2016

Phosphodiesterase Inhibition as a Mechanism of Ion Channel Modulation by Celecoxib

Christina Jean Robakowski
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

PHOSPHODIESTERASE INHIBITION AS A MECHANISM OF ION CHANNEL MODULATION BY CELECOXIB

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN MOLECULAR PHARMACOLOGY AND THERAPEUTICS

BY

CHRISTINA J. ROBAKOWSKI

CHICAGO, IL

MAY 2016

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my parents, Patty and Mike, for their endless support and encouragement throughout my academic career. They have always been my number one fans, and I can never thank them enough for raising me to be the hard-working person that I am today.

I would also like to thank my brother, Jimmy, my Uncle Rick, and my Aunt Debbie for their constant support and encouragement. After losing my father unexpectedly during my time here at Loyola, they have all done as much as possible to help me through this difficult time so that I could focus on finishing my project.

To Alex, who has been such a strong and positive presence in my life for the last four years. His support and constant encouragement have been invaluable to me in completing my thesis work.

To my lab mates, the best bunch of people around. To Jen Haick, without whom there would be no throwback Thursday radio, fiesta Friday lunches, or Backstreet Boys/NSYNC Pandora jam sessions. To Dr. Lioubov Brueggemann, for being so patient in training me in electrophysiology and for always being happy to help me out and share your expertise. You are a saint. And of course, a big thank you goes to my advisor, Dr. Kenneth Byron. I am so grateful for your support and guidance. Your determination to make me a better writer and

scientist is very much appreciated. Thank you all, you truly made life in lab an enjoyable experience.

I also want to thank Loyola University Chicago, especially the department of pharmacology for all of their advice, insight, assistance, and support over the last two years.

Finally, this thesis would not be possible without the support and guidance of my committee members: Dr. Kenneth Byron, Dr. Mitchell Denning, and Dr. Tarun Patel. Thank you for all of your understanding, thoughtful questions and excellent advice throughout my thesis project.

For my dad, my guardian angel

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AC	adenylyl cyclase
AKAP	A-kinase anchoring protein
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AVP	Arginine (8)- vasopressin
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
DAG	diacylglycerol
DMEM	Dulbecco's modified eagle medium
EC_{50}	half maximal effective concentration
E_{max}	maximum response in a dose-response curve
Fsk	forskolin; a direct activator of adenylyl cyclase
Gd^{3+}	gadolinium
GPCR	G-protein coupled receptor
G_q	guanine nucleotide binding protein that activates phospholipase C
G_s	guanine nucleotide binding protein that activates adenylyl cyclase
IBMX	3-isobutyl-1-methylxanthine
IP_3	inositol-1,4,5-trisphosphate

ISO	isoproterenol; a β-adrenergic agonist
K^+	potassium ion
KCNQ	gene that encodes Kv7 channel proteins
Kv	voltage-sensitive potassium channels
Kv7	class of voltage-sensitive potassium channels
mRNA	messenger RNA
NSAID	non-steroidal anti-inflammatory drugs
PDE	phosphodiesterase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
VSCCs	voltage-senstive calcium channels
VSMCs	vascular smooth muscle cells

CHAPTER 1

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen, and naproxen, are widely used to reduce pain, fever, and headaches associated with inflammation. The therapeutic benefits of these drugs are associated with the inhibition of cyclooxygenases 1 and 2 (COX-1 and COX-2). Of the two forms, COX-1 is the constitutively active isoform and is responsible for converting arachidonic acid into prostaglandins that are generally accepted to be “house-keeping” prostaglandins. COX-2 is the inducible part of the pathway that is responsible for converting arachidonic acid into inflammatory prostaglandins that result in pain, swelling, redness, and other inflammatory responses. Traditional NSAIDs non-specifically inhibit COX-1 and COX-2, meaning they inhibit both the inflammatory and house-keeping sides of the COX pathway. The non-specific nature of NSAIDs results in adverse effects that come about as a consequence of inhibiting the COX-1 pathway. The most noted adverse effects of COX-1 inhibition are gastric complications that result from the disruption of prostaglandin E2 production [1]. These gastric complications resulted in a population of patients who could not tolerate traditional NSAID use. These patients needed an alternative medication that could offer the anti-inflammatory properties of NSAIDs without the gastric complications.

