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Vascular Kv7 (KCNQ) Potassium Channels As Therapeutic Targets in Cerebral Vasospasm

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LOYOLA UNIVERSITY CHICAGO

VASCULAR Kv7 (KCNQ) POTASSIUM CHANNELS
AS THERAPEUTIC TARGETS IN CEREBRAL VASOSPASM

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR PHARMACOLOGY
AND THERAPEUTICS

BY
BHARATH K. MANI
CHICAGO, IL
MAY 2012
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To my Wife and Parents, for everything
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LIST OF ABBREVIATIONS

4-AP  4-aminopyridine
4α-PMA  4α-phorbol 12-myristate 13-acetate
4β-PMA  4β-phorbol 12-myristate 13-acetate
5HT  Serotonin
aCSF  Artificial cerebrospinal fluid
ANOVA  Analysis of variance
ATP  Adenosine triphosphate
AVP  Arginine8-vasopressin
Ba2+  Barium ion
BFNC  Benign familial neonatal convulsions
BK  Large conductance calcium-activated potassium channels
BOX  Bilirubin oxidation products
MAP  Mean arterial pressure
BSA  Bovine serum albumin
C  Capacitance
Ca2+  Calcium ion
[Ca2+]c  Cytoplasmic Ca2+ ion concentration
cAMP  Cyclic-adenosine monophosphate
CCB Calcium channel blocker
CGRPCalcitonin gene-related peptide
cGMP Cyclic-guanosine monophosphate
CICRCalcium induced calcium release
CI Chloride ion
COXCyclooxygenase
CSDCortical spreading depression
CSFCerebrospinal fluid
DAGDiacylglycerol
DMSODimethylsulfoxide
DRGDorsal root ganglia
EDTA Ethylenediaminetetraacetic acid
EC50Effective concentration 50
EGFEpidermal growth factor
ErevReversal potential
ETEndothelin
FRETFluorescence resonance energy transfer
GConductance
GmaxMaximal conductance
GABAγ-amino butyric acid
Gd³⁺Gadolinium
GPCRG-protein coupled receptor
HRHeart Rate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>I</td>
<td>Current</td>
</tr>
<tr>
<td>I-V</td>
<td>Current-voltage</td>
</tr>
<tr>
<td>I_M</td>
<td>M-currents</td>
</tr>
<tr>
<td>I_Ba</td>
<td>Voltage-sensitive barium currents</td>
</tr>
<tr>
<td>IP_3</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>K^+</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>K_ATP</td>
<td>ATP-sensitive potassium channels</td>
</tr>
<tr>
<td>K_Ca</td>
<td>Calcium-activated potassium channels</td>
</tr>
<tr>
<td>KCNQ</td>
<td>Name of gene family that encodes the Kv7 channel subunits</td>
</tr>
<tr>
<td>K_IR</td>
<td>Inward rectifier potassium channels</td>
</tr>
<tr>
<td>Kv</td>
<td>Voltage-gated potassium channels</td>
</tr>
<tr>
<td>Kv7</td>
<td>A family of voltage-gated K^+ channels encoded by the KCNQ genes</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>Na^+</td>
<td>Sodium ions</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>Po</td>
<td>Open probability</td>
</tr>
<tr>
<td>PSS</td>
<td>Physiological saline solution</td>
</tr>
<tr>
<td>Q</td>
<td>Charge</td>
</tr>
<tr>
<td>R</td>
<td>Resistance</td>
</tr>
<tr>
<td>REST</td>
<td>Repressor element 1-silencing transcription factor</td>
</tr>
<tr>
<td>RYR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>s</td>
<td>Slope</td>
</tr>
<tr>
<td>SAH</td>
<td>Subarachnoid hemorrhage</td>
</tr>
<tr>
<td>SOCE</td>
<td>Store-operated calcium entry</td>
</tr>
<tr>
<td>SPI</td>
<td>Specificity factor 1</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total internal reflection florescence</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine triphosphate</td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
</tr>
<tr>
<td>$V_{0.5}$</td>
<td>Voltage of half-maximal activation</td>
</tr>
<tr>
<td>$V_M$</td>
<td>Membrane voltage (also known as the membrane potential)</td>
</tr>
<tr>
<td>VSCC</td>
<td>Voltage-sensitive calcium channel</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
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ABSTRACT

Cerebral vasospasm, a grave sequel to subarachnoid hemorrhage (SAH), is characterized by prolonged severe constriction of arteries in the base of the brain, including the basilar artery. Spasmogens (serotonin, endothelin and vasopressin), elevated in response to SAH, induce persistent depolarization of the myocytes in the artery wall, leading to continuous influx of calcium (Ca\textsuperscript{2+}) through voltage-sensitive Ca\textsuperscript{2+} channels (VSCC) resulting in hyperconstriction (spasm). The spasm of the arteries restricts blood flow to the brain, inducing ischemic neurological deficits and consequential high morbidity and mortality. The cellular pathogenesis of cerebral vasospasm is poorly understood and the therapeutic options are limited.

Kv7 (KCNQ) potassium channels are reported to be critical determinants of resting membrane voltage in several excitable cells including neurons and vascular myocytes. Recent findings from our laboratory suggested that arginine\textsuperscript{8}-vasopressin exerts its vasoconstrictor effects on mesenteric arteries via suppression of Kv7 currents. Hence, we hypothesized that suppression of Kv7 currents by spasmogens is a central phenomenon in the pathogenesis of cerebral vasospasm and that Kv7 channel openers would be effective therapeutic agents to treat the vasospasm.

Expression of Kv7 channels evaluated by reverse transcriptase polymerase chain reaction revealed the presence of all five mammalian KCNQ channel subtypes (KCNQ1-5) in freshly isolated rat basilar artery myocytes. Kv currents recorded using the whole-
cell patch-clamp techniques were attributed to Kv7 channel activity based on their voltage dependence of activation, lack of inactivation, enhancement by flupirtine, retigabine (selective Kv7 channel activators), and inhibition by XE991 (a selective Kv7 channel blocker). XE991 depolarized the myocytes indicating that Kv7 channels are principal determinants of resting membrane voltage. Spasmogens suppressed Kv7 currents and depolarized the myocytes. The effects of spasmogens were significantly attenuated in the presence of retigabine. Celecoxib, a clinically used anti-inflammatory drug, not only enhanced Kv7 currents but also inhibited voltage-sensitive Ca\(^{2+}\) currents.

Functional responses to Kv7 channel modulators were studied in intact basilar artery segments using pressure myography. XE991 constricted basilar artery segments indicating that Kv7 channels are important determinants of contractile status. In the presence of XE991, spasmogens did not produce additive constrictor responses, suggesting that suppression of Kv7 currents is the predominant mechanism for spasmogen-induced membrane depolarization and constriction of basilar arteries. Kv7 channel activators dilated basilar artery segments pre-constricted with spasmogens, and were more effective than nimodipine, an L-type Ca\(^{2+}\) channel blocker used as standard care to relieve cerebral vasospasm in patients with SAH. Intraperitoneal administration of Kv7 channel openers, retigabine or celecoxib, significantly attenuated basilar artery spasm in rats with experimentally induced SAH. Both the Kv7 channel openers induced a transient drop in mean arterial pressure (MAP) generally lasting 30-60 min after administration, but mean 24h BP measured using radio-telemetry in normal rats was not altered.
In conclusion, Kv7 channels are expressed in basilar artery myocytes and function to maintain resting membrane voltage in basilar artery myocytes and hence oppose the constriction of the arteries. Suppression of Kv7 currents in myocytes is a mechanism by which spasmogens induce basilar artery constriction. Targeting vascular Kv7 channels using retigabine or celecoxib could provide a novel strategy to relieve basilar artery spasm without inducing hypotension.
CHAPTER 1

INTRODUCTION

Aneurysms are abnormal ballooning or outpouching of arteries at sites of weakness in the blood vessel wall. Arteries in the base of the brain are common predilection sites for aneurysms because of the vascular remodeling associated with hemodynamic stress induced by blood flow and the anatomy of the arterial system in the base of the brain. These intracranial aneurysms are very common, with a prevalence of up to 6% in the adult population. Rarely, rupture of the aneurysms occurs, which forces blood into the subarachnoid space resulting in hemorrhagic stroke. Aneurysmal subarachnoid hemorrhage (SAH) affects 30,000 Americans yearly and accounts for about 5% of all strokes (Bederson et al. 2009). The most common complication of SAH is cerebral vasospasm. As the name indicates, cerebral vasospasm is the intense constriction of cerebral arteries, mainly the large conduit arteries (basilar artery and arteries of the Circle of Willis) near the site of a ruptured aneurysm. Vasospasm is not usually detected until at least 48 hours after rupture of an aneurysm, but may be pronounced after 3 days of SAH and lasts up to 2 weeks after SAH. The consequent decrease in cerebral perfusion frequently results in stroke and death or prolonged disability in the SAH patients. Though considerable progress has been made in understanding the pathogenesis of cerebral vasospasm, the cellular events that lead to the delayed vasospasm and subsequent ischemic events remain poorly understood.
This basic science project aims to study the involvement of vascular Kv7 potassium channels in the pathogenesis of basilar artery vasospasm and to test the utility of selective Kv7 channel activators to treat cerebral vasospasm. The study utilizes laboratory rats as a model system to examine the presence and function of the channel. During the course of the project, we discovered that celecoxib, a clinically used drug, was a Kv7 channel activator. The scope of my dissertation project was therefore expanded to test celecoxib as a possible therapeutic agent to treat cerebral vasospasm. Finally, my dissertation work also attempted to provide insights into the mechanism by which celecoxib modulates Kv7 channels.

Chapter 2 of the document provides a review of the literature as background information for the proposed project. Chapter 3 states the hypotheses and research objectives of the project; scientific rationale for the stated objectives is also provided. Chapter 4 describes the methods used to test my hypotheses. Chapter 5 presents the detailed results of the experiments that were performed. Chapter 6 discusses the results, describes how the novelty of the findings fit with the existing broad range of literature, and states the advancements made with this study. Chapter 7 states the salient conclusions derived from the project. Chapter 8 documents potential future directions for the project.
CHAPTER 2
REVIEW OF LITERATURE

Arterial constriction and cerebral blood flow

Anatomy of the cerebral vasculature

The human brain is supplied with blood almost exclusively via two paired arteries- the left and right internal carotid and the left and right vertebral arteries. Three-fourths of the total blood supply is provided by the internal carotid artery and one-fourth by the vertebral artery (Scheel et al. 2000). The blood flowing through these four arteries join in an equalizing manifold of arteries called the Circle of Willis (Kramer 1912). The internal carotid artery is a branch of the common carotid artery that arises from the aortic arch. Branches of the internal carotid artery supply most of the cerebrum and terminally branch to the anterior and middle cerebral arteries, which form part of the Circle of Willis. The vertebral arteries supply the hindbrain including the cerebellum and brain stem, and portions of the cerebrum before coming together to form the basilar artery that anastamoses into the Circle of Willis.

Cerebral blood flow

Blood flow to the brain is regulated by multifarious overlapping mechanisms, which perhaps represent a natural adaptation to protect the brain from ischemia. Apart
from several humoral factors, cerebral blood flow is regulated by specialized structural and functional components that include myogenic arterial responses (cerebral blood pressure autoregulation), flow-metabolism coupling and the neurovascular unit comprised of endothelial cells, perivascular nerves and astrocytes (Peterson et al. 2011). The mechanisms that underlie the ability of the cerebral vasculature to maintain a constant blood flow despite variable mean arterial pressure (MAP), while rapidly adjusting the flow to meet the metabolic requirements of the brain, remain poorly understood.

All the aforementioned factors however modulate blood flow primarily by constriction/dilation of the arteries through which the blood flows. Arterial constriction is a function of the contractile state of the smooth muscle cells within the artery wall, which is in turn governed by intricate actions of various ion channels and the signaling cascades that affect the sensitivity of the contractile apparatus in the smooth muscle cells.

**Regulation of arterial constriction**

Vascular tone is primarily under the control of cytosolic free calcium ([Ca\(^{2+}\)]\(_{c}\)) concentration in the vascular smooth muscle cells (VSMCs). A rise in [Ca\(^{2+}\)]\(_{c}\) causes VSMC contraction by activating actin-myosin cross bridge formation. Activation of cell surface receptors by vasoconstrictor agonists or mechanical force due to flow of blood results in elevation of cytosolic calcium [Ca\(^{2+}\)]\(_{c}\). Elevation of [Ca\(^{2+}\)]\(_{c}\) above resting levels of around 100 nM leads to binding of free Ca\(^{2+}\) to an ubiquitous Ca\(^{2+}\) binding protein called calmodulin and resulting in its activation (Akata 2007b). The [Ca\(^{2+}\)]\(_{c}\) concentration
for half-activation of calmodulin is around 400 nM (Stull et al. 1998). The conformational change of calmodulin induced by Ca$^{2+}$ binding enables its binding to a serine-threonine protein kinase called myosin light chain kinase (MLCK), resulting in autophosphorylation and activation of the kinase. The active Ca$^{2+}$-calmodulin-MLCK complex catalyses the phosphorylation of serine-19 of the two 20-kDa light chains of myosin, triggering cross-bridge cycling and contraction of the VSMCs. Relaxation is induced by restoration of Ca$^{2+}$ levels and dissociation of calmodulin from MLCK, accompanied by reversal of the downstream events- dephosphorylation of myosin by phosphatases and detachment of myosin from actin (Somlyo and Somlyo 2003). The force of contraction: [Ca$^{2+}$]$_c$ ratio or the myosin light chain phosphorylation: [Ca$^{2+}$]$_c$ ratio is not linear. This suggests secondary regulation of contractile responses by several other factors that include RhoA/Rho kinase, protein kinase C and cyclic nucleotides (Somlyo and Somlyo 2003; Akata 2007a; Hirano et al. 2004; Hirano et al. 2003; Rembold 1992; Stull et al. 1991). These factors can modulate the sensitivity of the contractile apparatus, but elevation of [Ca$^{2+}$]$_c$ is the primary determinant of the contractile status of the artery.

**Regulation of cytosolic calcium concentrations in VSMCs**

An increase in [Ca$^{2+}$]$_c$ in cerebral artery myocytes, or in VSMCs in general, is brought about by either release of Ca$^{2+}$ from the sarcoplasmic reticulum (the central reservoir of Ca$^{2+}$ within the cell), or by increased influx of Ca$^{2+}$ from the extracellular space.
**Ca\textsuperscript{2+} release mechanisms**

Ca\textsuperscript{2+} release from the sacroplasmic reticulum is mediated through several channels, predominated by inositol 1,4,5-triphosphate receptor (IP\textsubscript{3}R) and ryanodine receptors (RyR) (Berridge et al. 2000). Activation of Gq-coupled seven transmembrane receptors leads to phospholipase C-mediated cleavage of the membrane-bound phosphatidylinositol 4,5-biphosphate (PIP\textsubscript{2}) to inositol 1,4,5-triphosphate (IP\textsubscript{3}), a soluble second messenger, and diacylglycerol (DAG) (Berridge and Galione 1988). IP\textsubscript{3} binds to its receptors on the sarcoplasmic reticulum to release Ca\textsuperscript{2+} from the stores. The Ca\textsuperscript{2+} released through IP\textsubscript{3} receptors induces a bimodal modulation of IP\textsubscript{3}R activity, with a low concentration (<1 \textmu M) stimulating further release and a higher concentration (>1-10 \textmu M) inhibiting it (Bootman and Lipp 1999). A continuous release of Ca\textsuperscript{2+} through IP\textsubscript{3} receptors and reuptake into the sarcoplasmic reticulum, independent of Ca\textsuperscript{2+} entry, have been reported to give rise to “Ca\textsuperscript{2+} waves” in some VSMCs (Foskett et al. 2007). These Ca\textsuperscript{2+} waves that normally occur physiologically as discrete events within the cell however may occasionally get synchronized to initiate vasomotion (Mufti et al. 2010; Peng et al. 2001). The Ca\textsuperscript{2+} waves have been reported to promote both depolarization, by activating Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels (Cl\textsubscript{Ca} channels), and hyperpolarization, by activating large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (BK channels) (Young et al. 2001), leading to enhanced or reduced Ca\textsuperscript{2+} entry through voltage-sensitive Ca\textsuperscript{2+} channels (VSCC) respectively. Activation of Gq-coupled receptors with uridine triphosphate (UTP) or norepinephrine have been shown to induce Ca\textsuperscript{2+} waves in VSMCs (Jaggar and Nelson 2000; Ruehlmann et al. 2000). However, physiological concentrations of Gq-coupled
vasoconstrictor agonists like vasopressin do not induce appreciable IP$_3$-mediated Ca$^{2+}$ release, implying minimal contribution of these events to global elevation of Ca$^{2+}$ (Byron and Taylor 1995). Therefore, the physiological significance of these waves remains uncertain (Hill-Eubanks et al. 2011).

Cytosolic Ca$^{2+}$ itself is the principal activator of RyR receptors in the sacroplasmic reticulum to release Ca$^{2+}$ by a process called Ca$^{2+}$-induced Ca$^{2+}$ release (CICR). While CICR is a predominant mechanism in cardiac myocytes, the amount of Ca$^{2+}$ required to induce CICR may be higher than what is achieved within smooth muscle cells during peak excitability, limiting the contribution of the RyR-mediated Ca$^{2+}$ release to vascular contractility (Sanders 2001). Quantal local release of Ca$^{2+}$ due to the opening of four to six RyR receptors, referred to as “Ca$^{2+}$ sparks”, activates closely juxtaposed large-conductance Ca$^{2+}$-activated K$^+$ channels (BK channels) increasing their open probability and producing hyperpolarization (Cheng and Lederer 2008; Jaggar et al. 2000). Ca$^{2+}$ overload increases the spark frequency, probably acting as a feedback mechanism to limit membrane depolarization and Ca$^{2+}$ influx through VSCC (Jaggar et al. 2000; Perez et al. 1999). Thus, RyR activation under physiological conditions seems to induce VSMC relaxation, opposite of the effect of IP$_3$ receptor activation, which is most commonly associated with VSMC contraction.

**Ca$^{2+}$ entry mechanisms**

Ca$^{2+}$ entry into the cell across the plasma membrane principally occurs through three different types of channels defined by their mechanism of activation as (i) receptor-
operated Ca\textsuperscript{2+} channels, (ii) store-operated Ca\textsuperscript{2+} channels and (iii) voltage-sensitive Ca\textsuperscript{2+} channels.

(i) **Receptor-operated Ca\textsuperscript{2+} entry:** The transient receptor potential “canonical” (TRPC) superfamily of non-selective cation channels are the predominant receptor-operated channels through which Ca\textsuperscript{2+} enters into VSMCs (Dietrich et al. 2010). These channels are not activated directly by receptor ligands, but through signal transduction events activated by Gq-coupled receptor stimulation. The functional channels are constituted by homo- or hetero-tetramers of the family members. Of the seven family members (TRPC1-7), TRPC3/6/7 are well characterized as receptor-operated channels (Maruyama et al. 2006). The channels formed by the TRPC3/6/7 subgroup are directly activated by diacylglycerol, a signal transduction intermediate in the Gq-PLC-pathway (Dietrich et al. 2005). These channels primarily conduct Na\textsuperscript{+} ions rather than Ca\textsuperscript{2+} ions. Influx of cations through these channels has been proposed as an important mechanism of membrane depolarization by vasoconstrictor agonists (Maruyama et al. 2006; Soboloff et al. 2005; Onohara et al. 2006).

The ATP-gated P2X receptor is the only direct receptor-operated cation channel that induces identifiable Ca\textsuperscript{2+} influx in VSMCs (Hill-Eubanks et al. 2011). These non-selective receptor-operated channels predominantly conduct Na\textsuperscript{+} ions, but also allow substantial influx of Ca\textsuperscript{2+} in VSMCs upon binding of ATP (Lamont and Wier 2002).

(ii) **Store-operated Ca\textsuperscript{2+} channels:** Store-operated Ca\textsuperscript{2+} entry (SOCE) is the influx of Ca\textsuperscript{2+} in response to depletion of intracellular stores. The Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} influx through SOCE serves as a mechanism to replenish Ca\textsuperscript{2+} stores. The molecular
identity of these channels in VSMCs remains controversial (Leung et al. 2008). TRPC channels, particularly TRPC1, have been identified as store-operated Ca\textsuperscript{2+} channels, accounting for at least part of the SOCE (Xu and Beech 2001; Sweeney et al. 2002; Brueggemann et al. 2006). Recent evidence, however, points to STIM1 as the transmembrane endoplasmic reticulum Ca\textsuperscript{2+} sensor that activates a cell surface channel of the Orai family to mediate the SOCE (Varnai et al. 2009). A recent study also suggested STIM1 and TRPC1 as components of SOCE (Takahashi et al. 2007). The role of SOCE in the regulation of vascular contractile status is minor under physiological conditions. SOCE however likely plays an important role in VSMC proliferation and remodeling under certain pathological conditions (Leung et al. 2008).

(iii) Voltage-sensitive Ca\textsuperscript{2+} channels: Calcium entry through voltage-sensitive Ca\textsuperscript{2+} channels (VSCC) is the primary contributor to global increase in [Ca\textsuperscript{2+}]\textsubscript{c} in VSMCs. These channels open in response to membrane depolarization induced by a variety of humoral, chemical, or mechanical stimuli. The voltage-sensitive Ca\textsuperscript{2+} currents also inactivate rapidly in response to membrane depolarization. The inactivation is both voltage- and Ca\textsuperscript{2+}-dependent. The activation and inactivation characteristics of VSCC are such that in a range of membrane voltage (around -60 to -20 mV) in which activation occurs, inactivation of the currents is incomplete. The remaining current is termed window current (Sanders 2001; Fleischmann et al. 1994). Though the magnitude of the sustained Ca\textsuperscript{2+} current within this “window” is small, the amount of Ca\textsuperscript{2+} influx driven by the steep difference in the concentration gradient is significant relative to the cell volume (Sanders 2001). Membrane depolarization of smooth muscles by about 15 mV from the
resting voltage (about -50 to -40 mV) activates VSCC and stimulates \( \text{Ca}^{2+} \) influx enough to elevate global \([\text{Ca}^{2+}]_c\) to \(\sim 300-400\) nM (Hill-Eubanks et al. 2011). VSCCs are composed of a pore-forming \( \alpha_1 \) subunit and regulatory subunits \( \beta, \alpha_2\delta \), and \( \gamma \) (Catterall 2000). The channels are classified based on the \( \alpha_1 \) subunits that encompass the pore, selectivity filter and the voltage-sensor. They are referred to as \( \text{Ca}_v1.1, \text{Ca}_v1.2, \text{Ca}_v1.3, \text{Ca}_v1.4 \) (L-type VSCC), \( \text{Ca}_v2.1 \) (P/Q-type VSCC), \( \text{Ca}_v2.2 \) (N-type VSCC), \( \text{Ca}_v2.3 \) (R-type VSCC), \( \text{Ca}_v3.1, \text{Ca}_v3.2, \text{Ca}_v3.3 \) (T-type VSCC) (Lacinova 2005). T-type channels activate at a more negative membrane voltage (around -60 mV, and hence are called low voltage-activated channels), while other types of channels activate at more positive voltages (around -40 mV, and hence are called high voltage-activated channels) (Perez-Reyes 2003). L-type and T-type \( \text{Ca}^{2+} \) channels are the most abundantly expressed subtypes in VSMCs. L-type \( \text{Ca}^{2+} \) channels inactivate relatively slowly, whereas T-type channels inactivate fast and have low unitary conductance, providing only a transient \( \text{Ca}^{2+} \) conductance. Though it is widely believed that L-type channels are the predominant \( \text{Ca}^{2+} \) channels in VSMCs, recent reports suggest that multiple types of \( \text{Ca}^{2+} \) channels are expressed in VSMCs derived from basilar artery (Nikitina et al. 2010; Navarro-Gonzalez et al. 2009).

The contribution of \( \text{Ca}^{2+} \) influx through each subtype of \( \text{Ca}^{2+} \) channel is difficult to ascertain because of several limitations, including lack of specific antagonists for certain subtypes, difficulty in knocking down a specific channel subtype without altering the expression profile of other channels, difficulty in electrophysiological isolation of currents through each subtype of \( \text{Ca}^{2+} \) channels (even though there are subtle differences
in their biophysical characteristics) and heterogeneity of expression of Ca$^{2+}$ channel subtypes between myocytes in the same artery.

Calcium influx is mediated by multiple types of VSCC, with L-type and T-type being the predominant functional channels expressed in basilar artery myocytes (Nikitina et al. 2010; Navarro-Gonzalez et al. 2009; Nikitina et al. 2007). Calcium channel blockers selectively targeting L-type Ca$^{2+}$ channels are used clinically to treat several cardiovascular disorders including hypertension, cerebral vasospasm, and angina (Kochegarov 2003).

**Electrical properties of membrane and membrane potential**

Ions are unequally distributed across the cell membrane, which acts as a charge storage device or capacitor. The electrical field or potential created by the charge separation across the membrane is given as $V= Q/C$, where $V$=voltage, $Q$=charge and $C$=capacitance. Membrane potential ($V_m$; also called membrane voltage) is the potential on the inside of the cell, equal to the difference between $V$ inside the cell and $V$ outside the cell. The normal resting membrane potential of most excitable cells is between -70 mV and -40 mV. Change in the membrane potential towards more negative voltages is termed hyperpolarization and a change towards more positive voltages is called depolarization.

Changes to the membrane voltage are governed by the simple laws of physics. As per Ohm’s law, voltage ($V$) = Current (I) * Resistance (R). The input resistance of excitable cells (with particular reference to VSMCs) is high, in the order of 5-15 GΩ, implying that even a small change in current will induce a significant change in voltage at
resting levels. At resting levels, $V_m$ is close to the reversal potential (a potential at which the current reverses direction of flow) of potassium ions ($K^+$) ($E_K = -85$ mV), indicating that under these conditions, the membrane voltage is predominantly controlled by $K^+$ current. The $Cl^-$ current, non-selective cation currents and voltage-sensitive $Ca^{2+}$ currents make only minor contributions (Nelson et al. 1990). Hence, at near resting conditions, modulation of very few $K^+$ channels would induce a significant change in membrane voltage.

**Potassium channels**

Potassium channel activity largely determines the resting membrane voltage, and thereby controls the activity of VSCCs, which conduct $Ca^{2+}$ into the cells to activate the contractile apparatus. Despite their importance as determinants of arterial smooth muscle contractility, $K^+$ channels, which constitute the largest class of ion channels, have surprisingly not emerged as clinically favored targets to modulate blood flow and blood pressure.

Four types of $K^+$ channels have been described in cerebral arteries; ATP-sensitive ($K_{ATP}$), inwardly rectifying ($K_{IR}$) $K^+$ channels, large conductance $Ca^{2+}$-activated ($B_{KCa}$), and voltage-dependent (delayed rectifier) $K^+$ channels (Faraci and Sobey 1998; Wellman 2006).

(i) **ATP-sensitive $K^+$ channels:** ATP-sensitive $K^+$ ($K_{ATP}$) channels in smooth muscle cells are formed by a 4:4 octomeric complex of pore forming Kir6.2 subunits, a member of the inward rectifier $K^+$ channel family, and SUR2B regulatory sulfonylurea
receptors (Aguilar-Bryan et al. 1998). These channels are not voltage-dependent, but
couple electrical activity of the cell to its metabolic status by activating in response to a
decrease in \([\text{ATP}]_i\) (Quayle et al. 1997). In VSMCs, \(K_{\text{ATP}}\) channels open in response to
hypoxic conditions, limiting the contractile force to conserve ATP (Daut et al. 1990).
Vasodilators that activate protein kinase A (PKA) increase the open probability of \(K_{\text{ATP}}\)
channels and vasoconstrictors that activate protein kinase C (PKC) inhibit these channels
(Quayle et al. 1997; Zhang et al. 1994; Brayden 2002; Quayle et al. 1994). The channels
are also activated by synthetic compounds such as levcromakalin, pinacidil, nicorandil,
and diazoxide, and inhibited by sulphonylureas like glibenclamide (Quayle et al. 1997).
Pharmacological evidence supports a role of \(K_{\text{ATP}}\) channels in the regulation of cerebral
blood flow (Hong et al. 1994; Rosenblum 2003). These channels may be more abundant
in smaller cerebral arteries than in larger arteries like the basilar artery (Rosenblum
2003). \(K_{\text{ATP}}\) channels were implicated in vasoconstrictor responses when VSMCs were
dialyzed in 0.1 mM ATP (Bonev and Nelson 1996). Whether these channels are active
under physiological conditions in VSMCs, however remains controversial, as
physiological concentrations (1.5 – 3 mM) of \([\text{ATP}]_i\) reduce the open probability of the
channels (Nelson and Quayle 1995).

