded in response to voltage steps ranging from -80 to 20 mV in 10 mV steps. Modified from: Salkoff et al. (TINS Vol. 15, No. 5, 1992)

Slo family of high conductance K⁺ channels

The high conductance K⁺ channels encoded by the Slo gene family are among the largest and most complex of the extended family of K⁺ channels. As discussed, they are members of the voltage-dependent potassium channel superfamily but they are also activated by intracellular ions and numerous additional intracellular factors. There are four members of the Slo family in mammals (Figure 4): Slo1 (BK or MaxiK channels) are activated by intracellular Ca²⁺, Mg²⁺ and other factors, as will be discussed; Slo2, Slo2.1 (Slick) and Slo2.2 (Slack), are two close paralogues which are both gated by intracellular Na⁺ and Cl⁻ and additional factors as well; and Slo3 channels which are modulated by intracellular pH. Slo1 and Slo2 channels are widely expressed all over the brain and they are present in many other tissues. In contrast, Slo3 is highly specialized to function exclusively in mammalian sperm.

Slo1 channels are high conductance voltage- and Ca²⁺-dependent potassium channels expressed in most human tissues (Butler et al., 1993) which play key roles in many important physiological processes, including smooth muscle contractile tone regulation (Nelson et al., 1995; Brenner et
Defects in Slo1 channels have been associated with hypertension (Petterson et al., 2002; Sausbier et al., 2005), autism and mental retardation (Laumonier et al., 2006), obesity (Jiao et al., 2011), asthma (Seibold et al., 2008), epilepsy (Du et al., 2005; Yang et al., 2010), and cerebellar ataxia (Sausbier et al., 2004).
Figure 4. Schematic representation of SLO α-subunits. The structural properties of SLO1–3 are compared to the α-subunit of a voltage-dependent channel (a). All channel subunits depicted have membrane-spanning domains S1–S6 surrounding an ionselective pore. SLO1 and SLO3 subunits each have an additional S0 membrane-spanning domain. SLO α-subunits also include an extensive cytosolic carboxy-terminal extension containing sites that sense cytosolic factors, which modify gating, such as the calcium bowl in SLO1 (b), which is absent in SLO3 (c) and greatly modified in SLO2 subunits in which some positive charges seem to have replaced negative charges present in SLO1 (d). SLO2 is also distinctive in having its amino terminus inside the cell and in its absence of positive charges in S4. Note that four α-subunits group together to form the pore of all channels depicted. RCK, regulators of conductance of K⁺ domain. Modified from Salkoff et al., 2006.

These channels were named BK or MaxiK because they have a very high single channel conductance (200 to 300 pS in 150 mM symmetrical K⁺) which is twenty to forty times greater than most K⁺ channels. Since Slo1 channels are regulated by transmembrane voltage and intracellular calcium, the Slo1 channel acts as a negative feedback for Ca²⁺ entry linking membrane depolarization and the increase in intracellular Ca²⁺. Upon depolarization Ca²⁺ channels are activated, depolarization and Ca²⁺ increase will activate Slo1 channels producing a membrane repolarization. This membrane repolarization will result in the closing of Ca²⁺ channels, consequently interrupting Ca²⁺ influx.

Slo2 channels are Na⁺-dependent high-conductance K⁺ channels. Slo2.1 (Slick) and Slo2.2 (Slack) are widely expressed in the brain but had been overlooked for decades. This high conductance channel was first reported in guinea pig cardiomyocytes (Kameyama, et al., 1984) and was thought to be a Slo1 channel. Inside-out patch recordings showed that it was not Ca²⁺ dependent but instead activated by intracellular Na⁺. The original report in heart cells and subsequent studies in neurons (Dryer et al., 1989; Haimann et al., 1990; Egan et al., 1992) showed that the sodium concentrations needed to activate these channels in inside-out patches, far exceeded the intracellular concentration of Na⁺ under normal physiological conditions. Thus,
it was proposed that Na\(^{+}\)-dependent K\(^{+}\) channels were an emergency conductance only activated under very special conditions such as during hypoxia or ischemia when Na\(^{+}\) levels increase inside the cell. However, other reports indicated that these channels could be active under normal physiological conditions where they had been proposed to contribute to action potential repolarization and setting membrane excitability (Bader et al., 1985; Dryer et al, 1991; Dale, 1991; Saito and Wu, 1991). Slo2 channels have very little voltage sensitivity, which is not surprising considering that they lack an obvious voltage sensor; the canonical positive charges present in the voltage sensor of all voltage-dependent channels are absent in Slo2 channels (Figure 4). Whether or not they play a role in hypoxia is still controversial. However, as will be presented, we have shown that they apparently are active in many neurons of the mammalian brain. In addition to the role they could play during ischemia it has been suggested that these channels are involved in accuracy of spike timing in auditory neurons (Yang et al., 2007). They may also be involved in the Fragile-X Mental Retardation Syndrome (Brown et al., 2010), and epilepsy (Barcia et al., 2012).

Slo3 channels are voltage- and pH-dependent K\(^{+}\) channels. Unlike Slo1 and Slo2, which are widely expressed in brain and present in many other tissues, Slo3 channels are exclusively expressed in mammalian sperm. It has been shown that Slo3 channels are crucial for sperm hyperpolarization during capacitation (a fundamental mechanism for fertilization). Slo3 knock-out mice are sterile, and their sperm are defective both with respect to hypermotility and the acrosome reaction (Santi et al., 2010). Slo3 channels are not considered in this dissertation.
**Slo Channel Structure**

As previously mentioned, Slo channels are members of the voltage-dependent K\(^+\) channel subfamily which are formed by tetramers of 6TM protein subunits. Unlike Slo2 and all other voltage-dependent K\(^+\) channels, Slo1 and Slo3 have an extra transmembrane segment (S0) which places the N-terminus on the extracellular side of the membrane (Meera *et al.*, 1997). S0 has been reported to be relevant for coupling with β subunits in Slo1 (Contreras *et al.*, 2012). The transmembrane segments S1 to S4 constitute the voltage sensor domain and S5-P-S6 the pore domain (Figure 4). Changes in membrane potential are sensed by charges in the voltage sensor domain (mainly positively charged residues in the S4 transmembrane segment) producing conformational changes that are transduced to the permeation gate within the pore domain. In this dissertation we will call the transmembrane domain (S0-S6 in Slo1) the “Core” or the voltage dependent core of the channel.

In contrast to all other voltage dependent channels, Slo channels have a very long cytoplasmic C-terminus domain which consists of more than two thirds of the protein. We refer to this as the “Tail” domain of the channel. The Tail is organized into two tandem domains called “regulators of the conductance of K\(^+\)” (RCK1 and RCK2, Jiang *et al.*, 2001). In the tetrameric channels, the four pairs of RCK domains form a large intracellular structure called the gating ring (Yuan *et al.*, 2010). As will be discussed later, binding of intracellular ions and other physiological factors to the gating ring will modulate Slo channel gating properties. In Slo1 each RCK domain is believed to have its own high-affinity calcium binding site. The site with the highest Ca2+ affinity is found in RCK2 and is referred to as the Calcium Bowl (Schreiber and Salkoff, 1999).

Not many structure-function studies have been undertaken in Slo2 channels, but it has been suggested that there is a sodium binding site in the RCK2 domain (Zhang *et al.*, 2010).
The atomic structures of the gating rings of Slo1 and Slo3 have been solved using X-ray diffraction of crystals obtained from the expression of the Tail domain alone (Yuan et al., 2010, Leonetti et al, 2012). In Slo1 the RCK domains are formed by two lobes, the larger N-terminal lobe forms a Rossmann fold that is connected to the smaller lobe. The two RCK domains in each subunit are folded tightly forming a “flexible interface” (Yuan et al., 2010). The structure of the Slo2 gating ring has not been solved yet at high resolution. A low resolution structure of Slo2 Tail domain tetramer was used to resolve the Slo1 tetrameric structure (Yuan et al., 2010). Molecular replacement modeling of monomeric Slo1 against Slo2.2 tetrameric diffraction data gave only one outstanding solution indicating that the gating ring structures of both channels must be similar (Yuan et al., 2010). The atomic structures may give us an idea of residue or domain localization within the gating ring but additional experiments are required to understand the conformational changes involved in ion channel function and regulation. The experimentally determined atomic structure of the Slo1 channel Core has not been solved yet. However, homology models (Figure 5) of the whole channel have been obtained using the atomic structure of the voltage sensor of Kv channels (Long et al., 2007), the pore structure of bacterial calcium-dependent MthK (Jiang et al., 2002), and the gating ring structure of Slo1 (Yuan et al., 2010). Of course, the details of these homology models should be taken with extreme caution and it is likely that parts of the channel are out of place (for example, the localization of S0 cannot be inferred in these models). However, homology models seem to be consistent with the size and shape of the channel obtained by electron cryomicroscopy (Wang and Sigworth, 2009).

The Core and Tail domains of Slo channels have to be very close together for functional interaction. In Slo1, Mg\textsuperscript{2+} coordination between residues in the voltage sensor domain and residues in RCK1 (Yang et al, 2008) suggests that “Core” and “Tail” domains are very close to
each other. In addition, the crystal structure of the Slo1 gating ring presents a “bump” that seems to fit perfectly in a “groove” observed in the Kv voltage sensor crystal structure (Yuan et al., 2010). Interestingly, in addition to the Mg2+ binding site, there is a group of mutations that affects calcium sensitivity of Slo1 that map to the putative area of contact between Core and Tail (Figure 5C).

**Figure 5. Model of the Slo1 channel structure.** A) Ribbon representation of a single subunit from the Slo1 model generated by superimposing the Slo1 gating ring (RCK1 in blue and RCK2 in red), the Kv voltage sensor and the MthK pore (both in green). B) Slo1 model from the side view with the transmembrane domain above. Each subunit of the tetramer is colored different. C) Gating ring and voltage sensor interaction. Mutations in the gating ring that affect the function were grouped by color, in red Calcium Bowl mutants, in green mutations near the flexible interface and in purple mutations in RCK1 close to the voltage sensor. Modified from Yuan et al., 2010.

Even in Slo1, one of the most studied K⁺ channels, it is largely unknown how the Tail (gating ring) functionally interacts with the Core of the channel. One logical way to start studying this question would be to express the Core in the absence of the Tail to observe its baseline
properties. Until now it has been not possible to perform this experiment. However, I will show in my thesis how we were able to express the Core of Slo1 with no gating ring, and reveal for the first time, the baseline properties of this channel.

**Slo1 calcium sensitivity is conferred by the Tail.**

Since the Slo1 channel was first cloned in *Drosophila* (dSlo1, Atkinson *et al.* 1991) the long C-terminus domain has been proposed as the place where the calcium sensing site might be localized. When the mammalian Slo1 channel was cloned (mSlo1; Butler *et al.* 1993) it was striking to find how highly conserved the mammalian protein was with the fly ortholog. However, there was one conspicuously non-conserved hydrophilic region separating the two RCK domains (Figure 4). It was discovered that the co-expression of the two conserved regions of the channel on both sides of this non-conserved region could generate functional channels without being covalently linked (Wei *et al.*, 1994). Using that knowledge and the different calcium sensitivities of dSlo1 and mSlo1, experiments conducted in our lab showed that the channels obtained by co-expressing the region including the Core and part of the Tail (from S0 through RCK1) from mSlo1 and the remaining distal part of the Tail domain (from RCK2 through the C-terminal) from dSlo1, had calcium sensitivity similar to the dSlo1 channel. This was the first result suggesting that the distal C-terminal region including RCK2 is important to determine calcium sensitivity. Replacement of this C-terminal region by the corresponding region of the non-calcium sensitive homolog Slo3 produces channels with greatly reduced calcium sensitivity (Schreiber *et al.*,1999). Further analysis of the C-terminal sequence showed the presence of a highly conserved region with five consecutive aspartate residues that was a likely candidate for calcium ion binding (subsequently we named this region the “Calcium
Bowl”). Mutation or deletion in the Calcium Bowl produced BK channels with its calcium sensitivity lowered (Schreiber and Salkoff, 1997). Direct radioactive binding of calcium ion to a fragment of the tail containing the Calcium Bowl has been measured (Bian et al., 2001, Braun and Sy, 2001) and substitution of five aspartates to aspargines produces a major reduction in calcium binding. More recently, the crystal structure of the gating ring was determined and a calcium ion is indicated to be bound at the center of the Calcium Bowl (Yuan et al., 2010). The Calcium Bowl is located in the RCK2 domain in a region where the crystal structure suggests is at the assembly interface between the two RCK domains (Yuan et al., 2010). The crystal structure of the gating ring was obtained in high calcium, and calcium ions were only observed bound to the Calcium Bowl.

The experiments with Calcium Bowl mutants provided evidence of a second calcium binding site that was sensitive to cadmium. The Calcium Bowl mutants were less calcium sensitive but still activated by calcium. However, like the wild-type Slo1 channel, cadmium ion still activated the channel, and there was no difference in cadmium sensitivity in the Calcium Bowl mutants (Schreiber and Salkoff, 1997). This second calcium binding site was also identified using structure-function experiments, with a focus on RCK1 (Xia et al., 2002). An additional lower affinity site able to bind magnesium was also identified in RCK1 (Xia et al., 2002; Shi et al., 2002). It is not clear why in the crystal structure calcium ions were not detected close to these other binding sites. A possible explanation is that the crystal was obtained in the absence of the Core domain and these calcium binding sites were predicted by the structure to be close to the voltage sensor. Perhaps the absence of the Core produces conformational changes of the gating ring affecting calcium binding. Also, the site that coordinates Mg$^{2+}$ includes residues in the
voltage sensor domain. Like that site, it is possible that amino acid residues in the Core domain are needed to coordinate Ca$^{2+}$ in the RCK1 high affinity calcium binding site.

**Slo2 sodium sensitivity**

High conductance K$^+$ channels activated by intracellular sodium (K$_{Na}$ channels) were first discovered in guinea pig cardiomyocytes (Kameyama, et al., 1984). Single channel analysis of these channels showed properties similar to Slo1 Ca-activated channels, but remarkably, the channels were insensitive to Ca$^{2+}$ and sensitive to Na$^+$ instead. K$_{Na}$ channels were also observed in different neural preparations (Dryer et al., 1989; Haimann et al., 1990; Egan et al., 1992; Dale, 1993; Koh et al., 1994). As in cardiomyocytes, the studies in neurons showed that the sodium concentrations needed to activate these K$^+$ channels exceeded the cytosolic physiological sodium concentration. As a consequence of the high [Na+] necessary to activate K$_{Na}$ channels some reports proposed that they might play a protective role during hypoxia or ischemia when intracellular Na$^+$ concentrations increase. The increase in intracellular Na$^+$ would activate the potassium channels to keep the cell hyperpolarized and to reduce its excitability.

The first Slo2 channel expressed in a heterologous system was cloned from *C. elegans* (Yuan et al., 2000), this channel was actually calcium dependent as Slo1 but also had chloride dependence. The finding of the *C. elegans* Slo2 channel led to the cloning of two Slo2 genes in mammals, Slo2.1 (Slick) (Bhattacharjee et al., 2003 and Slo2.2 (Slack) Yuan et al., 2003). Our laboratory discovered that, unlike the nematode Slo2 channels which were Ca$^{2+}$-dependent, mammalian Slo2 channels were dependent on sodium ion for their activation (Yuan et al, 2003). However, the property of chloride dependence was conserved between nematode and mammalian Slo2 channels. It soon became clear that Slo2 channels carried the sodium-
dependent $K^+$ current reported in cardiomyocytes and neurons. There are just a few structure-function studies on Slo2 channels. Studies by sequence comparison with the sodium binding site of an inward rectifier $K^+$ channel found many putative sodium binding sites in the Tail of Slo2.2 (Zhang et al., 2010). That report identified a sodium binding site using site directed mutagenesis in the RCK2 domain. The localization of the site in the RCK2 domain reinforces the idea of RCK domains playing an important role in coupling intracellular ion binding in the Tail with pore gating Slo channels. It was also shown that the activation of the channel by chloride was independent of sodium binding to this site (Zhang et al., 2010). The Slo2.2 channels with mutations in this putative binding site, still retained some sodium dependence, but higher concentrations of sodium were needed for channel activation. This could indicate that the mutant is reducing the affinity for sodium ion; alternately, it could be affecting the transduction of sodium binding to the Core. How chloride activates Slo2 channels is still a mystery. There is a group of positively charged residues in RCK2 (Figure 4) that has been suggested as a possible chloride binding site but experiments supporting this hypothesis have not been reported.

*Modulation of Slo1 and Slo2 channel gating by other intracellular factors*

Slo channels are widely expressed in brain and many other tissues. They present functional diversity which can be explained by alternative splicing, the presence of tissue specific auxiliary subunits, and modulation by many different physiological factors. There is an increasing number of reports about Slo channels being modulated by a wide spectrum of relevant physiological factors, many of them seem to be acting by binding or modifying the Tail domain of the channel, and transducing the conformational changes caused by the bound ligand to the Core.
**Slo1 channels:**

Slo1 channels have been reported to be subject to modulation by several biological factors such as phosphorylation, oxidation, steroid hormones, protons, heme, carbon monoxide (CO), lipids, reactive oxygen and nitrogen species (reviewed in Hou et al., 2009). Slo1 channel activity is modified by reversible protein phosphorylation of serine/threonine or tyrosine within the Tail of the channel. Slo1 channels also associate with several regulatory proteins. The dynamic interaction of competing kinases and phosphatases with Slo1 seems to be an important mechanism for tuning Slo1 channel response to calcium and voltage in smooth muscle (Schubert and Nelson 2001) and neurons (Widmer et al., 2003). Additionally to phosphorylation, modification of Tail residues by oxidation has been reported to modulate Slo1 channels. Reactive oxygen species are produced in all aerobic cells and are capable of oxidizing proteins and other cell components. Reactive molecules are vital signaling molecules in cells at low concentrations; however, reactive species at high concentrations are deleterious causing oxidative stress. Many reactive species such as H$_2$O$_2$ have been reported to affect Slo1 channels. Reducing and oxidizing agents have opposite effects when applied to inside-out patches of membranes expressing Slo1 channels (DiChiara and Reinhart, 1997). A cysteine residue close to the calcium bowl has been reported to be the target of H$_2$O$_2$ oxidation causing inhibition of Slo1. In contrast, oxidation of methionine residues located in the RCK domains increases open probability (Santarelli et al. 2006).