Coxibs were developed to meet the need of patients who could not tolerate traditional NSAID use. Coxibs specifically inhibit the inflammatory COX-2 pathway while leaving the COX-1 pathway relatively unaffected. This was meant to give patients the anti-inflammatory benefits of blocking the COX-2 pathway without the gastric complications associated with traditional NSAID use. The coxibs originally developed were celecoxib (Celebrex®), rofecoxib (Vioxx®), valdecoxib, etoricoxib, and lumiracoxib. The coxibs were available on the market just 5 years after animal model validation in 1994, making it one of the most rapid drug discovery and development efforts [2]. The need for these drugs was validated when celecoxib and rofecoxib garnered a combined sales total of almost 2 billion dollars in their first years on the market [2]. This initial success lead to an aggressive advertising campaign for both drugs. The advertising exposed many more people to a class of drugs that was originally developed for patients who could not tolerate traditional NSAIDs. This upsurge in popularity of coxibs lead to the launch of clinical trials that would add to the labeled uses of these drugs. One of these clinical trials was the Adenomatous Polyp Prevention on Vioxx (APPROVE) [2]. During this and two parallel trials, patients on rofecoxib were found to have an almost 2-fold increase in the occurrence of cardiovascular side effects; these side effects included myocardial infarctions and ischemic cerebrovascular events [3-5]. These findings caused Merck to withdraw Vioxx from the market within days of the report being released. The following year, valdecoxib was withdrawn from the market for similar reasons [2].

The negative publicity associated with the withdrawal of rofecoxib and valdecoxib from the market led to a push to determine the cardiovascular risk profiles of celecoxib (Celebrex®) and other coxibs still on the market.

A review of randomized clinical trials of COX inhibitors revealed that rofecoxib and diclofenac both significantly increased the risk of cardiovascular events [3], but a number of clinical trials were unable to establish the same increased cardiovascular risk with celecoxib [3, 6]. The mechanism behind this difference in cardiovascular risk is still unknown and the exact reason behind this difference is widely debated. Several studies reported celecoxib to exhibit vasorelaxant effects [7, 8] which may account for its differential risk profile compared to other COX-2 inhibitors. Our laboratory attempted to explore the mechanisms behind the vasodilatory actions and lower CV risk for celecoxib by examining the effects of celecoxib and other NSAIDs on vascular smooth muscle ion channels. Brueggemann et al. focused their attention on two types of ion channels that are important in determining the contractile state of vascular smooth muscle cells (VSMCs): KCNQ (Kv7 family) potassium channels that determine the resting membrane voltage and L-type voltage gated calcium channels, the activation of which induces calcium ion influx, smooth muscle contraction, and vasoconstriction [9]. In that study, our laboratory provided evidence that celecoxib, but not rofecoxib or diclofenac, activated Kv7 potassium channels and inhibited L-type calcium channels at comparable therapeutic concentrations, promoting vasorelaxation and decreasing vascular tone.

Celecoxib's ability to modulate these ion channels was also found to be independent of its COX-2 inhibitory effects. A celecoxib analog, 2,5-dimethylcelecoxib (DMC) which does not inhibit COX-2, mimicked both celecoxib's activation of Kv7 channels and inhibition of L-type channels [9]. That study ultimately led to the hypothesis that the COX-2 independent ion channel modulatory actions of celecoxib may account for the reduced cardiovascular risk in patients taking celecoxib compared to other coxibs.