(ii) Inwardly rectifying \(K_{\text{IR}}\) channels: Inwardly rectifying \(K^+\) (\(K_{\text{IR}}\)) channels are
tetramers of two membrane-spanning pore-forming domains encoded by \(K_{\text{IR}2.1}\) in
cerebrovascular smooth muscle cells (Zaritsky et al. 2000). Under voltage-clamp
conditions, inwardly rectifying \(K^+\) channels predominantly conduct inward currents rather
than outward currents like other \(K^+\) channels (Nelson and Quayle 1995). The voltage at
which these channels open is dependent on extracellular K⁺ concentrations. The sensitivity of Kᵢᵣ channels to extracellular K⁺ is observed experimentally by dilation of the cerebral arteries in response to a modest elevation of extracellular K⁺ concentration (Edwards et al. 1988). The rectification is due to block of the outward conductance by binding of cytosolic positively charged polyvalent ions, such as Mg²⁺ and polyamines, to the channel (Lopatin et al. 1994). Hence, membrane depolarization from resting membrane voltages results in small outward K⁺ currents, whereas hyperpolarization elicits large inward currents in VSMCs (Edwards et al. 1988). Enhanced neuronal activity increases extracellular K⁺ concentrations activating Kᵢᵣ channels expressed in cerebral artery myocytes, resulting in vasodilation and increased blood flow to meet the metabolic requirements for the enhanced neuronal activity (Dunn and Nelson 2010). Apart from the regulation of vascular tone to meet the metabolic needs of neurons, the physiological significance of Kᵢᵣ channels still remains poorly understood. Unlike most other K⁺ channels in the cerebral vasculature, activity of Kᵢᵣ was not regulated by protein kinases like, PKA, PKC, and PKG (Wellman 2006).

(iii) **Voltage-dependent K⁺ channels:** Voltage-dependent K⁺ channels are tetramers of identical or closely related pore forming six transmembrane α subunits that may be associated with 1 to 4 accessory β subunits (Cox 2005). Their versatile capacity for heteromeric assembly along with the influence of accessory subunits results in channels with diverse biophysical properties. However, most voltage-dependent K⁺ channels have similar pharmacological properties, making it difficult to determine which channels are functional in a given cell. Genes encoding voltage-dependent K⁺ channel
superfamily members constitute the largest of any ion channel family, with more than 70 genes identified in the human genome (Coetzee et al. 1999). The phylogenetic superfamily includes 8 subfamilies of Kv (Kv1-Kv6 and Kv8-Kv9), KCNQ (Kv7), and EAG (Kv10-12) subfamilies (Coetzee et al. 1999). The most commonly expressed Kv channel subunits in VSMCs are Kv1.1, Kv1.2, Kv1.4-1.6, Kv2.1, Kv9.3 (Standen and Quayle 1998). In dog basilar artery myocytes, messenger RNA (mRNA) for Kv1.2, Kv1.5, Kv2.2, Kv3.1, Kv3.4 and Kv4.3 were identified with expression of Kv1.5 and Kv2.2 being predominant (Aihara et al. 2004). Currents through these channels are distinguished from other K⁺ channels by their sensitivity to the pharmacological channel blocker 4-aminopyridine (4-AP) (Okabe et al. 1987). The open probability of these channels is voltage-dependent with an increase in open probability (Pₒ) increasing with membrane depolarization. Kv channels undergo varying degrees of inactivation with membrane depolarization depending upon the subunits involved. The voltage of half-maximal channel activation (Vₒ.5) for activation and inactivation of Kv currents in VSMCs are -9 to -6 mV and -35 (-45 to -25) mV respectively (Nelson and Quayle 1995). The Pₒ increases steeply at voltages positive to -30 mV, indicating that a window current should exist around the physiological range of membrane voltages (Nelson and Quayle 1995). The physiological role of the Kv channel varies with the vascular bed in which it is expressed. In small cerebral artery myocytes with membrane voltage around -40 mV, blockade of these channels leads to membrane depolarization and artery constriction (Albarwani et al. 2003). The resting membrane voltage in myocytes derived from most other vascular beds are more negative, ranging from -65 to -45 mV indicating that the Kv
channels in these vascular beds function to limit the membrane depolarization following a vasoconstrictor stimulus, with a relatively minor role under resting conditions. \( K_v \) channels in dog basilar arteries have \( V_{0.5} \) for activation of \(-1.3\) mV (Jahromi et al. 2008a), whereas the resting membrane voltage of basilar artery myocytes is around \(-35\) mV in dog (Jahromi et al. 2008a), and between \(-46\) and \(-60\) mV in rats (Chrissobolis and Sobey 2002; Weyer et al. 2006a). Hence, \( K_v \) channels are likely to provide negative feedback following membrane depolarization and vasoconstriction by hyperpolarizing the membrane.

**(iv) \( \text{Ca}^{2+} \)-activated \( K^+ \) channels:** \( \text{Ca}^{2+} \)-activated \( K^+ \) (\( K_{\text{Ca}} \)) channels, like \( K_v \) channels, form as tetrameric assemblies of six transmembrane pore forming \( \alpha \)-subunits (encoded by \( slo \) or \( SK \) genes) and regulatory \( \beta \)-subunits. Large-conductance \( K_{\text{Ca}} \) \( \alpha \)-subunits encoded by \( slo \) genes are expressed in VSMCs (Coetzee et al. 1999). Channels formed by these subunits exhibit large single-channel \( K^+ \) conductances (~250 pS in symmetrical \( K^+ \)), and hence are called “big \( K_{\text{Ca}} \) (BK) channels” or “maxi” channels (Nelson and Quayle 1995). The open probability of BK channels increases in response to membrane depolarization and/or increased \([\text{Ca}^{2+}]_c\). Hence, opening of BK channels serves as a negative feedback mechanism to limit the membrane depolarization and elevation of \([\text{Ca}^{2+}]_c\) that leads to vasoconstriction. The membrane hyperpolarization induced by BK channel opening also serves to limit the myogenic responses observed in resistance arteries and arterioles (Hill et al. 2010). A high \([\text{Ca}^{2+}]_c\), in the order of 1-10 \( \mu M \), is required to activate BK channels at a membrane voltage of \(-50\) mV (Hille 2001). Such high \( \text{Ca}^{2+} \) concentrations could be achieved in the subcellular environment of the
BK channels by transient local release of Ca$^{2+}$ (Ca$^{2+}$ sparks) from the sarcoplasmic reticulum through the opening of ryanodine receptors (Perez et al. 1999; Collier et al. 2000; Wellman et al. 2002). To the extent that Ca$^{2+}$ sparks occur under resting conditions, BK channels could potentially play a role in resting K$^+$ conductance (Wellman and Nelson 2003). BK channels are well studied as targets mediating the vasodilator actions of endothelin-derived relaxing factors like nitric oxide (NO) and epoxyeicosatrienoic acids (Dimitropoulou et al. 2007; Jiang et al. 1998; Kitazono et al. 1997). The channel activity is increased by elevated [Ca$^{2+}$]c, but also modulated by several different intracellular signaling intermediates activated following binding of vasoconstrictors to G-protein-coupled receptors (GPCRs) (Hou et al. 2009).

Pathophysiology and treatment of cerebral vasospasm

Subarachnoid hemorrhage

An estimated 1-5% of adults have weakened cerebrovascular blood vessel walls causing localized ballooning of arteries called cerebral aneurysm (King 1997). Most of the aneurysms are asymptomatic, but their rupture results in a devastating condition called subarachnoid hemorrhage (SAH). The extravasation of blood into the subarachnoid space is usually presented as a medical emergency with severe headache being the most common complaint, though sometimes accompanied by nausea or loss of consciousness. SAH affects as many as 30,000 people each year in North America alone and accounts for about 5% of all strokes (King 1997; Graf and Nibbelink 1974). Prognosis is disproportionately unfavorable for patients with SAH compared to ischemic
stroke (Laskowitz and Kolls 2010). SAH is typically diagnosed by non-contrast cranial computed tomography scan with the probability of detection of blood in the subarachnoid space proportional to the clinical grade and swiftness of performing the scan after SAH (Bederson et al. 2009; Vale et al. 1997). Cerebral vasospasm often occurs within a few days after SAH and is typically diagnosed by angiographic narrowing of the basilar artery or altered basilar artery blood flow as measured by transcranial Doppler ultrasonography (Zubkov and Rabinstein 2009).

Cerebral vasospasm is the leading cause of morbidity and mortality after SAH and affects 30-70% of patients with SAH (Heros et al. 1983; Kassell et al. 1985). The condition is characterized by sustained constriction of the large arteries in the base of brain – including the arteries that form the circle of Willis and the basilar artery. Though vasospasm implies reduction in diameter of the artery, in SAH, it involves a complex vasculopathy, impaired autoregulatory function, and focal reduction of cerebral perfusion to a point of causing ischemia (Keyrouz and Diringer 2007). Under normal conditions, the affected arteries account for 10-39% of resistance to blood flow to the brain (Heistad and Konotos 1983). The involvement of smaller diameter cerebral arteries in cerebral vasospasm is not clear because of the limitation of angiographic imaging. The onset of vasospasm is delayed, and usually starts 3-5 days after hemorrhage, and is sustained until it reaches a maximum between 5-14 days. The spasm resolves gradually within 2-4 weeks. Consequent to the narrowing of the arteries, poor blood perfusion distal to the affected artery causes ischemia and neurological deficits that may either resolve or result
in cerebral infarction and permanent disability or death (Keyrouz and Diringer 2007; Sehba et al. 2011).

**Etiology for development of cerebral vasospasm**

The etiology of vasospasm after SAH is not clear but appears to be multifactorial (Macdonald et al. 2007). The role of Ca\(^{2+}\) in cerebral vasospasm has been debated mainly because there is no known mechanism that causes a sustained increase in \([\text{Ca}^{2+}]_c\) over a period of several days (Hansen-Schwartz et al. 2008; Hansen et al. 2007; Tani 2002). There is evidence however that vasospasm in cerebral arteries is largely Ca\(^{2+}\) dependent and that Ca\(^{2+}\) influx is the major mechanism of elevated \([\text{Ca}^{2+}]_c\) (Wellman 2006; Tani 2002; Zuccarello et al. 1996a).

An impairment of cerebral auto-regulation has been observed in a number of experimental (Hauerberg et al. 1993; Hauerberg et al. 1995; Kamiya et al. 1983; Rasmussen et al. 1992; Takeuchi et al. 1991) and clinical studies (Lang et al. 2001; Soehle et al. 2004; Voldby et al. 1985) following SAH. The cause(s) for the impairment of the auto-regulation are difficult to ascertain but likely include a multitude of factors such as vasospasm itself, acidosis, and hypoxia after SAH (Voldby et al. 1985; Handa et al. 1992). Increased resistance due to vasospasm induced by altered neuronal and humoral influence and direct vasoconstictor action of the blood components results in reduced perfusion. Loss of auto-regulation after SAH may damage the cerebral microcirculation and increase cerebral edema.
Spasmogens involved in vasospasm

(i) Endothelin: Endothelin (ET) is a collection of endothelium derived vasoconstrictor peptides that exists as isoforms ET-1, ET-2, and ET-3. Endothelin induces its biological effects through its interaction with ET_A and ET_B receptors. ET-1 is the most potent of the isoforms and induces dose-dependent long-lasting constriction by binding to the ET_A receptors in the smooth muscle cells (Zimmermann and Seifert 1998). ET constricts rat basilar artery with the E_max that is 122% of that produced by K^+, ET has a pEC_{50} of 8.7 (Hansen-Schwartz et al. 2003b). ET is perhaps the most studied of the spasmogens that contribute to cerebral vasospasm. The involvement of ET has been demonstrated by several pieces of experimental evidence: changes in ET receptors (Hansen-Schwartz et al. 2003b; Hansen-Schwartz 2004; Hino et al. 1996), increased levels of ET in CSF and plasma (Juvela 2000; Kessler et al. 2005; Masaoka et al. 1989; Seifert et al. 1995; Suzuki et al. 2000; Yamaura et al. 1992), experimental induction of delayed vasospasm by administration of ET-1 (Asano et al. 1989; Ide et al. 1989; Kobayashi et al. 1991; Kobayashi et al. 1990), and attenuation of cerebral vasospasm by administration of ET receptor antagonists (Roux et al. 1997).

Physiologically, the vasoconstrictor actions of ET mediated by ET_A receptors in smooth muscle cells are antagonized by nitric oxide that is released by stimulation of ET_B receptors expressed in the endothelium. In cerebral vasospasm, the ET-dependent vasocontractility seems to be due to the decreased ET_B receptor function and increased sensitivity to ET_A receptor (Vatter et al. 2007; Vatter et al. 2011). Hence, ET_A selective antagonists were tested and found to attenuate vasospasm in pre-clinical studies (Roux et
al. 1997; Wanebo et al. 1998). One of the ET\textsubscript{A} antagonists, clazosentan, was found to reduce the frequency and severity of cerebral vasospasm in phase II clinical trials (Vajkoczy et al. 2005). However, a larger phase 3 placebo-controlled clinical trial failed to demonstrate a significant effect on mortality and vasospasm-related morbidity and functional outcome (Beck and Raabe 2011; Macdonald et al. 2011), indicating that ET is not the only spasmogen that contributes to vasospasm. Experimental evidence from a mouse model of SAH showed that clazosentan preserved cerebral blood flow, alleviated vasospasm, but did not reduce the neuronal injury. These data indicate that factors other than ET-induced vasospasm could still cause the neuronal injury (Sabri et al. 2010). The questionable reduction in the neurological deficits associated with administration of ET antagonists in patients were offset by adverse effects including hypotension and pulmonary complications limiting the use of these drugs to treat vasospasm (Kramer and Fletcher 2009; Vergouwen 2009).

(ii) Serotonin: Serotonin, also known as 5-hydroxytryptamine (5HT), is a well-recognized neurotransmitter and potent vasoconstrictor. 5HT exerts its physiological actions through diverse receptors comprising 7 classes with 15 subclasses (Nichols and Nichols 2008). The receptor classes are identified as 5HT\textsubscript{1-7}. All 5HT receptors except the 5HT\textsubscript{3} are G-protein coupled receptors; 5HT\textsubscript{3} are receptor-operated ion channels. 5HT induces a concentration-dependent constriction of rat basilar artery with an E\textsubscript{max} of 82% (compared to K\textsuperscript{+}) and pEC\textsubscript{50} of 7.2 (Hansen-Schwartz 2004). The 5HT receptors expressed in the rat basilar artery are 5HT\textsubscript{1B}, 5HT\textsubscript{1D}, and 5HT\textsubscript{2A} (Hansen-Schwartz 2004). 5HT\textsubscript{1B} and 5HT\textsubscript{2A} receptors mediate the constrictor responses and modulate the blood
flow through the arteries (Cambj-Sapunar et al. 2003; Nishimura 1996). The mRNA level of 5HT$_{1B}$ was found to be increased fivefold in basilar and cerebral arteries from a rat model of SAH compared to controls (Hansen-Schwartz 2004; Hansen-Schwartz et al. 2003a). The basilar artery or cerebral artery derived from SAH rats were more sensitive to the constrictor responses of 5HT and the responses were primarily mediated through 5HT$_{1B}$ receptors (Debdi et al. 1992; Mendelow et al. 1981; Nakagomi et al. 1987; Svendgaard et al. 1977). Cerebrospinal fluid 5HT levels are significantly elevated in SAH patients from a normal level of 60 ng/ml to 200 ng/ml (Chehrazi et al. 1989). Apart from the neuronal source, the majority of the 5HT increase is likely derived from aggregated thrombocytes after SAH, which contributes to the long-lasting constriction of the arteries in the brain (Allen et al. 1974; Satoh et al. 1991; Voldby et al. 1982). In summary, vasoconstriction induced by 5HT is a pivotal component in the pathogenesis of cerebral vasospasm.

(iii) Vasopressin: Vasopressin ([Arg$^8$]vasopressin; AVP) is a nine amino acid neuropeptide that exerts its physiological actions predominantly through 2 receptors- V$_1$ (V$_{1a}$, V$_{1b}$) and V$_2$ receptors. Vasoconstrictor responses of AVP are mediated through the V$_{1a}$ receptors that are widely expressed in VSMCs. The V$_2$ receptors, expressed in the renal collecting tubule, mediate the anti-diuretic effects of AVP. Plasma osmolarity is the principle stimulus for release of AVP in the circulation and CSF, but can be influenced by several other physiological and pathological conditions (Schrier et al. 1979). The normal concentration of AVP in CSF is 0.5-2.0 pg/ml in humans and 3.8-11.5 pg/ml in rats (Sorensen 1986). The concentration of AVP increases almost 3 times in the CSF of
patients during the incidence of vasospasm after SAH (Barreca et al. 2001; Jenkins et al. 1984; Mather et al. 1981). Intra cisternal injection of AVP into Brattleboro rats, that lack the capacity to produce AVP, resulted in an acute basilar artery vasospasm similar to that observed after injection of blood (Delgado et al. 1988). Rat basilar arteries showed a V1a-receptor-mediated enhancement of reactivity to AVP during the vasospasm phase after SAH (Nishihashi et al. 2005). Intravenous administration of SR49059, a V1a antagonist attenuated basilar artery vasospasm in a rat model of SAH, suggesting the participation of AVP in the development of cerebral vasospasm (Trandafir et al. 2004).

(iv) Other spasmogens: Other spasmogens including oxyhemoglobin (Asano 1999; Kolias et al. 2009; Macdonald and Weir 1991), bilirubin oxidation products (BOX) (Clark and Sharp 2006) from the hemolysate and eicosanoids (Kolias et al. 2009) also induce spasm or potentiate the constriction induced by other spasmogens. The increased activity of the spasmogens combined with the reduction in activity of physiological endothelium-derived relaxing factors like nitric oxide (Hanggi and Steiger 2006; McGirt et al. 2002; Pluta et al. 1996; Pluta 2006; Sobey and Quan 1999) likely causes the persistent spasm.

Membrane depolarization and elevation of cytosolic calcium

A continuous elevation of $[\text{Ca}^{2+}]_c$ (Kim et al. 1996; Tani and Matsumoto 2004) and myosin light chain (MLC20) phosphorylation (Tani and Matsumoto 2004; Bulter et al. 1996) has been observed in myocytes derived from spastic arteries, although one study failed to observe an increase in $\text{Ca}^{2+}$ levels (Yamada et al. 1994). Stimulation of
Gq-coupled receptors by spasmogens generates phospholipase C that hydrolyses PIP$_2$ to IP$_3$ and diacylglycerol, with IP$_3$ stimulating release of Ca$^{2+}$ from the intracellular stores (Tani 2002). However, prolonged elevation of [Ca$^{2+}$]$_c$ is sustained only by Ca$^{2+}$ influx through VSCC (Wellman 2006). Myocytes derived from basilar and cerebral arteries of rabbits or dogs with experimentally induced SAH were significantly depolarized (Jahromi et al. 2008a; Harder et al. 1987; Nystoriak et al. 2010; Waters and Harder 1985; Zuccarello et al. 1996b). Given the predominant role of Ca$^{2+}$ influx through VSCC in increasing [Ca$^{2+}$]$_c$, an increase in open-probability of VSCC in the depolarized vascular myocytes is proposed as the primary pathology in cerebral vasospasm (Wellman 2006; Marin 1988). The involvement of VSCC is supported by the beneficial effects of blockers of these channels in the treatment of cerebral vasospasm (Dorhout Mees et al. 2007; Allen et al. 1983). Nimodipine, a dihydropyridine Ca$^{2+}$ channel blocker with a preferential cerebrovascular action (Kazda and Towart 1982), remains the only recommended pharmacological treatment available to patients with cerebral vasospasm (Bederson et al. 2009). The beneficial effects of nimodipine in cerebral vasospasm however remain controversial, because unlike in animal studies, patients on nimodipine therapy have no demonstrable reduction in angiographic vasospasm in some studies (Allen et al. 1983; Jan et al. 1988), but had remarkable effects in other studies (Cho et al. 2011; Biondi et al. 2004). There are suggestions that the beneficial effect of nimodipine may be predominantly due to its direct neuroprotective effects (Allen et al. 1983; Mayberg et al. 1994).
Role of potassium channels in cerebral vasospasm

Since the membrane voltage is primarily regulated by $K^+$ conductance (Faraci and Sobey 1998), $K^+$ channels are postulated as mediators in the pathogenesis of cerebral vasospasm and have therefore been identified as possible therapeutic targets to treat the condition (Wellman 2006). $K^+$ conductance was found to be reduced in basilar arteries after SAH (Jahromi et al. 2008a; Harder et al. 1987; Jahromi et al. 2005). The roles of the major classes of $K^+$ channels expressed in basilar and cerebral arteries, $K_{ATP}$, $K_{IR}$, $BK_{Ca}$, and 4AP-sensitive-$K_V$ channels as contributors to membrane depolarization and hence cerebral vasospasm have been investigated.

$K_{ATP}$ channels expressed in basilar artery remain functional after SAH with their activity enhanced rather than inhibited. Pharmacological $K_{ATP}$ channel activators induced enhanced dilation of cerebral arteries from SAH animals (Zuccarello et al. 1996b; Sobey et al. 1996; Sugai et al. 1999). Reduced activity of the functional channels after SAH may develop due to reduced levels of endogenous $K_{ATP}$ channel activators like CGRP (Edvinsson et al. 1994; Edvinsson et al. 1990). Activation of $K_{ATP}$ channels with cromakalim relaxed pre-constricted basilar artery and attenuated basilar artery vasospasm in animal models of vasospasm (Zuccarello et al. 1996b; Sugai et al. 1999; Kwan et al. 2000; Kwan et al. 1998). However, administration of $K_{ATP}$ channel activators are associated with hypotension and tachycardia limiting its clinical utility (Spinelli et al. 1990). Alternatively, to avoid systemic hypotension, targeted delivery of CGRP to activate cerebrovascular $K_{ATP}$ channels by implantation of a time-released capsule in the CSF space (Ahmad et al. 1996; Inoue et al. 1996), adenoviral over expression of CGRP
in cerebral vasculature (Satoh et al. 2002; Toyoda et al. 2000a; Toyoda et al. 2000b) or intranasal delivery of CGRP (Sun et al. 2010) have been tested and found to be effective in animal models. Intravenous infusion of CGRP after SAH in patients failed to show any beneficial outcome in preventing neuronal deterioration, and produced consistent systemic hypotension (Bell 1995; Bell 1992). The efficacy of local delivery rather than systemic administration of $K_{ATP}$ channel activators remains to be verified in SAH patients.

Dysfunction of 4-AP-sensitive-Kv channels after SAH has been proposed as a mechanism contributing to cerebral vasospasm (Jahromi et al. 2008a; Ishiguro et al. 2006). A significant reduction in the mRNA of Kv channels- Kv2.2, Kv3.4 was observed in basilar artery myocytes derived from SAH dogs, and the changes in Kv2.2 mRNA correlated with the degree of vasospasm (Aihara et al. 2004). Protein levels of Kv2.1 and Kv2.2 channels were also significantly reduced in basilar artery myocytes of SAH dogs (Jahromi et al. 2008a). Kv currents measured from the basilar artery myocytes of SAH rats had diminished current amplitude, indicating dysfunction of Kv channels (Jahromi et al. 2008a; Jahromi et al. 2008c). Both half-activation and inactivation of Kv currents were also decreased in the myocytes (Jahromi et al. 2008c). The mechanisms that lead to the downregulation of expression of the Kv channels are not known. Oxyhemoglobin induces a tyrosine kinase-mediated endocytosis of Kv channels resulting in dysfunction of the channels (Ishiguro et al. 2006; Ishiguro et al. 2008). Tyrosine kinase activation by oxyhemoglobin involves matrix metalloprotease activation causing heparin binding of epidermal growth factor (EGF)-like growth factor shedding and increased epidermal
growth factor receptor tyrosine kinase activity (Koide et al. 2007). Kv channels have a high voltage threshold for activation ($V_{0.5} = -1.3$ mV in dog basilar arteries) (Jahromi et al. 2008a). Hence, dysfunction of Kv channels could lead to loss of a compensatory vasodilatory mechanism, potentiating the vasospasm.

The mRNA of the BK channel $\alpha$-subunit was not found to be altered in the basilar artery myocytes from SAH dogs (Aihara et al. 2004). Though mRNA of the $\beta_1$ subunit of the BK channels was found to be reduced, the protein levels were unaltered (Aihara et al. 2004). Function of the BK channels was preserved in vasospastic basilar artery myocytes from a dog model of SAH. There was no difference in the BK current density, kinetics, Ca$^{2+}$ and voltage sensitivity, single channel conductance or apparent Ca$^{2+}$ affinity after SAH (Jahromi et al. 2008c; Jahromi et al. 2008b). Heme breakdown products known as bilirubin oxidation end products (BOXs) present in the CSF after hemorrhage have been shown to reduce the open probability of BK channels in a heterologous expression system (Hou et al. 2011). The in vivo relevance of the modulation of BK currents by BOXs remains to be clarified. Pharmacological blockade of BK channels did not influence the membrane voltage in basilar artery myocytes from SAH dogs when compared to myocytes from control dogs (Jahromi et al. 2008b). Hence, the role of BK channel function in the pathogenesis of cerebral vasospasm, at least from the current understanding, is likely to be minimal.

$K_{IR}$ 2.1 mRNA was increased by more than 2-fold in basilar artery myocytes of dogs after SAH (Aihara et al. 2004; Weyer et al. 2006a). The mean conductance through $K_{IR}$ channels increased several fold, from 1.6 pS/pF to 9.2 pS/pF indicating an
upregulation of these channels (Weyer et al. 2006a). The increase in the channel expression and function indicate that $K_{IR}$ channels likely act as a compensatory mechanism to limit the depolarization induced by dysfunction of other ion channels (Aihara et al. 2004; Weyer et al. 2006a).

In summary, dysfunction of 4AP-sensitive Kv channels might be a contributing factor for the persistent membrane depolarization observed in artery myocytes after SAH though there are no available activators to use in clinical therapy. $K_{ATP}$ channel openers have been tested to relieve vasospasm, but were found to have questionable efficacy and increased the risk of hypotension. Hence, none of the $K^+$ channel subtypes known to be expressed in the cerebral vasculature has emerged as a successful target to treat cerebral vasospasm (Wellman 2006).

**Involvement of protein kinase C**

Stimulation of phospholipase C either by cell surface receptor activation or mechanical force leads to cleavage of PIP$_2$ to IP$_3$ and DAG. DAG can activate some members of the family of serine-threonine kinases, called protein kinase C (PKC). Twelve isoforms of PKC are known. They have been classified into 3 groups based on their regulation; conventional PKCs (comprised of PKC $\alpha$, $\beta$ and $\gamma$) requires both DAG and Ca$^{2+}$ for activation, novel PKCs (comprised of PKC $\delta$, $\varepsilon$, $\eta$ and $\theta$) requires DAG for activation, independently of Ca$^{2+}$, and atypical PKCs (comprised of $\lambda$, $\mu$ and $\zeta$) are insensitive to DAG, but activated by phosphatidyl serine and phorbol esters. The role of PKCs in mediating the VSMC contraction induced by cell surface receptor activation and
myogenic response is not entirely understood. PKC increases the Ca\(^{2+}\) sensitivity of the contractile apparatus, and therefore acts as a modulator to amplify the vasoconstrictor response to elevated [Ca\(^{2+}\)]\(_c\) in VSMCs (Laher \textit{et al.} 1989; Laher \textit{et al.} 1990). The kinase phosphorylates contractile proteins calponin and/or caldesmon leading to disinhibition of myosin ATPase thereby sensitizing the contractile apparatus.