The effect of pH on Slo1 channels has been debated because early studies in neurons showed an inhibitory effect by low intracellular pH (Church et al., 1998). More recent reports using heterologous expressed Slo1 channels show the opposite, that low pH increases channel activity (Avdonin et al., 2003). In this latter study, the effect of intracellular pH on Slo1 seems to be by
increasing open channel probability, producing a shift of the macroscopic G-V of \( \sim 50 \) mV to more negative potentials; the EC\(_{50}\) of the pH effect is Ph 6.5 (Avdonin et al., 2003). Two histidine residues in RCK1, close to the high affinity Ca\(^{2+}\) sensor, have been shown to be necessary for the pH effect (Hou et al., 2008a). The carbon monoxide effect has also been shown to be regulated by the same histidine residues in RCK1; thus, it is likely that the increase of H\(^{+}\) and CO mimics the binding of calcium to the RCK1 calcium sensor producing channel activation (Hou et al., 2008b).

**Slo2 channels**

Early studies on Slo2 channels endogenously expressed in neurons showed activity rundown over time in inside-out patches (Egan et al., 1992; Dryer 1993). This suggests that Slo2 channels are modulated by intracellular factors which are washed out by the experimental conditions. One factor that might be responsible for channel rundown in cell-detached patches might be NAD\(^{+}\) (nicotine amide dinucleotide). The existence of a putative NAD\(^{+}\) binding site in the Tail of Slo2 channels was discovered by sequence analysis. It was also shown that NAD\(^{+}\) increases the apparent Slo2.2 Na\(^{+}\) sensitivity, reducing the EC\(_{50}\) from \( \sim 50 \) to \( \sim 20 \) mM (Tamsett et al., 2009). This was shown by experiments in heterologously expressed Slo2.2 channels and in native KNa channels from DRG (dorsal root ganglion) neurons.

Some reports show factors which decrease Slo2 channel activity. It was reported that Slo2.1 is inhibited by intracellular ATP (Bhattacharjee et al., 2003) but ATP had no effect on Slo2.2 channels. This would indicate that ATP is binding to a domain in the Tail of Slo2.1 that is not present in Slo2.2; in fact, a consensus ATP binding site has been identified in SLO2.1
(Bhattachajee et al., 2003). Other studies using heterologous expression of Slo2.2 show that both low pH and carbon monoxide inhibit Slo2.2 channels (Ruffin et al., 2008).

In addition to the intracellular factors mentioned above, Slo2 channels can be modulated by the activation of neurotransmitter receptors via G-alpha-Q GPCR metabotropic pathways. It has been shown that activation of the M1 muscarinic receptor or the glutamate receptors mGluR1 modulates Slo2 channels when either receptor is co-expressed with Slo2 channels in Xenopus oocytes. Slo2.1 and Slo2.2 channel seem to be regulated in opposite ways; Slo2.1 is down-regulated while Slo2.2 is up-regulated by activation of either co-expressed receptor (Santi et al., 2006). In addition, Slo 2.2 currents were increased when PKC was activated by phorbol ester, while Slo2.1 currents were reduced. It has also been reported in Kenyon cells from mushroom bodies of cricket that K$_{Na}$ channel activity is modulated by the activation of octopamine and dopamine receptors. Single channel recordings from on-cell patches showed that the activation of the octopamine receptor increases the open probability of the high conductance Na$^+$-dependent K$^+$ channel (likely to be Slo2), while activation of the dopamine receptors reduces channel activity (Aoki et al., 2008). Thus, it was suggested that regulation of mushroom body cell excitability could be achieved by activating or inactivating Slo2 channels. Additionally, it has also been shown that the activity of Slo2 channels can be regulated by changing the quantity of channels in the cell membrane. It has been reported that activation of protein kinase A (PKA) could cause Slo2.2 channels to be internalized in DRG neurons (Newer et al., 2010).
**Slo Channel Modulation by auxiliary subunits**

Slo1 channels are tetramers formed by four α subunits encoded for a single gene. As explained in the previous section, the functional diversity of Slo1 channels in the different tissues is produced by many factors including the modulation of the channel by several biological factors, and the presence of auxiliary β and γ subunits (Contreras et al., 2012, Yan and Aldrich, 2012). In particular, regulation by auxiliary subunits is a key mechanism for obtaining tissue specific ion channel function. Slo1 auxiliary subunits are expressed in most tissues where Slo1 is present; these subunits show tissue specific expression, and they greatly modulate Slo1 macroscopic current kinetics and gating properties.

Four homologous β subunits, β1 to β4, have been cloned in mammals (Contreras et al., 2012). Structurally, they have two transmembrane segments linked together by a loop. The C- and N-terminals of all subunits are intracellular and consequently, the loop is extracellular. They present tissue specific expression with β1 expressed mainly in smooth muscle and β4 expressed mostly in brain (Contreras et al., 2012). The β1 and β2 subunit substantially increase the apparent calcium sensitivity of Slo1 channels. In addition, the β1 and β2 subunit subunits slow down macroscopic current kinetics (Orio et al., 2002). As well, β2 produces fast inactivation and also outward rectification. This latter possibility may result by interaction of the extracellular loop domain of the β2 subunit with the external mouth of the pore of the Slo1 α subunit (Chen et al., 2008). β3 subunits also produce inactivation and outward rectification of the channel, but to a smaller extent (Xia et al., 2000). Finally, the β4 subunit also slows down the macroscopic current kinetics. Interestingly, this subunit has an opposite effect on calcium dependence at low vs. high Ca\(^{2+}\) concentrations; at low [Ca\(^{2+}\)] it seems to decrease sensitivity in contrast to high [Ca\(^{2+}\)] where it increases the apparent Ca\(^{2+}\) sensitivity (Brenner et al., 2000).
More recently, a membrane leucine-rich repeat (LRR)-containing protein (LRRC26) was reported to be an auxiliary subunit of Slo1 (Yan and Aldrich, 2010). Three additional paralogs of LRRC26 were also reported to modulate Slo1 channel activity. This group of proteins is structurally and functionally different from the β subunits and was called the γ family of Slo1 auxiliary subunits (Yan and Aldrich, 2012). LLRC26 and its three paralogs seem to have a single transmembrane domain with the LRR domain on the extracellular side of the membrane. All of them have a hydrophobic N-terminal segment that is predicted to be a signal peptide which is cleaved in the mature protein and it is essential for the extracellular orientation of the LRR domain (Yan and Aldrich, 2012). LLRC26 produces a left-ward shift of 140 mV in the conductance-voltage relationship in the absence of Ca^{2+}. Consequently, the presence of this subunit could activate Slo1 channels in non-excitable cells without an increase in intracellular calcium (Yan and Aldrich, 2010). The other three γ subunits also cause significant shifts to more negative potentials in the half activation voltage of Slo1 channels (Yan and Aldrich, 2012). γ subunits are tissue specific: LRRC26 and LRRC38 are mainly expressed in secretory glands, while LRRC52 is expressed in testis and LRRC55 in brain.

There are no reports of auxiliary subunits for the other members of the Slo family. However, it was shown that LRRC52, which is mainly expressed in testis, regulates the functional expression and gating properties of human Slo3 channels when heterologously expressed in *Xenopus* oocytes (Leonetti *et al.* 2010). Also, one beta subunit, β4, was reported to express in sperm and increase currents and surface expression of the mouse Slo3 channel when heterologously expressed in *Xenopus* oocytes (Yang *et al.* 2009).
Modular structure and the allosteric model

As previously mentioned, functionally, Slo1 channels are gated by both voltage and intracellular calcium (as well as other intracellular factors). Structurally, they have a recognizably modular composition containing three distinguishable domains. Two of these domains are composed of transmembrane segments (the pore and voltage sensor domains), and form the Core of the channel; the third domain, which we call the Tail, is intracellular. The Tail confers sensitivity to calcium ion as well as other mentioned cytological factors. Hence, Slo1 channels are formed by recognizably separate structural domains with distinct functional contributions. In response to a change in voltage or the binding of cytological factors, these domains undergo conformational changes which are propagated through interdomain allosteric interactions (reviewed in Hoshi et al., 2012; Latorre et al., 2010).

The allosteric behavior of Slo1 has been summarized in a widely applied model (Figure 6) called the HA (Horrigan and Aldrich) model (2002). In this model, the gating of the pore is influenced both by the position of the voltage sensors and by binding of calcium to the calcium sensors. The movement of the voltage sensors (to the active state) by membrane depolarization increases the open probability of the pore; on the other hand, pore channel opening will facilitate voltage sensor movement upon depolarization. In a similar way, Ca\(^{2+}\) binding to the Tail sensors increases channel open probability; conversely, the opening of the channel increases the Ca\(^{2+}\) affinity of the sensors.

The HA model has been widely used and has shown to be useful in analyzing Slo1 channel gating in different studies. However, the HA model is incomplete, as it assumes a single Ca\(^{2+}\) sensor even though it has been well established that there are at least two Ca2+ sensors, with one in each RCK domain. In addition, the model does not consider the passive properties of the
gating ring; it has been shown that, in the absence of Ca\(^{2+}\) or Mg\(^{2+}\), the gating ring acts as passive spring modifying the voltage dependent gating (Niu et al., 2004). It has also been reported that Ca\(^{2+}\)-dependent conformational changes are inconsistent with the HA model (Savalli et al., 2012). I will address this problem in Chapter Two and present experiments that suggest ways for improving the HA model. The results I will present will give clues as to what changes may be made for making the model consistent with the experimental and structural data.

Figure 6. Schematic representation of the HA model of gating. The constants L,J and Q are equilibrium constants of the pore gate, the voltage sensor and calcium sensor, respectively. D,C and E are the allosteric interaction factors between the different domains. The model considers 4 voltage sensors that can be active or in repose and only 4 calcium sensor that can be bound to calcium or unbound. Modified from Horrigan and Aldrich, 2002.
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Introduction to Chapter 2

Disputing all the evidence of Ca\textsuperscript{2+} binding in the Tail, a paper by Pisokorowski and Aldrich (2002) reported that they were able to express Slo1 channels lacking the whole Tail which includes the gating ring and associated calcium binding sites. In their paper the authors showed that the properties of this truncated gating ring-less channel were almost identical to the full-length channel including both calcium and magnesium sensitivity. These controversial results implied that the calcium binding sites are located in the core of the channel. Our lab and many others attempted to reproduce these results with no success. A rigorous study of different Slo1 truncations subsequently showed that the construct used by Pisokorowski and Aldrich not only fails to produce currents, but also fails to form tetramers (Schmalhofer et al., 2005).

The function and the structure of Slo1 suggest that during evolution, a typical voltage dependent potassium channel became associated with a long C-terminal domain which binds calcium and ultimately conferred Ca\textsuperscript{2+}-dependence. This means that the two separate functions of the channel can be controlled by two structural domains. On the other hand, Slo1 channels are present from C. elegans to humans therefore; this could imply that these two structural domains have been evolving together for 500 million years making it impossible for one domain to be functional in the absence of the other. Our lab and several others had tried with no success to isolate the voltage sensor Core domain from the Tail to study its baseline properties in the absence of the gating ring.

In this chapter I will explain the strategy followed to obtain the expression of the Slo1 Core in the absence of the gating ring. The results obtained show that the Slo1 channel Core in the absence of the gating ring not only misses calcium and magnesium sensitivity but also, is
affected in other properties including single channel conductance, mean open time, and burst duration. Chapter 2 was done in collaboration with Karl Magleby and Yanyan Geng and it has been submitted as paper to PNAS with the same title, I am the first author and I contributed in the design and execution of experiments, analysis of the obtained data, and writing of the manuscript.

References


Chapter 2

Properties of Slo1 $K^+$ channels with and without gating ring
**ABSTRACT**

High-conductance Ca\(^{2+}\)- and voltage-activated K\(^+\) (Slo1 or BK) channels play key roles in many physiological functions. The structure of the Slo1 channel has two functional domains, a “Core” consisting of four voltage sensors controlling an ion-conducting pore, and a larger “Tail” that forms an intracellular gating ring thought to confer Ca\(^{2+}\) and Mg\(^{2+}\) sensitivity, as well as sensitivity to a host of other intracellular factors. Whereas the modular structure is known, the properties of the individual modules and the different transduction pathways among the modules are poorly understood because it has not been possible to study the modules in isolation. To answer these questions, we develop novel constructs that allow functional Cores of Slo1 channels to be expressed in the absence of the gating ring. This allows determination, for the first time, of the baseline properties of the Core without passive or active allosteric input from the gating ring. In experiments expressing the isolated Core without the gating ring we find that removing the gating ring removes all Ca\(^{2+}\)- and Mg\(^{2+}\)-sensitivity, greatly destabilizes the open state by reducing mean open channel and burst duration ~six fold; right-shifts the G-V relation, and reduces apparent single-channel conductance ~30%. These results show that the gating ring in the absence of Ca\(^{2+}\) and Mg\(^{2+}\) still has profound effects on the gating of Slo1 through allosteric transduction of its passive properties to the Core of the channel.
INTRODUCTION

Slo1 channels are expressed in most human tissues and play key roles in many important physiological processes, including smooth muscle contractile tone (Nelson et al., 1995; Brenner et al., 2000), neurotransmitter release (Robitaille et al., 1993), neuronal excitability (Montgomery et al., 2012), hair cell tuning (Fettiplace and Fuchs, 1999) and action potential termination (Shao et al., 1999). These channels are also named BK or MaxiK because of their very high single channel conductance (250 to 300 pS in 150 mM symmetrical K\(^+\)). Significantly, Slo1 channels are activated by both depolarization and intracellular calcium (Marty, 1981; Pallota et al., 1981; Latorre et al., 1982), linking these two activators in a negative feed-back system to restore the negative membrane potential which, in turn, closes voltage activated Ca\(^{2+}\) channels. The dual regulation by voltage and calcium led Hille to predict that BK channels functioned like the classical Hodgkin-Huxley “delayed rectifier” except that the range of voltage activation for Slo1 currents was set by the intracellular Ca\(^{2+}\) concentration (Hille, 2\(^{nd}\) edition). The cloning and analysis of the Slo1 channel structure seemed to validate this prediction in that Slo1 appeared to be modular in its construction, having a “Core” domain containing a voltage-sensor controlling a K\(^+\)-selective pore, and a long C-terminal “Tail” for sensing Ca\(^{2+}\) and transducing the effect of Ca\(^{2+}\)-binding through a gating ring structure consisting of four pairs of RCK (Regulators of K\(^+\) Conductance) domains (Atkinson et al., 1991; Butler et al., 1993; Yuan et al., 2010).
Figure 1. Slo1 channel constructs used in this study. The Slo1 channel constructs used in this study are based on the mouse mbr5 cDNA (12) and the mouse Shaker family Kv.4 channel (26). The Slo1 “Core” and “Tail” refer to the first 342 and the last 827 amino acid residues. The Kv1.4 “Tail” refers to the last 74 amino acid residues of Kv1.4 which has a defined motif for efficient processing and surface expression (26). The different channel constructs are designated as follows: Slo1-wt is Slo1 full length wild-type; Slo1C-KvT is Slo1 Core with 74 residue Kv1.4 Tail; Slo1C-Kv-minT is Slo1 Core with Kv1.4 11 residue mini Tail; Slo1C-KvT_{NAFQ} is Slo1 Core with 74 residue Kv1.4 Tail with NAFQ substituted for KKFR in the tail; Slo1C-KvT R207E is Slo1C-KvT with R207E in S4 in the Core.

One of the four identical α subunits that assemble to form the Slo1 channel is shown in Fig. 1 (upper diagram). For the mbr5 cDNA (Butler et al., 1993) used in this study, the “Core” consists of 342 residues including seven transmembrane segments (S0-S6) and the S6-RCK1 linker sequence, which is attached to a long “Tail” of 827 residues. The Tail sequence of Slo1-wt is distinct from the cytoplasmic domains of other members of the K^+ channel extended family. Structure-function studies of the Tail have shown the existence of two high affinity Ca^{2+} binding sites (Schreiber et al., 1997; Xia et al., 2002) and one low affinity Mg^{2+} site (Xia et al., 2002, Shi et al., 2002). Modulation of the channel occurs by additional biological factors, including
protons (Hou et al., 2008a), heme (Tang et al., 2003), carbon monoxide (Hou et al., 2008b), phosphorylation (Shubert and Nelson, 2001) and oxidation (Zhang et al., 2006), all of which may function via their interaction with the Tail. Thus, the large Tail accommodates a variety of regulatory domains which sense different intracellular factors, leading to pushing or tugging against the Core to facilitate or inhibit channel gating. These complicated allosteric interactions between Core and Tail almost certainly involve several transduction pathways (Niu et al., 2004; Lee and Cui, 2010; Yuan et al., 2012), all of which alter the baseline properties of the Core. Thus, a logical starting point to begin investigating the allosteric interactions would be to understand the baseline properties of the isolated Core. This approach, however, had been hampered by the inability to express the Core in the absence of the Tail. Previous in-depth analysis of many truncated expression constructs of Slo1 channels showed that their processing stalls in the ER, they are not assembled into tetramers, and/or they fail to be exported to the plasma membrane (Schmalhofer et al., 2005). We now show that isolated Core constructs can be expressed by including a short region required for subunit tetramerization and by appending a small domain which facilitates processing and efficient export to the plasma membrane. Thus, we are now able to reveal the baseline properties of the isolated Core of Slo1.
RESULTS

**Slo1 Core constructs express currents.** The constructs we have synthesized to achieve autonomous expression of the Slo1 Core are shown in Fig. 1. The upper diagram in Fig.1 shows the schematic structure of the wild-type Slo1 BK channel subunit (Slo1-wt) which has seven transmembrane segments containing a voltage sensor and a $K^+$ selective pore (labeled “Core”), and a larger cytoplasmic region containing two tandem regulators of $K^+$ conductance (RCK1 and RCK2) with two $Ca^{2+}$ sensors (labeled “Tail”). Two impediments to the functional expression of truncated Core constructs are their entrapment within the ER and their failure to form tetramers (Schmalhofer et al., 2005). We addressed the first of these problems by creating a construct (Slo1C-KvT) which contained a 74 amino acid C-terminal region (labeled Kv1.4 Tail in Fig. 1) from the Kv1.4 voltage-sensitive $K^+$ channel which contains a conserved motif (blue highlight) reported to greatly facilitate the efficiency of channel expression and export to the plasma membrane (Li et al., 2000). This added region is less than 10% the size of the normal Slo1-wt Tail domain (Fig. 1). The second problem was addressed by including the last 16 residues of the Core which extend into the cytoplasm from the base of S6 (Fig. 1, yellow highlight). This region was reported to be important to the formation of channel tetramers (Quirk and Reinhart, 2001; Schmalhofer et al., 2005). Amazingly, not only did this channel construct express currents, but the magnitude of the expressed currents was as large as that achieved by the expression of wild-type channels. Robust expression was observed for both whole cell currents and inside-out patches pulled from oocytes (Fig. 2; Slo1C-KvT currents). Curiously though, large slowly decaying current tails were present in these current records (Fig. 2 arrows) which were not seen in Slo1-wt channels. These current tails reversed at the potassium equilibrium potential indicating that the currents are the product of a potassium selective channel. Conceivably these
current tails might be an intrinsic property of the isolated Core liberated from constraints of the Tail, or it might be a property resulting from an added motif present in the 74 residue Kv1.4 C-terminal region.