The mechanism that underlies celecoxib's ion channel modulatory actions is still not known. As stated previously, it is believed that these actions are independent of celecoxib's COX-2 inhibitory activity [9]. Given the differential ion channel modulatory activities reported for celecoxib and rofecoxib, it stands to reason that the mechanism by which celecoxib activates Kv7 potassium channels would be stimulated by celecoxib and not by rofecoxib. One such mechanism is cyclic nucleotide phosphodiesterase (PDE) inhibition. Klein et al. reported that celecoxib and not rofecoxib, inhibited the PDE4 isoform, which specifically degrades cyclic adenosine monophosphate (cAMP) [8]. cAMP-specific cyclic nucleotide PDE inhibition results in higher cellular levels of cAMP which goes on to affect many downstream targets. One well known target of cAMP is protein kinase A (PKA). Activation of PKA by cAMP leads to the phosphorylation and activation of many downstream targets, one of which may be Kv7 potassium channels. In this thesis, I hypothesize that activation of the

cAMP/PKA pathway is necessary and sufficient to induce the activation of Kv7 potassium channels in vascular smooth muscle cells.

CHAPTER 2

REVIEW OF RELEVANT LITERATURE

KCNQ Channels

Discovery

Kv7 potassium channel subunits are encoded by five genes (KCNQ1-5) that were first cloned in the 1990s. The Kv7 nomenclature given to this family of channels was based on the deducted phylogenetic relationships [10] and is used interchangeably with KCNQ based on the Human Genome Organization's KCN nomenclature [11].

KCNQ1 was the first of the Kv7 channels to be discovered. It was originally named KvLQT1 because it was discovered in the heart and was found to co-assemble with MinK (which is encoded by the KCNE gene) to form slowly activating delayed-rectifier K^+ channels (IKs) whose dysfunction results in long-QT syndrome which can result in fatal cardiac arrhythmias. IKs currents modulate repolarization of cardiac action potentials [12].

M-currents in the brain are responsible for regulating the subthreshold excitability of neurons. KCNQ2 and KCNQ3's properties were determined and it was reported that the hetero-tetramerization of those two channel subunits is what underlies neuronal M-current. This conclusion was reached because of their kinetic properties, which were similar to previously recorded native M-currents,

and their sensitivity to muscarinic inhibition (where the M in M-current is derived) [13].

To date, KCNQ1-5 (which encode Kv7.1-7.5 channel subunits respectively) have all been cloned and the channels formed by these subunits have important functions in many types of excitable tissues [14].

Structure and composition of functional channels

The functional Kv7 channels resemble other Kv channels in that they are composed of homo- or hetero-tetramers of six transmembrane spanning (S1-S6) subunits [14]. The P-loop (single pore loop) formed between the S5 and S6 domains form the selectivity filter of the pore. The S4 domain consists of positively charged arginine residues and acts as a voltage sensor. Both the N- and C-terminus of the channel are present on the cytoplasmic side of the membrane. A distinct feature of Kv7 channels is that they have a long intracellular C-terminus [15]. The C-terminus contains a distinctive A-domain that determines the subunit specificity of the channels [16]. This distinctive C-terminus is important for channel gating, assembly, and trafficking [17].

The five channel subunits can assemble in a number of conformations. First, each channel is able to form a homo-tetramer which consists of four of the same alpha subunits. These channel subunits can also form hetero-tetramers, but the subunit combinations are limited. Kv7.1 is unable to form a hetero-tetramer with any of the other Kv7 channel subunits, but it has been suggested

that it co-assembles with KCNE1 which encodes the ancillary protein MinK [18]. Kv7.2 and Kv7.3 form a hetero-tetramer that underlies the M-current of the brain [13]. Kv7.4 can form a hetero-tetramer with Kv7.3 but not Kv7.2 or Kv7.1 [19, 20]. Kv7.5 can form a hetero-tetramer with Kv7.3 and Kv7.4 but not Kv7.2 [21, 22].

To date, there is no evidence of Kv7 channel subunits assembling with any other Kv channel subunits (such as Kv1, Kv2, or Kv4).

Expression

Originally, Kv7 channels were thought to be expressed only in neurons and cardiac myocytes, however, more recent evidence suggests a ubiquitous expression profile [23, 24]. Additionally, channel expression and assembly varies by tissue.