The phosphorylation targets mediating the response of PKC also include ion channels, pumps and exchangers as well as other kinases (Laher and Zhang 2001). Activation of PKC induces membrane depolarization by suppression of K\(^+\) channels resulting in Ca\(^{2+}\) entry through VSCC. Several potassium channels including K\(_{ATP}\), Kv and BK channels are sensitive to activation of PKC (Standen and Quayle 1998; Aiello \textit{et al.} 1996; Minami \textit{et al.} 1993; Shearman \textit{et al.} 1989). The sustained Ca\(^{2+}\) entry and sensitization of contractile apparatus by activated PKC plays a pivotal role in initiation and maintenance of chronic vasospasm.

Activation of PKC and/or increase in DAG has been observed in vasospastic arteries undergoing experimental chronic vasospasm (Fujikawa \textit{et al.} 1999; Sato \textit{et al.} 1997; Matsui \textit{et al.} 1991a; Nishizawa \textit{et al.} 1992; Nishizawa \textit{et al.} 1995; Sako \textit{et al.} 1993). Application of phorbol ester (12-0-tetradecanoylphorbol-13-acetate), an activator of PKC induced sustained constriction of dog basilar artery that lasted 3 days mimicking cerebral vasospasm; preemptive injection of staurosporine, a PKC inhibitor abolished the constriction induced by phorbol ester (Sako \textit{et al.} 1993).

Of the known PKC isoforms, \(\alpha\), \(\delta\), \(\eta\) and \(\zeta\) were the common isoforms present in the canine basilar artery (Nishizawa \textit{et al.} 2000; Nishizawa \textit{et al.} 2001). A chronological
analysis in dog basilar artery myocytes revealed that PKCδ translocated first followed by PKCα during the development of experimental cerebral vasospasm. Hence, these isoforms are considered important for the development and maintenance of vasospasm (Nishizawa et al. 2000). Treatment with topically applied PKC inhibitors, staurosporine, and H7, attenuated cerebral vasospasm in a canine model of SAH (Matsui et al. 1991b). However, PKC inhibitors failed to show similar beneficial effects in a rabbit model of SAH (Zuccarello et al. 1996b).

**Current treatments for cerebral vasospasm**

Since Ca$^{2+}$ entry through VSCC is the principle cause for vasospasm, it is not surprising that most of the therapeutic options available today attempt to minimize the Ca$^{2+}$ entry through these channels. Nimodipine, a selective L-type Ca$^{2+}$ channel antagonist, which preferentially dilates cerebral arteries, was shown to improve clinical outcomes in multiple clinical trials (Weyer et al. 2006b). The utility of nimodipine remains controversial because the improved outcome and reduction in cerebral infarction was not always associated with a significant decrease in angiographic vasospasm (Allen et al. 1983; Mayberg et al. 1994). However, nicardipine, another L-type Ca$^{2+}$ channel that was expected to work similarly to nimodipine, did not improve the outcome in patients with cerebral vasospasm in a randomized clinical trial (Haley et al. 1994). Magnesium, which acts as a Ca$^{2+}$ channel blocker and as an intracellular Ca$^{2+}$ antagonist, was found to reduce the delayed cerebral ischemia in some clinical studies (Wong et al. 2011), but most studies failed to show any beneficial effect (Suarez 2011).
A commonly practiced medical intervention to overcome cerebral vasospasm is triple-H therapy (hypervolemia, hypertension, and hemodilution) (Lee et al. 2006; Sen et al. 2003). This is achieved by increasing the volume status in patients accompanied by administration of vasoactive drugs like dopamine or phenylephrine (Sen et al. 2003). Increased blood volume, high perfusion pressure and reduced viscosity increases the cerebral perfusion reducing the risk of ischemia. Triple-H therapy however carries a substantial risk of pulmonary edema, myocardial ischemia, and hyponatremia, not to mention the risk of re-bleeding in patients after subarachnoid hemorrhage (Sen et al. 2003; Kassell et al. 1982; Solenski et al. 1995). The efficacy and safety of the triple H therapy remains a question, but it continues to be practiced due to the paucity of effective alternatives (Dankbaar et al. 2010; Treggiari and Deem 2009).

Clazosentan, a selective ET₄ endothelin receptor antagonist, which showed promise in several animal models of SAH, did not show a significant decrease in vasospasm in a large clinical trial (Macdonald et al. 2011). Nitric oxide donors met with a similar fate, limited by significant side effects, which included hypotension and increased intracranial pressure (Fathi et al. 2011). Intra-arterial papaverine (Liu and Couldwell 2005), administration of antioxidants (Trilizad mesylate), free radical scavengers (Nicaravin), anti-inflammatory serine protease inhibitors (nafamostat mesilate), immunosuppressive agents (cyclosporine, prednisolone) have demonstrated little to no beneficial effect on outcomes (Bederson et al. 2009).
Need for new treatment options

To date nimodipine is the only drug available with proven, but limited efficacy to treat cerebral vasospasm (Keyrouz and Diringer 2007; Weyer et al. 2006b; Velat et al. 2011). The limited efficacy may be due to the presence of heterogeneous Ca\(^{2+}\) channel subunits with varying sensitivities to nimodipine (Navarro-Gonzalez et al. 2009; Nikitina et al. 2007) and increased expression of nimodipine-insensitive T-type and R-type Ca\(^{2+}\) channels in vascular myocytes following SAH (Nikitina et al. 2010; Link et al. 2008). Hence, a drug that inhibits the activation of all VSCC subtypes either directly or indirectly (e.g. by activation of K\(^{+}\) channels and prevention of the membrane depolarization that activates VSCC) would be expected to be more efficacious than nimodipine. Therefore understanding the pathophysiological mechanisms that cause membrane depolarization and vasospasm could lead to discovery of alternative therapeutic strategies (Keyrouz and Diringer 2007; Deshaies et al. 2009).

Kv7 potassium channels

The following section reviews literature on Kv7 potassium channels with the emphasis on why Kv7 channels were chosen to test as candidate channels to control VSMC excitability and contractile status of the basilar artery.

Discovery of Kv7 channels

The KCNQ voltage-sensitive K\(^{+}\) channels (Kv7 channel family) are relatively newly found members of the diverse class of K\(^{+}\) channels. KCNQ genes that express Kv7
channel subunits were cloned in the 1990s along with several other genes cloned during the molecular biology revolution. The name for the family of channels was adopted as Kv7 based on the deducted phylogenetic relationships (Gutman et al. 2005), but still widely used interchangeably with KCNQ based on the KCN nomenclature developed by Human Genome Organization (HUGO) (White et al. 1997).

The first member of this family– KvLQT1 (later renamed KCNQ1) was discovered in the heart (Sanguinetti et al. 1996), where four of the Kv7.1 subunits combine to form channels that conduct the slowly activating delayed rectifier K⁺ current ($I_{Ks}$). The interest in Kv7 channels then exploded with the identification of Kv7.2/7.3 heterotetrameric channels as molecular correlates of the “M-currents” (Wang et al. 1998), which were recognized as regulators of membrane excitability in neurons. Wang and his colleagues demonstrated currents similar to neuronal M-currents by injecting cDNA encoding KCNQ2 and KCNQ3 in Xenopus oocytes.

M-currents ($I_M$), named by virtue of their sensitivity to muscarinic receptor stimulation, were first identified in frog and rat sympathetic neurons as non-inactivating K⁺ currents, slowly activated by membrane depolarization (Adams and Brown 1980; Constanti and Brown 1981). Identification of M-currents provided the first clear demonstration of control of neuronal excitability by voltage-sensitive K⁺ currents described by several investigators since the 1960s (Kobayashi and Libet 1968; Krnjevic et al. 1971; Kuba and Koketsu 1976; Weight and Votava 1970). At present, 5 KCNQ genes (KCNQ1-5), encoding Kv7.1-7.5 channel subunits, have been cloned and channels formed by these subunits have been found to have important functions in various
excitable tissues (Jentsch 2000). A great deal of research has focused on demonstrating the role of Kv7 channels in membrane excitability of neurons, and QT-interval regulation in cardiac myocytes (Delmas and Brown 2005; Robbins 2001).

**Structure and composition of functional Kv7 channels**

The molecular composition of Kv7 channels resembles other Kv channels, with each functional channel composed of homo- or hetero-tetramers of six transmembrane spanning (S1-S6) channel subunits (Jentsch 2000). The single pore loop (P-loop) formed between the S5 and S6 domain of each subunit forms the selectivity filter of the pore and S4 acts as voltage sensor. Both the amino (N)- and carboxyl (C)-terminus of the channel subunits lie within the cytoplasm. Kv7 channels are however distinct from other Kv channels in having a long intracellular C-terminus (Delmas and Brown 2005). The C-terminus has a distinctive conserved A-domain that determines the subunit specificity of the channels (Schwake et al. 2003). Apart from conferring subunit specificity, the C-terminal domain also plays a crucial role in channel gating, assembly, trafficking and scaffolding with signaling proteins (Haitin and Attali 2008).

While all the subunits can combine among themselves to form homo-tetramers, the formation of hetero-tetramers, as elucidated by co-expression studies, are restricted to certain combinations: Kv7.3 with Kv7.2 (Wang et al. 1998); Kv7.4 with Kv7.3, but not Kv7.2 or Kv7.1 (Bal et al. 2008; Kubisch et al. 1999); Kv7.5 with Kv7.3 and Kv7.4, but not Kv7.2 (Bal et al. 2010; Schroeder et al. 2000a). Kv7.1 subunits do not heterotetramerize with other known Kv7 channel subunits. At present, there is no
evidence to suggest existence of channels comprising Kv7 channel subunits and other Kv channel subunits (e.g. 4-AP-sensitive Kv channels of the Kv1, Kv2, and Kv4 subtypes).

**Expression of Kv7 channels**

Although expression was initially thought to be restricted to neurons and cardiac myocytes, recent evidence suggests that the expression of KCNQ channels is rather ubiquitous (Brown 2008; Mackie and Byron 2008). In the heart, homotetramers of Kv7.1 subunits co-assemble with two single-transmembrane ancillary protein called minK or IsK, that alters the gating characteristics of the $K^+$ current ($I_{Ks}$) (Sanguinetti *et al.* 1996; Barhanin *et al.* 1996). MinK is encoded by the KCNE1 gene which belongs to a family of 5 genes, KCNE1 through 5 (McCrossan and Abbott 2004). Interaction of KCNQ1 with KCNE1 in the heart enhances the amplitude and slows the activation of currents (Sanguinetti *et al.* 1996). Dominant mutations in cardiac Kv7.1 channels lead to long QT syndrome (LQTS1) also called Romano-Ward syndrome, leading to potentially fatal cardiac arrhythmia (Chiang and Roden 2000; Herbert *et al.* 2002; Jespersen *et al.* 2005). A recessive mutation identified in Kv7.1 or KCNE1 subunits however leads to a severe form of the disease called Jervell and Lange-Nielsen syndrome (JLNS), where cardiac arrhythmia is associated with congenital deafness (Neyroud *et al.* 1997). The deafness is due to defects in the trans-epithelial transport of $K^+$ through Kv7.1/KCNE1 channels in the inner ear (Neyroud *et al.* 1997).

Kv7.2/7.3 heterotetramers that underlie M-currents are the predominant subtype expressed in neurons (Wang *et al.* 1998). Channels comprising Kv7.5 and Kv7.4 subunits
also add diversification to the M-currents with many regions of brain showing overlapping expression of Kv7.2, Kv7.3, and Kv7.5 subunits (Schroeder et al. 2000a; Kharkovets et al. 2000). Unlike cardiac Kv7.1 channels, neuronal Kv7 channels do not appear to interact with ancillary subunits (Jentsch 2000). Mutations in Kv7.2 subunit or rarely in Kv7.3 subunit lead to an epileptic condition in infants called benign familial neonatal convulsions (BFNC) (Biervert et al. 1998; Singh et al. 1998). Kv7.4 channels are expressed in the inner ear cochlear sensory hair cells and vestibular cells. Mutation of Kv7.4 channels leads to slow degeneration of sensory hair cells resulting in progressive deafness (Kubisch et al. 1999; Kharkovets et al. 2000).

Recently, transcripts for all known Kv7 channel subunits and KCNE ancillary subunits were found to be expressed in murine gastrointestinal smooth muscle cells, with KCNQ4, KCNQ5 and KCNE4 being the predominant transcripts (Jepps et al. 2009). The authors proposed that the channels function similarly to neuronal Kv7 channels, in stabilizing the membrane voltage and limiting the gastrointestinal contractility. Apart from smooth muscle cells, Kv7 channels are expressed in epithelial cells of the colon where Kv7.1/KCNE3 channels function in the cyclic-adenosine monophosphate (cAMP)-stimulated secretion of Cl⁻ ions (Schroeder et al. 2000b).

K⁺ currents through Kv7 channels were also shown to induce a hyperpolarizing influence in urinary bladder interstitial cells of Cajal (Anderson et al. 2009), modulating the contractile status of urinary bladder (Rode et al. 2010). Expression of Kv7 channels varied with the stage of gestation in mouse and human myometrial smooth muscle cells and was proposed as a mechanism to modulate uterine contractions during pregnancy.
Expression of Kv7 channels in vascular smooth muscle cells are described in detail in a later section of the review of literature.

**Biophysical characteristics of Kv7 currents**

Outward $K^+$ currents conducted through Kv7 channels in neurons have unique biophysical characteristics - (1) threshold of activation around the resting membrane voltage, usually around -60 mV, (2) slow voltage-dependent enhancement of currents with membrane depolarization from resting voltages, (3) lack of inactivation. These characteristics of Kv7 currents tend to oppose membrane depolarization and hence act as a brake for neuronal excitation. Suppression of the currents increases input resistance that predisposes to increase in probability of action potential firing, responsiveness to synaptic input and spike frequency in neurons (Hernandez *et al.* 2008; Marrion 1997).

**Regulation of Kv7 channels**

(i) **Receptors involved in regulation of Kv7/M-current suppression:** The distinctive biophysical characteristics of the Kv7 channels dispose these channels to be targets of various physiological regulators in excitable cells. Since the discovery of the sensitivity of M-currents to muscarinic receptor ligands (Brown and Adams 1980), the channels were found to be regulated by a plethora of G-protein coupled receptors (Adams and Brown 1980; Marrion 1997). Stimulation of substance P (Jones 1985), luteinizing hormone releasing hormone (LHRH) (Adams and Brown 1980), purinergic P2Y (Adams *et al.* 1982), β-adrenergic receptors in bullfrog sympathetic ganglia (Akasu 1988);
muscarinic and AT₁ angiotensin receptors in rat sympathetic ganglia (Constanti and Brown 1981; Shapiro et al. 1994); M1/M3 muscarinic (Halliwell and Adams 1982), 5-HT₂ serotonin (Colino and Halliwell 1987), κ and δ opioid (Moore et al. 1994), and metabotropic glutamate (Charpak et al. 1990) receptors in hippocampal pyramidal neurons were found to suppress M-currents.

(ii) **PIP₂ mediated regulation:** A perceptible feature of all the aforementioned receptors is that they are all Gq-coupled receptors. Using a wide-range of approaches from molecular to knockout mouse models, it is now established that the M-current suppression is mediated by the Gaq subunit (Caulfield et al. 1994; Haley et al. 1998; Haley et al. 2000). Second messengers downstream of Gaq subunit activation, phospholipase(PLC)-β-mediated PIP₂ hydrolysis products (IP₃, and diacylglycerol), protein kinase C (PKC), elevation of cytosolic Ca²⁺ were each proposed as possible mediators of M-current suppression (Marriott 1997). The identity of the mediator however remained elusive even after knowledge about the molecular identity of M-currents. In 2002, Hille and his colleague showed the first evidence that the suppression of currents in response to muscarinic receptor stimulation was not mediated by the hydrolysis products of PIP₂, but by the depletion of membrane-bound precursor PIP₂ itself (Suh and Hille 2002). Since then, several studies have established that PIP₂ is required to keep the Kv7 channels open and that a reduction in the membrane PIP₂ concentration is the primary mechanism of receptor-induced M-current suppression (Suh and Hille 2007; Li et al. 2005; Winks et al. 2005; Zhang et al. 2003). A strong correlation was found to exist between the apparent affinity of the Kv7 channel subunits to PIP₂ and
the divergent maximal open probability ($P_o$) of the channels. Homotetrameric Kv7.3 channels with a $P_o$ of almost unity (Li et al. 2004b) had the maximum apparent affinity; $P_o$ was in the order of Kv7.3> Kv7.2/7.3> Kv7.2> Kv7.4 channels (Li et al. 2005). Taking advantage of the differential affinities of the Kv7 subunits, a recent study constructed chimeras of Kv7.3 and 7.4 subunits to identify C-terminal inter-helix linker as the site of PIP2 action (Hernandez et al. 2008).

(iii) **Signalosomes orchestrating Kv7 channel regulation:** The specificity and efficiency of signal transduction pathways to dynamically regulate the function of several cell surface proteins are mediated by signalosomes (Negro et al. 2008). Signalosomes are discrete multifunction protein complexes that compartmentalize signaling events by bringing together the regulatory intermediates and their substrates. Several lines of evidence suggest that Kv7 channels are part of a signalosome tethered together by a scaffolding protein, A-kinase anchoring protein (AKAP)79/150 in neurons. Named after its ability to bind to protein kinase A (PKA) holoenzyme (Rubin 1994), AKAP79/150 is a family of 3 known orthologs- human AKAP79, murine AKAP150 and bovine AKAP75 (Colledge and Scott 1999). Apart from PKA, AKAP binds to protein kinase C (Klauck et al. 1996), protein phosphatase 2B or calcineurin (Coghlan et al. 1995), calmodulin (Faux and Scott 1997), phosphodiesterases (Dodge et al. 2001) in a multiprotein complex along with Kv7 channels (Delmas and Brown 2005). AKAP79/150 constitutively binds to Kv7 channels; and suppression of M-currents by muscarinic receptors were dampened by perturbation of AKAP binding to Kv7 channels (Hoshi et al. 2003; Zhang et al. 2011). The requirement of AKAP was further supported by reduced M-current suppression in
response to muscarinic receptor stimulation in the sympathetic cervical ganglion neurons of AKAP150 knockout mice (Tunquist et al. 2008). The mice were resistant to seizures induced by pilocarpine, a non-selective muscarinic agonist (Tunquist et al. 2008). The interaction of Kv7 channels to AKAP was further clarified with the evidence that AKAP79 associated with KCNQ2-5, but not KCNQ1 using fluorescence resonance energy transfer (FRET) under total internal reflection fluorescence (TIRF) microscopy in a heterologous expression system (Bal et al. 2010).

Suppression of M-currents by activation of muscarinic receptors was thought to be mediated by PIP2 hydrolysis (Hernandez et al. 2008; Suh and Hille 2002; Suh and Hille 2007; Li et al. 2005; Zhang et al. 2003; Robbins et al. 2006) independent of diacylglycerol produced during activation of PLC, or the PKC that it might stimulate (Hille 1994; Suh and Hille 2006). Later studies however showed that at least part of the muscarinic suppression of currents is mediated by an AKAP-dependent pathway that involves PKC (Hoshi et al. 2003; Higashida et al. 2005). Knockdown of AKAP79/150 or expression of dominant negative AKAP150 that cannot bind PKC showed reduced suppression of M-currents with muscarinic receptor stimulation (Hoshi et al. 2003; Zhang et al. 2011; Hoshi et al. 2005). Though the PKC bound to AKAP is in the inactive form (Klauck et al. 1996; Faux et al. 1999), the anchored pool of PKC seems to be essential for muscarinic suppression of M-currents (Hoshi et al. 2005). The mechanisms by which muscarinic receptor stimulation activates PKC in the pool anchored to AKAP79/150 remain unclear. Calmodulin, apart from constitutively binding to the channel, binds to AKAP in a Ca$^{2+}$-dependent manner (Faux and Scott 1997). Calcium-calmodulin was
shown to regulate Kv7 currents by competing with and displacing the inactive PKC bound to AKAP, facilitating its activation by diacetylglcerol (Faux and Scott 1997).

Activation of PKC induces a large positive shift (~17 mV) in the conductance-voltage curve of Kv7 currents (Nakajo and Kubo 2005). PKC likely produces this effect by direct phosphorylation of serine or threonine residues in the intracellular loop or the C-terminal domain of the Kv7 channels subunits. Serine534 and 541 in KCNQ2 and T553 in KCNQ4 have been identified as critical amino acid residues for regulation of the channel activity by PKC (Bal et al. 2010; Hoshi et al. 2003).

Depletion of PIP2 or phosphorylation by PKC however does not account for Kv7 current suppression by all Gq-coupled receptor-PLC activation. Stimulation of bradykinin B2 receptors suppresses Kv7 currents by a distinct Ca2+-dependent mechanism. B2 receptor stimulation elevates [Ca2+]c by IP3-mediated Ca2+ release. The elevated cytosolic Ca2+ interacts with calmodulin bound to the long C-terminal domain of Kv7 channels resulting in suppression of the currents (Gamper and Shapiro 2003). Kv7 currents are exquisitely sensitive to elevations of [Ca2+]c with a IC50 of 70-100 nM, slightly above the resting Ca2+ levels in neurons (Gamper and Shapiro 2003; Selyanko and Brown 1996). Ca2+-calmodulin inhibited currents through channels formed by Kv7.2, Kv7.4 and Kv7.5, but not Kv7.1 and Kv7.3 demonstrating subtle subunit specificity (Gamper et al. 2005).

There are 2 non-continuous binding sites for calmodulin in the Kv7 channel subunits, with only the apo-calmodulin but not Ca2+ bound calmodulin capable of binding to it (Wen and Levitan 2002; Yus-Najera et al. 2002). Apo-calmodulin acts as an accessory subunit for the channel and is essential for the normal opening of the channel.
Regulation of the channel is likely to be complex with perturbation of calmodulin binding reported to either reduce or increase channel activity (Gamper and Shapiro 2003; Wen and Levitan 2002). The sensitivity of the channel to Ca\(^{2+}\) requires calmodulin, but the mechanism by which calmodulin modulates the channel remains to be understood, though modulation of phosphorylation has been ruled out (Selyanko and Brown 1996). Muscarinic receptor-induced suppression of Kv7 currents is independent of Ca\(^{2+}\)-calmodulin-mediated action, as \([\text{Ca}^{2+}]_{c}\) is not elevated after muscarinic receptor stimulation presumably because the muscarinic receptors and IP\(_3\) receptors exist in distinct “microdomains”. Hence, IP\(_3\) produced by PIP\(_2\) hydrolysis does not reach threshold levels near the endoplasmic reticulum to release Ca\(^{2+}\) through IP\(_3\) receptors (Delmas \textit{et al.} 2004). Apart from its role in regulation of Kv7 channels in the membrane, calmodulin appears to be a critical regulator for trafficking of the channel to the membrane (Etxeberria \textit{et al.} 2008; Alaimo \textit{et al.} 2009).

Tyrosine phosphorylation of Kv7 channels by a non-receptor tyrosine kinase Src also suppresses the currents by reducing the open probability of the channels. Src phosphorylation is subunit selective for Kv7.2, 7.3, Kv7.2/7.3 and 7.5 subunits (Gamper \textit{et al.} 2003). The tyrosine residues in Kv7.3 subunits were mapped as Y67 in the N terminus and Y349 in the C terminus, with simultaneous phosphorylation of both the residues a requirement for regulation by Src (Li \textit{et al.} 2004a). Src phosphorylation was however not a mechanism of M current suppression by muscarinic receptor activation in sympathetic neurons (Gamper \textit{et al.} 2003), and the physiological significance of Kv7 channel phosphorylation by Src is uncertain. Activation of receptor tyrosine kinases like
epidermal growth factor receptor (EGFR) and nerve growth factor (NGF) have also been reported to suppress Kv7 currents, likely through a tyrosine phosphorylation mechanism (Jia et al. 2007; Jia et al. 2008).

(iv) Transcriptional regulation: Most studies until recently focused on the regulation of channels present in the plasma membrane. Recently KCNQ2 and KCNQ3 genes were found to be regulated in dorsal root ganglia (DRG) neurons by two well known transcription factors- sp1 (specificity factor 1), a transcriptional activator and REST (Repressor element 1-silencing transcription factor), a transcriptional repressor (Mucha et al. 2010). Overexpression of REST reduced M-current density and increased excitability of DRG neurons, highlighting the significance of transcriptional regulation of Kv7 channels. Neuronal expression of REST increased in response to inflammatory mediators and correlates with a reduction in Kv7.2 subunit expression (Mucha et al. 2010). REST mediated transcriptional downregulation of Kv7.2 channel expression has been proposed as a mechanism for neuropathic pain (Rose et al. 2011). Peripheral application of flupirtine, a Kv7 channel opener, was able to alleviate neuropathic hyperalgesia in a partial sciatic nerve ligation rat model (Rose et al. 2011), possibly by activating the reduced population of Kv7 channels and opposing the membrane depolarization in neurons (Passmore and Delmas 2011).

Pharmacology of Kv7 channels

A number of pharmacological agents are available that modulate either all Kv7 channel subtypes or channels formed by select combinations of Kv7 subunits.
Linopirdine [DuP 996, 1,3-dihydro-1-phenyl-3,3-bis(4-pyridinylmethyl)-2Hindol-2-one], a phenylindolinone derivative originally developed as a cognitive enhancer and neurotransmitter release enhancer (Nickolson et al. 1990) was subsequently found to be an M-channel blocker (Aiken et al. 1995). The drug is selective for Kv7 channels and was without appreciable effects on other Kv channels (Wang et al. 1998). The IC50 for linopirdine block of pan-Kv7 channel varies between 1.2 - 36 µM depending on the heterogeneity in the molecular composition of the channels (Brown et al. 2002; Wickenden et al. 2001). Linopirdine and its potent analog XE991 [10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone] are not used clinically because of their epileptogenic effects produced due to the blockade of Kv7 channels (Zaczek et al. 1998). Linopirdine and XE991 are however used extensively in the laboratory for experimental purposes usually at concentrations \( \leq 10 \) µM as selective irreversible blockers of Kv7 channels (Schnee and Brown 1998; Wladyka and Kunze 2006). XE991 also moderately blocks other Kv channels reversibly albeit only at voltages positive to +20 mV (Zhong et al. 2010).

Flupirtine [D-9998, Katadol®], Ethyl 2-amino-6-((p-fluorobenzyl)amino)-3-pyridinecarbamate] is a triaminopyridine, clinically used as a non-opioid analgesic in Europe since 1984 even before the discovery of Kv7 channels. Though the analgesic effect was earlier thought to be due to its N-methyl-D-aspartate (NMDA) receptor-blocking and \( \gamma \)-amino butyric acid (GABA\(_A\)) receptor-potentiating effects, it was later found that the analgesic activity was due to its ability to enhance \( I_M \) currents within the therapeutic range (2-6 µM) (Kornhuber et al. 1999; Martire et al. 2004). Structural
modification of flupirtine yielded retigabine [known as ezogabine in United States; D-23129, ethyl N-[2-amino-4-[(4-fluorophenyl)methylamino]phenyl]carbamate], which was 10-30 times more potent as a Kv7 channel activator compared to its N-methyl-D-aspartate (NMDA) receptor-blocking and γ-amino butyric acid (GABA\textsubscript{A}) receptor-potentiating effects. Retigabine and flupirtine activate all channels formed by Kv7.2-7.5 subunits through interaction with residues in the S5 and S6 domains of which a tryptophan residue in S5 is a key element. Retigabine and flupirtine do not activate channels formed by Kv7.1 subunits, which lack the tryptophan residue in the S5 domain (Bentzen \textit{et al.} 2006; Schenzer \textit{et al.} 2005). Apart from increasing the open probability of the channel, retigabine also induces a hyperpolarizing shift in activation of Kv7 currents (Tatulian \textit{et al.} 2001; Tatulian and Brown 2003).

Recently, compounds were developed to target specific subtypes of Kv7 channels; ICA-27243 [N-(6-chloro-pyridin-3-yl)-3,4-difluoro-benzamide] selectively activates heteroteric channels formed by Kv7.2/7.3 subunits (Wickenden \textit{et al.} 2008); S(1) [(S)-N-[1-(3-morpholin-4-yl-phenyl)-ethyl]-3-phenyl-acrylamide [(S)-1]] preferentially activates channels formed by Kv7.4 and Kv7.5 subunits, but blocks homomeric channels formed by Kv7.1 subunits (Bentzen \textit{et al.} 2006; Wu \textit{et al.} 2003) and BMS-204352 selectively activates channels formed by Kv7.4 and Kv7.5 subunits (Dupuis \textit{et al.} 2002; Schrøder \textit{et al.} 2001). The binding site of ICA-27283 is in the S1-S4 voltage sensor domain rather than the pore domain in S5-S6 segment as observed with other non-selective Kv7 channel openers (Padilla \textit{et al.} 2009). The experiments described in this
dissertation utilize the prototypic commonly used Kv7 channel activators- flupirtine, retigabine and the inhibitor- XE991 at concentrations selective for Kv7 channels.