**Figure 2.** Slo1C- constructs without gating rings express large currents in *Xenopus* oocytes. (A) Representative whole-cell current recordings from oocytes injected with cRNAs of the indicated constructs (See Fig. 1 and legend). Slo1C-Stop indicates a stop codon added after the tetramerization domain at position 342 (see Fig. 1). With a two-electrode voltage clamp, oocytes were held at -70 mV and 20 ms step pulses were applied from -70 mV to 250 mV in 10 mV increments followed by a step to 0 mV to see outward tails currents. Not all traces are shown. (B) Currents recorded from inside-out macropatches pulled from oocytes injected with the same constructs as in A using asymmetric K⁺ (see Methods). A 50 ms prepulse to -100 mV was followed by 20 ms step pulses from -100 to 240 mV in 20 mV increments followed by a step to 0 mV for 10 ms. Only Slo1C-KvT expresses prominent tail currents at 0 mV, which are present in both whole-cell and inside-out recordings (arrows). The robust currents recorded for the Slo1C- constructs were not observed with the Slo1C-Stop construct. (C) Conductance/Voltage relationships for Slo1-wt compared to Slo1C-KvT and Slo1C-Kv-minT obtained from macropatch currents in symmetrical 140 mM K⁺.

To eliminate this latter possibility we made a second construct that includes only 11 residues of the Kv1.4 C-terminal sequence; the five residues reported to be essential for surface expression (Fig. 1, blue highlight) (Li *et al.*, 2000), and the last three residues of the Kv1.4 terminal sequence (Fig. 1, green highlight). Large macroscopic currents were also expressed by this
Slo1C-Kv-minT construct, but the expressed currents lacked the prominent tail currents seen when the full length Kv1.4 terminal sequence was present (Fig. 2, Slo1C-Kv-minT currents). We also observed that during longer step pulses Slo1C-KvT currents typically showed more macroscopic inactivation than Slo1C-Kv-minT currents.

Our interpretation of these results was that the full length Kv1.4 terminal sequence contained a possible “inactivation ball” (Zagotta et al., 1990) that was expelled upon repolarization, thus producing the prominent current tails seen in Fig. 2 (arrows). To test this hypothesis, we neutralized three positively charged residues grouped in the 74 residue Kv1.4 C-terminal region (Fig. 1, magenta highlight). Expression of this construct (Fig. 2, Slo1C-KvT NAFQ) eliminated the current tails seen in Slo1C-KvT channels and produced currents virtually indistinguishable from the Slo1C-Kv-minT construct.

The recorded currents are not from endogenous channels. Our ability to change the properties of the expressed currents by modifying the primary structure of the short Kv1.4 sequence added to the C-terminal of the Slo1 Core provided evidence that the currents we observed were from channels encoded by the cRNA injected into Xenopus oocytes. As an additional control we created a cDNA identical to the Slo1C-constructs except that all Kv1.4 sequence was omitted and a Stop codon added immediately following the 16 residue tetramerization domain (yellow highlight, Fig. 1). Injection of cRNA from this construct into Xenopus oocytes failed to produce any detectable currents (Fig. 2, Slo1C-Stop). In a further test to verify that the recorded currents resulted from the injected Tail-less constructs, we altered the voltage sensor of the Slo1C-KvT channels (R207E) and observed that the G-V curve shifted to the left and decreased its slope (Fig. 3). This is consistent with results reported for the same
mutation in Slo1-wt channels (Diaz et al., 1998; Ma et al., 2006) and gave us further confidence that we had indeed expressed the Slo1 Core without the gating ring.

**Voltage-sensitivity is retained and \( V_{1/2} \) is right shifted when the gating ring is removed.** One expectation might be that the baseline properties of the isolated Core would resemble those of the intact Slo1-wt channel in the absence of intracellular \( \text{Ca}^{2+} \) or \( \text{Mg}^{2+} \). As will be shown below, this is only the case for a subset of the properties of Slo1 channels. The retention of voltage-dependent gating in Slo1 channels without gating rings might be expected because the voltage sensor (S1-S4) is contained in the Core of the channel (Fig. 1). Indeed, as shown above, the manipulation of the voltage dependent gating of the Slo1C-K\( \text{vT} \) and Slo1C-K\( \text{v-minT} \) channels through mutation helped to verify that the currents were the products of the expressed constructs (Figs. 2 and 3). Although the voltage sensitivity of Slo1 channels without the gating ring was similar to Slo1-wt channels (similar slopes), the \( V_{1/2} \) was right shifted 27.8 ± 5.8 mV for Slo1C-K\( \text{vT} \) and 49.2 ± 3.0 mV for Slo1C-K\( \text{v-minT} \) relative to Slo1-wt in the absence of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) (Fig. 2C). These significant right shifts (P<0.001, n = 5 or 6) suggests that the passive gating ring (no \( \text{Ca}^{2+} \) or \( \text{Mg}^{2+} \)) allosterically facilitates open probability (Po), because more depolarization was required to achieve the same level of activation after removing the gating ring. Thus, the passive gating ring contributes to the baseline voltage gating properties of the Core by shifting \( V_{1/2} \).
**Figure 3. Verification that the Slo1 Core without the gating ring is expressed and functional.** The R207E mutation in S4 in the voltage sensor of Slo1-KvT left shifted the voltage-dependent activation of Slo1C-KvT as expected (23, 24), indicating that isolated Core of Slo1 channels without gating rings is being expressed. (A) Sequence of S4 in the voltage sensor of Slo1 (upper) indicating the R207E mutation (lower) reported to left-shift voltage G-V curves in Slo1-wt (23,24). (B) Currents from inside-out macropatches from oocytes injected with Slo1C-KvT and Slo1C-KvT-R207E. G-V plots (n=5) are shown on the right. The voltage protocol was -80 mV for 20 ms followed by a 40 ms voltage step of -80 to +295 mV (25 mV increments), followed by steps to 0 mV for 20 ms. Asymmetric K+ (see Methods). (C) Whole cell currents recorded from Slo1C-KvT and the Slo1C-KvT-R207E channels. Oocytes were held at -70 mV and 20 ms step pulses applied from -90 mV to 240 mV with a step back 0 mV. G-V plots are shown on the right with the expected left shift.
**Ca\(^{2+}\) and Mg\(^{2+}\) sensitivity is lost when the gating ring is removed.** A large number of structure-function studies have suggested that Ca\(^{2+}\) and Mg\(^{2+}\) activation of Slo1 channels works through the gating ring (Schreiber *et al.*, 1997; Xia *et al.*, 2002; Shi *et al.*, 2002; Niu *et al.*, 2004; Savalli *et al.*, 2012). We now test this directly by examining Ca\(^{2+}\) and Mg\(^{2+}\) sensitivity in Slo1-wt channels without gating rings using three different experimental approaches. In all cases, no significant sensitivity to Ca\(^{2+}\) or Mg\(^{2+}\) was observed. Single-channel recording showed that exposing inside out patches to 100 μM Ca\(^{2+}\) and 10 mM Mg\(^{2+}\) greatly increased Po in Slo1-wt channels 530 ± 109 and 52.9 ± 11.9 fold, respectively, compared to negligible effects on SloC-Kv-minT channels (Fig. 4A-B, Table S1, Fig. S1). When voltage ramps were applied to inside-out patches expressing either Slo1-wt, Slo1C-KvT, or Slo1C-KvT-minT channels, channel activity increased as the membrane potential was made more positive for all three channel types. However, application of 200 μM Ca\(^{2+}\) or 10 mM Mg\(^{2+}\) greatly increased channel activity for Slo1-wt channels while having no apparent effect on Slo1C-KvT or Slo1C-Kv-minT channels (Fig. 4C, note changed calibration bars for wt channels). A similar result was observed when G-V relationships were obtained in the absence and presence of intracellular Ca\(^{2+}\) and Mg\(^{2+}\); the V\(_{1/2}\) for Slo1-wt channels was left shifted towards more negative potentials 228 ± 5.5 mV with 200 μM Ca\(^{2+}\) and 51.2 ± 1.9 mV with 10 mM Mg\(^{2+}\) (Fig. 5A). In contrast, the V\(_{1/2}\) of Slo1C-Kv-minT was not shifted by either Ca\(^{2+}\) or Mg\(^{2+}\) (Fig. 5B), and normalized I-V curves for Slo1C-KvT channels were not shifted when exposed to 200 μM Ca\(^{2+}\) (Fig. S2). Hence, the gating ring is required for Ca\(^{2+}\) and Mg\(^{2+}\) sensitivity of Slo1 channels.
Figure 4. Slo1 channels without gating rings are insensitive to Ca\(^{2+}\) or Mg\(^{2+}\). (A) Representative excerpts of single channel current activity in Slo1-wt and Slo1C-Kv-minT. Inside out patches were held at +80 mV and exposed to Ca\(^{2+}\) or Mg\(^{2+}\) in the sequence indicated with a 0 Ca\(^{2+}\) and 0 Mg\(^{2+}\) solution bracketing each application. Open (O) and closed (C) current levels are indicated. 100 µM Ca\(^{2+}\) or 10 mM Mg\(^{2+}\) activate Slo1-wt channels while having little effect on Slo1C-Kv-minT channels. Observations were readily reversible. 10 mM Mg\(^{2+}\) decreased single-channel conductance (48) for both Slo1-wt and Slo1C-Kv-minT. (B) Ca\(^{2+}\) and Mg\(^{2+}\) significantly increase open probability (Po) in Slo1-wt channels (P<0.0001, n=5 for Ca\(^{2+}\) and P < 0.05, n=6 for Mg\(^{2+}\), paired t-test before normalization), while having
insignificant effects on Slo1C-Kv-minT channels (P > 0.1, n=4 in each case). The plotted values of the mean and SEM bars for Slo1C-Kv-minT have been multiplied by five before plotting so that the data and error bars can be differentiated from the axis. (C) Current traces from inside-out patches ramped from -90mV to 90 mV in the absence and presence of Ca^{2+} or Mg^{2+} as indicated. Slo1C-KvT and Slo1C-Kv-MinT currents are not detectably activated by 200 µM Ca^{2+} or by 10 mM Mg^{2+}. Note differences in scale bars in Slo1-wt. Increasing single channel activity in the ramps at positive voltages indicates voltage-sensitivity in all channel constructs.

![Graph showing mean open time](image)

**Figure S1.** Removing the gating ring destabilizes the open state. The mean open time from Table S3 is plotted for Slo1-wt channels and Slo1C-Kv-minT channels lacking gating rings. For Slo1-wt channels Ca^{2+} and Mg^{2+} significantly increased mean open time (P < 0.05, paired t-test, n = 4), while having insignificant effects on mean open time for Slo1C-Kv-minT channels (P > 0.09, n = 3).
Table S1. Ca\(^{2+}\) and Mg\(^{2+}\) no longer activate Slo1 channels after removing the gating ring

<table>
<thead>
<tr>
<th></th>
<th>Slo1-wt</th>
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<th>Slo1C-Kv-minT</th>
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<tr>
<td></td>
<td>0 Ca(^{2+})</td>
<td>100 μM Ca(^{2+})</td>
<td>10 mM Mg(^{2+})</td>
<td>0 Ca(^{2+})</td>
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<tr>
<td>nPo</td>
<td>0.0022 ± 0.0008</td>
<td>0.91 ± 0.01</td>
<td>0.13 ± 0.06</td>
<td>0.0037± 0.0008</td>
</tr>
<tr>
<td>Po normalized to 0 Ca</td>
<td>1.0</td>
<td>530 ± 110</td>
<td>53 ± 12</td>
<td>1.0</td>
</tr>
<tr>
<td>No.of opening in bursts</td>
<td>1.5 ± 0.2</td>
<td>28 ± 21</td>
<td>4.8 ± 1.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Open interval duration</td>
<td>0.76 ± 0.18</td>
<td>7.7 ± 2.1</td>
<td>2.1 ± 0.8</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Intraburst closed interval duration</td>
<td>0.23 ± 0.05</td>
<td>0.20 ± 0.07</td>
<td>0.16 ± 0.03</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>Burst duration</td>
<td>1.2 ± 0.2</td>
<td>*</td>
<td>11 ± 5</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>Mean duration of gaps between bursts</td>
<td>540 ± 100</td>
<td>*</td>
<td>77 ± 25</td>
<td>50 ± 13</td>
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Slo1-wt data are from four different patches each containing a single channel with n = 1 in nPo. Slo1C-Kv-minT data are from three different patches, each containing an unknown number of channels. For Slo1C-Kv-minT channels the mean duration of gaps between bursts for single channels would be greater by a factor of n for the unknown number of channels. The Po was sufficiently low for Slo1C-Kv-minT channels that openings seldom overlapped so that the second through fifth parameters could be determined with negligible error. *With 100 μM Ca\(^{2+}\) the Po is so high that the channel is essentially open all the time, so it is difficult to determine gaps between bursts or burst duration. Estimates for burst duration ranged from 21-1306 ms. Effective low-pass filtering of 4.47 KHz.
Figure 5. Ca$^{2+}$ and Mg$^{2+}$ no longer left shift the G-V plots after the gating ring is removed. (A and B) Currents recorded from inside-out macropatches from oocytes injected with Slo1-wt cRNA (A) or Slo1C-Kv-minT cRNA (B) were held at 0 mV, stepped to -100 mV for 50 ms and then stepped from -100 mV to 240 mV in 20 mV increments followed by a step to -80 mV to measure tail currents (left). Ca$^{2+}$ and Mg$^{2+}$ left shifted the G-V curves towards more negative voltages for Slo1-wt channels with no effect on Slo1C-Kv-minT channels.
Figure S2. Slo1-KvT currents show lack of calcium dependence in Inside-out macropatches. Macroscopic currents obtained from inside-out patches in asymmetrical K⁺ conditions were exposed to 0 Ca²⁺ or 200 µM Ca²⁺. Patches were held at 0 mV, a 50 ms prepulse to -100mV was applied and then stepped from -100 mV to 240 mV in 20 mV intervals, followed by a step back to 0 mV. (Right) Normalized currents were graphed in the absence (black) and presence (red) of Ca²⁺. The currents shown are reduced in the presence of calcium but the difference is not statistically significant.

Mean channel open time and burst duration are reduced when the gating ring is removed. Contrary to an expectation that the baseline properties of the isolated Core might resemble those of the intact Slo1-wt channel in the absence of intracellular Ca²⁺ or Mg²⁺, we observed that single-channel kinetics differed markedly from those of the Slo1-wt channels after removing the gating ring. Mean open channel duration and mean burst duration were decreased 5.6 and 6.4
fold for Slo1C-Kv-minT channels compared to Slo1-wt channels (Fig. 6, Table. S2). These marked changes in single-channel kinetics show that the gating ring even in the absence of Ca$^{2+}$ and Mg$^{2+}$ still has profound effects on channel gating by greatly increasing the stability of the open state. Thus, the gating ring contributes passively to the baseline properties of the channel.

**Single-channel conductance is reduced when the gating ring is removed.** The high conductance of Slo1-wt channels compared to other K$^+$ selective channels is one of the defining properties of Slo1-wt channels (Hille, 2nd edition). We found that removing the gating ring decreased the single-channel current amplitudes (Figs. 4A, Fig. 6), suggesting a decreased conductance. When measurements of currents were restricted to openings of sufficient duration that their amplitudes were not attenuated by the low-pass filtering, removing the gating ring decreased single-channel conductance ~30%, from 310 ± 4 pA (n = 3) for Slo1-wt channels to 213 ± 6 pA (n = 3) for Slo1C-Kv-minT channels (P < 0.001). The decreased single-channel conductance might be due to a structural feature missing when the gating ring is removed, unstable or partially open gates, or a conformational change produced by the absence of the gating ring that alters the conductance pathway. The observation of reduced conductance was unexpected because it is generally assumed that single-channel conductance is determined by the pore-gate domain of the Core and not by the gating ring (Brelidze *et al*; Nimigean *et al*., 2003)
Figure 6. Removing the gating ring destabilizes the open state of Slo1 channels. (A and B) Single-channel recordings at +80 mV from Slo1-wt channels (A) and from Slo1C-Kv-minT channels without the gating ring (B). Open interval duration and burst duration are greatly decreased by removing the gating ring. (C) Bar graphs of open interval duration and burst duration for Slo1-wt and Slo1C-Kv-minT.

Table S2. Removing the gating ring decreases mean open interval duration and mean burst duration for Slo1C-Kv-minT channels compared to Slo1-wt channels, destabilizing the open state

<table>
<thead>
<tr>
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<th>Slo1-wt</th>
<th>Slo1C-Kv-minT</th>
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<tbody>
<tr>
<td>Open interval duration</td>
<td>0.76 ± 0.18</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>No. of openings in bursts</td>
<td>1.5 ± 0.2</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Intraburst closed interval duration</td>
<td>0.23 ± 0.05</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>Burst duration</td>
<td>1.2 ± 0.2</td>
<td>0.19 ± 0.01</td>
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</table>

Data were obtained in 0 Ca\(^{2+}\) and 0 Mg\(^{2+}\). Slo1-wt data are from four different patches each containing a single channel. Slo1C-Kv-minT data are from three different patches, each containing an unknown number of channels. The Po was sufficiently low for Slo1C-Kv-minT channels that openings seldom overlapped so that the parameters could be determined. Open interval duration and burst duration were significantly decreased (P < 0.03) by removing the gating ring. Effective low pass filtering of 4.47 kHz.
Block by Iberiotoxin and TEA<sub>0</sub> is retained when the gating ring is removed. Because the single-channel conductance was unexpectedly reduced upon removal of the gating ring, the possibility arises that other properties of the pore-gate domain might also be altered. To explore this possibility we first tested the effect of the highly specific Slo1 channel blocker iberiotoxin (Galvez et al., 1990) on Slo1C-KvT channels (Fig. S3A). The application of 60 nM iberiotoxin to the external membrane surface of outside-out macropatches (+180 mV) reduced currents 78 ± 3% (n=5) for Slo1C-KvT channels, which was not significantly different from the 82 ± 3% (n=7, P=0.46) reduction for Slo1-wt channels. We next tested the effect of external application of the generic potassium channel blocker TEA, as Slo1 channels are known to be highly sensitive to block by external TEA (Blatz and Magleby, 1984; Vergara et al., 1984). We found that 2 mM TEA (+70 mV) reduced Slo1C-KvT whole-cell currents 81 ± 2%, which was not significantly different from the 85 ± 4% reduction seen on Slo1-wt currents (n=6, P=0.43); Fig. S3B). Both blocking agents were also tested on Slo1C-Kv-minT with similar effects. Hence, as might be expected, removing the intracellular gating ring had no significant effects on external block.