In the heart, Kv7.1 channel subunits form homo-tetramers that conduct the cardiac delayed rectifier current, IKs. This current is responsible for modulating cardiac repolarization. These Kv7.1 channels have been reported to co-assemble with MinK which is a single transmembrane spanning auxiliary β-subunit encoded by the KCNE1 gene. Assembly of MinK with KV7.1 channel subunits leads to slowing of Kv7.1 activation, an increase in unitary conductance, removal of inactivation, and an altered regulation and pharmacology of the complex [18]. Mutations in either KCNQ1 or KCNE1 genes can result in long QT syndrome (LQTS).

A study by Oliveras et al. suggested the formation of functional heteromeric complexes between Kv7.5 and Kv7.1 channels (possibly with KCNE1 and/or KCNE3 regulatory subunits) within the vasculature. This conclusion was based on the ability of Kv7.1, Kv7.5 and KCNE1 to co-immunoprecipitate in rat aorta myocytes. They also observed Kv7.1 and Kv7.5 forming functional channels with distinct biophysical and pharmacological properties in an expression system [25]. However, the functionality of Kv7.1 channels in arterial myocytes as homotetramers or in a heteromeric complex with Kv7.5 subunits requires further exploration.

Expression of KCNQ genes in smooth muscle will be discussed later in this chapter.

Biophysical properties

Outward potassium currents that are conducted through Kv7 channels have unique biophysical characteristics. First, the threshold of activation is approximately the same as the resting membrane voltage, usually around -60mV. Second, when the membrane depolarizes from resting voltages, there is a slow, voltage-dependent enhancement of the currents through the channels. Third, the currents do not inactivate [26, 27]. These biophysical characteristics suggest activation of Kv7 channels would oppose membrane depolarization and decrease the excitability of excitable cells. Therefore, inhibition of these channels

increases the likelihood of action potential firing, L-type calcium channel activation, calcium influx, and smooth muscle contraction.

Regulation

PIP_2 Mediated

Phosphatidylinositol-4,5- bisphosphate (PIP_2) plays a central role in the regulation of many voltage-sensitive potassium (Kv) channels. The Kv7 family of potassium channels is unique in that it has been reported to require PIP_2 in order to conduct current [28]. The best characterized of these interactions involve M-current KCNQ2/KCNQ3 in expression systems [29]. Agonists of $G_{q/11}$-coupled receptors cause M-current suppression and increase neuronal excitability. This suppression can be attenuated by phospholipase C (PLC) inhibitors and recovery from this suppression requires ATP [30]. Zhang and colleagues reported evidence that PIP_2 was required for activation of KCNQ channels. They reported application of PIP_2 to inside-out macropatches rescued both homomeric Kv7.2 and heteromeric Kv7.2/7.3 channel activities. Additionally, they tested the PIP_2 sensitivity of Kv7.1, Kv7.1/KCNE1, Kv7.4, and Kv7.5 and found that all of these family members were sensitive to PIP_2 since bath application of PIP_2 rescued the channel activities of all of these subtypes [31]. It has been suggested that this increase in activation of Kv7 channels by PIP_2 is the result of a PIP_2-mediated increase in the open probability of the channels. Li and colleagues reported that each channel is differentially sensitive to PIP_2 and this sensitivity is paralleled in the differential increase in open probability of each channel subtype [32].

Signaling Complexes

Signaling complexes have been implicated in regulating the specificity and efficiency of signal transduction pathways [33]. Signaling complexes mediate specificity of signaling pathways by bringing specific signaling molecules into the proximity of specific substrates, thereby compartmentalizing signaling events. There have been reports that Kv7 channels are involved in signaling complexes brought together by the scaffolding protein A-kinase anchoring protein (AKAP) 79/150 in neurons. AKAP79/150 is a family of 3 orthologs: human AKAP79, murine AKAP150, and bovine AKAP75 [34]. As its name suggests, AKAP is able to bind protein kinase A (PKA). It is also reported to bind to protein kinase C [35], phosphodiesterases [36], and Kv7 channels [15]. AKAP was reported to interact with Kv7.2-7.5 channels but not Kv7.1 using fluorescence resonance energy transfer (FRET) under total internal reflection fluorescence (TIRF) microscopy [22].