**Kv7 channels as therapeutic targets**

With increasing recognition that a number of human diseases involve alterations in Kv7 channel function, a variety of pharmacological modulators were developed to target Kv7 channels in the nervous system (Dalby-Brown *et al.* 2006). Flupirtine has been used for almost 3 decades in Europe as an analgesic with muscle relaxing properties to treat cancer, postoperative or traumatic pain without developing tolerance (Miceli *et al.* 2008). The more potent analog of flupirtine, retigabine is likely to become available to treat partial onset seizures in adults (Gunthorpe *et al.* 2012; Harris and Murphy 2011). ICA-27283 is under development to treat cortical spreading depression associated with migraine (Wu *et al.* 2003). Kv7 channel openers also hold promise to treat other diseases with disorders of excessive excitability such as anxiety (Korsgaard *et al.* 2005), neuropathic pain, cognitive disorders, urinary incontinence (Wickenden *et al.* 2004), Alzheimer’s disease and stroke (Czuczwar *et al.* 2010).

**Kv7 channels in vascular smooth muscle cells**

The first evidence of existence of Kv7 channels in VSMCs was provided by Ohya et al., who showed the expression of KCNQ1 transcripts in murine portal vein myocytes. They demonstrated a linopirdine-sensitive outwardly rectifying K⁺ current in the presence of 5 mM 4-aminopyridine (Ohya *et al.* 2003). The contribution of Kv7 channels to
membrane voltage in the myocytes was demonstrated in a follow up study from the same laboratory. Kv7 channel blockers, linopirdine and XE991 depolarized the membrane and augmented the spontaneous contractile responses in the portal vein (Yeung and Greenwood 2005). Using an analytical subtraction method the authors distinguished the voltage-step evoked currents through Kv7 channels from 4AP-sensitive Kv currents (Yeung and Greenwood 2005). Another major advancement was made by Brueggemann et al., who isolated Kv7 currents in cultured rat embryonic aortic smooth muscle (A7r5) cells and demonstrated the expression of KCNQ5 mRNA transcripts in both rat aortic smooth muscle cells and A7r5 cells, but KCNQ1 transcripts only in rat aortic smooth muscle cells and not in A7r5 cells (Brueggemann et al. 2007). Since then several studies have demonstrated the expression of KCNQ channels in different vascular beds, mesenteric, carotid, pulmonary, cerebral, femoral artery and aorta showing a consensus in the expression of KCNQ1, KCNQ4 and KCNQ5 mRNA transcripts with little or no expression of KCNQ2 and 3 (Zhong et al. 2010; Joshi et al. 2009; Mackie et al. 2008; Yeung et al. 2007).

A pivotal role of vascular Kv7 channels in the pulmonary arterial system was demonstrated by a concentration-dependent constriction of rat intrapulmonary artery by the Kv7 channel blockers, independent of endothelium or neuronal intervention (Joshi et al. 2006). The constriction produced was dependent on activation of voltage-sensitive Ca$^{2+}$ channels (Joshi et al. 2006) as a corollary to the membrane depolarization of pulmonary artery myocytes induced by Kv7 channel blockers (Joshi et al. 2009).
The expression and function of the Kv7 channels in human arteries were found to be similar to the rodent models validating the role of Kv7 channels in regulating vascular contractility in humans (Ng et al. 2011). Kv7.1 channels, whose expression was earlier thought to be restricted to heart was found to be expressed in all rodent and human vascular myocytes studied so far. However, K^+ channels formed by Kv7.1 subunits are not likely important contributors to regulation of vascular reactivity. The conclusions are based on the ability of retigabine and acrylamide S1 to dilate the pre-constricted rodent or human arteries and the inability of selective Kv7.1 channel blockers, chromanol 293B, L-768673 to constrict rodent or human arteries (Joshi et al. 2009; Mackie et al. 2008; Yeung et al. 2007; Yeung et al. 2007; Ng et al. 2011; Ng et al. 2011). Although channels formed by Kv7.1 subunits do not appear to contribute to the resting vascular tone, selective activation of these channels dilated pre-constricted arteries indicating that these channels may function in VSMCs under some conditions (Chadha et al. 2012).

The outwardly rectifying currents conducted through Kv7 channels isolated from A7r5 cells and mesenteric artery myocytes showed a threshold for voltage-dependent activation negative to -60 mV (Brueggemann et al. 2007; Mackie et al. 2008). Thus, Kv7 channels activate at resting membrane voltages, negative to the threshold for activation of the voltage-sensitive Ca^{2+} channels (~-40 mV), providing a hyperpolarizing influence that will tend to oppose the activation of VSCC. Kv7.5 channels are the only subtype of channels present in A7r5 cells (Brueggemann et al. 2007). Knockdown of the channels using an adenoviral vector expressing KCNQ5 short hairpin(sh)-RNA induced persistent membrane depolarization, triggering repetitive spontaneous Ca^{2+} spiking in A7r5 cells.
The Ca$^{2+}$ spiking responses observed in A7r5 cells were dependent on membrane depolarization and activation of VSCC (Byron and Taylor 1993). Hence K$^+$ current through Kv7 channels acts as a physiological “sub-threshold brake” in regulating Ca$^{2+}$ entry through VSCC and thereby determining the contractile status of VSMCs (Figure 2). The very negative threshold for voltage-dependent activation distinguishes Kv7 channels from other voltage-sensitive K$^+$ channels previously proposed to regulate vascular tone. These distinct properties of Kv7 channels may also enable them to serve as regulators of excitation of VSMCs.

Suppression of Kv7 channel activity has been proposed as a mechanism by which the vasoconstrictor hormone vasopressin induces its physiological constrictor effects (Brueggemann et al. 2007; Mackie et al. 2008). The suppression of Kv7 currents by vasopressin was dependent on protein kinase C activation (Brueggemann et al. 2007; Mackie et al. 2008), a common signal transduction intermediate of G$q/11$-coupled-receptor activation. Activation of protein kinase A has been shown to enhance the activity of certain Kv7 channel subtypes in expression system (Schroeder et al. 1998; Chambard and Ashmore 2005). If this is true in VSMCs, it is likely to induce vasodilation, for example in response to activation of G$s$-coupled $\beta$-adrenergic receptors. Kv7 channels are therefore well placed to function as common signal transduction effectors to regulate vascular tone in response to vasoconstrictors/dilators. The roles of signal transduction intermediaries including PIP$_2$ or Ca$^{2+}$-calmodulin in regulating Kv7 channels in VSMCs remain to be tested.
Figure 1: Knockdown of Kv7.5 channel depolarizes the membrane and induces Ca\(^{2+}\) spiking response in A7r5 cells. A. Effect of Kv7.5 channel knock down on membrane voltage measured in single cells using patch-clamp technique. Knockdown of Kv7.5 channels by expression of KCNQ5 shRNA was confirmed by immunofluorescence and abolishment of Kv7 currents (not shown). *Significant difference in membrane voltage between control cells (n=6) and cells expressing KCNQ5 shRNA (n=9) using Student’s “t” test, p<0.005. B. Representative charts show Ca\(^{2+}\) spiking in control A7r5 cells, cells expressing KCNQ5 shRNA or scrambled shRNA, measured using Ca\(^{2+}\) binding Fura-2 florescent dye. [Reproduced from Mani BK et al., Cell Calcium, 2009; 45(4): 400-11].
Figure 2: Kv7 channels act as a sub-threshold brake to prevent activation of voltage-sensitive Ca\textsuperscript{2+} channels. Outward K\textsuperscript{+} conductance through Kv7 channels (yellow color) maintains the membrane voltage (V\textsubscript{m}) negative to the threshold for activation of VSCC (red color) in vascular smooth muscle cells (VSMCs; top left). Current-voltage (I-V) relationship (right) shows the activation of Kv7 currents (yellow line) at voltages around the resting V\textsubscript{m} (rectangular column). Kv7 currents maintain V\textsubscript{m} negative to the threshold (~-40 mV) for activation of voltage-sensitive Ca\textsuperscript{2+} currents (broken red line). Inhibition of the Kv7 currents depolarizes the membrane to voltages more positive to -40 mV, activating the steeply voltage-dependent VSCC to allow Ca\textsuperscript{2+} influx and the ensuing contraction of VSMCs (bottom left). [Reproduced from Mani BK and Byron KL., British Journal of Pharmacology, 2011; 162: 38-41].
Cardiovascular effects of cyclooxygenase inhibitors

Cyclooxygenase (COX)-2 inhibitors

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed drugs to treat inflammation and pain. NSAIDs exert their anti-inflammatory effect by blocking cyclooxygenase enzymes. These enzymes catalyze the biotransformation of arachidonic acid to prostaglandin endoperoxide intermediates PGG2 and PGH2. These intermediates are then converted to a variety of prostanoids including prostaglandins, thromboxanes, and prostacyclins by action of isomerases and synthases. After purification of cyclooxygenase-1 (COX-1) several years ago (Hemler and Lands 1976), a second isoform of the enzyme (COX 2) was discovered (Fletcher et al. 1992; Xie et al. 1991) with a distinct expression and distribution pattern (Willoughby et al. 2000). COX-1 was recognized as a constitutive form of the enzyme, and COX-2 as an inducible isoform that catalyzes the production of prostaglandins involved in inflammation and cancer (Grosser et al. 2006). It is appreciated that labeling COX-1 as “constitutive” and COX-2 as “inducible” form is an oversimplification. However, it is more certain that COX-2 is the predominant form elaborated during inflammatory responses (FitzGerald and Patrono 2001).

An approach called the “COX-2 hypothesis” lead to selective targeting of the COX-2 isoform to treat inflammation and pain. The concept gained traction in an effort to avoid the adverse gastrointestinal intolerance associated with COX-1 inhibition. The gastrointestinal complication associated with COX-1 inhibition was thought to be due to the reduced COX-1 derived PGE2 and PGI2 in gastrointestinal epithelium that protects
the mucosa from acid irritation. Though there is some evidence that constitutive actions of COX-2 are also involved in the mucosal inflammation and ulcer healing, the incidences of GI complications were fewer with COX-2 selective inhibitors compared to the non-selective COX inhibitors. Three selective COX-2 inhibitors, celecoxib (Celebrex™, Pfizer, New York, NY), rofecoxib (Vioxx™, Merck, Whitehouse station, NJ) and valdecoxib (Bextra™, Pfizer, New York, NY) were approved within a short span between 1998 and 2001, based on data from small short-term clinical studies (Psaty and Furberg 2005).

**Cardiovascular risk with COX-2 inhibitors**

After only a few years into the market, rofecoxib and valdecoxib were withdrawn (in 2004 and 2005 respectively) due to observations of an increased incidence of myocardial infarction and stroke with prolonged use. However, celecoxib along with other “traditional” non-selective COX inhibitors continue to be available to treat inflammatory pain, but they are marketed with a “black box” warning to highlight the small risk of myocardial infarction and stroke associated with these classes of drug (Grosser et al. 2006). The increased cardiovascular risk with use of selective COX-2 inhibitors has been proposed to be due to increase in synthesis of thromboxane A2, a potent platelet activator and vasoconstrictor and reduction in the synthesis of vasodilatory and antiaggregatory prostacyclins (Mukherjee et al. 2001).
Divergence in cardiovascular risk profile among COX-2 inhibitors

A systematic review and meta-analysis of controlled observational studies revealed a dose-related cardiovascular risk with rofecoxib and diclofenac (another NSAID, that was found to have COX-2/COX-1 selectivity similar to celecoxib), but not with celecoxib at commonly used doses (Juni et al. 2004; McGettigan and Henry 2006; Zhang et al. 2006). The heterogeneity in the risk profile despite belonging to the same class of drugs remains poorly understood. The divergence was attributed to differences in pharmacokinetic and pharmacodynamic properties, altered metabolism, inhibition of carbonic anhydrase and ensuing diuresis (FitzGerald and Patrono 2001; Aw et al. 2005; Knudsen et al. 2004; Weber et al. 2004). A meta-analysis of nineteen randomized controlled trials involving 45,451 participants revealed an increase in blood pressure with COX-2 inhibitors (Aw et al. 2005). A consistently greater increase in systolic and diastolic blood pressure was observed with rofecoxib in head-to-head trials vs celecoxib, consistent with the increased cardiovascular risk observed with rofecoxib (Aw et al. 2005; Whelton et al. 2001; Whelton et al. 2002). A disproportionate rise was observed in systolic blood pressure, widening the pulse pressure with rofecoxib. A steep relationship observed between systolic BP and incidence of cardiovascular complications in the Framingham study (Kannel 2000a; Kannel 2000b) indicates that the change in blood pressure could be a single large risk factor responsible for the increased incidence of cardiovascular events associated with rofecoxib.
Vasodilatory effects of celecoxib

Several recent studies demonstrated vasodilatory effects of celecoxib, but not rofecoxib (Hermann et al. 2003; Klein et al. 2007; Widlansky et al. 2003) indicative of a vasodilatory compensatory mechanism associated only with celecoxib opposing the COX-2 mediated increase in blood pressure. The vasodilatory effect of celecoxib in humans was dependent on the endothelial function (Widlansky et al. 2003; Chenevard et al. 2003). Using guinea pig coronary artery and aortic rings, Klein et al., suggested that phosphodiesterase (PDE) 5 enzyme inhibition and amplification of endothelium-dependent nitric oxide/ cyclic-guanosine monophosphate (cGMP) signaling as a mechanism for the vasodilatory effects of celecoxib (Klein et al. 2007). The study also revealed that celecoxib, but not rofecoxib inhibited PDE isoforms 4 and 5 in VSMCs at low micromolar concentrations. A recent study from our laboratory provided evidence that celecoxib dilated rat mesenteric artery independently of endothelial actions by activation of Kv7 channels and inhibition of VSCC in arterial myocytes (Brueggemann et al. 2009). It remains to be seen if the ion channel modulatory effects of celecoxib is due to inhibition of the PDE enzymes.

Vascular effects of phosphodiesterase enzyme inhibition

Phosphodiesterase enzymes

Phosphodiesterases are isoenzymes that cleave the phosphodiester bond in the second messenger molecules cAMP and cGMP. Based on the amino acid sequence, the overall domain structure, catalytic and regulatory characteristics, 11 distinct PDE enzyme
families (PDE1-11) are known to exist (Omori and Kotera 2007). The PDE enzymes differ considerably in their substrate specificity. The cAMP-specific enzymes include PDE4, 7 and 8; the cGMP-specific enzymes include PDE 5, 6 and 9, while PDE1, 2, 3, 10 and 11 cleave both cAMP and cGMP (Omori and Kotera 2007; Maurice et al. 2003).

cAMP and cGMP are key components of intracellular signaling regulating numerous cellular functions including metabolism, contractility, motility and transcription in almost all cells including VSMCs (Maurice et al. 2003; Antoni 2000; Klein 2002). Modulation of PDE enzyme activity is therefore expected to influence a myriad of cell signaling pathways in diverse cell systems. The relative expression of the isoenzymes across cell types confer some selectivity to target specific systems.

**Vasodilatory effects of phosphodiesterase enzyme inhibition**

Of the known PDE isoforms, PDE1, PDE3, PDE4 and PDE 5 are the predominant enzyme families expressed in VSMCs (Polson and Strada 1996; Rybalkin et al. 2003). PDE4 and PDE5 are targeted pharmacologically to treat several clinical conditions. PDE4 inhibitors (eg. rolipram, rofulimilast) are used to treat asthma and chronic obstructive pulmonary disease (O'Byrne and Gauvreau 2009). PDE5 inhibitors (eg. Sildenafil, tadalafil) are used to treat erectile dysfunction and pulmonary hypertension (Arif and Poon 2011; Sandner et al. 2007). A common observation with the inhibition of PDE4 and 5 isoenzymes is a slight reduction in blood pressure owing to its ability to dilate arteries (Jackson et al. 1999; Kloner 2004; Reffelmann and Kloner 2005; Webb et al. 1999). The signal transduction mechanisms that lead to relaxation of VSMCs in the arteries (Atalay
et al. 2006; Gokce et al. 2010; Han et al. 2012; Inoha et al. 2002; Khajavi et al. 1997; Nishiguchi et al. 2010) are not clearly understood. Activation of $K^+$ channels and blocking of $Ca^{2+}$ currents have been proposed as a mechanism of relaxation induced by PDE inhibitors, with activation BK currents shown to contribute at least in part to the enhanced $K^+$ conductance (Bardou et al. 2002; Fellner and Arendshorst 2010; Prieto et al. 2006; Salom et al. 2008).

Cerebral vasospasm and phosphodiesterase enzymes

Several studies have proposed inhibition of PDE5 or PDE3 by using milrinone or sildenafil respectively as an effective therapeutic strategy to treat cerebral vasospasm (Atalay et al. 2006; Gokce et al. 2010; Han et al. 2012; Inoha et al. 2002; Khajavi et al. 1997; Nishiguchi et al. 2010). Vasospastic basilar artery isolated from SAH dogs showed robust increased expression of PDE5 isoenzyme compared to control non-spastic arteries (Inoha et al. 2002). Milrinone, a selective inhibitor of PDE3 that hydrolyses both cAMP and cGMP has been shown to attenuate cerebral vasospasm in human subjects (Arakawa et al. 2001; Arakawa et al. 2004; Fraticelli et al. 2008; Shankar et al. 2011). Knowledge of downstream targets, ion channels in particular that regulate the contractile response mechanisms can facilitate identification of pharmacological targets to attenuate cerebral vasospasm.
CHAPTER 3
RESEARCH OBJECTIVES AND RATIONALE

Significance of the project: The project aims to identify the functional existence of Kv7 channels in basilar artery myocytes. Identification of the function of these channels is expected to reveal the mechanism(s) involved in the pathogenesis of vasospasm and potentially identify therapeutic targets to treat cerebral vasospasm.

Main hypothesis: Suppression of Kv7 currents by spasmogens is a central phenomenon in basilar artery vasospasm after SAH; direct Kv7 channel openers will reverse basilar artery spasm (Figure 3).

The hypothesis was tested by pursuing the following specific aims.

Specific aim 1: To record Kv7 currents from freshly isolated rat basilar artery myocytes and to determine whether spasmogens – ET-1, 5-HT, and AVP suppress Kv7 currents using whole cell electrophysiology.

Rationale: Previous studies identified membrane depolarization and Ca^{2+} influx through VSCC in myocytes as the primary mechanism for elevation of [Ca^{2+}]_{c} in vasospastic arteries. Outward currents through Kv7 channels, as shown in neurons and mesenteric artery myocytes, stabilize membrane voltage of excitable cells. The existence and possible involvement of the Kv7 channels in membrane voltage regulation in basilar
Figure 3: Hypothetical role of Kv7 channels in the pathogenesis of basilar artery vasospasm. We hypothesize that Kv7 (KCNQ) channels are expressed in basilar artery myocytes and function to maintain the resting membrane potential. Spasmogens elevated in response to SAH bind to cell-surface receptors to suppress Kv7 currents through a signal transduction mediated mechanism. Suppression of Kv7 currents induces membrane depolarization (ΔV_m), which activates voltage-sensitive Ca^{2+} channels (VSCC) leading to continuous influx of Ca^{2+} resulting in vasospasm. Direct Kv7 channel activators may be able to oppose the actions of spasmogens to relieve the vasospasm.
artery myocytes will help to elucidate the mechanisms involved in development of vasospasm. Knowledge of the ability of Kv7 channel openers to reverse the spasmogen-induced suppression of currents will provide evidence at the cellular level about the feasibility of Kv7 channel openers to reverse vasospasm. Apart from utilizing selective Kv7 channel openers, the study also utilized celecoxib, a recently reported dual Kv7 channel opener and VSCC blocker. The drug was tested as a possible therapeutic agent to relieve vasospasm, as it is expected to be more effective because of its dual ion channel modulatory action.

PKC is a common signal transduction intermediate in the constriction induced by spasmogens. Activation of PKC in myocytes derived from vasospastic myocytes is reported in several studies. Hence, experiments were undertaken to test if activated PKC suppresses Kv7 currents.

Experiments: Sprague-Dawley rats were used as a model to test our hypothesis. Myocytes derived from basilar artery, one of the major conduit arteries in the base of brain, which undergoes vasospasm after SAH were used in the studies. Single-cell patch-clamp electrophysiology techniques were utilized to isolate and identify Kv7 currents and to test their role in regulating membrane voltage in rat basilar artery myocytes. The following experiments tested our hypothesis at a cellular level:

1. Isolation and identification of Kv7 currents based on biophysical characteristics and their modulation by selective Kv7 channel modulators.
2. Sensitivities of Kv7 currents to spasmogens involved in vasospasm were examined.
3. Effects of Kv7 channel blockers and spasmogens on membrane voltage were tested.
(4) Abilities of direct Kv7 channel openers to reverse the spasmogen-induced suppression of Kv7 currents and membrane depolarization in basilar artery myocytes was tested.

(5) Effect of celecoxib on Kv7 currents and voltage-sensitive Ca^{2+} currents in basilar artery myocytes was examined.

(6) The effect of PKC activation on Kv7 currents was tested.

**Specific aim 2:** To evaluate the ability of Kv7 channel opener(s) to reverse/prevent the vasoconstriction induced by spasmogens in pressurized rat basilar arteries.

Rationale: We hypothesize that the potent constrictor actions of spasmogens on basilar artery are mediated by suppression of Kv7 currents, membrane depolarization and activation of VSCC. Selective small molecule activators of Kv7 channels should hence be able to reverse the constriction induced by spasmogens. The electrophysiology experiments described in aim 1 tested our hypothesis at the level of individual myocytes, whereas the experiments proposed in this aim examined if these cellular mechanisms are integrated to induce functional basilar artery constriction/dilation.

Experiments: Pressure myography was used to examine the role of Kv7 channels on the contractile status of basilar artery. The following experiments tested our hypothesis at the organ level:

(1) The effect of a Kv7 channel blocker on the contractile status of basilar artery was examined.

(2) We hypothesized that vasoconstriction induced by spasmogens involve Kv7 current suppression and Ca^{2+} influx through VSCC. Therefore, additivity experiments were
performed to reveal whether spasmogen-induced constriction involves suppression of Kv7 currents.

(3) The ability of selective Kv7 channel openers—flupirtine and/or retigabine, VSCC blocker—nimodipine and dual Kv7 channel opener and VSCC blocker—celecoxib to reverse/prevent spasmogen-induced constriction were tested.

(4) The ability of PKC activators to constrict basilar artery segments was investigated.

**Specific aim 3:** Evaluate the ability of Kv7 channel openers to attenuate basilar artery vasospasm in a rat model of SAH.

Rationale: We hypothesize that consequent to SAH, suppression of Kv7 currents in basilar artery myocytes by elevated concentrations of spasmogens in cerebrospinal fluid is a basic pathology in vasospasm. Hence, administration of Kv7 channel openers is expected to overcome Kv7 current suppression and attenuate vasospasm after SAH. Sustained hypotension may limit the utility of K⁺ channel openers or CCBs in the effective treatment of cerebral vasospasm. Therefore, the effect of Kv7 channel openers on mean arterial pressure (MAP) was measured in normal rats.

Experiments:

(1) SAH was experimentally induced in rats by injection of autologous blood into the rat's cisterna magna to simulate the presence of blood after SAH. SAH rats were administered either the Kv7 channel opener—retigabine or dual Kv7 channel opener and VSCC blocker—celecoxib after hemorrhage. The ability of Kv7 channel
openers to attenuate vasospasm was assessed by measurement of basilar artery diameter 48 hours after hemorrhage.

(2) The effect of Kv7 channel openers on MAP was measured in normal rats using radiotelemetry. The dosage schedule for MAP measurements mimicked the drug administration in the diseased animal model.

Specific aim 4: To identify the mechanism by which celecoxib induces ion channel modulatory activity.

Rationale: Celecoxib is clinically utilized as a COX-2 inhibitor to treat inflammation and pain. Celecoxib is a unique COX-2 inhibitor that induces vasodilation and a modest reduction in blood pressure whereas other drugs that belong to the same class have a tendency to increase blood pressure. We presented evidence that celecoxib-induced Kv7 current enhancement and voltage-sensitive Ca$^{2+}$ current inhibition could account for the anti-hypertensive effects of celecoxib. Celecoxib was reported to inhibit phosphodiesterase (PDE) enzyme isoforms 4 and 5 in VSMCs, physiologically critical targets among the several other known non-specific molecular targets of celecoxib. Therefore, we tested if celecoxib induces its ion channel modulatory effect by virtue of phosphodiesterase enzyme inhibition. These experiments therefore are also expected to reveal if activation of Kv7 channels is the mechanism by which several clinically employed PDE inhibitors induce vasodilation and modest reductions in blood pressure.
Experiments:

(1) Determine the effect of clinically utilized PDE inhibitors—rolipram (prototype PDE4 inhibitor), sildenafil (prototype PDE5 inhibitor), papaverine (non-selective PDE inhibitor) on Kv7 currents in rat aortic smooth muscle (A7r5) cells.

(2) Determine if PDE inhibitors also mimic the effect of celecoxib as a voltage-sensitive Ca\(^{2+}\) channel blocker were also tested using A7r5 cells.

(3) Determine the effect of the PDE inhibitors on AVP-induced Ca\(^{2+}\) entry, measured in fura-2 loaded A7r5 cells.

(4) Determine the effect of PDE inhibition on mesenteric artery segments pre-constricted with AVP to validate the dilation induced by PDE inhibitors.
CHAPTER 4
MATERIALS AND METHODS

Animal Welfare and Housing

All animals used in the study were purchased from Harlan laboratories, Indianapolis, Indiana. Animals were housed in pairs in standard plexiglass cages, fed with standard rat chow under 12 hours dark-light cycle. All procedures described in this study were approved by the Institutional Animal Care and Use Committee, Loyola University Medical Center, Maywood, IL and were carried out according to the guidelines for animal welfare (Guide for the Care and Use of laboratory Animals, National Research Council, National Academy press, Washington D.C, Rev. 1996).

Basilar artery dissection

Male Sprague-Dawley rats, 275-325g, were euthanized by cardiac extirpation (surgical removal of heart) under 4% isoflurane anesthesia. The brain was removed immediately and placed in ice-cold dissection solution (in mM - 145 NaCl, 4.7 KCl, 1.2 NaH$_2$PO$_4$, 1.2 MgSO$_4$, 2 CaCl$_2$·2H$_2$O, 2 pyruvic acid, 0.02 EDTA dihydrate, 3 MOPS, and 5 D-glucose with 1% bovine serum albumin (BSA), pH 7.4 at 0° C, osmolality adjusted to 300 mOsm/L using D-glucose). The basilar artery was dissected free from the
brain and used for isolation of myocytes for patch-clamp procedures, polymerase chain reaction (PCR) or pressure myography.

**Isolation of basilar artery myocytes**

Myocytes were isolated from the basilar artery by modifying the procedure described by Berra-Romani *et al.* for mesenteric artery myocytes (Berra-Romani *et al.* 2005). The dissected basilar artery was cut into 2 or 3 segments and placed in low-Ca\(^{2+}\) physiological saline solution (low-Ca\(^{2+}\) PSS, in mM): 140 NaCl, 5.36 KCl, 0.34 Na\(_2\)HPO\(_4\), 0.44 K\(_2\)HPO\(_4\), 10 HEPES, 1.2 MgCl\(_2\), 0.05 CaCl\(_2\), and 10 D-glucose, pH adjusted to 7.2 at 37°C using NaOH, osmolarity adjusted to 298 mOsm/L using D-glucose) for 30 min. The arterial segments were then subjected to enzymatic digestion in low-Ca\(^{2+}\) PSS containing (in mg/ml) 2 collagenase type XI; 0.16 elastase type IV, and 2 BSA (fraction V, protease-free) at 37°C for 30 min. The digested segments were repeatedly washed with ice-cold low-Ca\(^{2+}\) PSS to remove the enzymes and then the individual myocytes or clusters of myocytes were released from the segments by trituration using fire-polished Pasteur pipettes.