The gating ring is not required for β1 subunits to slow the activation of Slo1 channels. Several β auxiliary subunits are reported to modulate the properties of Slo1-wt channels (Contreras et al., 2012). The β subunits are integral membrane proteins with two transmembrane segments that interact with Slo1-wt α subunits and alter various channel properties such as kinetics. Whereas some of the β subunits may interact with both the Core and Tail of Slo1-wt channels, the β1 subunit may interact only with the Core (Morrow et al., 2006; Liu et al., 2010), slowing the rate of activation of the current (Contreras et al., 2012). Consistent with the previous results for the β1 subunit, we observed that co-expression of β1 with Slo1-wt produced currents
that activated more slowly than those of Slo1-wt α subunits alone (Fig. S3C). In a similar manner, co-expression of the β1 subunit with the Slo1C-KvT and Slo1C-Kv-minT constructs also produced currents that activated significantly more slowly than in the absence of the β1 subunits (Fig. S3C). In addition, the slow current inactivation observed after activation was absent for all three constructs (Slo1-wt, Slo1C-KvT, and Slo1C-Kv-minT) when co-expressed with β1 subunits (Fig. S3C). Thus, the gating ring is not required for β1 subunits to functionally interact with Slo1 channels.
Figure S3. Slo1C-KvT has properties characteristic of the full length SLO1-wt channel. A) (Left) Extracellular application of 60nM iberiotoxin blocks Slo1-wt and Slo1C-KvT currents similarly in outside-out macropatches of oocytes injected with cRNA. Currents were evoked at 180 mV in the absence (black) and presence (red) of 60 nM extracellular iberiotoxin. (Right) Normalized current vs voltage plots are shown before and after exposure to iberiotoxin. The blocking effect is virtually the same for wild-type Slo1 and Slo1C-KvT (n=6; p=0.42). B) (Left) 2mM TEA similarly blocks Slo1-wt and Slo1C-KvT currents. WT and Slo1C-KvT whole-cell currents evoked at +70 mV before (black) and after (red) 2 mM extracellular TEA. (Right) Normalized current vs voltage plots are shown before and after exposure to TEA, The blocking effect is virtually the same for wild-type Slo1 and Slo1C-KvT (n=6; p=0.32). C) The auxiliary subunit β1 similarly modulates Slo1-wt and Slo1C-KvT currents. Co-expression of β1 with Slo1-wt or with Slo1C-KvT produces slower activating currents and less inactivation. Red traces are with β1 subunits; black are without β1 subunits. Depolarizing pulses are to 60mV in two microelectrode whole cell clamp, and to 240mV in macropatches. The coexpression of β1 produces a significant difference in the activation τ for both channels: The macropatch current rising phase was fitted with a single exponential; Slo1-wt τ=1.85±0.28 and 7.26±0.56 in the presence of β1 (n=4 and p=0.0001); Slo1C-KvT τ=1.12±0.07 and 2.94±0.37 in the presence of β1 (n=6 and p= 0.0006). The Slo1C-Kv-MinT construct is also modulated by β1, there is a significant 3 fold change of the activation tau (n=5 p=0.001; τ= 0.91±0.09 and 2.61±0.32).
DISCUSSION

The Core can be expressed without the Tail. The function and the structure of Slo1 suggests that during evolution, a rather typical voltage dependent potassium channel became associated with a long C-terminal domain which bound calcium and ultimately conferred Ca\(^{2+}\)-dependence of channel gating. The fact that Slo1 channels are conserved in invertebrates as well as vertebrates implies that Core and Tail have been associated for over 500 million years, raising the possibility that Core and Tail have become so interdependent that the Core can no longer function without the Tail. Our laboratories and others had tried to express the Core without the gating ring but without success. An analysis of several truncated Slo1 constructs which failed to express currents indicated difficulties in protein processing, tetramerization, and export from the ER to the plasma membrane (Schmalhofer et al., 2005, Quirk and Reinhart, 2001). We achieved robust expression of Slo1 Cores by preserving the tetramerization domain of Slo1 (Fig. 1) and attaching either the last 74 residues of the KV1.4 C-terminus, or by attaching a much shorter 11 residue C-terminus which included the five residue motif from KV1.4 for processing and surface expression (Li et al., 2000), and also the last three residues of the C-terminus of KV1.4 (Fig. 1).

Functions of Core and Gating Ring. Our experiments directly show the functions of the Core and gating ring of Slo1 channels: 1) voltage sensitivity, block by iberiotoxin and TEA, and slowed activation with \(\beta1\) subunits are all conferred by the Core; 2) Ca\(^{2+}\) and Mg\(^{2+}\) sensitivity is entirely determined by the gating ring. These observations confirm previous prognostications (Hoshi et al., 2013).

Unexpectedly, however, we found that the passive gating ring (in the absence of Ca\(^{2+}\) or Mg\(^{2+}\)): 1) facilitated activation by shifting the \(V_{1/2}\) to more negative voltages (Fig. 1C); 2) stabilized the open state by greatly increasing mean open interval duration and burst duration (Fig. 6C, Table
S2); and 3) was required to maintain the large single-channel conductance (Fig. 6). Thus, the passive gating ring has profound effects on gating and conductance. The facilitation of voltage dependent activation and stabilization of the open state is consistent with the passive gating ring allosterically interacting with the Core to decrease the effective energy barriers for channel opening and increase the effective energy barriers for channel closing, respectively. (Possible mechanisms for the reduced conductance were considered in the Results.)

**Comparison of Slo1 to MthK.** The functional expression of the Slo1 Core alone has now revealed that the baseline properties of the pore-gate domain and voltage sensors of Slo1 differ substantially from those of the full length Slo1-wt channel with a passive gating ring (i.e. in the absence of Ca\(^{2+}\) and Mg\(^{2+}\)). This result contrasts with a previous experiment removing the gating ring from the MthK channel. The prokaryotic Ca\(^{2+}\)-dependent K\(^{+}\) channel MthK (from *Methanobacterium thermoautotrophicum*) has an intracellular gating ring formed by four pairs of RCK1 and RCK2 domains like Slo1, but with differences in gating ring structure (Jiang *et al.*, 2001; Yuan *et al.*, 2010; Yuan *et al.*, 2012). In experiments removing the gating ring from MthK channel, it was shown that the intrinsic properties of the MthK Core closely resembled those of the intact MthK channel in the absence of Ca\(^{2+}\); the mean open times in MthK were little changed by removing the gating ring, and both the intact channel and the core alone have Ca\(^{2+}\)-independent fast kinetic intraburst closed intervals (flickers) that were shown to be an intrinsic property of the Core (Li *et al.*, 2007). Thus, the gating ring-less MthK is similar to the full length MthK channel in the absence of Ca\(^{2+}\) (pH 7.5). This is in marked contrast to our results that show that the properties of the Slo1 Core differ markedly from those of full length Slo1 in the absence of Ca\(^{2+}\) and Mg\(^{2+}\). These contrasting results might be related to the fact that, unlike Slo1 channels, the MthK Core only includes a pore-gate domain without a voltage sensor domain. The
difference in results between MthK and Slo1 may indicate that possible interactions between the gating ring and voltage sensor domain in Slo1 channels (Yuan et al., 2010; Yuan et al., 2012) may stabilize the open state of the Slo1.

**Implications for Allosteric Gating.** Different allosteric models have been used to explain the gating of Slo1 channels and to understand the relationship between voltage, calcium binding, and channel opening (Magleby, 2003; Horrigan, 2012). Remarkably, neither Ca$^{2+}$ nor voltage is absolutely needed to activate the channel. The channel can be activated without calcium at very high membrane potentials (Horrigan et al., 1999) or in the presence of high Ca$^{2+}$ over the range of physiological voltages (Marty, 1981; Pallota et al., 1981; Latorre et al., 1982). The binding of Ca$^{2+}$ or Mg$^{2+}$ produces conformational changes of the gating ring that are transduced to the Core of the channel to open the gates. The conformational changes and the transduction pathways between Tail and Core are still not well understood. The experimental data and resulting allosteric models for the gating suggest that the Core and gating ring strongly interact, such that Ca$^{2+}$ binding can move the voltage sensors, and reciprocally, that voltage sensor movement can change the calcium binding affinity (Hoshi et al., 2013, Horrigan, 2012; Sweet and Cox, 2009). Many open questions regarding these complex interactions between Core and Tail may now be pursued with our novel constructs, as the complex allosteric modifications and interactions might be easier to understand if the Core is studied without the pushes and shoves normally provided by the gating ring. Our results do show that the “passive” gating ring (no Ca$^{2+}$ or Mg$^{2+}$) is, in fact, not passive. Even in the absence of Ca$^{2+}$ and Mg$^{2+}$ the attached gating ring left-shifts the $V_{1/2}$ for opening to more negative potentials and increases mean open interval duration and burst duration, suggesting that the passive gating ring facilitates channel opening and stabilizes the open state. Consistent with this conclusion, effectively removing an allosteric connection of the
gating ring to the gates by lengthening the RCK1-S6 linker decreased Po (Niu et al., 2004), suggesting that passive gating ring may apply spring tension to facilitate gate opening. The fact that we can now recognize the baseline properties of the Slo1Core in the total absence of input from the gating ring now provides the opportunity for future experiments to selectively dissect the different allosteric pathways between Core and Tail.

MATERIALS AND METHODS

Constructs

The Slo1C-KvT (mSlo1 Core plus Kv1.4 Tail) construct was made by first PCR amplifying the C-terminal 74 amino acids and stop codon of Kv1.4 from mouse genomic DNA using primers that added a BsiW1 site to the 5’ end and a Hind3 site following the stop codon, then inserting the PCR fragment in frame into a version of the MBr5 variant of mSlo1 (Butler et. al., 1993) which has a BsiW1 site following mSlo1 amino acid #342 (previously added using Stratagene’s Quick-Change Mutagenesis Kit) and a Hind3 site in the 3’ polylinker region. The Slo1C-Kv-minT (mSlo1 Core plus Kv1.4 Minimal Tail) construct was made by first annealing complementary 5’-phosphorylated oligos corresponding to the amino acid sequence shown in Fig.1 with the addition of a BsiW1 site at the 5’ end and a stop codon and Hind3 site at the 3’ end and then subcloning the annealed fragment in frame into the same mSlo1 Bsiw1 and Hind3 sites described in the above construct. The Slo1C-KvT construct with the substitutions KKFR->NAFQ was made by PCR amplification using a sense primer located 5’ of the same Bsiw1 site used previously and an antisense primer containing the required mutations and extending far enough 3’ into the Kv1.4 tail region to include the unique restriction site Pas1, then inserting this piece into the BsiW1 and Pas1 sites of Slo1C-Kv-minT. The Slo1C-Kv-minT with S4 R->E
substitution was made by using overlap extension PCR to create a fragment of mSlo1 containing
the S4 R→E substitution in between the unique restriction sites Age1 and Pst1 then subcloning it
into the Slo1C-Kv-minT construct using those sites.

Xenopus oocytes

Defolliculated oocytes were injected with 50 nl of cRNA (1 to 3 µg/µl) using a Drummon
Scientific nanoinjector (Broomal, PA). Injected oocytes were incubated at 18°C in ND96
complete medium (ND96: 96mM sodium chloride, 2mM potassium chloride, 1.8 calcium
chloride, 1mM magnesium chloride and 5mM HEPES, pH 7.5; ND96 complete: ND96 +
2.5mM sodium piruvate and Pen-Strep 1 ml/100 ml). The oocytes were electrophysiologically
analyzed 2 to 5 days after injection.

Electrophysiology

Two-microelectrode voltage clamp recordings were obtained in ND96 with 1 mm DIDS to block
the endogenous chloride conductances. The currents were obtained with an Oocyte Clamp OC-
725C (Warner Instrument Corp.) amplifier. The electrodes were made with WPI borosilicate
glass capillaries pulled with a Sutter instrument Co. puller (model P-87) and filled with 3 M KCl.

For patch-clamp experiments, the vitelline membrane was mechanically removed before
recording. For single-channel recordings, the 0 Ca$^{2+}$ and 0 Mg$^{2+}$ (control) solution contained: 150
mM potassium chloride, 5 mM TES, 1 mM EGTA, and 1 mM HEDTA. Estimated free Ca$^{2+}$ was
~0.02 µM, 5-fold less than required to affect the Po of BK channels (Nimigean and Magleby,
2000 JGP 115, 719-734.). 11.266 mM magnesium chloride was added to this solution to give a
calculated free Mg$^{2+}$ of 10 mM, and 2.0637 mM CaCl$_2$ was added to give a calculated free Ca$^{2+}$
of 100 μM. The pH of these solutions was adjusted to 7.0 with 1 M KOH. Inside-out macropatches were recorded while perfusing the intracellular side of the membrane with a solution containing 140 mm potassium methanesulfonate, 10 mm HEPES, 1 EGTA and the calcium chloride and magnesium chloride needed to obtain the desired free ion concentrations. In the experiments with symmetrical potassium conditions, the pipette solution was 140 mM potassium methanesulfonate, 2 mM potassium chloride, 2 mM magnesium chloride and 10 mm HEPES, and in the experiments with asymmetrical K+ the solution contained 140 mM sodium methanesulfonate, 10 mM potassium chloride and 10 mM HEPES. The pH of these solutions was adjusted to 7.2 using KOH. Pipette tip resistance ranged from 0.8 to 1.5 megaohms. Macropatch pipettes were obtained by pulling borosilicate glass from Warner Instruments. Drugs and pharmacological agents used in this study were purchased from Sigma with the exception of iberiotoxin that was bought from Tocris Bioscience.

The traces were acquired with an Axopatch 200B (Molecular Devices), digitized at 10 kHz (macroscopic currents) or at 100 kHz (single-channel), and filtered at 2 kHz or 20 kHz respectively. The data war be analyzed using pClamp 9 (Molecular Devices), SigmaPlot (Jandel Scientific) and Origin (Microcal Software, Northampton, MA). The conductance-voltage (G-V) relationships of the wild type and mutant channels were obtained from the tail currentsin most cases and by converting the current values at steady state to conductances using the equation $G = I/(V_m - E_{rev})$, where $I$ is the K$^+$ current at steady state, $V_m$ is the test pulse potential, and $E_{rev}$ is the potassium equilibrium potential. The $G$-$V$ curves were fitted with the Boltzmann equation, $G = G_{max} / (1 + e^{-(V - V_{1/2})/k})$, where $V_{1/2}$ is the voltage for the channel at half-activation, $k = RT/zF$, $z$ is the number of equivalent gating charges, and $F$, $R$, and $T$ have their usual meanings.
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Introduction to Chapter 3

After the discovery of high conductance $K_{\text{Na}}$ channels in guinea pig cardiomyocytes (Kameyama et al., 1984) many reports showed that these channels were also expressed in neurons (Dryer et al., 1989; Egan et al., 1992; Haimann et al., 1992; Dale 1993; Koh et al., 1994). Using inside-out patches many of these studies reported that the sodium concentrations needed to activate high conductance $K_{\text{Na}}$ channels largely exceeded the reported intracellular concentration of sodium. Consequently, it was proposed that $K_{\text{Na}}$ channels were only active during special conditions such as hypoxia or schema where intracellular sodium concentrations will increase.

Following the initial reports of $K_{\text{Na}}$ channels in inside-out patches, transient $K_{\text{Na}}$ currents were reported (Bader et al., 1985; Dryer et al., 1989). These transient $K_{\text{Na}}$ currents were observed immediately after the transient sodium currents evoked by membrane depolarization. The application of the highly selective sodium channel blocker TTX removed not only the inward sodium current but also the transient outward current following it. These results were taken with skepticism. It was argued that the outward current could be attributed to voltage clamp artifact and also that the fast diffusion of sodium would make it impossible to increase the local intracellular sodium concentration to the needed levels to activate $K_{\text{Na}}$ channels (Dryer et al., 1991).

Delayed rectifying $K_{\text{Na}}$ currents were reported in spinal neurons of Xenopus (Dale, 1991) and in Drosophila neuron cultures (Saito and Wu, 1991). In contrast to the transient $K_{\text{Na}}$ currents, the sustained $K_{\text{Na}}$ currents can not be attributed to instrumental artifact but these reports did not generate much interest. In addition, consistent to the reports of sustained $K_{\text{Na}}$ currents, several reports indicate that long lasting slow afterhyperpolarization in different neurons is caused by a
Na$^+$-dependent K$^+$ current (Schwindt et al., 1989; Kubota and Saito, 1991; Safronov and Vogel, 1996; Franceschetti et al., 2003; Zhang et al., 2010). Even though, the physiological role of KNa channels have been controversial and many thought they were only active under special conditions.