Phosphorylation

Phosphorylation of Kv7 channels by the non-receptor tyrosine kinase, Src, was reported to inhibit currents by reducing the open probability of the channels. Src phosphorylation is selective for Kv7.2, Kv7.3, Kv7.2/7.3, and Kv7.5 channel subunits [37]. The specific tyrosines phosphorylated by Src on the Kv7.3 subunit were reported to be Y67 on the N-terminus and Y349 on the C-terminus. Simultaneous phosphorylation of these two residues was reported to be required

for Src regulation of the channel [38]. The physiological significance of Kv7 channel regulation by Src phosphorylation is unknown.

Transcriptional

Rose and colleagues reported transcriptional regulation of KCNQ2 in dorsal root ganglia neurons by repressor element 1-silencing transcription factor (REST) [39]. They reported that an upregulation of REST in situations such as neuropathic pain and injury (typified by an overexcitability of the neurons) leads to a downregulation of KCNQ2. This gene encodes for the Kv7.2 channel subtype that, together with Kv7.3, form functional channels that underlie the M current of the brain and control neuronal excitability. They showed that application of the Kv7 channel activator, flupirtine, was able to alleviate the neuropathic hyperalgesia in their rat model, further highlighting the importance of Kv7 channels in neuronal excitability [39].

Pharmacology of KCNQ channels

Pharmacological agents have been developed that modulate Kv7 channel activity. Linopirdine [DuP, 1, 3-dihydro-1-phenyl-3,3-bis(4-pyridinylmethyl)-2*H*indol-2-one] is a derivative of phenylindolinone and was developed to be a cognitive enhancer [40]. It and its more potent analog, XE991 are effective blockers of all five subtypes of Kv7 channels. Activators of the Kv7 channels have been developed as well. Flupirtine [D-9998, Katadolon®, ethyl 2-amino-6-((p-fluorobenzyl)amino)-3-pyridinecarbamate] is a triaminopyridine and is used clinically as non-opioid analgesic in Europe. Modification of flupirtine's structure

yielded the antiepileptic drug, retigabine [also known as ezogabine in the United States; D-23129, ethyl N-[2-amino-4-[(4-fluorophenyl) methylamino) phenyl)carbamate]. Retigabine was reported to augment GABA-ergic signal transduction in the central nervous system, however, it was reported to be a much better activator of potassium channels at therapeutic ranges [41]. Both retigabine and flupirtine activate Kv7 potassium channels Kv7.2-7.5 through interaction with residues in the S5 and S6 pore forming domains. Kv7.1 is the only Kv7 channel subtype that is insensitive to regulation by these Kv7 channel activators. There exists a tryptophan residue in the S5 domain of subtypes Kv7.2-7.5 that is critical for channel activation that Kv7.1 subunits lack that render them unaffected by these typical pharmacological Kv7 channel activators [42].

Kv7 channel activators like retigabine do not distinguish amongst the Kv7 channel subunits and can give rise to possible off-target effects when activating potassium channels in tissues outside of the central nervous system. In recent years, drugs have been developed to specifically target certain Kv7 channel subunits over others. ICA-27243 (N-(6-chloro-pyridin-3-yl)-3, 4-difluoro-benzamide) was found to be a 20-fold more potent activator of Kv7.2/7.3 heteromeric channels than of homomeric Kv7.4 channels. It was reported to have no effect on Kv7.3 homomeric channels and only elicit weak activation of Kv7.3/7.5 heteromeric channels.