**Polymerase chain reaction**

Reverse transcription polymerase chain reaction (PCR) was used to detect the KCNQ gene products (Kv7 channel mRNA transcripts). Myocytes were freshly isolated from basilar artery segments by enzymatic digestion and total RNA was isolated using RNeasy Kit (Qiagen, Valencia, CA). PCR was performed essentially as described
previously (Brueggemann et al. 2007). cDNA was synthesized with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), and then one-tenth of the cDNA product was used for PCR. PCR was carried out using Platinum PCR Supermix (Invitrogen) and 10 pmols of forward and reverse primers at the appropriate annealing temperature (dependent on primer pair). Primers were adapted from previous publications: KCNQ1–3 and KCNQ5 (Ohya et al. 2002) and KCNQ4 (Yeung et al. 2007) to correspond to rat sequences (Table 1).

**Electrophysiology**

Freshly isolated myocytes or A7r5 cells were allowed to attach to glass coverslips for 10-15 minutes at room temperature or at 37°C respectively. Whole-cell currents or membrane voltage were measured by using perforated patch configuration (120 μg/ml Amphotericin B was included in the internal solution) under voltage-clamp or current-clamp conditions. All measurements were made with continuous perfusion of bath solution at room temperature.

**(A) Kv7 currents in basilar artery myocytes:** Whole cell Kv7 currents were recorded from basilar artery myocytes as described previously (Mackie et al. 2008; Brueggemann et al. 2009). Bath solution contained (in mM): 140 NaCl, 5.36 KCl, 1.2 MgCl₂, 2 CaCl₂, 10 HEPES, 10 D-glucose. Bath solution pH and osmolarity was adjusted to 7.3 and 298 mOsm/L using NaOH and D-glucose respectively. Internal (pipette) solution contained (in mM): 135 KCl, 5 NaCl, 10 HEPES, 0.05 K₂EGTA, 1 MgCl₂, 20 D-glucose. Internal solution pH and osmolarity was adjusted to 7.2 and 298 mOsm/L.
using KOH and D-glucose respectively. Currents were recorded by application of 5 s voltage steps from a -4 mV holding voltage to test voltages ranging from -84 mV to +16 mV. Gadolinium chloride (GdCl₃; 100 µM) and spermine (100 µM; to inhibit the inwardly-rectifying K⁺ currents) were included to the bath solution. Apart from blocking L- and T-type Ca²⁺ channels (Beedle et al. 2002; Biagi and Enyeart 1990) and non-selective cation channels (Miyoshi et al. 2004; Setoguchi et al. 1997; Zhang et al. 2000), Gd³⁺ shifts activation of 4-AP-sensitive Kᵥ currents to voltages positive to -20 mV (Figure 4) enabling isolation of Kv7 currents at voltages negative or equal to -20 mV. Whole-cell K⁺ currents were digitized at 2 kHz and filtered at 200 Hz. Whole-cell capacitance was compensated. The steady-state K⁺ currents recorded during the last 1000 ms (2000 points) of each voltage step were averaged and normalized to cell capacitance to obtain the current-voltage (I-V) relationship. In experiments with variations in current density, I-V relationships were derived by normalizing the sustained currents measured at each voltage to the mean current measured at -20 mV before treatment. Time-course of enhancement or inhibition of isolated Kv7 currents was recorded by continuous measurement of outward currents at -20 mV holding potential.

(B) Membrane voltage measurements in basilar artery myocytes: Membrane voltages were recorded from individual myocytes or clusters of myocytes in current clamp (I = 0) mode. The bath solution and the internal solution were the same as used to record Kv7 currents, but performed in the absence of GdCl₃ and spermine. Membrane voltage was recorded for at least 5 min before treatment. The effect of treatment on
Figure 4: Addition of gadolinium enables isolation of Kv7 currents. A. Current-voltage curves show response of 4-AP-sensitive Kv currents to the addition of 100 µM Gd\(^{3+}\). B. Normalized conductance plots of 4-AP-sensitive-Kv currents fitted by a single Boltzmann function shows positive shift in activation of the currents with the addition of Gd\(^{3+}\) (half maximal activation of currents is shifted by approximately 15 mV, n=3). This enabled isolation of Kv7 currents at voltages ≤ -20 mV, without contribution from 4-AP-sensitive Kv currents. [Reproduced from Mani BK et al., British Journal of Pharmacology, 2011; 164: 237-249].
membrane voltage was determined by taking the mean of the values recorded over at least a 1 min period, prior to treatment, and at the end of treatment.

(C) Voltage-sensitive calcium currents in basilar artery myocytes: To record currents through voltage-sensitive Ca\(^{2+}\) channels in basilar artery myocytes, 10 mM Ba\(^{2+}\) was used as a charge carrier. Inclusion of Ba\(^{2+}\) as a charge carrier is routinely used in patch-clamp experiments to measure voltage-sensitive Ca\(^{2+}\) currents for two reasons: (i) switching from Ca\(^{2+}\) to Ba\(^{2+}\) as the charge carrier changes the rapidly inactivating currents to a more slowly inactivating and larger currents, (ii) use of Ba\(^{2+}\) as charge carrier avoids entry of Ca\(^{2+}\), which would activate the contractile apparatus and constrict the myocytes. The bath solution contained (in mM): 140 NaCl, 2.7 KCl, 10 BaCl\(_2\), 10 HEPES. Bath solution pH and osmolarity was adjusted to 7.3 and 298 mOsm/L using NaOH and D-glucose or water respectively. Internal solution contained (in mM): 135 CsCl, 10 HEPES, 10 Cs\(_2\)EGTA, 2.5 MgCl\(_2\), 10 D-glucose. Internal solution pH and osmolarity was adjusted to 7.2 and 298 mOsm/L using CsOH and D-glucose respectively. Whole-cell capacitance was compensated. Ba\(^{2+}\) currents were recorded in isolation by application of a 300 ms voltage step protocol from -90 mV holding voltage to test voltages ranging from -85.2 to +44.8 mV. Whole-cell voltage-sensitive Ba\(^{2+}\) currents were digitized at 10 kHz and filtered at 5 kHz and the peak inward current was measured and normalized to cell capacitance.

(D) Kv7 currents in A7r5 cells: Whole-cell Kv7 K\(^+\) currents were recorded in A7r5 cells, essentially as described previously (Brueggemann et al 2009). The standard bath solution contained (in mM): 5 KCl, 130 NaCl, 10 HEPES, 2 CaCl\(_2\), 1.2 MgCl\(_2\), 5 D-
glucose. Bath solution pH and osmolarity was adjusted to 7.3 and 268-270 mOsm/L using NaOH and D-glucose respectively. Standard internal (pipette) solution contained (in mM): 110 K gluconate, 30 KCl, 5 HEPES, 1 K₂EGTA. Internal solution pH and osmolarity was adjusted to 7.2 and 268-270 mOsm/L using KOH and D-glucose respectively. GdCl₃ (100 μM) was added to external solution to isolate Kv7 currents. The currents were recorded using a 5 s voltage steps from a -74 mV holding potential to test potentials ranging from -94 mV to +36 mV. Whole-cell capacitance was compensated. Whole-cell K⁺ currents were digitized at 2 kHz and filtered at 200 Hz. The last 2000 points recorded during each voltage step (corresponding to 1000 ms recording time) were averaged and normalized by cell capacitance to obtain end pulse steady-state K⁺ current for each voltage step. In experiments with variations in current density, I-V relationships were derived by normalizing the sustained currents measured at each voltage to the mean current measured at -20 mV before treatment. Time course of changes in isolated Kv7 currents with treatment were recorded by continuous measurement of outward current at -20 mV holding potential.

(E) Voltage-sensitive calcium currents in A7r5 cells: To record voltage-sensitive Ca²⁺ currents in isolation in A7r5 cells, a Cs⁺-containing internal solution was used (in mM): 110 Cs aspartate, 30 CsCl, 5 HEPES, 1 Cs₂EGTA, pH 7.2. Internal solution pH and osmolarity was adjusted to 7.2 and 268 mOsm/L using CsOH and D-glucose respectively. Voltage sensitive Ca²⁺ currents were recorded using 10 mM Ba²⁺ as a charge carrier (in mM): 120 Tris-MES, 10 BaCl₂, 5 HEPES, 1 MgCl₂, pH 7.3, 268 mOsm/L. Isolated Ca²⁺ currents were recorded with a 300 ms voltage step protocol from -90 mV holding
potential. Whole-cell Ca\(^{2+}\) currents were digitized at 10 kHz and filtered at 5 kHz; peak inward current was measured and normalized by cell capacitance. In experiments with variations in current density, I-V relationships were derived by normalizing the peak currents measured at each voltage to the maximal peak inward current measured before treatment.

For all electrophysiology measurements, liquid junction potentials were calculated using Junction Potential Calculator provided by PCLAMP8 software and subtracted off-line. At least two control recordings were made to ensure that the currents were stable before treatment. Current-voltage (I-V) curves for the K\(^{+}\) currents and voltage-sensitive Ca\(^{2+}\) currents were derived after leak subtraction using a procedure described by Passmore et al. (Passmore et al. 2003). To analyze the voltage-dependence of channel activation of Kv7 currents, the conductance was calculated from steady-state K\(^{+}\) currents according to the equation \(G=I/(V-E_{rev})\) and normalized by maximum conductance for each experiment. In the equation, \(I\) is the steady-state current, \(V\) is the step potential and \(E_{rev}\) is the reversal potential (Wickenden et al. 2001). \(E_{rev}\) for K\(^{+}\) was calculated to be -86 mV using the Nernst equation. Normalized conductance was fitted by a Boltzmann distribution: \(G/G_{max}=1/[1+exp(V_{0.5}-V)/s]\), where \(G/G_{max}\) is fractional maximal conductance, \(V_{0.5}\) is the voltage of half-maximal activation and \(s\) is the slope factor.
[Ca^{2+}]_c measurements in A7r5 cells using Fura-2

Changes in the [Ca^{2+}]_c were measured using fura-2/acetoxyethyl ester at room temperature as described previously (Mani et al. 2009). A7r5 cells cultured to confluent monolayers in six-well plates were washed twice with modified Kreb’s medium (135 mM NaCl, 5.9 mM KCl, 1.5 mM CaCl_2, 1.2 mM MgCl_2, 11.5 mM D-glucose, and 11.6 mM HEPES, pH adjusted to 7.3 using NaOH) and then incubated in the same medium with 1 μM fura-2 acetoxyethyl ester (Invitrogen), 0.1% bovine serum albumin, and 0.02% Pluronic F127 detergent for 60 min at room temperature (22–25°C) in the dark. Changes in [Ca^{2+}]_c with treatment were measured using a Biotek Synergy HT plate reader (340- and 380-nm excitation, 510-nm emission) (BioTek Instruments, Winooski, VT). Frequency of spiking was calculated as the number of spikes per minute from the time of onset of repetitive Ca^{2+} spiking. Each “n” represents the mean of triplicate wells.

Pressure Myography

The effects of various treatment conditions on basilar artery diameter were studied using a pressure myograph system (DMT-USA, Atlanta, GA) as described previously (Henderson and Byron 2007). The pipette contained physiological saline solution (in mM - 145 NaCl, 4.7 KCl, 1.2 NaH_2PO_4, 1.2 MgSO_4, 2 CaCl_2·2H_2O, 2 pyruvic acid, 0.02 EDTA dihydrate, 3 MOPS, and 5 D-glucose with 1% BSA, pH 7.4 adjusted at 37°C with NaOH, osmolarity adjusted to 300 mOsm/L with D-glucose) and the bath contained the same solution without BSA. The artery segment was transferred to the pressure myograph system and secured to the glass pipettes using nylon sutures. By
gradually increasing the pressure using a pressure column, the arteries were pressurized to 80 mmHg (the pressure that would be expected in the basilar artery in vivo) (Weyer et al. 2006a). The bath solution was gradually warmed to 37°C. The viability of the vessel was confirmed by observation of a quick constrictor response to the depolarization induced by transient application of 60 mM KCl saline solution. The artery segment was then allowed to equilibrate and arteries with diameters that were stable for at least 30 min were used in the study. Arteries that developed myogenic tone (a decrease in diameter coinciding with pressurization to 80 mmHg) or that failed to respond to KCl were discarded. The concentration of spasmogens was chosen based on the EC₅₀ concentrations to constrict basilar artery (Nishimura 1996; Katori et al. 2001; Mayhan 1998). The vasoconstrictor/vasodilator responses to various treatments were measured and the results are presented as changes in outer vessel diameter, in micrometers.

Pressure myography experiments utilizing mesenteric artery segments were also performed similar to the basilar artery pressure myography experiments described above. Third or fourth order mesenteric artery segments were utilized for the study.

**SAH-induced cerebral vasospasm model**

SAH in humans is produced primarily by the spontaneous rupture of aneurysms commonly found in cerebral arteries. The syndrome is not reported in animals, but animal models of SAH are developed by introduction of blood into the subarachnoid space. Two common methods of introduction of autologous blood are stereotactic injection of blood into the cisterna magna or endovascular puncture of cerebral blood vessels (Megyesi et
al. 2000; Titova et al. 2009). While dogs and rabbit were commonly used previously, many investigators have recently moved to rat model of SAH for ease of use (Nystoriak et al. 2010; Titova et al. 2009; Marbacher et al. 2011).

In the present study, SAH-induced cerebral vasospasm was induced in rats by introduction of blood using a posterior cranio-cervical approach under aseptic conditions. Male Sprague-Dawley rats (300-350g) were used for a single-injection SAH model using procedures similar to those described previously (Gules et al. 2002). The left or right femoral artery was first cannulated under 3% isoflurane anesthesia. The rat was then anesthetized by intramuscular administration of ketamine (80 mg/kg) and xylazine (8 mg/kg) and secured in a stereotaxic apparatus. A small sub-occipital incision was made to expose the arch of the atlas, the occipital bone, and the atlanto-occipital membrane. The cisterna magna was tapped to remove 0.1 ml of CSF followed by 2 min slow injection of 0.3 ml of artificial cerebrospinal fluid (aCSF; solution contained, in mMol/L - 125 NaCl, 2.5 KCl, 1 MgCl\(_{2}\)\(\cdot\)6H\(_2\)O, 1.25 NaH\(_2\)PO\(_4\), 2 CaCl\(_2\)\(\cdot\)2H\(_2\)O, 25 NaHCO\(_3\) and 25 D-glucose, pH 7.3 at 37° C, 300 mOsm/L) or autologous blood drawn from the femoral artery. Immediately after injection, the hole was sealed with glue to prevent fistula. The animal was tilted at a 30° angle for 30 min in a head lowered position to permit distribution of blood (or aCSF) in the subarachnoid space. Pain was relieved by subcutaneous injection of buprenorphine (0.03 mg/kg). The rats were returned to the cage after suturing the skin to allow recovery from anesthesia. Of the 51 rats that underwent the surgery, 13 rats, all of which were injected with arterial blood into the cisterna magna, died during the surgery. The surviving rats were grouped randomly and treated with intra-peritoneal
administration of retigabine (7.5 mg/kg), celecoxib (20 mg/kg) or the solubilizing vehicle (DMSO; 1 ml/kg) starting an hr after SAH and repeated twice a day until 48 hr (4 doses in total). Doses of retigabine and celecoxib were adopted from the previous studies in which the drugs were utilized for their anti-seizure and anti-inflammatory properties, respectively (Cuzzocrea et al. 2002; Blackburn-Munro et al. 2005). Two SAH rats, one treated with vehicle and the other treated with retigabine, died during the course of treatment. Body temperature was measured rectally using a digital thermometer (Warner Instruments, CT).

Body temperature measurements were made every 24 hr after the surgical induction of SAH before the impending drug treatment. The neurological endpoints were assessed using behavioural tests used in ischemic stroke models (Garcia et al. 1995). The functions assessed were spontaneous activity, symmetry in movement of limbs when suspended by tail, forepaw out reaching, climbing on a wire cage, body proprioception, and response to vibrissae touch. All assessments were conducted by personnel who were blinded to the treatments given to the rats. The rats were humanely euthanized with 100% CO₂ inhalation 48 hr after surgery (or 12 hr after last drug treatment). The brain was removed immediately and the basilar artery was photographed using a dinocapture digital system (AnMo Electronics Corporation, Taipei, Taiwan). Measurements of the outer diameter of the basilar artery were made at 10 places along the length of the basilar artery. The measurements were averaged to obtain the basilar artery diameter of each rat and expressed in micrometers.
Blood pressure measurements

The catheter tips of previously used Physiotel® PA-C40 or C50-PXT transmitters (data Science International, St. Paul, MN) were re-gelled, and the pressure and temperature offsets were checked as per the instructions provided by the manufacturer. The transmitters were then sterilized using Cidex® OPA solution (0.55% Orthophthalaldehyde), rinsed and stored in saline prior to implantation. Thirteen male Sprague-Dawley rats (275-325g) were aseptically implanted with PhysioTel® PA-C40 or C50-PXT radio-telemetry transmitters (Data Sciences International (DSI), St. Paul, MN) to allow continuous recording of blood pressure in freely moving rats. Under 3% isoflurane anesthesia, a ventral midline laparatomy was performed and the abdominal contents were gently exteriorized into a moist and warm sterile gauze. Blood flow through the abdominal aorta was briefly interrupted using a bulldog clamp. The catheter of the radio-transmitter was inserted through a puncture made posterior to the blockade of blood flow. The puncture hole was immediately sealed with medical glue and the blood flow was restored. The abdominal contents were placed back into the peritoneal cavity. The transmitter body was placed in the cavity and secured with abdominal sutures. Rats were administered buprenorphine 0.03 mg/kg s/c and ampicillin 50 mg/kg i/m every 8-12 hours for 2 days or as needed after surgery. The rats were allowed to recover from surgery for 10 days and then distributed into 2 groups such that MAP measured before treatment was approximately equal in each group. Blood pressure (mm Hg) was measured in each rat for 10 seconds every 10 minutes for 72 hours (Dataquest A.R.T 3.1 Gold Telemetry System, DSI, with RPC1 receivers). Each group underwent 2 treatments...
separated by a 72-hour recovery period. The first treatment was control (no treatment) or vehicle treatment (DMSO, 1ml/kg i.p, every 12 hours) and the second treatment was retigabine (7.5 mg/kg i.p, every 12 hours) or celecoxib (50 mg/kg i.p, every 12 hours). Prior to each treatment, baseline blood pressure was recorded for 24 hours. This was followed by a 48-hour recording during treatment. Data were exported offline using Dataquest ART analysis software (DSI) to a spreadsheet for further analysis.

**Data presentation and Statistics**

Data are presented as group mean ± standard error of the mean. Data were analyzed using Clampfit (Axon Instruments) and SigmaStat (Systat Software, Inc.) software programs. Student's ‘t’ test was used for comparison between 2 groups, Student's paired ‘t’ tests were used for comparisons of parameters measured before and after treatments. Data derived by multiple treatments on the same system were statistically compared by using repeated measures analysis of variance (RM-ANOVA) followed by Holm-Sidak post hoc test. Comparisons among multiple independent treatment groups were evaluated by analysis of variance (ANOVA) followed by Holm-Sidak post hoc test. Differences with P-value ≤ 0.05 were considered statistically significant.

**Materials**

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of the following. Amphotericin B and nimodipine were from Calbiochem (San
Diego, CA), BSA fraction V was from Boehringer Mannheim (Germany), celecoxib were from LKT Laboratories, Inc. (St. Paul, MN), retigabine was from LGM Pharma, Inc. (Boca Raton, FL), XE991 was from Ascent Scientific (Princeton, NJ). Drugs were prepared as stock solutions in vehicle for patch-clamp and pressure myography experiments and used at dilutions between 1:1000 and 1:10,000.
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**Table 1: Primers used for reverse-transcriptase polymerase chain reaction.** Presence of KCNQ mRNA was assessed in isolated rat basilar myocytes using the primers listed. Positive (+) sequences represent the 5’ to 3’ sense strand and the negative (-) sequences represent the 5’ to 3’ antisense strand.
CHAPTER 5
RESULTS

Expression of KCNQ genes in rat basilar arteries

Reverse transcriptase-PCR was used to evaluate the gene expression pattern of Kv7 channels in basilar artery myocytes. The mRNAs of all five mammalian KCNQ genes (KCNQ1-5, encoding Kv7.1-Kv7.5 channels) were detected (Figure 5).

Kv7 currents in basilar artery myocytes

To assess the presence of functional channels, Kv7 currents were recorded in basilar artery myocytes using patch-clamp electrophysiology under voltage-clamp conditions (Figure 6A). Kv7 currents were isolated by using a pharmacological approach and by utilizing the distinct biophysical properties of Kv7 currents (activation at very negative membrane voltages of around -60 mV, non-inactivation). GdCl$_3$ (100 μM) was included to the bath solution to block L- and T-type Ca$^{2+}$ channels, non-selective cation channels, and to shift activation of 4-AP-sensitive Kv currents to voltages positive to -20 mV (Figure 4; (Brueggemann et al. 2012). Outwardly rectifying currents were recorded with a threshold for voltage-dependent activation < -60 mV and half-maximal activation ($V_{0.5}$) at ~ -34 mV (Figure 6C and D). Addition of 10 μM flupirtine, a selective Kv7 channel activator, significantly enhanced the Kv7 currents at all voltages tested from -49.2 to +5.8 mV; at -20 mV, the voltage at which we observed near-maximal activation,
Figure 5: Kv7 channels are expressed in basilar artery myocytes. Reverse transcriptase (RT)-PCR detection of KCNQ1 through KCNQ5 from mRNA extracted from basilar artery myocytes. Expected sizes of each reaction product are KCNQ1, 453 bp; KCNQ2, 372 bp; KCNQ3, 424 bp; KCNQ4, 359 bp; and KCNQ5, 240 bp. Molecular weight marker (100 base-pair ladder, New England Biolabs, Ipswich, MA., is shown in the left lane). The reaction products were confirmed by sequencing. [Reproduced from Mani BK et al., British Journal of Pharmacology, 2011; 164: 237-249].
Figure 6: Kv7 currents in basilar artery myocytes. A. Representative current traces recorded from a basilar artery myocyte (capacitance = 38.72 pF) under control conditions (left, untreated), treatment with 10 µM flupirtine (middle) and 10 µM XE991 (right; dotted lines indicate zero current). Inset shows the schematic of the voltage protocol used to record the Kv7 currents. B. Representative time course of Kv7 currents recorded by continuous holding at -20 mV in a single basilar artery myocyte before and during the application of 10 µM flupirtine. The voltage-activated currents were enhanced with the application of flupirtine and restored to control levels after wash out of flupirtine (Bars above the trace indicate the duration of treatment or wash out. C. Summarized I-V curves show the outwardly rectifying Kv7 currents (control; □) and the response to application of 10 µM flupirtine (○) and 10 µM XE991 (▽). *Significant enhancement of
Kv7 currents at all tested voltages from -49.2 to +5.8 mV compared to control (Paired Student's ‘t’ test, p < 0.05; n = 4). D. Normalized conductance plots fitted by a single Boltzmann function shows no significant shift in the steady-state voltage dependence of activation of currents with addition of flupirtine; G/Gmax, fraction of maximal conductance. [Reproduced from Mani BK et al., British Journal of Pharmacology, 2011; 164: 237-249].
currents more than doubled with the addition of flupirtine (0.04 ± 0.01 pA/pF before (control) compared to 0.09 ± 0.02 pA/pF after addition of flupirtine (n=4; Figure 6C). The effect of flupirtine was reversible on wash out (Figure 6B). There was no significant shift in the steady-state voltage-dependence of activation of the currents with addition of flupirtine (V0.5 for flupirtine was -36.8 ± 0.8 mV compared to control -33.9 ± 1.5 mV; Figure 6D). XE991 (10 µM), a selective Kv7 channel blocker (Wang et al 1998) effectively abrogated the currents after wash out of flupirtine between voltages of -60 to -20 mV (Figure 6A, C). Outward currents recorded positive to -20 mV are contributed by currents through other K⁺ channels including the 4-AP-sensitive-Kv channels and hence were not sensitive to XE991 (Figure 6C).

Retigabine, an analog of flupirtine also robustly enhanced Kv7 currents in freshly isolated rat basilar artery myocytes and the effect was completely reversible on wash out (Figure 7A). Enhancement of currents with retigabine (10 µM) was significant at all voltages tested from -59.2 mV to +0.8 mV (Figure 7B). At -20 mV, the voltage where near maximal activation of currents were observed (Figure 7C), currents increased from 0.2 ± 0.09 pA/pF before treatment to 0.46 ± 0.17 pA/pF during treatment with retigabine, n=5. Retigabine, unlike flupirtine, induced a significant leftward shift in the steady-state voltage dependence of activation; V0.5 (control) was -30.2 ± 1.4 mV, V0.5 (retigabine) was -41.4 ± 0.5 mV (Figure 7C).
Figure 7: Retigabine enhances Kv7 currents in freshly isolated rat basilar artery myocytes. A. Time-course of XE991-sensitive Kv7 currents with the addition of 10 µM retigabine, recorded from a single basilar artery myocyte by continuous holding at -20 mV. B. Current-voltage curves (I-V) of mean steady-state Kv7 currents before (control; ●) and during treatment with 10 µM retigabine (○) and 10 µM XE991 (▼), normalized to control current measured at -20 mV before treatment. *Significantly different from control at indicated voltages using paired Student’s “t” test, p<0.05, n=5. C. Normalized conductance plotted against membrane voltage was fitted by a single Boltzmann function to show the effect of retigabine on steady-state voltage dependence of activation, *Significantly different from control using paired Student’s “t” test, p<0.05, n=5.
Contribution of Kv7 channels to membrane voltage and contractile status of basilar artery

The Kv7 currents have a very negative threshold for voltage-dependent activation (Figure 6D, 7C), suggesting that K⁺ conductance through Kv7 channels should contribute to maintenance of resting membrane voltages. To evaluate this possibility, we measured the effect of the Kv7 channel blocker XE991 on whole cell membrane voltage under physiological ionic conditions. Addition of 10 µM XE991 significantly depolarized the basilar artery myocytes (by ~22 mV; from -57.5 ± 6.3 mV mean resting voltage to -35.3 ± 1.8 mV, n=4; Figures 8A, B). Vehicle for XE991 (deionized water) did not induce significant depolarization, n=4 (Figure 8B). To confirm that the depolarization of myocytes’ membrane translates functionally into a constrictor response, we assessed if blockade of Kv7 channels would constrict the intact basilar artery using pressure myography. Application of 10 µM XE991 to pressurized basilar arteries induced a significant constrictor response (15.1 ± 1.6 % compared to 0.25 ± 0.06 % constriction with vehicle treatment, n=5; Figure 9A, B), which was almost completely reversed by addition of the L-type Ca²⁺ channel blocker nimodipine (2 µM, Fig. 9A).

Spasmogens suppress Kv7 currents and induce membrane depolarization in basilar artery myocytes

Myocytes derived from basilar artery of SAH-induced animal models are persistently depolarized (Jahromi et al. 2008a; Harder et al. 1987), an effect generally attributed to increased local concentrations of spasmogens. The cellular mechanism(s) by
Figure 8: Kv7 currents determine resting membrane voltage in basilar artery myocytes. A. Representative time course of membrane voltage recorded in current-clamp mode from a basilar artery myocyte before and during the application of selective Kv7 channel blocker XE991 (10 µM). B. Mean membrane voltage values in basilar artery myocytes before (control; white bars) and during the addition of vehicle or 10 µM XE991 (black bars). *significantly different from respective control using paired Student’s ‘t’ test, p < 0.05; N.S – not significant. n = 4 each). #Change in the membrane potential induced by addition of the vehicle of XE991 (3.9 ± 0.96 mV) and XE991 (-22.2 ± 4.69 mV) were significantly different, using Student’s ‘t’ test, p < 0.05. [Reproduced from Mani BK et al., British Journal of Pharmacology, 2011; 164: 237-249].
Figure 9: Kv7 currents determine contractile status of basilar artery. A. Representative trace shows XE991 (10 µM)-induced constriction of a pressurized basilar artery segment. The constriction was reversed with the addition of 2 µM nimodipine, an L-type Ca^{2+} blocker. B. Bar graph shows the percentage change in the basilar artery outer diameter with the addition of 10 µM XE991 or vehicle. *significant constriction compared to vehicle using paired Student’s ‘t’ test, p < 0.05; n = 5. [Reproduced from Mani BK et al., British Journal of Pharmacology, 2011; 164: 237-249].
which the spasmogens induce membrane depolarization is not clear. Since K\(^+\) conductance through Kv7 channels is a critical determinant of resting membrane voltage in rat basilar artery myocytes and artery tone (Figure 8, 9) we tested if Kv7 currents are regulated by spasmogens. We tested three spasmogens (ET-1, 5HT and AVP) that have been implicated in cerebral vasospasm. All three spasmogens significantly suppressed Kv7 currents measured in the physiological voltage range (Figure 10, 11). Currents measured at -20 mV were suppressed by 56%, 49% and 37% with the addition of serotonin (100 nM), ET-1 (250 pM) and AVP (250 pM) respectively. The current amplitudes at -20 mV were 0.18 ± 0.04 pA/pF before and 0.07 ± 0.02 pA/pF during application of 5HT (n=6), 0.12 ± 0.02 pA/pF before and 0.06 ± 0.01 pA/pF during application of ET-1 (n=4), 0.37 ± 0.13 pA/pF before and 0.27 ± 0.12 pA/pF during application of AVP (n=6). Kv7 current amplitude was more than fully restored with addition of 10 µM retigabine in the presence of spasmogens. In the presence of 5HT, ET-1 and AVP at the concentrations indicated above, 10 µM retigabine increased current amplitude (measured at -20 mV) to 72%, 65% and 78% above the control levels, respectively.