In this chapter I will present results showing that $K_{Na}$ currents are a major component of the outward currents in different neuron types under physiological conditions. This chapter was published in Nature Neuroscience using the same title (Budelli G, Hage TA, Wei A, Rojas P, Jong YJ, O’Malley K, Salkoff L. Nat Neuro 2009, 12:745-750). I did most experiments in medium spiny neurons and all the control experiments with Slack-HEK cell line. I also contributed in the design and execution of experiments, analysis of the obtained data, and writing of the manuscript.
Chapter 3

Na$^+$-activated K$^+$ channels express a large delayed outward current in neurons during normal physiology
ABSTRACT

One of the largest components of the delayed outward current that is active under physiological conditions in many mammalian neurons, such as medium spiny neurons of the striatum and tufted-mitral cells of the olfactory bulb, has gone unnoticed and is the result of a Na\(^+\)-activated K\(^+\) current. Previous studies of K\(^+\) currents in mammalian neurons may have overlooked this large outward component because the sodium channel blocker tetrodotoxin (TTX) is typically used in such studies. We found that TTX also eliminated this delayed outward component in rat neurons as a secondary consequence. Unexpectedly, we found that the activity of a persistent inward sodium current (persistent \(I_{\text{Na}}\)) is highly effective at activating this large Na\(^+\)-dependent (TTX sensitive) delayed outward current. Using siRNA techniques, we identified SLO2.2 channels as being carriers of this delayed outward current. These findings have far reaching implications for many aspects of cellular and systems neuroscience, as well as clinical neurology and pharmacology.
INTRODUCTION

The original discovery of high-conductance sodium-activated potassium channels (K$_{\text{Na}}$ channels) in the heart (Kameyama et al., 1984) and brain (Dryer et al., 1989; Egan et al., 1992) presented a conundrum, as studies of single channel properties in inside-out patches showed that they respond to very high levels of Na$^+$, far exceeding those that are present in the normal intracellular bulk cytosol (Kameyama et al., 1984; Dryer et al., 1989; Egan et al., 1992; Dryer, 1991; Koh et al., 1994). Thus, it was suggested that this channel class represents a reserve conductance that can be activated during times of stress resulting from ischemia or hypoxia, when sodium ions accumulate in cells (Kameyama et al., 1984; Dryer, 1994). However, other studies have indicated that K$_{\text{Na}}$ channels may be active under normal physiological conditions (Dryer, 1994; Bhattacharjee & Kaczmarek, 2005; Wallen et al., 2007; Yang et al., 2007) and the effectiveness of sodium entry through voltage-dependent sodium channels in activating K$_{\text{Na}}$ channels remains in dispute (Dryer 1994; Bhattacharjee & Kaczmarek 2005). To explore these questions, we undertook a study of the action of TTX on outward currents in several types of rat neurons. Unexpectedly, we found that many neuronal cell types had a large TTX-sensitive delayed outward current that decayed only slightly over a time course of a second.
RESULTS

We assumed that the observed TTX sensitivity of the delayed outward current was a secondary consequence of the block of inward Na\(^+\) current by TTX, and found this to be the case in a variety of experiments (Fig. 1). To demonstrate the effectiveness of Na\(^+\) entry through TTX-sensitive sodium channels in activating the delayed outward current, we adjusted the intracellular concentration of Na\(^+\) to very low levels by removing Na\(^+\) from the intracellular pipette recording solution; thus, any intracellular Na\(^+\) would be a minor residual. Under these conditions, we applied voltage step pulses to voltage clamped neurons and compared the delayed outward current before and after the addition of TTX (examples are shown of a tufted/mitral cell, a medium spiny neuron of the striatum (MSN) and a cortical pyramidal cell; Fig. 1a–c). We plotted the delayed outward current component as the average current during the interval of 150–250 ms after the initiation of step pulses. The addition of TTX reduced the delayed outward current by 43.3\% \pm 2.5\% (n = 14, \(P \leq 0.01\)) in MSNs and 57.2\% \pm 3.6\% (n = 21, \(P \leq 0.01\)) in tufted/mitral cells. In cortical pyramidal cells, the reduction was smaller, but still represented a substantial component in about half of the cells (Fig. 1c). To further validate that the TTX-sensitive outward current was evoked by the influx of sodium, we repeated the experiment, removing extracellular Na\(^+\) instead of adding TTX, and the results were similar to those obtained by adding TTX, with the delayed outward current being reduced by 49\% (Figs. 1d and 2a). The effect of eliminating extracellular Na\(^+\) was readily reversible, although perhaps not totally (Fig. 2a). As an additional method of showing the selective activation of the delayed outward current by Na\(^+\) entry, we substituted equimolar lithium ion for external Na\(^+\). Although Li\(^+\) is carried by voltage-gated sodium channels, prior studies (Dryer, 1994) have shown that K\(_{\text{Na}}\) channels are
insensitive to Li\textsuperscript{+}. We found that substituting Li\textsuperscript{+} for Na\textsuperscript{+} had a similar effect to adding TTX, reducing the delayed outward current in MSNs by 41.6% versus a 43% reduction by TTX (Fig. 2b). This result not only shows the specificity of Na\textsuperscript{+} influx for activation of the delayed outward current, but also eliminates the possibility that the change in the current is an artifact resulting from changing space-clamp conditions by TTX addition or Na\textsuperscript{+o} removal.
Figure 1. The TTX-sensitive (Na⁺-dependent) delayed outward current and its elimination by Slack-siRNA. (a–c) The TTX-sensitive delayed outward current in tufted/mitral cell (a), MSN (b) and cortical pyramidal cell (c), respectively. The top traces show the family of control currents evoked from a holding potential of -70 mV. The middle traces show the outward current remaining after addition of TTX (1 μM). The bottom traces show the TTX-sensitive current (TTX-sensitive K⁺ current) that is the difference between the control currents before TTX and the remaining currents after the addition of TTX. The current values shown in the I-V are average values measured in the interval of 150–250 ms after the initiation of the voltage step. The intracellular pipette solution in these whole-cell patch-clamp experiments contained no Na⁺ and no Ca²⁺ was present in the extracellular recording solutions. Some current traces in our experiments showed unusual kinetics at the initiation of the voltage-clamp step pulse. It is likely that these were anomalies resulting from the fact that TTX was not present, and very rapid inward Na⁺ currents therefore are opposing rapid transient outward currents at the initiation of the pulse in a cell in which the space clamp is not perfect (which is why TTX is so often used in studies of outward currents). (d) The removal and subsequent replacement of extracellular Na⁺ revealed the Na⁺-dependent delayed K⁺ current in an MSN. Control indicates the family of control currents evoked from a holding potential of -70 mV, Na⁺ indicates the outward current remaining after removal of external Na⁺, Na⁺-dependent K⁺ current indicates the difference between the control currents before Na⁺ removal and the remaining currents after Na⁺ removal, and recovery indicates the current after reintroduction of extracellular Na⁺. The intracellular pipette solution in these whole-cell patch-clamp experiments contained no Na⁺. (e) An example of an MSN treated with Slack-siRNA that had a smaller TTX-sensitive outward component. This supports the hypothesis that the TTX-sensitive delayed outward current is carried by Slack channels. See Figure 2 for statistical information.
Inhibition of Na+-dependent delayed outward current by the removal of extracellular Na+ and the substitution of external Li+ for Na+. (a) The removal of extracellular Na+ reduced the delayed outward current by 49.9% ± 2.3% (n = 8, P ≤ 0.01) in MSNs. Recovery after reintroduction of external Na+ was 85.7% ± 3.4% (n = 7, P = 0.047) compared with starting control currents. This indicates that recovery after reintroduction of [Na+]o may not have been quite complete. (b) The substitution of external Li+ for Na+ reduced the delayed outward current by 41.6% ± 3.1% (n = 11, P ≤ 0.001). Recovery after reintroduction of external Na+ was 103% ± 8.0% (n = 6, P = 0.78) compared with starting control currents. Error bars represent standard errors.

Tufted/mitral cells of the rodent olfactory bulb had been used in classic studies (Egan et al., 1992a; Egan et al., 1992b) to determine the properties of Na+-activated potassium channels in
mammalian neurons because they are abundant in these cell types. We previously found that the *Kcnt1* (also known as *Slo2.2* and *Slack*) gene encoded a Na\(^+\)-activated potassium channel (Yuan *et al.*, 2003) and it has been demonstrated by immunocytoLOGY and *in situ* techniques that Slack channels are expressed in those cell types (Bhattacharjee *et al.*, 2002). Thus, the *Slack* gene is a prime candidate to carry the Na\(^+\)-dependent delayed outward current that we observed. An immunocytochemical survey of many areas of the rodent brain found that the *Slack* gene was widely expressed in both cortical and subcortical regions (Bhattacharjee *et al.*, 2002). In contrast with olfactory bulb cells, Slack channel expression in MSNs has not been well studied. As we had previously detected that a substantial fraction of the delayed outward current was Na\(^+\)-dependent, we sought to verify that Slack channels are expressed in MSNs and that Slack channels carry the large Na\(^+\)-dependent delayed outward current in those cells. We positively identified Slack channel expression in MSNs using a variety of techniques, including western blot and reverse transcription PCR (Supplementary Fig. 1). The expression of Slack channels in MSNs was also independently verified in another study by *in situ* hybridization (Berg *et al.*, 2007). To investigate whether Slack channels actually carried the Na\(^+\)-dependent delayed outward current in MSNs, we designed siRNA primers to knockdown Slack expression in these cells, with the expectation that Slack-siRNA treatment would remove or reduce the Na\(^+\)-dependent outward current that is present in MSNs (siRNAs were designed on the basis of a previous study (Pei & Tuschl, 2006). As a control, we used a HEK cell line stably transfected with the *Slack* gene (Supplementary Fig. 2). We observed the efficient knockdown of Slack channel expression by siRNA to *Slack* by both immunocytophological staining and physiological recordings (Supplementary Fig. 2). We then used the siRNAs validated by these experiments to knockdown Slack expression in primary cell cultures of MSNs (Fig. 1e). In MSNs transfected
with Slack-siRNA (and a green fluorescent protein (GFP)-expressing vector), TTX reduced the delayed outward current by only 16.8% ± 3.2% (n = 8), whereas it reduced the current in MSNs transfected with a control siRNA (Slick) (and a GFP-expressing vector) by 34.0% ± 3.9% (n = 8, P ≤0.01).

Supplementary Figure 1. SLO2.2 (Slack) expression in striatal neurons as shown by Western Blots (left) and rtPCR (right) from primary cultures of rat striatal neurons. 

Left. Western blots showing Slack protein expression in indicated developmental stages of striatum (P1-P15). The antibody used in these Western studies was previously characterized (15). Western blots also showed Slack expression in adult striatum (not shown). 

Right. Slack transcripts detected by rtPCR in striatal primary neuronal cultures (rat) and whole brain (mouse). Slack-specific primers were designed to amplify a 390bp fragment. Control rtPCRs were performed with β-actin primers (772bp product) and with primers alone. First-strand syntheses were performed using 2.0 μg of total RNA isolated from rat striatal primary neuronal cultures, and 5.0 μg of total RNA from whole mouse brain. A fraction of each first strand reaction served as template for rtPCRs; rat striatal neuronal cultures (~5.0%) and mouse whole brain (~2.0%). rtPCR assays were cycled 35 times, and the products electrophoresed on a 1.5% agarose gel with molecular weight markers.
a Slack (red) stable transformed cell line

b Cell line transfected with Slack siRNA and GFP marker (green)

c HEK cells  |  HEK- Slack cells  |  HEK- Slack cells + siRNA
Supplementary Figure 2. siRNA knock-down of Slack channel expression in a HEK cell line stably transformed with Slack. a. Slack (stained red with an anti-Slack antibody) is abundantly expressed in all the cells of this line. b. Knock-down of Slack expression after transfection with anti-Slack siRNA. Transfection of siRNAs into cells included co-transfection of a plasmid expressing GFP as a fluorescent marker (green) to identify transfected cells and assess transfection efficiency. Upper left, Nomarski image of cells in culture. Upper right, transfected GFP expressing cells (green) lack expression of Slack (red) as seen in the lower left panel. Lower left, Slack (red) expressing cells which have not been transfected. Lower right, composite image of upper left, upper right, and lower left panels. If siRNA-transfected cells expressed Slack they would be yellow in this image. Lipofectamine (Invitrogen) was used to transfect HEK cells, and a high efficiency electroporation method was used to transfect neurons in primary cell culture (Mouse Neuron Nucleofector Kit from Amaxa, Inc.). Transfection efficiency of siRNAs was monitored by co-transfection with a plasmid expressing green fluorescent protein. c. Anti Slack siRNA effectively knocks down Slack expression in a stably transfected HEK-Slack cell line. Left traces, representative current records from a control HEK cell that has not been transfected with Slack. Middle traces, current records from a HEK-Slack stably transfected cell. Right traces, current records from a HEK-Slack cell after transfection with siRNA. All experiments had 10 mM Na+ in the intracellular pipette solution. Control cells 217.3 +/- 30.8 pA; n=8. [Control non-transfected HEK-Slack cells 730.5 +/- 89.9 pA; n=3, pairwise with Control HEK-Slack cells (+GFP) 798 +/- 95 pA; n=4, P=0.43]; [HEK-Slack cells (+GFP + Slack siRNA) 272 +/- 30 pA, n=10 pairwise with Control HEK-Slack cells (+GFP). P<=.01]. Measured step pulse = +40 mV.

A persistent Na⁺ current activates outward current

One indication that persistent Na⁺ entry might be involved was the observation that the sodium-dependent delayed outward current persisted long after the transient sodium current was fully inactivated. Indeed, the sodium-dependent delayed outward current showed only a minor decay even during step pulses lasting for 1,000 ms (Fig. 1b). We examined the importance of the persistent I_{Na} component relative to the transient I_{Na} component in activating the sodium-dependent delayed outward current using two separate methods. First, we employed the pharmacological agent riluzole, which preferentially blocks the persistent I_{Na} over the transient component of the sodium current (Hebert et al., 1994). Riluzole is known to block the persistent
sodium current by stabilizing the inactivated state of the sodium channel, thereby preventing reopenings (flickering) of the channel (Hebert et al., 1994). A large fraction of the delayed outward current was reduced by the application of riluzole (20 μM; Fig. 3a), but the transient sodium current was not reduced (Fig. 3b). We examined the I-V relationship for the subtracted component and found that the subtracted (riluzole sensitive) component also included a persistent inward current, as well as the delayed outward component (Fig. 3c). Note that the currents represent average values at 150–250 ms, long after the cessation of the transient inward component. Thus, riluzole removed the sodium-dependent outward current and the persistent I$_{Na}$, but not the transient I$_{Na}$. This indicates that the persistent I$_{Na}$ is the more important inward component coupled to the Na$^+$-dependent delayed outward component. Control experiments in our stably Slack-transfected HEK cell line showed no reduction of the Slack delayed outward current by riluzole (Fig. 3). In MSNs, riluzole (20 μM) was slightly less effective than TTX in reducing the delayed outward current, reducing it 30.1% (±3.09%, n = 9, P < 0.01). Thus, because a large fraction of the transient I$_{Na}$ remained after the application of riluzole, the transient sodium current does not appear to be the major factor in activating the sodium-dependent delayed outward current.
Figure 3. Evidence that a persistent Na\textsuperscript{+} current activates the sodium-dependent delayed outward current. Riluzole removed the persistent $I_{\text{Na}}$, but not the transient $I_{\text{Na}}$, and also removed a delayed outward current. (a) Currents before and after the application of riluzole (20 $\mu$M) and the subtracted (riluzole sensitive) component recorded from a tufted-mitral neuron. The currents shown on the left are plotted with respect to voltage. Plotted currents were average amplitudes measured during the 150–250-ms interval after initiation of the step pulse. (b) Transient sodium currents in a at higher resolution showed no reduction by riluzole. (c) The base of the plotted current curves from the graph in a is shown at higher
resolution. The arrow indicates a riluzole-sensitive persistent inward current. Also note the TTX-sensitive persistent inward current in Figure 1e. Note that we did not find any block of Slack currents in control experiments by recording delayed outward currents before and after applying riluzole (20 μM) to our stably Slack-transfected HEK cell line. The addition of 20 μM riluzole to the cell line resulted in a slight statistically insignificant increase in delayed outward current (4.83% ±3.02%, n = 3). Currents were measured 200 ms after voltage step to +40 mV. The effect of riluzole was similar across all voltages tested (-90 mV to +80 mV). (d) The TTX-sensitive current shown from a holding potential of -50 mV. Except for the depolarized holding potentials, other experimental details are as described in Figure 1a. No transient inward sodium current was noted in this cell, but the TTX-sensitive delayed outward current was well over half the total outward current. (e) The base of the plotted current curves from the graph in d is shown at higher resolution. The arrow indicates a TTX-sensitive persistent inward current.

Our second method for examining the importance of the persistent $I_{Na}$ was to use a relatively depolarized holding potential (-50 mV) to inactivate the transient $I_{Na}$ component. In these experiments, the TTX-sensitive delayed outward current was still present, as determined by the substantial reduction of the delayed outward current after the addition of TTX (Fig. 3d). The TTX-sensitive outward component was larger than the remaining outward current. At a holding potential of -50 mV, persistent sodium currents are active in most neuronal cell types, including MSNs (Alzheimer et al., 1993; Taddese & Bean, 2002; Chao & Alzheimer, 1995). The amplitudes of persistent sodium currents are often only a fraction of peak transient sodium currents, but are active over a broader voltage range (Alzheimer et al., 1993; Taddese & Bean, 2002; Chao & Alzheimer, 1995; Huang & Trussell, 2008). It is now widely accepted that several of the voltage-dependent sodium channel types that carry fast transient Na$^+$ currents, also carry persistent sodium currents (Alzheimer et al., 1993; Taddese & Bean, 2002), but we have not established the genetic identity of the persistent Na$^+$ currents in the cells that we have been studying. Our experiments here indicate that the persistent Na$^+$ current is the largest factor in
activating the Na\(^+\)-dependent delayed outward current, but we cannot rule out the possibility that
the transient Na\(^+\)-current is a contributing factor.

Experiments to measure the persistent Na\(^+\) current in MSNs (Fig. 4) revealed a current that was a
small fraction of the peak transient Na\(^+\) current, as has been previously reported (Chao &
Alzheimer, 1995). Also, as previously reported, we found the persistent Na\(^+\) current to be active
over a wider voltage range than the transient Na\(^+\) current, with some current being seen at
negative potentials of at least -70 mV. Note that we adjusted the Na\(^+\) equilibrium potential in
these experiments to approximately +23 mV to improve voltage control by raising internal [Na\(^+\)]
(Fig. 4). The lower driving force on Na\(^+\) resulted in a smaller sodium current than would be seen
under normal physiological conditions and showed a reversal potential close to \(E_{Na}\). Experiments
raising \(E_{Na}\) by lowering internal [Na\(^+\)] indicated little inactivation of the persistent \(I_{Na}\) at voltages
exceeding +23 mV.
Figure 4. Transient and persistent Na⁺ currents in MSNs. (a,b) Transient Na⁺ current. (c,d) Persistent inward Na⁺ current. The persistent Na⁺ current was plotted as the mean current during the 150–250-ms interval after the transient inward Na⁺ current, as indicated in c (n = 3). We used 100 mM cesium ion in the internal pipette solution and 40 mM tetraethylammonium chloride (TEA) in the extracellular saline to block the potassium conductances. To isolate the TTX-sensitive components, we recorded currents before and after applying TTX. We then subtracted the residual currents after TTX application from the currents recorded before TTX application. Thus, the currents that are shown represent both the TTX-sensitive transient and persistent components. The internal pipette solution contained 40 mM Na⁺ to reduce the driving force of Na⁺ and to gain better voltage control. This also reduced the amplitudes of the currents. The approximate Na⁺ equilibrium potential was +23 mV. Error bars represent standard errors.