The only drug reported to distinguish between Kv7.4 and Kv7.5 channel was diclofenac, an NSAID with an affinity for COX-2 similar to that of celecoxib. Diclofenac enhanced Kv7.4 channel currents, suppressed Kv7.5 channels currents, and produced an intermediate effect on Kv7.4/7.5 heteromeric channel currents expressed in A7r5 cells [43]. ML213 (N-mesitybicyclo[2.2.1]heptane-2-carboxamide) was a newer clinically available drug that claimed to be a selective activator of Kv7.2 and Kv7.4. Brueggemann et al. conducted a rigorous electrophysiological exploration of this claim by testing ML213's effect on hKv7.4, hKv7.5, and hKv7.4/7.5 channel currents exogenously expressed in A7r5 cells. In contrast to what was previously reported [44], Brueggemann et al found that ML213 robustly enhanced the currents of all three channel assemblies with no apparent specificity. Additionally, they tested ICA-069673 (N-(6-chloropyridin-3-yl)-3,4-difluorobenzamide) and found that it more effectively enhanced Kv7.4 channel currents compared to Kv7.5 channel currents but in a comparable manner to Kv7.4/7.5 heteromeric channel currents [45]. Finding pharmacological agents that can distinguish between the different Kv7 channel subunits is still an important endeavor that needs to be explored further.

Channels as therapeutic targets

It is becoming increasingly clear that Kv7 modulation can be utilized as a therapeutic target for a variety of diseases. In particular, pharmaceutical agents can be developed to relieve ailments that involve overexcitability of the cells such

as anxiety [46] and neuropathic pain [39], since activation of these Kv7 channels decreases the excitability of the cells by promoting membrane hyperpolarization.

Channels in vascular smooth muscle cells

Ohya and colleagues were the first to report the existence of Kv7 channels in vascular smooth muscle cells when they reported the expression of KCNQ1 transcripts in murine portal vein myocytes [47]. This same group then published a follow up study where Kv7 channel blockers, linopirdine and XE991 were applied to the portal vein and induced membrane depolarization and increased spontaneous contractile responses [48]. These studies demonstrated the importance of Kv7 channels and their contribution to vascular smooth muscle membrane voltage.

Another important finding for Kv7 channels in vascular smooth muscle cells was reported by Brueggemann et al. who isolated Kv7 currents in cultured rat embryonic aortic smooth muscle cells (A7r5 cells). They also reported KCNQ5 mRNA expression in both A7r5 cells as well as rat aortic smooth muscle cells. KCNQ1 mRNA transcripts were only expressed in rat aortic smooth muscle cells and were not expressed in A7r5 cells [49].

There have been several studies since Brueggemann et al. that demonstrated KCNQ expression in different vascular beds such as mesenteric, pulmonary, carotid, cerebral, aorta, and femoral artery. The general consensus of KCNQ expression shown by these studies is KCNQ1, KCNQ4, and KCNQ5

mRNA transcripts predominating with little to no expression of KCNQ2 or KCNQ3 [50-53].

The expression and function of Kv7 channels reported in human arteries were found to be similar to rodent models, thus validating the role of Kv7 channels in regulating vasoconstriction and vascular tone in humans [54]. Kv7.1 channels were originally thought to only be expressed in the heart but have been found in all human and rat myocytes studied to date. However, it is unlikely that channels formed by Kv7.1 channel subunits contribute significantly to vascular tone. This conclusion is based on the ability of Kv7.2-5 activator, retigabine, to dilate pre-constricted human or rat arteries and the inability of the Kv7.1-specific channel blocker, chromanol 293B to constrict human or rat arteries [50, 52-54]. While channels formed by Kv7.1 subunits do not contribute to resting vascular tone, Chadha et al. reported that selective activation of Kv7.1 channels dilated pre-constricted arteries and suggested that these channels may function under certain conditions in VSMCs [55].