Since Kv7 currents are active at resting membrane voltages (Figure 6, 7) and Kv7 current suppression induces membrane depolarization (Figure 8), we tested if the spasmogens also induce membrane depolarization of myocytes. As expected, all three spasmogens induced significant depolarization: 5HT, from -57.1 ± 6.3 mV to -35.4 ± 2.4 mV, n=4; ET-1, from -62.1 ± 2.3 mV to -42.8 ± 1.3 mV, n= 4; AVP from -53.2 ± 4.2 mV to -37.9 ± 1.4 mV, n=4 (Figure 12 A, C). More importantly, simultaneous addition of 10
Figure 10: Serotonin suppresses Kv7 currents in freshly isolated rat basilar artery myocytes. A. Kv7 current amplitude measured in a single basilar artery myocyte by continuous holding at -20 mV during application of 100 nM 5HT and subsequent addition of 10 µM retigabine as indicated. B. I-V curves of steady-state Kv7 currents before (Control; ●), during treatment with 5HT (○) and following addition of 10 µM retigabine in the continued presence of 5HT (▼). Currents at each voltage step are normalized to control currents measured at -20 mV before treatment. 5HT significantly suppressed currents at voltages from -34.2 mV to -14.2 mV (using repeated-measures (RM) one-way ANOVA followed by post hoc Holm-Sidak test, p<0.05, n=6). C. Summary of 5HT-induced suppression of Kv7 currents measured at -20 mV. Addition of retigabine enhanced Kv7 current amplitude in the presence of 5HT. * Significantly different from control using RM one-way ANOVA followed by post hoc Holm-Sidak test, p<0.05, n=6.
Figure 11: Vasopressin and endothelin suppresses Kv7 currents in freshly isolated rat basilar artery myocytes. A. Current-voltage curves (I-V) of steady-state Kv7 currents before (Control; □), during treatment with 250 pM AVP (○) and following addition of 10 µM retigabine in the continued presence of AVP (▼) (currents at each voltage step are normalized to control currents measured at -20 mV before treatment). AVP significantly suppressed currents at voltages from -49.2 mV to 5.8 mV (using repeated-measures (RM) one-way ANOVA followed by post hoc Holm-Sidak test, p<0.05, n=6). B. Summary of AVP-induced suppression of Kv7 currents measured at -20 mV. Addition of retigabine enhanced Kv7 current amplitude in the presence of AVP. *: Significantly different from control using RM one-way ANOVA followed by post hoc Holm-Sidak test, p<0.05, n=6. #: Significantly different from AVP treatment using RM one-way ANOVA followed by post hoc Holm-Sidak test, p<0.05, n=6.
Holm-Sidak test, p<0.05, n=6. C. Current-voltage curves (I-V) of steady-state Kv7 currents before (Control; ●), during treatment with 250 pM ET-1 (○) and following addition of 10 µM retigabine in the continued presence of ET-1 (▼) (currents at each voltage step are normalized to control currents measured at -20 mV before treatment). ET-1 significantly suppressed currents at voltages from -39.2 mV to -4.2 mV (using repeated-measures (RM) one-way ANOVA followed by post hoc Holm-Sidak test, p<0.05, n=4). D. Summary of ET-1-induced suppression of Kv7 currents measured at -20 mV. Addition of retigabine enhanced Kv7 current amplitude in the presence of ET-1. * Significant different from control using RM one-way ANOVA followed by post hoc Holm-Sidak test, p<0.05, n=4.
Figure 12: Spasmogens depolarize membrane voltage in basilar artery myocytes; Kv7 channel opener retigabine inhibits spasmogen-induced membrane depolarization. A. Time-course of change in the membrane voltage with the addition of 100 nM 5HT as indicated, in the absence (top) or presence of 10 µM retigabine (bottom). B. Summarized result of membrane voltage measured before (control; white bars) or approximately 25 min after (grey bars) addition of 5HT in the absence (n=4) or presence (n=3) of 10 µM retigabine as indicated. *Significantly different from control using paired Student’s “t” test, p<0.05; N.S— Not significant. #Change in the membrane potential induced by addition of 100 nM 5HT alone (21.8 ± 4.1 mV) was significantly different from the change induced by addition of 5HT in the presence of retigabine (0.5 ± 1.3 mV), using Student’s ‘t’ test, p < 0.05. C. Summarized result of membrane voltage measured before (control; white bars) or after (grey bars) addition of 250 pmol/L AVP or 250 pM ET-1 as indicated. *Significantly different from control using paired Student’s “t” test, p<0.05, n=4 each.
µM retigabine to enhance activation of Kv7 currents prevented membrane depolarization by 5HT (Figure 12 A, B). The voltage measured was -65.2 ± 4.6 mV before and -64.7 ± 4.3 mV after combined treatment with 10 µM retigabine and 100 nM 5HT, n=3. These results indicate that activation of Kv7 currents can oppose the membrane depolarization induced by spasmogens involved in cerebral vasospasm.

**Basilar artery constriction induced by spasmogens involve Kv7 current suppression**

Pharmacological inhibition of Kv7 channels with XE991 was sufficient to constrict rat basilar arteries and this effect was almost completely reversed by application of the L-type CCB, nimodipine (Figure 9A). Nimodipine (1 µM) also dilated basilar artery segments pre-constricted by spasmogens (Figure 13). This finding implicates membrane depolarization and Ca\(^{2+}\) influx through VSCC as the predominant mechanism for constriction induced by spasmogens at the concentrations measured following SAH. Since spasmogens involved in cerebral vasospasm suppressed Kv7 currents and induced membrane depolarization (Figure 10, 11 and 12), we sought to determine whether spasmogen-induced basilar artery constriction involves suppression of Kv7 currents. To address this question, we tested whether addition of spasmogens in the presence of XE991 (10 µM, a concentration that fully suppresses Kv7 currents; Figure 6C, 7B) induces additive basilar artery constrictor responses. Of the three spasmogens tested, 100 pM AVP and 100 pM ET-1 induced a slight but non-significant additional constriction in the presence of XE991; addition of 75 nM 5HT to XE991 induced a constrictor response
Figure 13: Spasmogen-induced basilar artery constriction is dependent on activation of voltage-sensitive calcium currents. A. Representative time-course shows the reversal of 100 pmol/L AVP-induced basilar artery constriction with addition of 1 µmol/L nimodipine, a selective L-type Ca\(^{2+}\) channel blocker. B. Mean nimodipine-induced dilation in basilar artery segments pre-constricted with spasmogens as indicated. Numbers within bracket indicates the number of experiments in each group.
that was significantly more than the constriction induced by either XE991 alone or the spasmogen alone.

The percentage constriction induced by XE991 before addition of AVP was 15.1 ± 1.6%, AVP in the presence of XE991 was 20.2 ± 2.1% and AVP alone was 15.0 ± 2.7%; XE991 before addition of 5HT was 17.5 ± 2.3%, 5HT in the presence of XE991 was 28.3 ± 1.7% and 5HT alone was 19.4 ± 2.5%; XE991 before addition of ET-1 was 19.0 ± 2.6%, ET in the presence of XE991 was 22.0 ± 1.8% and ET-1 alone was 16.7 ± 1.0%, n = 4-8 (Figure 14). As the values indicate, none of the spasmogens tested induce an additive constrictor response when combined with Kv7 channel blocker XE991 (10 µM). i.e., percentage constriction induced by the combined presence of spasmogen and XE991 is less than the sum of constrictor response induced by spasmogens alone and XE991 alone. This indicates involvement of Kv7 current suppression in the constriction induced by spasmogens.

**Activation of protein kinase C (PKC) suppresses Kv7 currents in basilar artery myocytes**

Myocytes isolated from spastic arteries derived from SAH animal models showed activation and translocation of PKC to the plasma membrane (Laher and Zhang 2001). Inhibition of PKC was previously found to attenuate rat basilar artery constriction induced by each of the three spasmogens used in the present study (Murray et al. 1992a; Murray et al. 1992b), indicating that activation of PKC is a common signal transduction intermediate in spasmogen-induced basilar artery constriction. PKC was previously
Figure 14: Kv7 current suppression is sufficient to induce basilar artery constriction; lack of additivity with basilar artery spasmogens. A. Representative time-course of change in basilar artery outer diameter shows constriction induced by application of Kv7 channel blocker, XE991 (10 µM) and subsequent addition of 100 pM AVP (left) or 75 nM 5HT (right) during the continued presence of XE991. B. Summarized bar graph shows the percentage basilar artery constriction induced by 10 µM XE991, spasmogens (100 pM AVP, 75 nM 5HT, 100 pM ET-1) alone and addition of spasmogens in the continued presence of 10 µM XE991. *Significant additional constriction compared to constriction induced by XE991 alone and 5HT alone using one-way ANOVA followed by post hoc Holm-Sidak test, p<0.05. Addition of 100 pM AVP or 100 pM ET-1 in the presence 10 µM XE991 did not induce significant additional constriction, n=4-8 in each group.
implicated as a mediator of the AVP-induced suppression of Kv7 currents in A7r5 rat aortic smooth muscle cells (Brueggemann et al. 2007). Therefore, we tested whether activation of PKC is sufficient for suppression of Kv7 currents in basilar artery myocytes and constriction of the basilar artery, using a direct activator of PKC: phorbol-12-myristate-13-acetate (PMA; 10 nM). PMA significantly suppressed Kv7 currents at voltages from -44.2 mV to +15.2 mV (Figure 15) and PMA (100 nM) also constricted rat basilar artery segments (Figure 16). An inactive phorbol ester did not constrict basilar artery segments (Figure 16).

**Kv7 channel activators dilate basilar artery segments pre-constricted with spasmogens involved in cerebral vasospasm**

To assess the functional consequences of Kv7 current enhancement, we studied the efficacy of flupirtine or retigabine in reversing the constriction induced by spasmogens involved in the pathogenesis of cerebral vasospasm: 5HT, ET-1 and AVP. Flupirtine induced a concentration-dependent relaxation of basilar arteries pre-constricted with 100 pM ET-1, 100 pM AVP or 75 nM serotonin (Figure 17A, B). The EC₅₀ of relaxation induced by flupirtine was ~40-60 µM (Figure 17B). Flupirtine (100 µM) did not reverse 5HT-induced constriction in the presence of 10 µM XE991 (flupirtine induced 2.8 ± 2.5 % dilation in the presence of XE991 compared to 97.4 ± 1.7 % dilation in the absence of XE991; data not presented) indicating that the dilation induced by flupirtine was specifically through activation of Kv7 channels. We then assessed whether flupirtine is more efficacious than nimodipine in dilating the basilar artery. Application of
Figure 15: Activation of protein kinase C (PKC) suppresses Kv7 currents in basilar artery myocytes. A. Current-voltage curves (I-V) of steady-state Kv7 currents recorded before (control; ●) and in the presence of selective PKC activator, phorbol 12-myristate 13-acetate (PMA, 10 nM; ○). Currents at each voltage step were normalized to control currents measured at -20 mV. *Significantly different from control at indicated voltages using paired Student’s “t” test, p<0.05, n=5.
Figure 16: Activation of PKC is sufficient to constrict basilar artery. A. Representative time-course shows change in basilar artery outer diameter with addition of 100 nM PMA (top) or 100 nM 4α PMA (negative control for PMA, bottom). B. Summary of basilar artery constriction induced by 4α PMA (n=4) and PMA (n=5), *Significant constriction compared to addition of 4α PMA using Student’s “t” test, p<0.05.
Figure 17: Flupirtine reverses the constriction induced by spasmogens in basilar artery. A. Representative trace shows the concentration-dependent effect of flupirtine on a basilar artery segment pre-constricted with a 75 nM 5HT. B. Mean concentration-response curves of the percentage relaxation induced by flupirtine in arteries pre-constricted with the spasmogens: 5HT, AVP and ET-1. C. Representative time course trace shows the dilation induced by nimodipine and flupirtine when pre-constructed with 5HT. Increasing the concentration of nimodipine above 1 µM did not induce additional dilation. However addition of flupirtine induced additional dilation in the same artery. D. Summary of the percentage dilation of pre-constricted basilar artery with the application of 2 µM nimodipine alone, 2 µM nimodipine + 100 µM flupirtine and 100 µM flupirtine alone. *Flupirtine either alone or when added along with nimodipine induced significantly more dilation compared to nimodipine alone (One-way ANOVA followed by post hoc Holm-Sidak test, p < 0.05, n = 3-6). [Reproduced from Mani BK et al., British Journal of Pharmacology, 2011; 164: 237-249].
100 µM flupirtine either alone or combined with nimodipine induced a significant additional dilation of basilar artery compared with nimodipine alone (Figure 17C, D). Retigabine, an analog of flupirtine and a Kv7 channel activator (Figure 7) also induced 92.8% reversal of the constriction induced by 5HT with the maximally used concentration of 100 µM. The EC$_{50}$ retigabine was 23.7 µM, n=5 (Figure 18).

**Celecoxib enhances Kv7 currents and inhibits voltage-sensitive Ba$^{2+}$ currents in basilar artery myocytes**

Celecoxib was recently reported to be a dual Kv7 channel activator and VSCC blocker in cultured rat aortic smooth muscle cells and rat mesenteric artery myocytes (Brueggemann *et al* 2009). The ion channel modulatory effects of celecoxib were tested in basilar artery myocytes. Celecoxib (10 µM) significantly enhanced the Kv7 currents in basilar artery myocytes at all voltages tested from -34.2 to +0.8 mV; at -20 mV the currents more than doubled in amplitude, from 0.08 ± 0.02 pA/pF to 0.19 ± 0.04 pA/pF (Figure 19). Celecoxib did not induce a significant shift in the voltage-dependence of activation (V$_{0.5}$ for celecoxib was -35.6 ± 1.6 mV compared to control -29.8 ± 2.1 mV; Figure 19D).

Voltage-sensitive Ca$^{2+}$ channels were recorded using Ba$^{2+}$ as a charge carrier. Celecoxib (10 µM) inhibited I$_{Ba}$ at all voltages from -20.2 to +39.8 mV (Figure 20A, B). The peak inward currents were reduced from 1.78 ± 0.1 pA/pF to 0.08 ± 0.05 pA/pF with the addition of 10 µM celecoxib. The effects of celecoxib on the I$_{Ba}$ were slightly reversible after wash out of celecoxib (Figure 20B).
Figure 18: Retigabine reverses serotonin-induced basilar artery constriction. A. Representative time-course shows the effect of increasing concentrations of retigabine on outer diameter of a basilar artery segment pre-constricted with 5HT (75 nM). B. Bar graph shows concentration-dependent retigabine-induced basilar artery dilation *Significant dilation compared to absence of retigabine, using RM one-way ANOVA followed by post hoc Holm-Sidak test, p<0.05, n=5.
Figure 19: Celecoxib enhances Kv7 currents in basilar artery myocytes. A. Representative Kv7 current traces before and during the application of 10 µM celecoxib. B. Representative time course of Kv7 currents recorded at -20 mV in a single basilar artery myocyte before and during the application of 10 µM celecoxib. C. Summarized I-V curves show the Kv7 currents before and during application of 10 µM celecoxib. *Significant enhancement of the voltage-activated Kv7 currents at all tested voltages from -34.2 to +0.8 mV (Paired Student's ‘t’ test, p<0.05, n=7). D. Normalized conductance plots fitted by a single Boltzmann function shows no significant shift in the steady-state voltage dependence of activation with addition of celecoxib. G/Gmax= fraction of maximal conductance. [Reproduced from Mani BK et al., British Journal of Pharmacology, 2011; 164: 237-249].
Figure 20: Celecoxib inhibits voltage-sensitive Ba\textsuperscript{2+} currents in basilar artery myocytes. A. Representative voltage-sensitive Ba\textsuperscript{2+} current (I\textsubscript{Ba}) traces before and during the application of 10 µM celecoxib. Inset shows the schematic of the voltage protocol used to record I\textsubscript{Ba}. B. Summarized I-V curves show the effect of 10 µM celecoxib on I\textsubscript{Ba}. *Celecoxib significantly inhibited I\textsubscript{Ba} at all tested voltages from -20.2 to +39.8 mV (Student's ‘t’ test, p < 0.05, n = 5). [Reproduced from Mani BK et al., British Journal of Pharmacology, 2011; 164: 237-249].
Celecoxib is a more efficacious voltage-sensitive calcium channel blocker than nimodipine

Since nimodipine is the standard therapeutic agent used to treat patients with cerebral vasospasm, we compared the ability of nimodipine to inhibit $I_{Ba}$ with that of celecoxib. The concentration of nimodipine used (2 µM) was found to be maximally effective in dilation of basilar artery segments pre-constricted with vasoconstrictor agonists (Figure 17C, 22C). Celecoxib was significantly more effective in inhibiting $I_{Ba}$ in basilar artery myocytes than was 2 µM nimodipine (Figure 21). The inhibition of peak inward $Ba^{2+}$ currents with application of 2 µM nimodipine alone was 85.5 ± 2.9%; with 2 µM nimodipine + 10 µM celecoxib it was 97.0 ± 0.3%; and with 10 µM celecoxib alone, it was 96.0 ± 2.2%.

Celecoxib reverses/prevents basilar artery constriction induced by spasmogens involved in cerebral vasospasm

Celecoxib, the dual Kv7 channel activator and VSCC blocker, was a more potent dilator of the basilar artery compared to flupirtine. Basilar artery segments pre-constricted with ET-1, AVP or 5HT were dilated by celecoxib in a concentration-dependent manner, with an $EC_{50}$ of relaxation between 14 and 17 µM (Figure 22A, B). Celecoxib (30 µM) either alone or when combined with nimodipine (2 µM) induced significant additional dilation of basilar arteries compared to dilation induced by nimodipine alone (Figure 22D, E).
Figure 21: Celecoxib is a more effective voltage-sensitive Ca\textsuperscript{2+} blocker than nimodipine. A. Time course shows the blockade of peak inward I\textsubscript{Ba} with addition of 2 µM nimodipine followed by addition of 10 µM celecoxib in the presence of nimodipine. B. Percentage inhibition of peak I\textsubscript{Ba} with the application of 2 µM nimodipine alone, 2 µM nimodipine + 10 µM celecoxib and 10 µM celecoxib alone. *Celecoxib either alone or when added along with nimodipine produced significantly more inhibition of I\textsubscript{Ba} compared to nimodipine alone (One-way ANOVA followed by post hoc Holm-Sidak test, p < 0.05; n = 5-6).
Figure 22: Celecoxib reverses the spasmogen-induced constriction in basilar artery more effectively than nimodipine. A. Representative trace shows the concentration-dependent effect of celecoxib on a basilar artery segment pre-constricted with a 75 nM 5HT. B. Mean concentration-response curves of the percentage relaxation induced by celecoxib in arteries pre-constricted with the spasmogens: 5HT, AVP and ET-1. C. Representative time course trace shows the dilation induced by nimodipine and celecoxib when pre-constructed with 5HT. Increasing the concentration of nimodipine above 1 µM did not induce additional dilation. However addition of celecoxib induced additional dilation in the same artery. D. Summary of the percentage dilation of basilar artery with the application of 2 µM nimodipine alone, 2 µM nimodipine + 10 µM celecoxib and 10 µM celecoxib alone. *Celecoxib either alone or when added along with nimodipine induced significantly more dilation compared to nimodipine alone (One-way ANOVA followed by post hoc Holm-Sidak test, p < 0.05, n = 3-6). [Reproduced from Mani BK et al., British Journal of Pharmacology, 2011; 164: 237-249].
Development of cerebral vasospasm is delayed and is appreciable usually 3 days after SAH. Therefore, we also sought to address whether celecoxib could prevent the development of vasospasm when administered preemptively. In order to simulate that in an acute experimental system, the ability of a spasmogen to constrict basilar artery in the presence of celecoxib was tested. Serotonin was not able to constrict basilar artery segments pre-treated with 30 µM celecoxib, but after wash out of both 5HT and celecoxib, re-addition of 5HT in the presence of the vehicle of celecoxib induced a robust constriction in the same artery (Figure 23A). The mean constriction induced by 5HT in the presence of celecoxib was 0.11 ± 0.34% compared to 17.3 ± 1.7% induced in the presence of vehicle for celecoxib in the same arteries (Figure 23B). The constriction induced by 5HT in the presence of vehicle was subsequently overcome by application of 30 µM celecoxib (Figure 23A).

**Kv7 channel openers attenuate basilar artery vasospasm in a rat model of SAH**

Single-cell electrophysiology and pressure myography studies strongly indicated the ability of Kv7 channel openers to attenuate spasmogen-induced membrane depolarization and basilar artery constriction (Figure 12, 17, 18, 22 and 23). We therefore tested the efficacy of Kv7 channel openers to relieve basilar artery vasospasm *in vivo* using a rat model of SAH. We used an established single hemorrhage model that induces biphasic vasospasm of basilar artery, reaching its peak at day 2 after initiation of SAH (Delgado *et al.* 1985; Nikaido *et al.* 2004). Rats were treated with either retigabine (7.5 mg/kg, i.p) or celecoxib (20 mg/kg, i.p) twice daily starting an hour after hemorrhage and
Figure 23: Celecoxib prevents spasmogen-induced constriction in basilar artery. A. Time-course shows that 75 nM 5HT was not able to constrict the basilar artery segment in the presence of 30 µM celecoxib. However after wash out, serotonin was able to constrict the same artery in the absence of celecoxib. Re-application of celecoxib completely reversed the 5HT-induced constriction (representative of n=3). B. Summary of the percentage constriction induced by 5HT in the presence of 30 µM celecoxib and vehicle of celecoxib. *Significant constriction by serotonin in the presence of vehicle compared to constriction induced in the presence of 30 µM celecoxib (Student’s ‘t’ test n=3, p < 0.05). [Reproduced from Mani BK et al., British Journal of Pharmacology, 2011; 164: 237-249].
continuing for 48 hours (4 doses in total). Basilar artery outer diameter measured at 48 hours (12 hours after the last injection) was used as the endpoint criterion to assess the severity of vasospasm and efficacy of treatments. SAH induced by an intracisternal injection of autologous blood induced significant basilar artery vasospasm compared to control rats that had no surgery or that were injected with artificial cerebrospinal fluid (aCSF) instead of blood (Figure 24). Both retigabine and celecoxib significantly attenuated the basilar artery spasm when compared with vehicle control in the rat model of SAH (Figure 24). The mean diameter of basilar artery in the treatment groups were—no surgery control: 147.0 ± 9.2 µm, sham surgery (aCSF) group: 143.5 ± 6.7 µm, SAH group: 114.9 ± 9.9 µm, SAH + retigabine treatment group: 158.4 ± 9.0 µm and SAH group + celecoxib treatment group: 176.8 ± 10.7 µm, n=9 in each group.

A striking observation was a pronounced hypothermia with administration of retigabine in SAH rats. Rats administered retigabine had significant hypothermia even 12 hours after drug administration. The rectal temperature of rats in the retigabine group (33.1 ± 1.4 °C) was approximately 5°C lower than the mean temperature in all untreated control groups (Figure 25). Celecoxib also tended to induce mild hypothermia, though this effect was not statistically significant. Rats treated with retigabine and celecoxib demonstrated a significant reduction in locomotor activity. Both the ambulatory movements (scored by consecutive breaks of adjacent photobeams) and fine movements (scored by consecutive break in a single photobeam) were reduced with treatment (Figure 26). Neurological endpoints assessed using behavioural tests used in ischemic stroke...
Figure 24: Kv7 channel openers attenuate basilar artery spasm in a rat model of SAH. A. Illustrative photographs show basilar artery in the base of brain from rats in different treatment groups. Brains were photographed 48 hours after injection of blood or aCSF and compared with brains of control rats (no surgery). B. Bar graph shows the mean basilar artery outer diameter measured in each of the treatment groups—Control (no surgery), aCSF control (sham surgery), SAH rats treated with vehicle (DMSO, 1ml/kg, ip, bid), SAH rats treated with retigabine (7.5 mg/kg, ip, bid) and SAH rats treated with celecoxib (20 mg/kg, ip, bid). *Significant difference between the treatment groups as indicated using one-way ANOVA followed by post hoc Holm-Sidak test, p<0.001, n=9 in each group.
Figure 25: Retigabine induces hypothermia in the rat model of SAH. A. Rectal temperature of rats in different groups (as indicated), measured 48 hours after induction of SAH or sham surgery to inject aCSF, along with control rats (no surgery). *Significantly different from controls (open bars) and SAH rats treated with vehicle, using one-way ANOVA followed by post hoc Holm-Sidak test, p<0.05, n=9 in each group. In treatment groups, body temperature was measured 12h after last treatment time.
Figure 26: Kv7 openers dampen locomotor activity performance of SAH rats. Line plot of fine (A) and ambulatory (B) movements of rats belonging to various treatment or control groups, measured on days before and after surgery (day 0 is the day of surgery). *significant reduction in activity compared to vehicle treated SAH rats (One-way ANOVA test followed by post hoc Holm-Sidak test, p<0.05, n=9) in each group.
models (Garcia et al. 1995) showed no significant change in neurological function compared to rats injected with aCSF into the cisterna magna.