Na⁺-activated K⁺ channels and Na⁺ channels may be clustered

Given the high [Na⁺], requirements for Na⁺-activated K⁺ channel activation (Kameya et al., 1984; Koh et al., 1994; Yuan et al., 2003), it seems unusual that such a small Na⁺ current could
effectively activate these $K^+$ channels. However, during a depolarizing step pulse, the transient $I_{Na}$ is only maximal for 1 to 2 ms, whereas the persistent $Na^+$ current, although smaller, is active indefinitely as long as sufficient depolarization is maintained. It may also be continuously active at or near cell resting potentials, albeit to a smaller degree. Conceivably, both $Na^+$-activated $K^+$ channels and sodium channels might be tightly clustered in a microdomain that permits an increased concentration of $Na^+$ relative to the bulk cytosol, similar to that seen for $Ca^{2+}$ microdomains, in which calcium channels and $Ca^{2+}$-dependent $K^+$ channels are clustered (Fakler & Adelman, 2008). Considering the high diffusion constant for $Na^+$, we considered the possibility that a $Na^+$ microdomain might consist of an 'unstirred layer' (Abriel & Horisberger, 1999; Pohl et al., 1998) or 'fuzzy space' (Barry, 1993; Carmeliet 1992) that restricts the diffusion of $Na^+$, but such a space would also restrict the movement of $K^+$, thus lowering the $K^+$ conductance of $Na^+$-activated $K^+$ channels. However, an alternative mechanism for the creation of a $Na^+$-rich microdomain might be an electrostatic environment that concentrates $Na^+$ at a level that is higher than at the bulk cytoplasm. Such an electrostatic microdomain for $K^+$ enrichment is present at the entrance to the intracellular vestibule of the BK (SLO1) channels, which have a ring of eight negatively charged glutamate residues (Brelidze et al., 2003; Nimigean et al., 2003). This ring of charge has been shown to double the conductance of SLO1 channels by increasing the local concentration of $K^+$ in the vestibule through an electrostatic mechanism. Notably, the concentration of $K^+$ in the vestibule by the ring of charge has been calculated to be equivalent to that achieved by increasing the $K^+$ in the bulk intracellular solution from 150 to 500 mM (Brelidze et al., 2003; Nimigean et al., 2003). Such a simple electrostatic mechanism might function alone or in conjunction with an unstirred layer or fuzzy space to raise the local concentration of $Na^+$ to a higher concentration than that of the bulk cytoplasm.
Our experiments (Figs. 1,2,3) were designed to determine the activation of delayed outward current by Na\(^+\) influx into a cell in which [Na\(^+\)]\(_i\) had been adjusted to minimal levels. However, we also undertook experiments to determine the activation of delayed outward current by Na\(^+\) influx into a cell in which [Na\(^+\)]\(_i\) had been adjusted to higher levels. Thus, we raised the internal [Na\(^+\)] by filling whole cell–patch recording electrodes with intracellular recording solutions containing 20, 30 and 40 mM Na\(^+\). In these experiments, we found that, even though [Na\(^+\)]\(_i\) was elevated, additional Na\(^+\) influx during voltage-clamp step pulses and/or at rest produced an incremental increase in the delayed outward current, noted as a TTX-sensitive component (Fig. 5a). This was true even though sodium entry during voltage-clamp step pulses was unlikely to appreciably raise the concentration of bulk [Na\(^+\)].

One possible explanation for the effectiveness of sodium entry into Na\(^+\)-loaded cells in activating additional Na\(^+\)-dependent outward current is that persistent sodium entry may occur in close proximity to Slack channels. Hypothetically, if an inward Na\(^+\) current in close vicinity to Slack channels can raise the local [Na\(^+\)] to a higher level than the bulk intracellular solution, then an outward Na\(^+\) current in close proximity to the Slack channels might deplete the local [Na\(^+\)] sensed by Slack channels. A prediction of this hypothesis was that removing extracellular Na\(^+\) would remove a larger component of Na\(^+\)-dependent outward current than by simply adding TTX. This is because the removal of extracellular Na\(^+\) from Na\(^+\)-loaded neurons should result in an outward Na\(^+\) current in close proximity to Slack channels, whereas adding TTX should simply block the Na\(^+\) current. To test this, we raised [Na\(^+\)]\(_i\) in a cell by filling the intracellular pipette with 40 mM Na\(^+\) (Fig. 5a). After applying a series of control voltage-clamp step pulses to a maximum of +90 mV, we removed extracellular Na\(^+\). After approximately 1.5 min, we repeated the series of voltage-clamp step pulses and observed a substantially diminished delayed outward
current. Finally, we added TTX to the extracellular solution containing 0 Na\(^+\), and, after approximately 1.5 min, we again repeated the series of voltage-clamp step pulses. At this final condition, the delayed outward current was larger than the current with 0 mM \([\text{Na}^+]_o\). Our interpretation of this result is that Na\(^+\) entry in the first series of control voltage-clamp step pulses raised the \([\text{Na}^+]\) in the vicinity of Slack channels to a higher level than that present in the bulk intracellular solution. However, after removing extracellular Na\(^+\), sodium moved in the outward direction across the membrane, diluting the intracellular Na\(^+\) in the immediate vicinity of Slack channels to a lower concentration than would be present if the outward flow of Na\(^+\) was blocked by TTX. Finally, after adding TTX to the extracellular solution containing 0 Na\(^+\), the outward flow of Na\(^+\) was reduced and the local intracellular concentration of Na\(^+\) in the vicinity of Slack channels rose to a level that was intermediate between control conditions, when the Na\(^+\) current was inward and the condition in which the Na\(^+\) current was outward.

We also determined the residual delayed outward currents obtained by subtracting the currents recorded after removal of external Na\(^+\) from the control currents recorded in normal \([\text{Na}^+]_o\), and the residual currents obtained by subtracting the currents recorded after addition of TTX to 0 mM external Na\(^+\) from the control currents recorded in normal \([\text{Na}^+]_o\) (Fig. 5a). Notably, these residual currents declined at higher voltages. This is probably a result of the fact that Slack channels at higher voltages are particularly vulnerable to block by \([\text{Na}^+]_t\) (Dryer, 1994; Yuan et al., 2003). This could also be a result of the fact that the sodium current was outward at the higher voltage steps for the control currents recorded in normal \([\text{Na}^+]_o\), and the outward flow of Na\(^+\) could therefore partially deplete the local concentration of Na\(^+\) sensed by SLO2 channels.
Figure 5. Experiments in cells loaded with high [Na⁺]. (a) A greater amount of delayed outward current was blocked by removing extracellular Na⁺ than by blocking Na⁺ flux with TTX. Current-voltage plots are shown from a cell in which the intracellular pipette solution contained 40 mM Na⁺. A series of control voltage-clamp step pulses were applied at 10-mV intervals to a maximum of +90 mV. Control
indicates the delayed outward currents plotted from the cell with normal $[\text{Na}^+]_o$. -$\text{Na}^+_o$ indicates the outward currents plotted from the same cell after the removal of external Na$^+$. -$\text{Na}^+_o + \text{TTX}$ indicates the outward currents plotted from the same cell after subsequently adding TTX to the -$[\text{Na}^+]_o$ condition. This plot shows the delayed outward current in response to the same series of voltage-clamp step pulses after reducing the outward movement of Na$^+$. These experiments were repeated seven times in MSNs and three times in tufted/mitral cells with similar results. (b) Conductance/voltage relationships showing the normalized incremental conductance increases in the delayed outward current resulting from Na$^+$ influx in cells loaded with different levels of $[\text{Na}^+]_i$. Tufted/mitral cells loaded with different intracellular concentrations of Na$^+$, as indicated, were subjected to a series of voltage-clamp step pulses. The incremental conductance/voltage curves were constructed from residual currents obtained by subtracting currents recorded after removal of external Na$^+$ from control currents recorded in normal $[\text{Na}^+]_o$ (as in a, 1–2). As $[\text{Na}^+]_i$ increased, the incremental conductance curves shifted leftward to more negative voltages and also had steeper slopes. This seems consistent with the hypothesis that, at higher concentrations of bulk $[\text{Na}^+]_i$, the sodium influx substantially increases the local internal Na$^+$ concentration to a level above that of the bulk cytoplasm. Conductances ($g$) were calculated for individual cells at the three indicated concentrations, normalized to the maximum value and then averaged. Regions of the curves to the left of maximum conductance ($g_{\text{max}}$) were fit by a Boltzmann equation (red). For 0 mM $[\text{Na}^+]_i$, $V_{1/2} = 11.8 \text{ mV}, n = 3$; 20 mM $[\text{Na}^+]_i$, $V_{1/2} = 1.40 \text{ mV}, E_{\text{Na}} = 50.8 \text{ mV}, n = 3$; 30 mM $[\text{Na}^+]_i$, $V_{1/2} = -11.0 \text{ mV}, E_{\text{Na}} = 40.5 \text{ mV}, n = 4$.

However, we noted that the reduction of outward current that occurred after TTX was added (as in Fig. 1a–c) was not immediate and required approximately 1 to 2 min before the current was stabilized at the lower level. Thus, the local concentration of Na$^+$ sensed by SLO2 channels may only be partially changed during shorter voltage-clamp step pulses.

These results clearly suggest that, even if the internal bulk concentration of Na$^+$ is raised to as high as 40 mM, there appears to be an additional rise in the Na$^+$ concentration in the vicinity of Slack channels as long as the driving force on Na$^+$ is inward. To explore this further, we conducted a series of experiments on cells 'loaded' with different intracellular concentrations of Na$^+$ and subjected to a series of voltage-clamp step pulses. We then plotted the incremental rise in the Na$^+$-dependent delayed outward conductance resulting from Na$^+$ influx augmenting the
level of Na$^+$ already present in the presumed microdomain (Fig. 5b). These incremental conductance-voltage curves were constructed from the residual currents obtained by subtracting the currents recorded after removal of external Na$^+$ from the control currents recorded in normal [Na$^+$]$_o$ (as in Fig. 5a). An examination of the normalized incremental conductance curves for 0, 20 and 30 mM [Na$^+$]$_i$ (Fig. 5b) revealed two important features: as [Na$^+$]$_i$ increases, the incremental conductances shifted leftward to more negative voltages, and the incremental conductances had a steeper slope. This seems to be consistent with the hypothesis that, at higher concentrations of bulk [Na$^+$]$_i$, the sodium influx is augmenting the local Na$^+$ concentration to an even higher level. These observations also seem to be consistent with earlier studies of the Na$^+$ sensitivity of cloned Slack channels in inside-out patches, which found that there is a steep and accelerating relationship between Slack channel activity and [Na$^+$]$_i$ (Yuan et al., 2003). That study showed that raising [Na$^+$]$_i$ from 10 to 30 mM increased Slack channel activity by only 20%, but raising [Na$^+$]$_i$ from 30 to 50 mM increased channel activity by 70%. In the neurons that we studied, we asked how high the concentration of Na$^+$ reached in the local region sensed by Slack channels. To answer this question, we loaded neurons with increasing concentrations of internal Na$^+$ by including Na$^+$ in the internal pipette solution. We found that, as long as the driving force on Na$^+$ was inward, we could still detect a TTX-sensitive delayed outward current until the internal pipette solution contained approximately 70 mM Na$^+$. This suggests that the TTX-sensitive Na$^+$ flux could still substantially increase the local concentration of Na$^+$ in the vicinity of Na$^+$-activated K$^+$ channels until the bulk intracellular [Na$^+$] approached 70 mM. This, however, could be an underestimate, as loading cells with high intracellular concentrations of Na$^+$ causes a substantial block of most K$^+$ channels (Bezanilla & Armstrong, 1972).
DISCUSSION

Na\(^+\)-activated K\(^+\) channels are difficult to study because their activity is influenced by many factors, including their Na\(^+\) sensitivity, weak voltage sensitivity, channel run-down, modulation by second messenger systems and, not least of which, the mechanism and kinetics of Na\(^+\) delivery to the channels themselves. Nevertheless, although the precise mechanisms that are responsible for the local rise of [Na\(^+\)] have not been fully established, our data strongly suggest that Na\(^+\) entry through TTX-sensitive Na\(^+\) channels, most likely carrying a persistent Na\(^+\) current, activates a Na\(^+\)-dependent delayed outward current that is a large component of outward conductance in some neurons under normal physiological conditions. This result further suggests a very special relationship between Na\(^+\)-activated K\(^+\) channels and TTX-sensitive sodium channels. Even at relatively high internal bulk concentrations of Na\(^+\), for example, 40 mM, an inward Na\(^+\) current can still activate a substantial incremental component of delayed outward current. Thus, local sodium entry appears to be highly effective at raising the local [Na\(^+\)]\(_i\), regardless of the concentration of the internal bulk solution. Although a diffusion barrier model might permit a local increase in Na\(^+\), an electrostatic microdomain model might explain not only a local rise in [Na\(^+\)], but a unique requirement for persistent sodium entry as well. A putative electrostatic microdomain would most likely be nonselective and would elevate both K\(^+\) and Na\(^+\) in relative proportion to their bulk intracellular concentrations. However, closely related persistent Na\(^+\) entry might displace a substantial amount of K\(^+\), elevating the local [Na\(^+\)] to a higher level. Notably, Slack channels are similar to SLO1 channels in having a double ring of negative charges surrounding the inner vestibule of the channel (Brelidze et al., 2003). They also have other cytoplasmic domains with high net negativity, but the relevance of these features, if at
all, to the mechanism of Na\(^+\)-dependent gating of Slack channels must await further studies.

Regardless of the exact nature of the Na\(^+\)-rich microdomain, our results suggest that Na\(^+\) is normally maintained at a higher concentration in the microdomain than in the bulk cytoplasm. TTX-dependent persistent Na\(^+\) entry appears to occur over a wide range of voltages and there appears to be sufficient entry of Na\(^+\) at a holding potential of -70 mV such that neurons maintained at that holding potential for long periods still show a TTX-sensitive component of outward current even when test steps are taken to the Na\(^+\) reversal potential. Thus, the possibility that a small contribution of persistent inward Na\(^+\) current operates continuously at cell resting potentials and maintains the local intracellular concentration of Na\(^+\) at a higher level than that of the bulk cytoplasm at the resting state cannot be excluded.

The potential role of the sodium channel–Na\(^+\)-activated K\(^+\) channel–coupled system may differ widely in different neuronal cell types, but in some, its effect may be profound. The relative contribution of the sodium-dependent delayed outward current relative to the total outward current may increase as membrane resting potential becomes more positive because some voltage-dependent K\(^+\) currents inactivate (for example, A-type currents (Connor & Stevens, 1971), whereas Na\(^+\)-activated K\(^+\) currents and persistent sodium currents do not. These findings of a major, but previously unseen, K\(^+\) conductance have far reaching implications for many aspects of systems and cellular neuroscience, as well as clinical neurology and pharmacology. In systems and cellular neuroscience, studies of 'up-down' states of neuronal excitability, spike adaptation, synaptic integration and other aspects of neuronal physiology may have to be reexamined taking this system into consideration. In clinical and pharmacological studies, this previously unseen current system that is active during normal physiology represents a new and promising pharmacological target for drugs dealing with seizure and psychotropic disorders.
METHODS

Cell preparation and recording methods.

Dissociated MSNs and tufted-mitral cells were cultured from postnatal day 2 rat pups and recorded from 4–7 d post-plating. Recording solutions used an internal (pipette) \([\text{Na}^+]\) solution as indicated in the figure legends and an external (bath) \([\text{Na}^+]\) solution of 150 mM or as indicated in the figure legends. Experiments requiring the removal of external \(\text{Na}^+\) replaced sodium with choline in the extracellular solution. Experiments that elevated \(\text{Na}^+\) in the internal pipette solution removed equimolar \(\text{K}^+\). The 140 or 150 mM \(\text{Na}^+\) bath solution was composed of 150 mM NaCl, 5 mM KCl, 2 mM MgCl\(_2\), 10 mM dextrose, 10 mM HEPES, pH 7.4 with NaOH. The 0 mM \(\text{Na}^+\) bath solution contained 150 mM choline chloride, 5 mM KCl, 2 mM MgCl\(_2\), 10 mM dextrose, 10 mM HEPES, pH 7.4 with KOH. Data were acquired using an Axopatch 200A or Axopatch 200B amplifier (Molecular Devices), digitized at 10 kHz using a Digidata 1440A (Molecular Devices), filtered at 5 kHz and collected using pCLAMP 10. Recording pipettes had tip resistances of 3–6 M\(\Omega\). In experiments using TTX or riluzole, drugs were added to external solutions at concentrations of 1 and 20 \(\mu\text{M}\), respectively. Sodium-dependent potassium currents sometimes showed 'rundown' in the whole-cell or detached patch-recording modes. We did see rundown during the initial phase of recording currents in some cells. However, we applied test voltage steps over time, before the application of TTX or other treatments, and only initiated experiments after test pulses produced delayed outward currents that were stable over time (in general, the current stabilized within \(\sim 2\) min).
Statistical analysis.

We performed statistical analyses using previously described tools (Kirkman, T.W. Statistics to use. <http://www.physics.csbsju.edu/stats/>; 1996). For comparison between two groups, we used paired Student's t tests for the same procedures before and after applied treatments.

Animal welfare.

It is the policy of Washington University Medical School that all research involving animals be conducted under humane conditions, with appropriate regard for animal welfare. Washington University Medical School is a registered research facility with the United States Department of Agriculture (USDA) and is committed to complying with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services), the provisions of the Animal Welfare Act (USDA) and all applicable federal and state laws and regulations. At Washington University Medical School an Animal Care Committee has been established to insure compliance with all applicable federal and state regulations for the purchase, transportation, housing and research use of animals. Washington University Medical School has filed appropriate assurance of compliance with the Office for the Protection of Research Risks of the US National Institutes of Health.