**Kv7 channel openers induce transient hypotension, but do not alter long-term mean arterial pressure**

One of the limitations in the use of direct CCBs like nimodipine to treat cerebral vasospasm is its propensity to induce sustained hypotension (Porchet et al. 1995). Systemic hypotension reduces the perfusion pressure to the brain further exacerbating ischemia (Ahmed et al. 2000). We tested the effect of the Kv7 channel opener retigabine (7.5 mg/kg i.p) and the dual Kv7 channel opener and CCB celecoxib (50 mg/kg i.p) on systemic mean arterial pressure (MAP) in conscious rats (MAP was monitored by radiotelemetry). The drugs were administered intraperitoneally every 12 hours for 48 hours (4 doses in total) to mimic the schedule of test drug administration in SAH rats. Both the drugs induced a transient drop in MAP lasting for 30-60 min after each administration (Figure 27). By comparison vehicle administration tended to increase MAP transiently (Figure 27A). Despite the differences in MAP immediately after drug administration, the 24 h MAP (Figure 27B) and heart rate (Figure 28) were not significantly different between vehicle- and drug-treated groups.
Figure 27: Kv7 channel openers induce a transient drop in blood pressure. A. Change in mean arterial pressure (MAP) during treatment, compared to the 24h baseline MAP measured before treatment. Areas in grey shade are dark hours and areas in white are light hours during the 12h dark/light cycle. Arrows indicate treatment times. Each data point represents the average change in BP during a 30 min interval. Standard error is not represented for clarity. *Significantly different from vehicle group, using one-way ANOVA followed by post hoc Holm-Sidak test, p<0.05, n=6-7. B. Line graph shows the 24h mean BP recorded in untreated rats (control) or rats treated with vehicle (DMSO, 1ml/kg, ip, bid), retigabine (7.5 mg/kg, ip, bid) or celecoxib (50 mg/kg, ip, bid), N.S— no significant difference from control, n=6-7.
Figure 28: Kv7 channel openers do not alter 24h heart rate in rats. A. Change in mean heart rate (HR) during treatment, compared to the 24h baseline HR measured before treatment. Areas in grey shade are dark hours and areas in white are light hours during the 12h dark/light cycle. Arrows indicate treatment times. Each data point represents the average change in HR during a 30 min interval. Standard error is not represented for clarity. *Significantly different from vehicle group, using one-way ANOVA followed by post hoc Holm-Sidak test, p<0.05, n=6-7. B. Line graph shows the 24h mean HR recorded in untreated rats (control) or rats treated with vehicle (DMSO, 1ml/kg, ip, bid), retigabine (7.5 mg/kg, ip, bid) or celecoxib (50 mg/kg, ip, bid), N.S— no significant difference from control, n=6-7.
Phosphodiesterase inhibitors activate Kv7 channels and inhibit voltage-sensitive calcium currents in A7r5 cells

Celecoxib is prescribed in clinical practice to treat prostaglandin-mediated inflammation and pain (Frampton and Keating 2007). Celecoxib induces its anti-inflammatory effect by inhibiting a specific class of prostaglandin producing enzyme called cyclooxygenase (COX)-2. We tested if the ion channel-mediated vasodilatory effect of celecoxib is due to its ability to inhibit COX-2. Rofecoxib, a more potent and selective COX-2 inhibitor than celecoxib (FitzGerald and Patrono 2001), did not dilate basilar artery segments pre-constricted with 5HT (75 nM). Application of celecoxib at equimolar concentrations (30 µM) in the same arteries almost completely reversed the 5HT-induced constriction (Figure 29A, C). Dimethyl celecoxib, an analog of celecoxib, which is devoid of COX-2 inhibitory activity (Kardosh et al. 2005), but retains the Kv7 channel and VSCC modulatory activity (Brueggemann et al. 2009), mimicked the vasodilatory effects of celecoxib (Figure 29B, C). These results indicate that celecoxib-induced ion channel-mediated vasodilatory effects are independent of its COX-2 inhibitory effects, therefore not a “class-effect” (Figure 29).

Celecoxib, but not rofecoxib, was reported to be an inhibitor of phosphodiesterase enzyme isoform 4 and 5 (Klein et al. 2007). Hence, we hypothesized that celecoxib induces the ion channel modulatory effects (Kv7 channel activation and VSCC block) through inhibition of the PDE enzyme(s). To that end, we tested the effect of selective PDE enzyme inhibitors on Kv7 currents in A7r5 cells. Rolipram, a selective PDE4 inhibitor, induced a concentration dependent enhancement of Kv7 currents in A7r5 cells
Figure 29: Vasodilatory effect of celecoxib is not dependent on COX-2 inhibition. Representative time course trace shows that 30 µM rofecoxib failed to dilate basilar artery segments pre-constricted with a 75 nM serotonin. Application of 30 µM celecoxib (CXB) completely reversed the serotonin-induced constriction of the same artery. B. Representative time course trace shows that 30 µM dimethyl celecoxib (DMC) completely reversed the serotonin-induced constriction of basilar artery. C. Summary of the percentage dilation induced by rofecoxib and dimethyl celecoxib. *Significant dilation compared to 30 µM rofecoxib (One-way ANOVA followed by post hoc Holm-Sidak test, p < 0.05, n = 3 each). [Reproduced from Mani BK et al., British Journal of Pharmacology, 2011; 164: 237-249].
(Figure 30). Rolipram at concentrations as low as 1 nM induced about 50% enhancement of Kv7 currents in A7r5 cells. The concentration response curve for enhancement of Kv7 currents was bell shaped, with a maximal 3.4-fold enhancement of currents with addition of 100 nM rolipram and suppression of currents at a concentration above 100 nM (Figure 30B, C). Rolipram also mimicked the voltage-sensitive Ca\(^{2+}\) current inhibitory action of celecoxib (Figure 31). Rolipram inhibited I\(_{\text{Ba}}\) with a maximal suppression of peak inward currents at a concentration of 100 nM. The concentration response curves for enhancement of Kv7 currents and inhibition of voltage-sensitive Ca\(^{2+}\) currents with rolipram showed similar concentration-dependence (Figure 30C, 31C). Sildenafil, a PDE5 inhibitor and papaverine, a non-selective PDE inhibitor also enhanced Kv7 currents in A7r5 cells (Figure 32, 34). Sildenafil induced a maximal 2.7-fold enhancement of Kv7 currents at a concentration of 10 μM and papaverine induced a 3.1 fold enhancement of the currents at a maximally tested concentration of 100 μM. Sildenafil also inhibited voltage-sensitive Ca\(^{2+}\) currents similar to the effects of celecoxib and rolipram (Figure 33). These results indicate that inhibition of either PDE4 or PDE5 enzyme is sufficient to simulate the ion channel modulatory action of celecoxib.

**Phosphodiesterase inhibitors attenuate AVP-induced calcium spiking response in A7r5 cells; dilate mesenteric artery segments**

We then tested if the ion channel modulatory effects of PDE inhibitors translated functionally to inhibition of Ca\(^{2+}\) entry in A7r5 cells. Physiological concentrations of AVP (10-100 pM) induces Ca\(^{2+}\) spiking responses attributed to Kv7 current suppression,
Figure 30: Rolipram enhances Kv7 currents in A7r5 cells. A. Current traces show enhancement of non-inactivating outward Kv7 K⁺ currents with the addition of rolipram, a selective PDE4 inhibitor. B. Current-voltage curves of steady-state Kv7 currents recorded before (control) and during treatment with increasing concentrations of rolipram as indicated (currents recorded at each voltage step were normalized to control currents measured at -20 mV before treatment). C. Concentration-response relationship demonstrates a more than 3-fold maximal enhancement of Kv7 currents with the addition of rolipram. Currents measured at -20 mV in the presence of rolipram were normalized to currents measured at -20 mV before the addition of rolipram. *significantly different from pre-treatment levels, using repeated measures one-way ANOVA followed by Holm-Sidak post hoc test, p < 0.001, n= 4-5).
Figure 31: Rolipram inhibits voltage-sensitive $\text{Ba}^{2+}$ currents in A7r5 cells. A. Inward $\text{Ba}^{2+}$ current traces recorded before (control) and during treatment with 100 nM rolipram. B. Current-voltage curves show the mean $I_{\text{Ba}}$ recorded across the voltages tested with the addition of different concentrations of rolipram. (Currents at each voltage step were normalized to the peak inward $I_{\text{Ba}}$ measured before treatment). C. Summarized concentration-response relationship shows significant suppression of peak inward $I_{\text{Ba}}$ with increasing concentrations of rolipram. Peak inward $I_{\text{Ba}}$ measured in the presence of rolipram were normalized to the peak inward $I_{\text{Ba}}$ measured before treatment. (*significantly different from pre-treatment levels, repeated measures one-way ANOVA followed by Holm-Sidak post hoc test, $p < 0.001$, n=3-5).
Figure 32: Sildenafil enhances Kv7 currents in A7r5 cells. A. Current-voltage curves of the steady-state Kv7 currents recorded before (control), during treatment with increasing concentrations of sildenafil as indicated (currents recorded at each voltage step were normalized to control currents measured at -20 mV before treatment). B. Concentration-response relationship demonstrates enhancement of Kv7 currents with the addition of sildenafil. Currents measured at -20 mV in the presence of sildenafil were normalized to currents measured at -20 mV before the addition of sildenafil. *significantly different from pre-treatment levels, using repeated measures one-way ANOVA followed by Holm-Sidak post hoc test, p < 0.05, n= 4 each).
Figure 33: Sildenafil inhibits voltage-sensitive Ba\textsuperscript{2+} currents in A7r5 cells. A. Current-voltage curves show the mean I\textsubscript{Ba} recorded across the voltages tested with addition of different concentrations of sildenafil. (Currents at each voltage step were normalized to the peak inward I\textsubscript{Ba} measured before treatment). B. Summarized concentration-response relationship shows significant suppression of peak inward I\textsubscript{Ba} with increasing concentrations of sildenafil. Peak inward I\textsubscript{Ba} measured in the presence of sildenafil were normalized to the peak inward I\textsubscript{Ba} measured before treatment. (*significantly different from pre-treatment levels, RM one-way ANOVA followed by Holm-Sidak post hoc test, p < 0.05, n=3-5).
Figure 34: Papaverine enhances Kv7 currents in A7r5 cells. A. Current-voltage curves of steady-state Kv7 currents recorded before (control) and during treatment with 2 different concentrations of papaverine (10 and 100 µM) (currents recorded at each voltage step were normalized to control currents measured at -20 mV before treatment). B. Concentration-response relationship demonstrates enhancement of Kv7 currents with the addition of papaverine. Currents measured at -20 mV in the presence of papaverine were normalized to currents measured at -20 mV before the addition of papaverine. *significantly different from pre-treatment levels, using repeated measures one-way ANOVA followed by Holm-Sidak post hoc test, p < 0.001, n= 4 each).
membrane depolarization and Ca$^{2+}$ entry through VSCC in A7r5 cells (Brueggemann et al. 2007; Mani et al. 2009; Byron 1996). Pre-treatment with 100 nM rolipram (Figure 35), 10 μM sildenafil or 100 μM papaverine (not shown) significantly attenuated AVP (25 pM)-induced Ca$^{2+}$ spiking responses in A7r5 cells. Rolipram and sildenafil also induced concentration-dependent relaxation of mesenteric artery segments pre-constricted with 30 pM AVP, indicating that the ion channel modulatory effects of PDE inhibitors translate functionally into vasodilatory response (Figure 36).
Figure 35: Rolipram inhibits calcium spiking responses in A7r5 cells. A. Representative traces show Ca\textsuperscript{2+} spiking in response to addition of 25 pM AVP (indicated by arrow) in fura-2 loaded A7r5 cells pretreated with vehicle or 100 nM rolipram for 30 min. B. Bar graph summarizes the effect of rolipram on AVP-induced Ca\textsuperscript{2+} spiking response. **significant reduction in the Ca\textsuperscript{2+} spiking frequency in cells pre-treated with 100 nM rolipram, Student’s ‘t’ test, p < 0.001, n=4 (Data generated by Kakad).
Figure 36: Phosphodiesterase4/5 inhibitors reverse AVP-induced constriction in rat mesenteric artery. A. Representative time course shows rolipram-induced concentration-dependent dilation of mesenteric artery pre-constricted with 30 pM AVP. Summarized dose-response relationship of the dilation induced by rolipram (B) and sildenafil (C) in arteries pre-constricted with AVP. **Significant dilation with addition of rolipram or sildenafil compared to absence of the drugs, using RM one-way ANOVA followed by Holm-Sidak post hoc test, p < 0.001, n=4-5.
CHAPTER 6
DISCUSSION

This study presents several novel findings:

(1) Kv7 channels are expressed and functional in rat basilar artery myocytes.

(2) K⁺ conductance through the Kv7 channels maintains hyperpolarized resting membrane voltages, limiting the activation of VSCC in myocytes, thereby regulating the contractile status of basilar artery.

(3) Suppression of Kv7 currents is a central mechanism by which multiple spasmogens implicated in SAH-induced cerebral vasospasm induce membrane depolarization and constrict basilar artery.

(4) Direct Kv7 channel openers (retigabine or flupirtine) are able to restore the Kv7 current amplitude in the presence of spasmogens. This action attenuates the spasmogen-induced membrane depolarization and artery constriction.

(5) Celecoxib is a dual Kv7 channel activator and VSCC blocker in basilar artery myocytes. Celecoxib was able to reverse/prevent spasmogen-induced basilar artery constrictor responses.

(6) Flupirtine and celecoxib are more effective in dilating basilar artery than the L-type-calcium channel blocker, nimodipine, which is currently used as the standard care for patients with cerebral vasospasm.
Retigabine and celecoxib are effective in preventing the sustained basilar artery vasospasm in a rat model of SAH. These drugs do not alter long-term mean arterial pressure.

These findings suggest that Kv7 channels are critical mediators of cerebral vasospasm and that clinically used drugs that enhance Kv7 channel activity may be effective in reducing vasospasm and preventing or attenuating stroke following SAH.

**Expression of Kv7 channels in rat basilar artery myocytes**

Message transcripts of all the known KCNQ genes (KCNQ1-5, encoding Kv7.1-7.5 channels) were detected in rat basilar artery myocytes (Figure 5). A quantitative assessment of the expression of KCNQ genes in rat basilar artery and middle cerebral artery myocytes by Zhong et al. also revealed the presence of all five KCNQ mRNAs, though the mRNAs of KCNQ2 and KCNQ3 were much less abundant than those of KCNQ1, KCNQ4 and KCNQ5 (Zhong et al. 2010). Kv7.1 channels are insensitive to drugs (e.g., flupirtine, retigabine, and N-ethyl maleimide) that activate the other Kv7 channel subtypes (Gamper et al. 2005; Munro and Dalby-Brown 2007) and Kv7.1 subunits do not heteromerize with other Kv7 channel subunits (Schwake et al. 2003). Considering that the whole cell Kv7 currents measured in basilar artery myocytes were robustly enhanced by flupirtine and retigabine (Figure 6, 7), the contribution of outward K⁺ conductance through homomeric Kv7.1 channel is likely to be minimal. Using selective pharmacological modulators for Kv7.1 channels, a recent study indicated that Kv7.1 channels may be functional, but do not contribute to the resting tone of mesenteric
arteries (Chadha et al. 2012). Hence, the functional Kv7 channels that contribute to the resting tone in basilar artery myocytes are likely to be predominantly constituted by homo- or hetero-tetramers of Kv7.4 and Kv7.5 channel subunits.

Expression profiles of KCNQ channel transcripts in VSMCs from various vascular beds reveal expression of KCNQ1, KCNQ4 and KCNQ5 subunits, and either absence or very little expression of KCNQ2 and KCNQ3 subunits (Zhong et al. 2010; Joshi et al. 2009; Mackie et al. 2008; Yeung et al. 2007). This indicates that the expression profile of KCNQ genes in basilar artery and VSMCs in general is different from neurons where the predominant expression is KCNQ2 and KCNQ3 (Jentsch 2000; Delmas and Brown 2005).

**Kv7 currents in rat basilar artery myocytes**

This study presents the first successful isolation of Kv7 currents in the cerebral vasculature (Figure 6, 7). Whole cell Kv currents were recorded using voltage-clamp conditions. Currents recorded at test voltages between -60 mV and -20 mV can reasonably be attributed to Kv7 channel activity based on several observations: 1) the currents measured under our recording conditions were robustly enhanced by 10 µM flupirtine or retigabine, selective Kv7 channel activators, 2) the currents were completely inhibited by 10 µM XE991, a selective Kv7 channel blocker, 3) the currents had electrophysiological characteristics of Kv7 currents (non-inactivating, voltage-dependent with a very negative threshold for activation and half-maximal activation ($V_{0.5}$) of ~ -34 mV (Wickenden et al. 2001; Brueggemann et al. 2007; Mackie et al. 2008; Adams and
Brown 1982). Currents recorded at voltages more positive than -20 mV were not completely blocked by XE991 suggesting that at these more depolarized voltages we were recording a mix of currents, with likely contributions of other K⁺ channels, such as 4-AP-sensitive Kv channels and Ca²⁺-activated K⁺ channels (KCa) channels.

In the present study, both Kv7 channel activators—retigabine and flupirtine—significantly enhanced Kv7 currents over the physiological voltage range, with more than 100% enhancement of Kv7 currents measured at -20 mV (Figure 6, 7). Retigabine induced a significant hyperpolarizing shift in voltage-dependence of activation of the currents (Figure 7C). The voltage of half-maximal activation of the currents (V₀.₅) shifted with addition of 10 μM retigabine from -30.2 ± 1.4 mV in control conditions to -41.4 ± 0.5 mV. Retigabine induced a similar hyperpolarizing shift in activation of currents through Kv7.2/Kv7.3 heteromeric channels expressed in CHO cells; V₀.₅ shifted from -28.7 mV to -40.1 mV with addition of retigabine (Tatulian and Brown 2003). Flupirtine only induced a small non-significant shift in the voltage-dependence of activation of currents (Figure 6D), similar to our previous observation in mesenteric artery myocytes (not shown). Flupirtine, however, induced a significant hyperpolarizing shift in voltage-dependence of activation of Kv7 currents (~ 11 mV) measured in guinea pig airway smooth muscle cells (Brueggemann et al. 2012). The reason for the difference is not immediately apparent, but could be due to the difference in cell type and differential expression of Kv7 channel subtypes. Retigabine increased the maximal Pₒ of single Kv7.2/Kv7.3 channels expressed in CHO cells from 0.13 to 0.38 (Tatulian and Brown 2003). Hence, the increase in whole cell current amplitude with addition of Kv7 channel
openers in basilar artery myocytes is likely due to increase in open probability ($P_o$) of the channels.

The subunits constituting the functional channel is difficult to ascertain because of the lack of subunit-selective pharmacological activators and the difficulty in using molecular approaches. The present study utilized well established Kv channel activators retigabine and flupirtine, both of which activate all Kv7 channel subtypes, except Kv7.1 channel homomers. Recently, several classes of Kv7 channel activators have started to emerge with the identification of a new target site in these channels (Xiong et al. 2008). Most of these compounds were tested only against neuronal Kv7.2/7.3 heterotetramers. While some recent compounds showed relative selectivity to certain channel subtypes, compounds that can serve as tools to identify channel subunits and/or for selective cardiovascular or neuronal therapy remain to be identified (Bentzen et al. 2006; Wickenden et al. 2008; Gribkoff 2008; Brueggemann et al. 2011).

**Kv7 channels are critical contributors to resting membrane voltage and basilar artery contractile status**

The resting membrane voltage of basilar artery myocytes measured in the current study was $-57.5 \pm 6.3$ mV, within the range reported in previous studies for basilar artery myocytes (Jahromi et al. 2008a; Chrissobolis and Sobey 2002; Allen et al. 2002). The maximal density of $K^+$ currents through Kv7 channels is small ($< 0.3$ pA/pF, Figure 6C) compared to maximal current densities reported for 4-AP-sensitive-K$_V$ (36.9 pA/pF) and K$_{Ca}$ channels ($\sim 140$ pA/pF) (Jahromi et al. 2008a; Jahromi et al. 2008b). However, Kv7
channels are likely to play an important role in determining the resting membrane voltage, more so than $K_V$ and $K_{Ca}$ channels, as they are activated at negative membrane voltages than 4-AP-sensitive-$K_V$ and $K_{Ca}$ channels [$V_{0.5}$ for Kv7 currents is -34 mV (Figure 6D), compared to $V_{0.5}$ of -1.3 mV for 4-AP-sensitive-$K_V$ currents (Jahromi et al. 2008a) and +86.8 mV for large-conductance $K_{Ca}$ currents at 200 nM $[Ca^{2+}]_c$ (Jahromi et al. 2008b)]. This is supported by the evidence that blockade of Kv7 channels with XE991 significantly depolarized the cell membrane (Figure 8). This brought the membrane voltage from -58 mV, at which L-type VSCC have very low activity, to -35 mV, which is in the range of membrane voltage where VSCC activity increases in a steeply voltage-dependent manner (Figure 8B). Our results shown in Figure 9 support this hypothetical mechanism: XE991 robustly constricted the basilar artery and this effect was reversed by the L-type VSCC blocker nimodipine. Kv7 channels have a well-established role in stabilizing resting membrane voltages and suppression of their activity is a common depolarizing stimulus in neurons and arterial myocytes (Joshi et al. 2009; Mackie et al. 2008; Adams and Brown 1982).

**Suppression of Kv7 currents as a mechanism of vasoconstriction by spasmogens**

Though it is well established that basilar artery spasmogens depolarize the vascular myocytes and trigger $Ca^{2+}$ influx through VSCC to induce constriction, the signal transduction events that lead to membrane depolarization remain poorly understood. Most previous evidence points to activation of non-selective cation channels as a mechanism for spasmogen-induced membrane depolarization (Albert and Large
Our results show that spasmogens suppress Kv7 currents (Figure 10, 11) that are active at resting membrane voltages, an effect which is associated with significant membrane depolarization (Figure 8, 12). Direct inhibition of Kv7 currents by XE991 was sufficient to induce constriction of basilar arteries (Figure 9), suggesting that resting inward leak currents are sufficient to induce membrane depolarization. Suppression of the K⁺ channel activity at resting voltages would be expected to increase membrane resistance and thereby amplify the voltage change induced by inward currents, further enhancing membrane depolarization. The additional constriction induced by 5HT when Kv7 currents were already fully inhibited by XE991 (Figure 14B, C) could be due to activation of inward currents (non-selective cation currents and/or chloride currents) that induces further membrane depolarization (Albert and Large 2006) and/or direct activation of VSCC (Worley et al. 1991). Neither ET-1 nor AVP induced significant additional constriction in the presence of XE991, suggesting that Kv7 current suppression may be sufficient to account for their constrictor actions at the concentrations tested (Figure 14C).

**Protein kinase C as a mediator of the spasmogen-induced suppression of Kv7 currents**

The signal transduction intermediates involved in the spasmogen-induced suppression of Kv7 currents are not currently known. Activation of PKC, a common signal transduction intermediate following activation of G_{q/11}–coupled receptors, is consistently observed in vasospastic myocytes after SAH (Laher and Zhang 2001).
Previous studies reported that PKC inhibitors attenuate spasmogen-induced constrictor responses in rat basilar artery, indicating activation of PKC as an intermediate for the constrictor response to spasmogens (Murray et al. 1992a; Murray et al. 1992b). In our experiments, activation of PKC using phorbol-12-myristate-13-acetate (PMA) suppressed Kv7 currents (Figure 15) and constricted basilar artery (Figure 16), mimicking the effects of the direct Kv7 channel blocker XE991. Hence, PKC activated during vasospasm is likely mediating its constrictor responses at least in part via the suppression of Kv7 currents in basilar artery myocytes. Suppression of the Kv7 currents by PKC could be due to either direct phosphorylation of the channels (Higashida et al. 2005) or through activation of other kinases like Src (Gamper et al. 2003; Li et al. 2004a) that can phosphorylate the channels.

**Kv7 current enhancement attenuates spasmogen-induced membrane depolarization and vasoconstriction**

Retigabine reversed the spasmogen-induced suppression of Kv7 current amplitude (Figures 10, 11) and attenuated membrane depolarization in basilar artery myocytes (Figure 12). These effects translated functionally into dilation of the basilar artery when it was pre-constricted by spasmogens (Figure 18). Retigabine alone more than doubled the amplitude of resting Kv7 currents (Figure 7). In the presence of the spasmogens, when Kv7 currents were suppressed, retigabine still enhanced currents to about 65-80% above control levels (an approximately 3-fold increase in current compared with current levels measured in the presence of spasmogen alone (Figure 10, 11)). The increase in Kv7
current amplitude was accompanied by a hyperpolarizing shift of more than 10 mV in the voltage dependence of steady-state current activation (Figure 7C). Thus, in the presence of retigabine at physiological resting membrane voltages, Kv7 channels would have much greater probability of opening and hence provide a greater stabilization of negative membrane voltage. This would effectively oppose spasmogen-induced membrane depolarization (Figure 12) and opening of L-type voltage-sensitive Ca\textsuperscript{2+} channels. The latter is the primary stimulus for constriction of the basilar artery at physiological concentrations of vasoconstrictor agonists (Figure 13). This mechanism can explain why retigabine and flupirtine oppose the constrictor effects of spasmogens (Figure 17, 18). The actions of retigabine and flupirtine might be due to enhanced voltage-dependent activation of the proportion of Kv7 channels that were not suppressed by the spasmogens. Alternatively, (or perhaps in addition) the Kv7 channel openers may reverse the suppression of the Kv7 channels by spasmogens by virtue of their direct binding to the channel. Our findings do not distinguish between these two possibilities.

Addition of flupirtine in the presence of nimodipine induced additional dilation of basilar artery segments (Figure 17C, D). We speculate that flupirtine induced an additional vasodilatory effect by opposing the membrane depolarization induced by the spasmogens, and thereby preventing the activation of both nimodipine-sensitive and nimodipine-insensitive VSCC. Our results shown in Figure 21 are consistent with this idea in that we can detect Ba\textsuperscript{2+} currents in basilar artery myocytes that are activated by membrane depolarization, but not fully blocked by 2 µM nimodipine.
The spasmogen and Kv7 channel opener concentrations used in the patch-clamp experiments and the vascular reactivity studies in pressure myography differ because of the varying sensitivities of the two systems used (Figures 6, 7, 10-14, 17-23). The differential sensitivity is likely because in intact arteries depolarization of a small proportion of smooth muscle cells leads to depolarization of the adjacent smooth muscle cells connected by gap junctions. Hence, lower concentrations of Kv7 current suppressors (spasmogens) and higher concentrations of Kv7 channel openers were required in artery preparations compared to patch clamp experiments.

**Celecoxib is a Kv7 channel activator and voltage-sensitive calcium channel blocker**

Celecoxib (Celebrex®) is marketed as a selective inhibitor of the cyclooxygenase-2 (COX-2) enzyme and is widely prescribed to treat pain and inflammation. However, our present findings indicate that celecoxib is also a robust Kv7 channel activator (like flupirtine and retigabine) (Figure 19). Enhancement of the currents (Figure 19C) was not accompanied by a significant shift in the voltage-dependence of activation (Figure 19D). Previous work from our laboratory demonstrated similar effects of celecoxib in mesenteric artery myocytes and provided evidence that these effects are apparent at concentrations of celecoxib that could be achieved with clinical therapy (Brueggemann et al. 2009). Potassium currents conducted by human Kv7.5 channels expressed in VSMCs were also shown to be activated by celecoxib further indicating the translational relevance for the action of celecoxib (Brueggemann et al. 2009). Using an expression system, Du et al., expanded the repertoire of Kv7 channels activated by celecoxib.
Celecoxib activated Kv7.2-7.4, Kv7.2/7.3 and Kv7.3/7.5 channels, but inhibited Kv7.1 and Kv7.1/KCNE1 channels (Du et al. 2011). The actions of celecoxib on Kv7 channels in that study were concentration-dependent with a half-maximal concentration between 2-5 μM, within the concentration range used in the present study.

Incidentally, Kv7 channels are not the only K⁺ channels modulated by celecoxib. Celecoxib has been shown to block 4AP-sensitive Kv1.5, Kv4.3 and Kv2.1 channels apart from blocking Kv7.1 channels (Frolov et al. 2010; Macías et al. 2010). Celecoxib inhibited Kv2.1 currents by modifying the gating kinetics and by inducing open- and closed-channel block (Frolov et al. 2010). It is also worthy to note that celecoxib is not the first COX inhibitor known to activate Kv7 channels. Several N-phenylanthranilic acid derivatives, that are mostly non-selective COX-inhibitors including meclofenamic acid, flufenamic acid, mefenamic acid, niflumic acid and diclofenac activate Kv7 currents (Brueggemann et al. 2011; Abitbol et al. 1999; Busch et al. 1994; Peretz et al. 2005). These compounds activate Kv7 currents by direct binding to the channel, and the activity is independent of its COX inhibitory effects (Peretz et al. 2007). Celecoxib is a benzenesulfonamide, structurally unrelated to the N-phenylanthranilic acid derivatives. The mechanism(s) by which celecoxib activates Kv7 channels are currently not known. Du et al., suggested possible direct binding of celecoxib to Kv7 channels (Du et al. 2011), but that remains to be validated.

Celecoxib also inhibited voltage-sensitive Ca²⁺ currents in basilar artery myocytes independently of its enhancement of Kv7 currents (Figure 20). Celecoxib was more effective as a VSCC blocker than 2 μM nimodipine in basilar artery myocytes (Figure
21). The concentration of nimodipine (2 µM), which induced significantly less suppression of VSCC than did 10 µM celecoxib, is more than 1000-fold higher than concentrations achieved in cerebrospinal fluid after nimodipine administration in patients (Allen et al. 1983). A similar Ca²⁺ channel blocking activity of celecoxib was observed in rat undifferentiated pheochromocytoma cells derived from adrenal gland (Zhang et al. 2007).

As expected from its dual ion channel effects, celecoxib very effectively reversed the constriction induced by the spasmogens (Figure 22A, B). And, like flupirtine, celecoxib was a more effective dilator of basilar artery than nimodipine (Figure 22C, D). The additional dilation induced by celecoxib can be plausibly due to two mechanisms: 1) enhancement of Kv7 currents, thereby limiting the voltage change necessary to activate all of the VSCCs; or 2) Kv7 channel-independent inhibition of nimodipine-insensitive VSCC. Our results provide evidence for both of these mechanisms. The finding that flupirtine induces an additional dilation in the presence of nimodipine supports the former (Figure 17D) and that celecoxib induces additional inhibition of voltage-sensitive Ba²⁺ currents in the presence of 2µM nimodipine supports the latter (Figure 21). Celecoxib also prevented the constrictor effect of 5HT, indicating that celecoxib can potentially be employed as a pre-emptive therapy to prevent vasospasm (Figure 23).

Rofecoxib, a more potent COX-2 inhibitor than celecoxib (FitzGerald and Patrono 2001), but devoid of the ion channel modulatory activity (Brueggemann et al. 2009; Du et al. 2011), did not induce vasodilatory responses (Figure 29A, C). Dimethyl celecoxib, a structural analog of celecoxib modified to eliminate COX-2 activity
(Kardosh et al. 2005), that retained the ion channel modulatory actions of celecoxib (Brueggemann et al. 2009), almost completely dilated spasmogen pre-constricted artery segments (Figure 29B, C). Hence, the vasodilatory effects observed with celecoxib are independent of its ability to inhibit the COX-2 enzyme. Disruption of endothelium did not attenuate the vasodilatory effect of celecoxib in pressurized mesenteric arteries (Brueggemann et al. 2009). Hence, the vasodilatory action of celecoxib are mediated through action on vascular smooth muscle cells rather than modulation of endothelial function as implicated previously (Klein et al. 2007). Though Kv7 channel activation and VSCC blockade in vascular smooth muscle cells are likely the principle mechanisms for vasodilatory responses observed with celecoxib, it is not possible to rule out modulation of other ion channels or intracellular Ca$^{2+}$ mobilization pathways by celecoxib that may also contribute in part to the responses.

**Kv7 channel activators prevent basilar artery vasospasm in a rat model of SAH**

Since Kv7 channel openers were able to reverse and/or prevent the constrictor responses of spasmogens (Figure 17, 18, 22 and 23), we tested the efficacy of two Kv7 channel openers, retigabine and celecoxib, as potential therapies to reduce basilar artery vasospasm in a rat model of SAH. We used a single hemorrhage model that produces biphasic vasospasm of basilar artery that peaks at day 2 after injection (Delgado et al. 1985). The basilar artery is the most common artery that undergoes spasm after injection of arterial blood into cisterna magna (Gules et al. 2002). In our disease model, injection of autologous arterial blood (but not aCSF) into the cisterna magna induced a sustained
constriction of the basilar artery that persisted 48 hours after the initial injury (Figure 24). Both the Kv7 channel openers, retigabine (7.5 mg/kg, i.p) and celecoxib (20 mg/kg, i.p), when administered twice daily, significantly attenuated the sustained basilar artery vasospasm in this model (Figure 24). Basilar artery outer diameter measured at 48 hr (12 hr after the last injection) was used as the endpoint criterion to assess the severity of vasospasm and efficacy of treatments.

Celecoxib offers a dual action (Kv7 current enhancement and voltage-sensitive Ca$^{2+}$ current inhibition), which may account for its tendency to induce a greater dilation of the basilar artery in the SAH model (Figure 24), translating its ex vivo vasodilatory efficacy (Figure 22, 23). Apart from modulating the activities of vascular ion channels, celecoxib is likely to provide neuroprotective and anti-inflammatory effects due to its clinically utilized COX-2 inhibitory activity. Cytokine-mediated upregulation of COX-2 expression has been observed in endothelial cells of basilar artery in several animal models of SAH contributing to the inflammatory response after SAH (Tran Dinh et al. 2001; Osuka et al. 2006; Osuka et al. 1998). COX-2 inhibition has been demonstrated to significantly reduce neuronal deterioration in a mouse model of aneurysmal SAH (Ayer et al. 2011). Thus, celecoxib may have multiple beneficial effects for patients suffering from SAH-induced cerebral vasospasm.

The limitation of the rat animal model is that the rats did not exhibit the neurological deficits (Gules et al. 2002; Germano et al. 1994) observed in humans. A double hemorrhage model, involving a second injection of blood into cisterna magna 48 hours after the first injection, induces a more sustained basilar artery constriction
simulating the timeline of vasospasm development in humans (Gules et al. 2002). Despite that improvement, rats in the double hemorrhage model did not exhibit a neurological profile similar to humans (Gules et al. 2002; Jeon et al. 2009). We attempted to assess the neurological endpoints using behavioural tests used in ischemic stroke models (Garcia et al. 1995). The SAH rats did not exhibit any significant change in neurological function compared to rats injected with aCSF into the cisterna magna (not shown). Assessment of neurological endpoints were further limited due to the significant reduction in the activity of rats with treatment of retigabine and celecoxib (Figure 26). A limitation in most of the neurological tests used in this study and elsewhere in the literature is that these neurological tests were developed to detect deficits caused by focal ischemia as seen in traumatic brain injury models (Jeon et al. 2009). Focal ischemic lesions are very uncommon in patients after SAH. The patients suffer from deficits in neurobehavioural function such as memory, visuospatial/construction ability and executive function (Jeon et al. 2009; Al-Khindi et al. 2010; Anderson et al. 2006; Kreiter et al. 2002). Since focal deficits are rare in animal models of SAH (Jeon et al. 2009), it is not surprising that none of the neurological tests showed a change with SAH. Impaired memory and behavior deficits after SAH in rodents can be assessed using paradigms like morris water maze, matching-to-sample tests, and open field behavior. However, these assessments were not feasible because of the significant reduction in the locomotor activity of rats treated with retigabine or celecoxib (Figure 26).

**Direct neuroprotective effects of Kv7 channel activators:** Emerging evidence suggests that cortical spreading depression (CSD) is a major contributing factor to
cerebral ischemia after SAH (Leng et al. 2011). CSD is manifested as slow (2-5 mm/min) waves of depolarization in cerebral gray matter that propagate across the brain (Leao 1944). The spreading depression is characterized by near-complete depolarization of neurons (from -70 mV resting voltage to -10 mV) and a maximal shunt in membrane resistance in neurons (Canals et al. 2005; Dreier 2011). The ensuing depolarization-induced hypermetabolic rate of neurons results in disrupted neurovascular coupling, hypoxia, and toxic accumulation of metabolic end products. This cascading events lead to an inverse hemodynamic effect in response to depolarization of neurons and a reduction of regional cerebral blood flow (Dreier 2011). A vicious cycle is likely established- the spreading depolarization maintains vasoconstriction and ischemia, and the ischemia maintains spreading depolarization (Dreier 2011). Hence, CSD is likely a co-morbid pathology with chronic vasospasm that contributes to delayed cerebral ischemia in patients (Bosche et al. 2010; Dreier et al. 2009). Electrocorticography recordings detected spreading depolarizations in 72% of patients after SAH and recurrent spreading depolarizations had a time locked correlation to cerebral ischemia (Lauritzen et al. 2011). The events that instigate CSD (and vasospasm) after SAH remain unclear. The fact that endothelin can induce CSD (Dreier et al. 2002) and vasospasm (Asano et al. 1990) indicates there might be some common etiological factors involved.

Neuronal Kv7 channels may be important regulators of CSD, based on previous findings that inhibition of Kv7 channels with linopirdine induced spreading depression in hippocamal slices (Okada et al. 2003) and S(1)-acrylamide, a Kv7.2-selective channel opener, prevented CSD induced by K+ in a rat model (Wu et al. 2003). Thus, a Kv7.2-7.5
channel opener like retigabine is expected to reduce cerebral ischemic deficits by exerting
direct neuroprotective effects by antagonizing CSD after SAH. Direct neuroprotective
effects of retigabine and flupirtine have been reported in several studies (Boscia et al.
2006; Gamper et al. 2006; Seyfried et al. 2000). Therefore, Kv7 channel openers will
likely reduce neuronal deficits after SAH through direct neuroprotective effects as well as
from their direct vasodilatory actions mediated by Kv7 channels in the basilar artery
myocytes (Figure 24).

**Kv7 channel activators induce hypothermia in a the rat model of SAH:**
Increased body temperature after SAH is associated with vasospasm, increased functional
disability and cognitive impairment, and high mortality in patients (Badjatia et al. 2010;
Fernandez et al. 2007; Oliveira-Filho et al. 2001). Mild hypothermia (33-34°C) has been
reported to have a neuroprotective effect and increases the functional outcome in patients
with delayed cerebral vasospasm (Gasser et al. 2003; Maekawa et al. 1994; Nagao et al.
2000; Nagao et al. 2003) and in a rat model of SAH (Torok et al. 2009). Induction of
moderate hypothermia also reduced the hypoperfusion-induced metabolic alterations in a
rat model of SAH (Schubert et al. 2008). Therapeutic hypothermia has emerged as a
potent and practicable neuroprotective treatment for ischemic and hemorrhagic stroke and
intracerebral hemorrhage (Feigin et al. 2002; Hemmen and Lyden 2009; Linares and
Mayer 2009). The mechanism for the neuroprotective effects likely includes reduced
metabolic demand of the brain parenchyma (Erecinska et al. 2003; Jiang et al. 1992),
stabilization of the blood-brain barrier (Jiang et al. 1992; Smith and Hall 1996), reduced
post-ischemic cerebral edema (Kurasako et al. 2007), reduction of ischemia-induced
neurotoxic glutamate release (Busto et al. 1989; Globus et al. 1995) and free radical production (Globus et al. 1995; Lee et al. 2009).

We found that retigabine induced significant hypothermia in the rat model of SAH, even 12 hours after administration (Figure 25). Celecoxib (20 mg/kg) also tended to lower body temperature. A recent study also observed hypothermia with intraperitoneal administration of Kv7 channel openers: retigabine, ICA-27243, S(1)-acrylamide and BMS-204352 in mice (Kristensen et al 2011). The hypothermic response in mice (about 4°C reduction in temperature) with retigabine administration was rapid in onset and lasted only up to an hour after administration (Kristensen et al. 2011). The reason for the long lasting hypothermia observed in the rat model of SAH in not clear, but could involve modulation of Kv7 channels in the hypothalamus that encompasses the thermoregulatory center (Cooper et al. 2001; Weber et al. 2006). Nevertheless, the hypothermic effect observed with Kv7 channel activators is likely to improve the functional outcome in patients after SAH. Severe hypothermia produces several complications ranging from electrolyte imbalances to multiple organ failure in patients with cerebral vasospasm (Seule et al. 2009). Therefore, a careful individualized examination of the risk: beneficial effects of the Kv7 channel activation-induced hypothermia need to be undertaken if administered to patients with cerebral vasospasm.

**Kv7 channel activators do not induce sustained hypotension**

Sustained hypotension may limit the utility of other classes of $K^+$ channel openers (e.g. $K_{ATP}$ channel openers) or CCBs in the effective treatment of cerebral vasospasm.
(Spinelli et al. 1990; Porchet et al. 1995). In contrast, intraperitoneal administration of either retigabine (7.5 mg/kg) or celecoxib (50 mg/kg) induced only a transient drop in MAP without any appreciable change in the 24-hour MAP (Figure 27) or heart rate (Figure 28). The reason for resiliency to effects of Kv7 channel openers on MAP is likely because (1) these channels are active under physiological conditions in a sub-threshold voltage range (-60 to -40 mV, Figure 6) in which VSCCs are not appreciably active; (2) the whole-cell conductance through Kv7 channels is very small compared to 4-AP-sensitive Kv and BK channels (Jahromi et al. 2008c).

The half-life of retigabine in male rats is 2 h and that of celecoxib is 3.7 h (Mazarati et al. 2008; Paulson et al. 2000). Both the drugs accumulate significantly in the brain, exceeding the plasma concentrations when administered to rats (Rostock et al. 1996; Pfizer Canada 2007). This could also account for the short-lived effect on systemic arterial blood pressure, but long-lasting vasodilatory effect on basilar artery.

The vascular effects (vasodilation) of enhanced Kv7 channel activity would primarily result from relaxation of myocytes with depolarized membrane voltages (e.g. basilar artery myocytes following SAH), with little effect on the systemic vasculature where arterial myocytes have resting voltages between -60 and -40 mV. In this regard, the function of Kv7 channels in vascular myocytes is analogous to their function in the excitation of neurons, where dysfunction, either by suppression of M-currents, or inactivating mutations of Kv7 channels, results in hyperexcitability precipitating seizures (Jentsch 2000). In arteries, activation of Kv7 currents relaxes the constricted arterial myocytes (Figure 24) without producing marked systemic hypotension (Figure 27).
similar to the effects of retigabine used as an anti-epileptic agent, where it dampens neuronal hyperexcitability with little effect on the normal function of the nervous system (Gunthorpe et al. 2012).

**Kv7 channel activators as candidates to treat cerebral vasospasm**

A number of pharmaceutical companies are currently developing novel Kv7 channel openers to treat epilepsy and neuropathic pain (Miceli et al. 2008). Retigabine was recently approved by the US Food and Drug Administration as an adjunctive treatment for partial-onset seizures in adult patients (Gunthorpe et al. 2012; Harris and Murphy 2011; Stafstrom et al. 2011). More than 20 patents for selective openers of Kv7.2-7.5 channels have been issued and an estimated more than 100 applications are currently under review (Castle 2010; Wulff et al. 2009). Therefore, it is reasonable to expect that Kv7 channel openers with better pharmacodynamic and pharmacokinetic profiles will be available soon. Our findings suggest that, in addition to their direct neuroprotective effects (Boscia et al. 2006), these drugs with established safety profiles (Blackburn-Munro et al. 2005; Frampton and Keating 2007; Brodie et al. 2010; French et al. 2011; Ueberall et al. 2011) can be readily adopted to prevent or limit cerebral vasospasm after SAH. Based on the pronounced antivasospastic effect of Kv7 channel openers reported here, we would add cerebral vasospasm to the expanding repertoire of conditions potentially treatable with Kv7 channel openers.
Phosphodiesterase inhibitors activate Kv7 channels and block voltage-sensitive calcium channels in A7r5 cells

We utilized cultured aortic smooth muscle (A7r5) cells derived from rat embryos to study the mechanism by which celecoxib modulates ion channels. Since a previous study suggested that celecoxib is a PDE4 and 5 inhibitor (Klein et al. 2007), we tested if selective PDE inhibitors simulate celecoxib’s action on ion channels. Selective pharmacological inhibition of PDE4 (with rolipram), PDE5 (with sildenafil) or a non-selective PDE inhibition (with papaverine) significantly enhanced the voltage-activated Kv7 currents (Figure 30, 32 and 34). Similarly, all these PDE inhibitors also inhibited voltage-sensitive Ca^{2+} currents (Figure 31, 33, (Han et al. 2007)) in vascular smooth muscle cells. Application of either rolipram or sildenafil attenuated AVP-induced Ca^{2+} spiking responses in A7r5 cells (Figure 35; sildenafil results not shown) and dilated pre-constricted mesenteric artery segments (Figure 36), again simulating the effect of celecoxib (Brueggemann et al. 2009). However, whether inhibition of PDE enzymes accounts for all (or even any) of the ion channel modulatory effects of celecoxib is not known. Experiments undertaken to address that question using whole cell patch-clamp experiments were inconclusive because of the bell-shaped dose-response for the ion channel modulatory actions of both celecoxib and the PDE inhibitors (Figure 30, 31 (Brueggemann et al. 2009)). Another possible mechanism for the ion channel modulatory effects of celecoxib could be due direct binding of the drug to the channels as proposed for Kv7 channels (Du et al. 2011). The ability of celecoxib to enhance single channel currents through overexpressed Kv7 channels would be an approach to test whether
activation of Kv7 channels by celecoxib is due to direct binding of the drug to the channels or mediated through a signal transduction pathway as proposed here. Nevertheless, these present experiments suggest enhancement of Kv7 currents and inhibition of voltage-sensitive Ca\(^{2+}\) currents as likely contributors to the vasodilatory responses observed with PDE inhibitors. Further experiments are needed to confirm whether inhibition of PDE enzymes fully accounts for ion channel modulatory effects of celecoxib.

All the aforementioned PDE inhibitors are used clinically to treat a variety of clinical conditions. Rolipram was used to treat depression and chronic obstructive pulmonary disease (O'Byrne and Gauvreau 2009), sildenafil is used to treat erectile dysfunction (Sandner et al. 2007) and papaverine is used to treat cerebral vasospasm (Liu and Couldwell 2005). Despite their extensive clinical utility and the well-characterized vasodilatory responses and reduction in blood pressure associated with their use (Oliver et al. 2006; Zusman et al. 1999), understanding of the cellular mechanisms leading to vascular responses is nascent.

PDE4 selectively hydrolys...
Furthermore, activation of the downstream kinases by the nucleotides: PKA by cAMP and PKG by cGMP, while selective, are known to cross-activate the other kinase (Keef et al. 2001). Therefore, a simplistic model with both or either of the nucleotides regulating the channel through activation of a vertical downstream signal transduction cascade may be unlikely.

In neurons, Kv7 channels exist in a signaling complex along with PKC, PKA, and protein phosphatase 2B (PP2B) held together by a multivalent scaffolding protein, AKAP (Hoshi et al. 2003; Higashida et al. 2005; Hoshi et al. 2005). AKAP has also been shown to immunoprecipitate with Kv7.5 channels in A7r5 cells and is proposed to be in a signaling complex that includes PKC (Hummert et al. 2011), similar to the existence in neurons. PDE4 enzymes have been also demonstrated to be constitutively associated with AKAP in VSMCs (Dodge et al. 2001; Raymond et al. 2009; Houslay et al. 2007). Hence, there is a possibility that AKAP tethers Kv7 channels in a signalosome, along with PDE4 and PKA, thereby facilitating phosphorylation of the channels by the cAMP-activated PKA to enhance Kv7 currents.

Functional analysis in cardiomyocytes indicated that PDE4 and cAMP-activated-PKA are in close proximity, and are held together by AKAP (Dodge et al. 2001). Enhanced activity of PDE4 was found to reduce PKA activity; elevated cAMP and kinase activity had a negative feedback stimulation of PDE4 enzyme in cardiac or vascular myocytes (Dodge et al. 2001; Rose et al. 1997). Another study also showed that long-term elevation of cAMP by exogenous administration of cAMP-elevating drugs to rats stimulated transcription of PDE 3 and 4 enzymes in vascular myocytes (Tilley and
This highlights the complex overlapping feedback loops at different levels, which enables a tight control of the cAMP levels and the downstream PKA activity. The possible existence of the signalosome and the negative feedback mechanisms in vascular myocytes could contribute to the bell shaped concentration response curves observed here (Figure 30, 31).

The mechanism by which sildenafil regulates the Kv7 channels is not known and requires further investigation. As an inhibitor of PDE5, sildenafil is expected to increase cGMP levels. Voltage-sensitive Ca$^{2+}$ channels are known to be modulated by cyclic nucleotides, cAMP and cGMP. Elevation of cGMP using a membrane-permeable analog, 8Br-cGMP or nitric oxide (NO) donors have been shown in several studies to inhibit VSCC in a PKG-dependent manner (Keef et al. 2001). PKG-mediated VSCC blockade may therefore explain the sildenafil effect observed here. PKG has been shown to phosphorylate both the Ca$_1$1.2 $\alpha$1c subunit and the accessory $\beta$2 subunit of VSCC in a heterologous expression system; phosphorylation of a serine residue in the $\beta$2 subunit accounted for the PKG-induced inhibition of the currents (Yang et al. 2007). Thus, a direct phosphorylation of VSCC by PKG could account for the inhibition of voltage-sensitive Ca$^{2+}$ currents observed with sildenafil.

In the present study, addition of rolipram, which is expected to increase cAMP, inhibited voltage-sensitive Ca$^{2+}$ currents. Regulation of voltage-sensitive Ca$^{2+}$ currents by cAMP and the downstream kinase (PKA) in VSMCs is controversial, with several studies showing either enhancement, inhibition, or no effect on the currents (Keef et al. 2001). Elevation of intracellular cAMP by stimulation of Gs-coupled seven transmembrane
receptors results in dilation of intact arteries (Satake and Shibata 1997), consistent with our electrophysiological and functional results with rolipram. However, further studies are needed to elucidate the downstream effectors that lead to inhibition of VSCC with addition of rolipram.

An increase in the expression of PDE5 enzyme and enhanced PDE5 enzyme activity was observed in vasospastic canine basilar artery myocytes compared to control basilar artery myocytes (Inoha et al. 2002). Another study using a mouse model of SAH found increased PDE5 enzyme activity, but no increase in the expression of the enzyme in vasospastic arteries (Han et al. 2012). Treatment with sildenafil attenuated the basilar artery vasospasm in the canine, rat and mouse models of SAH (Gokce et al. 2010; Han et al. 2012; Inoha et al. 2002). The fact that sildenafil is a Kv7 channel activator (Figure 32) indicates that at least part of its anti-vasospastic effects might be mediated through activation of Kv7 channels.
CHAPTER 7

CONCLUSIONS

This comprehensive dissertation provides the first evidence for the presence of Kv7 channels in basilar artery and a central role of the channels in regulating the contractile status of the artery. The results show that known basilar artery spasmogens, including the $G_{q/11}$-coupled receptor agonists, serotonin, endothelin, and vasopressin, potently suppress the activity of Kv7 channels in basilar artery myocytes and induce membrane depolarization. This may account for the depolarization that has been observed in myocytes following SAH. This study also demonstrates for the first time that celecoxib is an effective dilator of basilar artery by activating Kv7 channels as well as by blocking voltage-sensitive $Ca^{2+}$ channels. Pharmacological Kv7 channel activators oppose the effect of spasmogens on Kv7 currents and membrane depolarization, and relax pressurized basilar arteries that are pre-constricted with these spasmogens. We also demonstrate that clinically used Kv7 channel activators, retigabine, and celecoxib were effective in relieving basilar artery spasm \textit{in vivo} in rats with experimentally-induced SAH. Hence, these results provide evidence supporting the use of Kv7 channel activators as a novel class of drugs to treat patients who develop or are likely to develop cerebral vasospasm (Figure 37).

Our laboratory was the first to propose that Kv7 potassium channels are signal transduction intermediates in the vasoconstrictor actions of physiological concentrations
of vasopressin. This dissertation provides the first evidence that this mechanism is shared by multiple vasoconstrictors that activate Gq/11-coupled receptors in basilar artery myocytes. As such, its significance extends beyond the role of these channels in the cerebral circulation, providing mechanistic insights into the signal transduction processes that contribute to physiological and pathological regulation of blood flow and blood pressure in general. This dissertation also provides the first evidence that activation of Kv7 channels is a mechanism by which phosphodiesterase inhibitors induce vasodilation and reduction in blood pressure (Figure 37).

In summary, Kv7 channels in basilar artery myocytes are critical regulators of the vasoconstrictor/dilator response. Apart from revealing a potential novel therapeutic target for treatment of cerebral vasospasm, identification of the role of Kv7 channels in the vasculature is likely to aid in the development of alternative treatment options for other dreadful cardiovascular disorders like coronary vasospasm, hypertension, and septic shock, which are conditions characterized by dysregulated vasoconstriction. This dissertation project, thus, identifies a possible solution to contain several epidemic cardiovascular diseases that engulf our society and continue to expand.
Figure 37: Spasmogens suppress Kv7 currents to induce basilar artery vasospasm; Kv7 channel activators oppose the spasm. Spasmogens, by binding to their specific receptors, suppress the Kv7 currents through a signal transduction mediated mechanism. Suppression of the Kv7 currents depolarizes the membrane, activating voltage-sensitive Ca\(^{2+}\) channels, resulting in influx of Ca\(^{2+}\) and, initiation and maintenance of constriction. Administration of the Kv7 channel activator, retigabine, or the dual Kv7 channel activator and voltage-sensitive Ca\(^{2+}\) channel blocker, celecoxib, prevents/overcomes the spasmogen-induced vasospasm.
CHAPTER 8
FUTURE DIRECTIONS

The dissertation project presents the first evidence for the presence and function of Kv7 channels in rat basilar artery myocytes. This study adds to the previous findings that Kv7 channels play a pivotal role in maintaining the resting membrane voltage of excitable cells. Single-cell patch-clamp and pressure myography studies provided evidence for the mechanism by which Kv7 channel activators could be a novel class of drugs to treat patients with cerebral vasospasm. The study also provided “proof-of-concept” evidence that Kv7 channel activators attenuate basilar artery vasospasm in an acute model of SAH. However, the study does not address whether the anti-vasospastic effects of Kv7 channel activators would result in a favorable clinical outcome in terms of reduction in neurological deficits and perhaps reduction in mortality. This is not an easy question to address in the current scenario, given that there are no good animal models that can predict the neurological outcome in patients. Alternatively, since both the Kv7 channel activators employed here are clinically utilized drugs with established safety profile, a clinical study with a small cohort of patients can be performed.

The findings from the study, if they hold in humans, could have immense translational value and offer a possible effective alternative therapeutic approach for cerebral vasospasm. However, additional information is needed to realize the potential of the current study. For one thing, there is a paucity of information regarding the
expression of Kv7 channels in the human cerebral vasculature and even vascular myocytes in general. To date there is only one report, which shows the existence of Kv7 channels in human vasculature (Ng et al. 2011). Evidence for the expression and function of these channels in human cerebral vasculature is therefore required before initiation of clinical trials using Kv7 channel activators to treat cerebral vasospasm.

The study provides strong evidence that inhibition of phosphodiesterase enzymes(s) using clinically utilized drugs activate Kv7 currents in cultured A7r5 cells. The findings need to be validated in myocytes derived from human vasculature to address the translational relevance of the presence findings. In a classic case of reverse pharmacology, the findings opens the door for understanding signal transduction mediated regulation of ion channels, including Kv7 channels and voltage-sensitive Ca\(^{2+}\) channels. As mentioned in the discussion section, there is a very high likelihood that Kv7 channels are in a signalosome that includes PKA and PDE, held together by AKAP (Figure 38). Studying the mechanisms by which celecoxib and phosphodiesterase inhibitors regulate Kv7 channels is therefore expected to shed more light on the regulation of vascular tone and therefore potentially identify new targets to modulate vascular tone.
Figure 38: Hypothetical mechanism by which celecoxib and rolipram regulate Kv7 currents in vascular smooth muscle cells. Activation of the membrane-bound enzyme adenyl cyclase synthesizes cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). The cytosolic levels of cAMP is dynamically regulated by degradation to adenosine monophosphate (AMP) by cAMP-selective phosphodiesterase (PDE) enzymes, like PDE4. PDE4 is likely tethered in a signalasome that also encompasses protein kinase A (PKA), and Kv7 channels, held together by a scaffolding protein, A-kinase anchoring protein (AKAP). Inhibition of PDE4 enzyme by celecoxib or rolipram results in accumulation of cAMP, which activates PKA. PKA likely phosphorylates the channels in setine/threonine residue(s) to activate the Kv7 channels. Sildenafil similarly enhances cyclic guanosine monophosphate (cGMP) levels, protein kinase G (PKG) activity, which could modulate the Kv7 channel activity either by direct phosphorylation of the channels or by modulating the cAMP-PKA pathway.
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VITA

Bharath Mani was born on April 02, 1978 in Namakkal, a small town in the southern state of Tamil Nadu, India to Subraya Gounder Mani and Rajeswari Mani. After completing his grade school education in 1995, Bharath entered the Bachelor of Veterinary Sciences program at Veterinary College and Research Institute located in his hometown. After graduating in 2001, he moved to Chennai, India to pursue a Master’s degree, majoring in pharmacology. During his Master’s program he worked on a thesis project investigating the effects of ionophores on ruminant digestion. In 2004, he joined the cardiovascular drug discovery program at Torrent Research Centre, Ahmedabad, India, where he was involved in conducting pre-clinical efficacy and toxicity studies of new chemical entities.

In July of 2006, Bharath moved to Chicago and enrolled in the pharmacology graduate program at Loyola University Medical Center. After his research rotations, Bharath joined the laboratory of Dr. Kenneth Byron in the fall of 2007. Bharath has since been working to understand the regulation of vascular tone by ion channels in smooth muscle cells. Bharath’s dissertation research was funded by American Heart Association (2009-2011) and Arthur J. Schmitt Foundation (2011-2012).

Bharath is married since 2007 to Thejashree and they have a lovely daughter, Aditi.