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The activating effect of most divalent cations on Slo1 is well documented, but the effect of divalent cations on Slo2.2 channels is largely unstudied. In this chapter I present results showing that many of the divalent ions that activate Slo1 channels have the opposite effect on Slo2.2 channels; they reduce channel activity. Our results indicate that the effect of divalent ions on Slo2.2 is not by blocking the pore but most likely by allosteric modification. We show that the blocking effect of divalent cations on Slo2.2 channels is conserved in the orthologous channel from *Drosophila* which has been cloned in our lab. In addition, we have located a residue involved in the interaction of divalent cations with the Slo2.2 channel. This chapter is the draft for a paper in preparation in which I will be the first author. I did most of the experiments, some of them with the help of Qi Sun (an undergraduate student at the lab), I also designed the experiments together with Dr. Salkoff, I analyzed the obtained data, and wrote the following draft.
Chapter 4
Opposite regulation of Slo1 and Slo2.2 channels by divalent cations
ABSTRACT

The Slo family of channels are among the largest and most complex voltage-dependent K+ channels. They are activated by voltage and modulated by intracellular ions and a host of different intracellular factors. Two members of this family are Slo1 and Slo2.2. Slo1 is activated by calcium and many other divalent cations to different degrees. Slo2.2 is activated by sodium but its modulation by divalent cations had not been explored. We studied the effect of different divalent cations on the Na+-dependent potassium channel Slo2.2 using inside-out patches. In the presence of intracellular sodium, the addition of divalent cations significantly reduced Slo2.2 currents recorded in macropatches. Among the divalent cations studied, the relative effectiveness in reducing channel activity was Zn²⁺ > Cd²⁺ > Ni²⁺ > Ba²⁺ > Ca²⁺ > Mn²⁺ > Mg²⁺. Zn²⁺, the most effective of these, has an IC₅₀ of 8 µM, in contrast to Mg²⁺ which is the least effective with an IC₅₀ close to 1.5 mM. Our results indicate that the effect of divalent cations on Slo2.2 is not by blocking the pore but more likely by an allosteric modification. Site-directed mutagenesis implicates a histidine residue down-stream of S6 as a possible binding site. Slo2.2 channels with a glutamine substituted for the histidine are less sensitive to divalent cation inhibition. The inhibition of Slo2 channels by divalent cations is a conserved property; we show that Drosophila Slo2 channels also show a reduction in activity when exposed to intracellular divalent cations.
INTRODUCTION

The Slo family channels are high conductance K\(^+\) channels that are gated both by voltage and intracellular ions. Structurally they resemble voltage gated channels but have additional conserved intracellular domains appended on the C-terminal that allows them to be gated by different intracellular ions (reviewed in Salkoff et al, 2006). Two members of this family are Slo1 which is activated by Ca\(^{2+}\), and Slo2.2 (also known as Slack) which is activated by Na\(^+\). Both channels are widely expressed in the brain and other tissues in many species from C. elegans to humans. The activating effect of divalent ions on Slo1 is well documented, but their effect on Slo2.2 channels is largely unstudied. In exploring this question we were surprised to observe that all of the divalent ions that were reported to activate Slo1 channels, inhibit Slo2.2 channels by reducing channel activity. Slo2 channels are widely expressed in mammalian brain. Using immunohistochemistry (Bhattacharjee et al., 2002) and inside out patch recordings (Eagan et al., 1992) it has been shown that these channels are present in the soma and dendrites of neurons. The somatodendritic localization of Slo2 channels and their modulation by neurotransmitter receptors (Santi et al, 2005) make Slo2 channels good candidates for regulating intrinsic neuronal excitability. Slo2 are high conductance and weakly voltage dependent, and if active, will hyperpolarize the soma of a neuron making it more difficult for summed postsynaptic potentials to initiate an action potential. Thus, because of their properties and strategic location, we believe that Slo2 channels could play a “gate keeper” role in synaptic integration, and its modulation may contribute to use-dependent mechanisms of plasticity in the mammalian brain. The modulation of Slo2 channels by some divalent cations may be physiologically relevant. Some have an IC\(_{50}\) such that the physiological range of intracellular concentrations could
conceivably be relevant in regulating the intrinsic excitability of some neurons. The effect of divalent ions on Slack is not by blocking the pore. Supporting this claim is evidence showing the lack of voltage dependence of inhibition, and the fact that the conductance of the channel remains unchanged at the concentrations of divalent ions that close the channel. Other evidence to discount a pore blocking effect is the fact that the block is smaller when we apply the divalent ions in the presence of saturated concentration of sodium, suggesting a possible competition. Interestingly, Ba\(^{2+}\) which has been reported to block Slo1 channels also inhibits Slo2.2 channels but, unlike all other divalents, has an obvious pore-blocking effect and shows an increasing block at more positive membrane potentials. By site-directed mutagenesis a histidine residue downstream of S6 was implicated in the divalent cation inhibition of Slo2.2 channels. Because of the position of the histidine and the similar affinity for Ni\(^{2+}\) our results resemble divalent cation inhibition of cyclic nucleotide channels which involve a histidine residue at a similar position (Gordon and Zagotta, 1995). In addition, the inhibitory effect of divalent cations on Slack are conserved; we report here that the *Drosophila* Slo2 channel is also activated by sodium ion, and *Drosophila* Slo2 channels are also inhibited by divalent cations.
RESULT

The inhibitory effect of the divalent cations is reversible

Inside-out patch recordings obtained from a HEK cell line constitutively expressing Slo2.2 channels were used to study the effect of intracellular divalent cations on Slo2.2 channels. It has been reported that calcium ions inhibit native Na\(^+\)-dependent K\(^+\) channels, likely to be Slo2.2, in thick ascending limb of mouse kidney (Paulais et al., 2006) but the effect of calcium has not been studied in heterologously expressed Slo2.2 channels. We observed that calcium inhibits Slo2.2 channels in the presence of 80 mM sodium and the inhibition is concentration dependent (Figure 1). The blocking effect of calcium ion is not voltage dependent in the range of voltages studied and it is almost completely reversible.

Different divalent cations inhibit Slack channels at very different concentrations

The effect of different divalent cations applied to the cytoplasmic surface of inside-out patches expressing Slo2.2 channels was tested in the presence of sodium. The range of concentrations used was adjusted in accordance to the observed blocking effect of the individual cation. All divalent cations tested inhibited Slo2.2 currents (Figure 2). In all cases the effect was reversible and all except for Ba\(^{2+}\) (as discussed below) showed similar blocking effects at positive and negative potentials. Zinc cation was the strongest inhibitor of the Slack channel. As Figure 2 illustrates the relative strength of inhibition was: Zn\(^{2+}\) > Cd\(^{2+}\) > Ni\(^{2+}\) > Ba\(^{2+}\) > Ca\(^{2+}\) > Mn\(^{2+}\) > Mg\(^{2+}\).
Figure 1. Family of I-V curves for an inside-out macropatch at different internal calcium concentrations. Representative I-V curves obtained from inside-out macropatches in the presence of 80 mM Na\(^+\) and exposed to different calcium concentrations. The experiments were performed in symmetrical K\(^+\), the patch was held at 0 mV and 200 ms steps from -100 to 100 mV were applied in 10 mV increments. The currents in the plot are averages of the last 50 ms of the trace where the currents are relatively stable. Notice that 5 mM calcium reduces the current to levels close to the leak current obtained in 0 mM Na\(^+\).
Figure 2. Different divalent cations inhibit Slo2.2 channels at different concentrations. All divalent cations tested show an inhibitory effect on Slack currents when applied to the intracellular side of the membrane. The effect of internal divalents was measured at -50mV in the presence of 80 mM sodium at different divalent concentrations. The most effective divalent was Zn$^{2+}$ followed by Cd$^{2+}$, Ni$^{2+}$, Ba$^{2+}$, Ca$^{2+}$, Mn$^{2+}$ and Mg$^{2+}$. The concentrations used were in the range of the apparent affinity. The inside-out patches were exposed to the same solution with increasing divalent cation concentrations. The current obtained in the presence of zero intracellular sodium was subtracted from all the recordings and currents were normalized to the current obtained with 80 mM Na$^{+}$ in the absence of divalent cation. The plot shows the averages of the normalized currents at different divalent concentrations fitted with the logistic function for all divalents tested. The number of patches for each point was between 5 and 8 for all divalent cations.
The effect of divalent cations on Slo2.2 currents is not voltage dependent

Among all the divalent cations tested only Ba$^{2+}$ showed more block at positive potentials than at negative (Figure 1 and Figure 3). We should expect, for a charged molecule blocking the pore of the channel, more block when electrostatic force is applied pushing the molecule further into the pore. In our case, divalent cations on the inside should block more effectively at positive potentials. However, this was not observed and the absence of a voltage-dependent block suggests that the effect of cations is not by blocking the pore. Interestingly, Ba$^{2+}$, a known pore blocker of K+ channels, is the one exception, and its blocking effect is indeed voltage dependent. Ba$^{2+}$ is a well known pore blocker of Slo1 BK channels (Vergara and Latorre, 1983; Miller et al., 1987). It has been recently reported that barium has a dual effect; not only does it block the pore of Slo1 channels but also activates the channel by binding to the calcium bowl (Zhou et al., 2012). Similarly, we will show that Ba$^{2+}$ also has a dual effect on Slo2.2 channels; it blocks with a voltage-dependent effect at positive potentials but also inhibits the channel in a non-voltage-dependent manner.

Single channel conductance is not decreased by divalent cation application

We studied the effect of divalent cations on Slo2.2 using single channel recordings. Our results show that the application of divalent cations did not produce a reduction of the single channel conductance nor flickering of the channel (Figure 4). Instead, the reduction of the current is produced by a decrease in open channel probability. These results suggest that the effect of divalent cations on Slo2.2 channels is by allosteric modification of the channel and not by blocking of the pore.
Figure 3. Among divalent cations tested, only Ba$^{2+}$ showed a voltage dependent block. Ramps from -90mV to 90mV were applied to inside-out patches expressing Slo2. Note that the block shown at higher voltages in the control traces (black) is due to the high intracellular Na$^+$ (80 mM) which is necessary to activate these sodium-activated channels (Yuan et al., 2002). **Left:** When nickel is added to the intracellular solution the decrease in the current observed is similar for positive and negative potentials. **Right:** voltage dependent block of Ba$^{2+}$. Barium was the only cation we tested that had a voltage dependent effect in this voltage range. Barium has also been reported to block the pore of Slo1 as well as other K$^+$ channels.
Figure 4. Divalent cations decrease open probability but not the single channel conductance.
Single channel currents of an inside-out patch containing at least four channels. The open probability of Slack channels decreases in the presence of divalent cations but the single channel conductance remains constant. The recordings shown here were performed at -80 mV using symmetrical potassium and high internal sodium in the absence and presence of 5 mM magnesium.

Divalent cation inhibition is smaller at saturating sodium concentrations
Experiments were performed in inside-out macropatches to test the inhibiting effect of Zn$^{2+}$ at different Na$^+$ concentrations. Our results showed that Zn$^{2+}$ is a more effective inhibitor of the channel at low sodium concentrations. When the same patch is exposed to different sodium concentrations where the concentration of Zn$^{2+}$ is held constant we observed that at saturating sodium the inhibitory effect of Zn$^{2+}$ is noticeably smaller (Figure 5).
Figure 5. Plots of normalized currents from inside-out macropatches recorded at -40mV with different sodium concentrations in the absence and presence of Zinc. Averaged normalized currents are shown which were recorded under symmetrical potassium conditions. Results were fitted with the Hill equation. Hill coefficients indicate cooperativity for sodium binding which is little changed by the presence of Zn$^{++}$ (Zn$^{++}$ experiment: $h$ (control) = 2.8; $h$ (0.01 Zn$^{++}$) = 2.7; $h$ (0.05 Zn$^{++}$) = 3.3). In the presence of zinc EC$_{50}$’s for sodium ion are significantly increased but estimated $V_{max}$ values were similar ($V_{max}$ (control) = 1.03; $V_{max}$ (0.01 Zn$^{++}$) = 0.95; $V_{max}$ (0.05Zn$^{++}$) = 1.01). This may indicate competitive inhibition. However, this does not necessarily mean that the site of sodium and divalent ion binding are the same.
**Slo2.2 channels are pH dependent**

Since Zn\(^{2+}\) was the most effective inhibitor of the divalent cations tested we investigated residues usually present in its coordination. We focused our attention on histidine or cysteine residues which are often present in Zn\(^{2+}\) binding sites (Auld, 2001). Our first approach was the use of histidine and cysteine modifiers to detect whether the modification of one or more residues of either class might alter gating when modified. When the cysteine modifier MTSEA was applied to inside-out patches no change in currents or in divalent cation effect was observed. In contrast the histidine modifier diethyl pyrocarbonate decreased the macroscopic currents when applied to the inside of the membrane. Also, as previously reported (Ruffin et al., 2008), we confirmed that low pH inactivates Slo2.2 in inside-out macropatches (Figure 6). Thus, both of these techniques might reveal the importance of a histidine residue in channel gating.

**A histidine residue downstream of the S6 transmembrane segment affects divalent cation inhibition of Slo2 channels**

Since our results indicated that a histidine could be present in divalent cation binding we undertook mutagenesis of histidine residues in the C-terminal domain of the Slo2.2 channel. We found that substitution of two histidines by arginines (His823Arg and His824Arg) in a putative Slo2 sodium binding site (Zhang et al., 2010) had no effect either on the effectiveness of sodium activation of the channel, or by inhibition of the channel by low pH.
Figure 6. Effect of intracellular pH on Slo2.2 currents. Inside-out macropatch currents of Slo2.2 channels were recorded in the presence of 80 mM sodium at indicated pH. At low pH the inhibition of current shows an IC$_{50}$ for protons of pH 6.8.

Additionally, these residue alterations had no effect on divalent cation inhibition of the mutant channel. Subsequently a comparative analysis of the sequence of Slo2.2 and Slo1 channels revealed a histidine present in Slo2.2 channels on the cytoplasmic side of S6 that is not present in Slo1. Interestingly, this histidine residue is conserved in a cyclic nucleotide gated channel (Figure 7), and was shown to be involved in the inhibition of those channels by Ni$^{2+}$ (Gordon and Zagotta, 1995). As was found for cyclic nucleotide gated channels the substitution of this
histidine by glutamine significantly decreases the inhibitory effect of Zn\(^{2+}\) on Slo2.2 currents recorded in inside-out macropatches (Figure 7). We also tested the effect of Ba\(^{2+}\) on this mutant. Remarkably, at the concentration tested, barium had no effect at negative potential, reducing the current only at positive potentials (Figure 8). This result suggests that barium inhibits the channel by an allosteric effect only in the wild-type channel and not in the mutant channel, while it blocks the pore in a voltage sensitive manner in both wild-type and mutant channels.

**Figure 7. A histidine residue downstream of S6 affects zinc inhibition of Slo2.** Above: sequence alignment for CNGSlo2 and Slo1 of the region including the transmembrane segment S6 and the S6-RCK1 linker. Below: Gap-free recordings of Slo2 WT and Slo2 mutant in the presence of different zinc concentrations. The voltage was maintained at -40 mV and the intracellular sodium concentration was alternated between 0 mM to 80 mM. Increasing zinc concentrations were added as noted. On the right, plot showing the significant difference in percentage of current reduction in the presence of 10 μM zinc for WT and Slo2H>Q (n>4, P<0.01).
Figure 8. Effect of Barium in Slo2.2 WT and in the Slo2.2_{H>Q} mutant. Left (WT): Currents evoked by a voltage ramp from -90 to 90 mV in the presence of 80 mM sodium before (black) and after application of 0.1 mM barium (red). Right(Slo2.2_{H>Q}): The same experiment was performed in macropatches expressing the histidine mutant. Note that the application of barium produces no effect at negative potential but blocks the currents.

The divalent cation inhibition of Slo2 channels is conserved

We cloned and functionally expressed the *Drosophila* Slo2 channel (dSlo2). Our results show that the *Drosophila* Slo2 channel has sodium sensitivity similar to that of mammalian Slo2 channels and it was also similarly inhibited by divalent cations (Figure 9).
Figure 9. Ni$^{2+}$ inhibition of Slo2 channels in mammals and flies. Above: a representative recording of rat Slo2.2 currents in inside-out patches when exposed to 0 Na$^+$ (red symbols), 80 mM Na$^+$ (blue) and 80 mM Na$^+$ with 0.5 mM Ni$^{2+}$ (green). At the right are the plotted currents of the experiments showed in the left. Below: The same experiment was performed on patches expressing Drosophila Slo2 channels. Results obtained with Drosophila Slo2 channels were similar to those obtained with rat Slo2 channels, the one exception being that the current amplitudes are different.
DISCUSSION

All the divalent cations tested in this study showed to be effective in inhibiting Slo2.2 channels. The properties of the inhibition indicate that it is not by blocking the pore. First, the divalent cation effect is not voltage dependent as expected for a positive charge interacting with the pore of the channel. Second, the conductance of the Slo2.2 channel is not affected in the presence of divalent cations. Third, the effect is smaller at saturating concentration of sodium. All this indicates that the effect of divalent cations is by allosteric modification.

Magnesium is the least effective divalent cation tested in inhibiting Slo2.2 channels. Nevertheless, is the only one that is clearly modulating Slo2.2 channels at its physiological range of concentrations. Interestingly, as Slo2.2 channels respond to very high levels of Na\(^{+}\), it has been suggested that Slo2.2 represents a reserve conductance that can be only activated during times of stress resulting from ischemia or hypoxia, when sodium ions accumulate in cells (Kameya et al., 1984; Dryer, 1994). The results presented in this work would indicate the opposite, it is well established that the concentration of many divalent cations increase during hypoxia or ischemia. In particular, the free concentration of magnesium is elevated due to the decrease in ATP which buffers Mg\(^{2+}\).

Among the divalent cations tested, the most effective in inhibiting Slo2.2 channels was Zn\(^{2+}\) with a IC\(_{50}\) close to 8 μM. Such intracellular Zn\(^{2+}\) concentrations are probably never reached in the cytoplasam. However, as has been observed in calcium measurements (Fakler and Adelman, 2008), it is possible that intracellular Zn\(^{2+}\) concentrations increase in microdomains (close to channels permeable to Zn\(^{2+}\) or other Zn\(^{2+}\) sources) high enough to modulate Slo2.2 activity. It is possible that some channels or receptors permeable to Zn\(^{2+}\) co-localize with Slo2.2 channels in
neurons. In Slo1, which is activated by similar concentrations of Zn$^{2+}$, co-expression with TRP channels that are permeable to Zn$^{2+}$ increased Slo1 channel activity when extracellular Zn$^{2+}$ was present (Hou et al., 2009).
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Chapter 5

Conclusions and Future Directions
In the previous chapters of this dissertation I have presented results about structure, function and physiology of Slo1 and Slo2 channels. As all the experimental results presented in chapters 2-4 of this dissertation have been analyzed and discussed within the chapters, I will center the focus of this final chapter in the future directions opened by my research.

**Chapter 2**

*We have demonstrated that the Core of Slo1 channels can be expressed in the absence of the Tail (gating ring). The results show the properties of the Slo1 Core and how they are affected by the gating ring. Some properties of the full-length channel are retained in the channels formed by the isolated Core: they are voltage-dependence, block by iberiotoxin and low external TEA, and a slowing of activation is observed when co-expressed with β1 subunits. Other properties are determined or modified by the gating ring: Slo1-Core channels are not activated by Ca\(^{2+}\) or Mg\(^{2+}\). However, we observed other changes that seem to be dependent on the interaction of the gating ring with the Core of the channel: a decrease in the mean open time duration and the burst time duration. In addition, there is a decrease in single channel conductance which was totally unexpected. Thus, the gating ring not only determines the Ca\(^{2+}\) and Mg\(^{2+}\) sensitivity but also, has a profound effect on Slo1 channel gating and conductance.*
**Contribution of the gating ring to the conductance properties of Slo1**

The results presented in Chapter 2 demonstrate that removing the gating ring reduces single-channel conductance in Slo1 channels ~30%. We could explore if the reduced conductance occurs because the gates are only partially open by substituting tryptophan or tyrosine in the S6 gates at the entrance to the inner conduction pathway, which should give a greater decrease in conductance for the partially open gates than for fully open gates in wt channels modified with the same mutations (see rational in Geng et al., 2001). An alternative explanation for the conductance decrease might be that the gating ring electrostatically attracts K\(^+\) to the entrance of the inner conduction pathway. If this were the case, then, replacement of negatively charged amino acids in the gating ring in the general region of the Pore domain with neutral amino acids should reduce conductance in wild-type Slo1 channels (see similar approach in Brelidze et al., 2003 where removing negative charge on S6 at the entrance to inner conduction pathway decreased conductance). The homology model structure can be used to identify additional negatively charged residues in the gating ring positioned close to the Pore of the channel.

**Effect of removing the gating ring on block and selectivity**

Although changes in blocking by toxins and ion selectivity might not be expected if the gating ring is removed, the changes in conductance we observed were also not expected. Consequently, we could test for changes in blocking with TEA and iberiotoxin and changes in selectivity for K\(^+\) over other cations.

We showed that the Slo1 Core channels are blocked by 60 nM iberiotoxin and 2 mM TEA, but the block effect of both drugs on the Core channel was smaller than that observed on Slo1-wt.
However, with only a small number of experiments undertaken the difference was not statistically significant. To show if there are differences in the block we should undertake a more rigorous analysis and do dose-response curves comparing the IC₅₀’s for Core and wt. A change in blocking properties of TEA or iberiotoxin could indicate that interactions of the gating ring with the Core are allosterically altering the structure of the conduction pore in the region where block occurs (Candia et al., 1992).

In our experiments the currents and tail currents of the Slo1 Core constructs reverted very close to the equilibrium potential of potassium but we did not do a thorough analysis of the selectivity of these channels. To do that we could study the relative selectivity of K⁺ over Tl⁺, Rb⁺ or Na⁺ using standard techniques (Blatz and Magleby, 1984). A change in selectivity could indicate that an allosteric effect might progress from the S6 gates to the selectivity filter, altering its structure.

**Characterize the contribution of the gating ring to the voltage dependent gating of Slo1**

We showed that in the gating ring-less channels, the conductance-voltage (G-V) relationships are shifted to more positive potentials. The slope of the G-V relation seems to be similar to the one obtained for Slo1-wt but more experiments going to higher potentials (to reach the point of clear saturation) are needed to be sure that the slope is not changed by the absence of the gating ring. We also observed a decrease in the open time duration and the burst duration of Slo1 Core channels compared to Slo-wt. To examine if the gating ring might increase open channel stability through direct contact with the voltages sensors, the voltage sensors could be held down (deactivated) with very negative voltages in Slo1 wt channels and in Slo1C-Kv-minT channels. If mean open time is still ~six fold longer in wt channels compared to the channels without
gating rings, then this would suggest that the gating ring is not acting to increase open stability by elevating (activating) the voltage sensors. A similar series of experiments could be used to determine whether the gating ring alters the stability of the closed state.

To understand how the gating ring is affecting the voltage gating of the channel in the absence of calcium we could study the gating currents. If the allosteric transduction pathways between gating ring and the Core inhibit or facilitate the movement of charge in the voltage sensors, then a change in gating charge movement would be expected. We could explore whether the gating ring contributes any cooperativity in voltage sensor movement. If the gating ring imparts some degree of cooperativity in charge movement in the four voltage sensors, then removing the gating ring would be expected to lead to reduced amplitude and broadening of the ON gating current. For Slo1-wt channels, the ON and OFF gating charge movement occurs in multiple phases thought to be associated with various processes involved in the gating (Horrigan and Aldrich, 1999). For example, ON gating following a depolarizing step can be described with a fast component associated with the initial outward movement of charge, mainly through the movement of S4, in the voltage sensors followed by a slow component that reflects the further movement of S4 associated with gate opening (Horrigan and Aldrich, 1999). The OFF gating charge of Slo1 induced by a hyperpolarizing voltage step decays with three components: fast, thought to reflect gating charge movement in closed channels; intermediate, thought to reflect gating charge movement in open channels before they close; and slow, thought to reflect gating charge movement associated with open channels closing (Horrigan and Aldrich, 1999). We could also determine whether the gating ring changes the relationship between gating charge movement and gate opening by comparing the charge-voltage (Q-V) and G-V curves obtained from channels with and without the gating ring. The charge movement is always faster than the
activation of macroscopic currents thus, the ON gating charge precedes channels opening. The finding that this relationship changes, as well as the Q-V and G-V curves, could indicate changes in the coupling between voltage sensors and gates (Horrigan and Aldrich, 1999; Horrigan and Aldrich, 2002).

Determine the allosteric transduction pathways from the gating ring to the Core domain for Ca\textsuperscript{2+} activation of Slo1 channels

This could be done by identifying which transduction pathways are required for activation by the RCK1 and RCK2 sensors acting both individually and jointly. A number of observations suggest that there may be separate transduction pathways for each of the Ca\textsuperscript{2+} binding sites; Ca\textsuperscript{2+} binding to the RCK1 site is voltage dependent, whereas Ca\textsuperscript{2+} binding to RCK2 is not (Sweet and Cox, 2008); the RCK2 site mainly accelerates activation of channels whereas the RCK1 site influences both activation and deactivation (Zeng et al., 2005); the RCK2 site is required for allosteric facilitation of voltage sensor movement, whereas the RCK1 site is not (Savalli et al., 2012). These results indicate that Ca\textsuperscript{2+} activates Slo1 channels through at least two distinct transduction pathways that couple Core and gating ring. We could try to functionally uncouple each of the two pathways in the intact channel by site-directed mutagenesis at critical points in the transduction pathways. One pathway is likely to be through the contact between the RCK1 ridge in the gating ring and the VSD which appears to be associated with the RCK1 Ca\textsuperscript{2+} site. The second pathway arises from the RCK1-S6 linkers which appear to be associated with the RCK2 Ca\textsuperscript{2+} bowl sensor. To eliminate these pathways in channels with the gating ring, the RCK1 sensor pathway could be disrupted by mutations in regions which the crystal structure
predicts are contacts between Core and Tail (Yuan et al., 2010). The RCK2 sensor pathway can be disrupted by adding 6-12 amino acids to the RCK1-S6 linker to remove any pull on the S6 gate through the linker, as it has been done before (Niu et al., 2004). The specific transduction pathways involved can then be determined by disrupting the pathways individually or in combination until the baseline state properties of Slo1 Core are reached (this is, until the full-length mutant channel behaves as the Slo1 Core channel). If the baseline properties are not reached, then the amino acid scan may identify additional pathways that are involved. Of course there are potential problems associated with mutational analysis, disruption of potential pathways should also be made on both the Core and gating ring sides of the pathway as a check on pathway disruption versus local functional disruption.

We actually found a couple of mutations in the predicted contact region between Core and Tail, which seem to disrupt the RCK1 pathway. These mutants make the Slo1 channel completely insensitive to magnesium and reduce calcium sensitivity ~ 70%. To test if the remaining calcium sensitivity is by the activation of the RCK2 domain many experiments can be performed using the available mutants and the different properties of the two calcium binding sites.

Expanding the HA model

The expanded HA model should include the two known types of Ca\(^{2+}\) sites and their related parameters. The unmodified HA model is inconsistent with FRET measured gating ring movement in Slo1 (Miranda et al., 2013). It is likely that having the two known Ca\(^{2+}\) sites will resolve (or help resolve) this issue. The identified transduction pathways could also provide some structural basis for the HA parameters. The results presented in Chapter 2 and the
experiments proposed above should make a major contribution in quantifying the role of the gating ring to the gating of Slo1 channels in terms of an expanded HA allosteric model for gating.

Chapter 3

In chapter 3, I presented experiments demonstrating that Na\(^+\)-dependent K\(^+\) currents are a major component of the outward currents in neurons. We showed that the influx of sodium activates \(K_{Na}\) currents in different neuron types in culture. Our results suggest that the persistent sodium current and not the transient sodium current activates \(K_{Na}\) channels. Using siRNA we showed that \(K_{Na}\) currents are carried by Slo2.2 channels in medium spiny neurons of the striatum.

Determine the physiological roles of Slo2 channels

As described before, there are reports suggesting that Slo2 channels contribute to action potential repolarization and membrane excitability (Bader et al., 1985; Dryer et al., 1991; Dale, 1993; Saito and Wu, 1991). Also, they contribute to the after-hyperpolarization following an action potential or a burst of action potentials (Schwindt et al., 1989; Kubota and Saito, 1991; Safronov and Vogel, 1996; Franceschetti et al., 2003; Zhang et al., 2010). Our results show that they are a major component of the delayed outward current in different neuron types. It has been suggested that they are involved in Fragile-X mental retardation syndrome (Brown et al., 2010) and epilepsy (Barcia et al., 2012). Also they are expressed in other body tissues such as, heart and
kidney. To fully understand the role of Slo2 channels in these processes more evidence is required. Unfortunately, there are not specific blockers for Slo2 channels which would be important to study the contribution of Slo2 channels in all these processes.

Since direct and specific pharmacology on Slo2 channels is not yet available, one alternative is the use of knock out (KO) animals for Slo2 channels. As Slo2.1 and Slo2.2 are conserved and have similar sodium sensitivities there is a risk of compensatory effects of one by the other if we only knock out one of the genes. Consequently, the strategy should be to obtain both single KOs and the double mutant. In the double mutant animals we could repeat the experiments we performed in chapter 3 and it is predicted that we would not see a TTX effect on the outward currents. Also, we could study action potential duration, membrane excitability and after hyperpolarization in the neurons where Slo2 channels had been suggested to contribute to these processes. As Slo2 channels are widely expressed, it is possible that the double KO mouse will not be viable or that the loss of the Slo2 channels will be compensated by other potassium channel which could hide some phenotypes. To avoid any of the two possibilities, conditional knock outs should be constructed.

*Studying the coupling between Slo2 and voltage-dependent Na\(^+\) channels (NaV)*

Our results show that sodium entering through TTX sensitive sodium channels activates Slo2 channels in neurons. As it was discussed in chapter 3 it is likely that the Slo2 and sodium leak channels are very close together to permit the activation of Slo2 by the persistent sodium current. Many things are unknown and could be studied about this association. One important question to resolve is the identity of the NaV channel(s) responsible for carrying the Na\(^+\) currents that
activate Slo2 channels. TTX blocks many NaV channels at the concentrations we used in our experiments (NaV1.1 to NaV1.7) it is possible that only a particular NaV channel associates with Slo2 or it may be a combination of them. To determine the genetic identities of the TTX-sensitive persistent sodium current which activate Slo2 channels in different neuronal types we could use rtPCR as well as immunocytochemistry. Also, siRNA against specific NaV channels as well as pharmacology where available could be used to knock down the activity of distinct components to uncouple the system. It is possible that more than one NaV channel activates Slo2 and in addition, auxiliary subunits or scaffolding proteins may be required.

It has been reported, that Slo1 and voltage dependent Ca\(^{2+}\) channels form protein complexes (Berkefeld et al., 2006). We can study if the same type of interaction occurs between Slo2 and NaV channels by using co-immunoprecipitation. The results obtained by co-IP should be consistent with the results disrupting the system by the use of siRNA against the different NaV channels. Therefore, if a NaV channels, when knocked down by siRNA, decreases the TTX effect in a particular type of neuron, this channel should co-precipitate with Slo2 from a homogenate made from these neurons if they actually form complexes. We can also determine if other proteins co-immunoprecipitate with the complex or if the channels interact directly without the need of auxiliary subunits or other proteins. A problem that we can face while doing Co-IP experiments is that steps required for the purification of the membrane proteins may disrupt the integrity of the complexes, especially if cytoplasmic or cytoskeletal proteins are associated.
Reconstitute and study the association between NaV and Slo2.2 channels in heterologous systems

If we are able to determine the genetic identity of the NaV channels (and auxiliary subunits if needed) that form complexes with Slo2 in neurons, we could try to reconstitute the system by co-expressing these proteins in a heterologous system and study the bases of the association. In such a system it may be possible to identify the stoichiometry and the domains of the two channels involved in the complex formation by directed mutagenesis. To verify the results it may be necessary to mutate both channels (both sides of the interaction) to confirm that we are disrupting the interaction and not affecting the function of one of the channels in a way consistent with disrupting the complex. Also, it is possible, if a physical association exists, that the interaction between the two channels produces allosteric effects which alter channel gating in one or both channels. The reconstitution of a heterologous system would let us control many conditions better than in native cells and avoid unknown complexities to study all these questions.

Chapter 4

In chapter 4, I show experiments demonstrating that divalent cations inhibit Slo2.2 channels. Interestingly, most of these cations activate Slo1. The results suggest that the effect of divalent cations on Slo2.2 channels is by allosteric modification. We also showed that the effect is conserved; for the first time we cloned and functionally expressed the Drosophila Slo2 channel and demonstrate that it is activated by sodium and inhibited by divalent cations.
Structure-function studies of Slo2 to determine other residues involved in channel inhibition by divalent cations

As Zn$^{2+}$ was the most efficient divalent cation tested, we focused our attention on studying amino acid residues that are usually present in the coordination of these ions, in particular histidine and cysteine residues (Auld, 2001). We were able to identify a histidine residue that, when substituted by a glutamine residue, significantly decreases divalent cation inhibition. We could continue this line of research to identify all the residues involved in divalent cation coordination. Basically the same strategy can be followed, producing mutant channels by site directed mutagenesis and testing the effect of the mutation by divalent cation inhibition of the currents. If we succeed in finding other residues that affect divalent cation inhibition we can produce the double or triple mutants and test for the completed removal of inhibition or if the effect of these mutants is additive.

Determine the binding sites in the Tail of Slo2.2 that confer CF and pH dependence

The structure and function of Slo2 channels has been only poorly studied. There is a report of a site in RCK2 that confers Na$^+$ sensitivity to Slo2.2 (Zhang et al., 2010). It was found by sequence homology to the Na$^+$ coordination site present in the Na$^+$-dependent inward rectifier potassium channels (Ho and Murrell-Lagnado, 1999; Rosenhouse-Dantsker et al., 2009). Substitution of an aspartic acid or a histidine residue (five aminoacids downstream, D818 and H823) greatly reduces sodium affinity (Zhang et al., 2010). Remarkably, the site is not conserved in Slo2.1 channels which present very similar sodium sensitivity (Yuan et al., 2003; Bhattacharjee et al.). In addition, in the preliminary histidine screening we performed looking for the divalent cation
binding site, the substitution of the histidine (H823) did not have any effect in sodium sensitivity (not published). It would be interesting to confirm if this site is actually the Na+ coordinator in Slo2.2 and to understand why our results are in disagreement with the report of Zhang and collaborators.

The sites in the tail of Slo2 channels that confer Cl− and pH sensitivity are unknown. It has been shown that low intracellular pH decreases Slo2.2 activity (Ruffin et al., 2008). Also, we observed that application of histidine modifier diethyl pyrocarbonate, reduces Slo2.2 currents in inside-out patches mimicking the low pH effect. These two results suggest that Slo2.2 pH dependence is mediated by histidine residues in the Tail of the channel. We did some histidine substitutions when we were looking for the divalent cation binding site but none of the mutants had a clear effect in pH sensitivity. To identify the histidine residue or residues that confer pH sensitivity on Slo2.2 we could replace each conserved histidine residue by arginine and test the pH dependence of the currents in each mutant.

Both Slo2.1 and Slo2.2 have been reported to be Cl− dependent (Yuan et al., 2003; Bhattacharjee et al.). The mechanism of chloride activation of Slo2 channels is completely unknown. Remarkably, there is a group of positively charged residues in the RCK2 domain of Slo2 channels in the region that corresponds to the calcium bowl in Slo1 but, mutation or deletion of those residues did not have an obvious effect on the chloride sensitivity of nematode Slo2 channels (unpublished). In mammalian Slo2.2, chloride alone is not able to activate the channel and the channel can be activated in the absence of chloride. It is possible that instead of having a binding site, chloride actually helps to coordinate sodium producing an increase in channel activity.
Determine if Slo2.2 can be modulated by divalent cations entering the cell through other channel pathways

Among the divalent cations tested, the most effective in inhibiting Slo2.2 channels was Zn$^{2+}$ with a IC$_{50}$ close to 8 μM. Such intracellular Zn$^{2+}$ concentrations are probably never reached in the cytoplasm. However, as has been observed in calcium measurements (Fakler and Adelman, 2008), it is possible that intracellular Zn$^{2+}$ concentrations increase in microdomains (close to channels permeable to Zn$^{2+}$ or other Zn$^{2+}$ sources) high enough to modulate Slo2.2 activity. It is possible that some channels or receptors permeable to Zn$^{2+}$ co-localize with Slo2.2 channels in neurons. We can co-express Slo2.2 channels with channels that are permeable to Zn$^{2+}$ and study if the increased activity of these channels in the presence of elevated extracellular Zn$^{2+}$ decreases the activity of Slo2.2 channels (similar to the experiments done for Slo1 by Hou et al., 2009).
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