While more is being discerned about the expression and function of Kv7 channels in the vasculature, there is still much that is unknown about any changes in expression of KCNQ during development. There has been a report, however, that the KCNQ4 gene was downregulated in the aorta of a rat model of hypertension which correlated to a decrease in Kv7.4 channel protein [56]. This downregulation was not observed in the same rat model within the skeletal or left coronary arteries [57, 58]. Mesenteric artery expression proved to be even more

inconsistent. Zavaritskaya and colleagues reported a reduction in levels of Kv7.4 channels within mesenteric arteries of spontaneously hypertensive rats (SHR) relative to the same arteries from normotensive Wistar rats, but Khanamari and colleagues did not detect any changes in KCNQ gene expression between hypertensive and normal rats [57, 58]. Despite the lack of change detected in mRNA levels, a reduction of Kv7.4 channel protein was reported in mesenteric arteries of hypertensive rats [56, 57]. A reduction in Kv7.4 channel proteins has also been reported in renal arteries of hypertensive rats [59]. In contrast to the multiple reports of Kv7.4 expression changes in hypertensive animal models, there is not a lot known about Kv7.5 expression in hypertension. Initial reports by Jepps and colleagues showed an increase in KCNQ5 gene expression in hypertension but later reports by this same group showed an insignificant reduction of KCNQ5 gene expression. The protein levels of Kv7.5 were not examined in either report [56, 57].

The Kv7 currents isolated from A7r5 cells and mesenteric artery myocytes were outwardly rectifying and showed a threshold of activation around -60mV [49, 53]. Kv7 channels activate at resting membrane voltages which is more negative than the threshold of activation of voltage-sensitive calcium channels (VSCCs; ~ -40mV) and promote membrane hyperpolarization which opposes activation of VSCCs in vascular smooth muscle cells. Kv7.5 monomeric channels are the only subtype of Kv7 channel expressed in A7r5 cells [43] and knockdown of these channels via KCNQ5 short hairpin(sh)-RNA resulted in membrane

depolarization which in turn, induced repetitive spontaneous Ca^{2+} spiking [60]. The Ca^{2+} spiking response was dependent on membrane depolarization and activation of VSCCs [61]. Therefore, Kv7 channels regulate the contractile status of VSMCs by regulation of membrane voltage which determines activation of VSCCs.

Kv7 channel suppression has been suggested to be the mechanism through which the hormone arginine vasopressin elicits its vasoconstrictor effects [49, 53]. This reported suppression was dependent on the activation of protein kinase C which is a common signaling intermediate in the $G_{q/11}$-coupled receptor signaling pathway [49, 53]. In contrast, activation of protein kinase A has been shown to enhance the activity of certain Kv7 channel subtypes in expression systems [62, 63]. PKA activation is a known consequence of G_s-coupled receptor signaling. The β-adrenergic receptor is one such G_s-coupled receptor whose activation could potentially lead to vasodilation in VSMCs by regulation of Kv7 channels.

Phosphodiesterases

Signaling

Cyclic nucleotide concentrations within the cell are determined by the balance between their synthesis (which is catalyzed by cyclases) and their degradation (which is catalyzed by phosphodiesterases (PDEs)). When a stimulatory ($G_{\alpha S}$) G-protein coupled receptor (GPCR) is activated, it goes on to activate adenylyl cyclase, which is responsible for synthesizing cyclic adenosine

monophosphate (cAMP). Certain PDEs degrade cAMP and thereby attenuate cAMP-dependent cell signaling [64]. Inhibition of cAMP-specific PDEs results in an attenuation of cAMP degradation thereby increasing the overall cellular cAMP concentration. This increase in cAMP concentration leads to enhanced downstream signaling of many cAMP effectors, the best characterized of which is protein kinase A (PKA) [65]. PKA is a symmetrical complex containing two regulatory and two catalytic subunits. Activation occurs when cAMP binds to sites on the regulatory subunits, causing a dissociation from the catalytic subunits [66]. PKA is a serine/threonine kinase that, once activated, goes on to phosphorylate its own downstream targets. One such target of PKA could be the Kv7 potassium channels of interest in this thesis. This question will be addressed by later experiments.

Characteristics

There are 11 members of the PDE family encoded by different genes [64]. Each member is able to specifically degrade cAMP, cyclic guanosine monophosphate (cGMP), or both. The cAMP-specific isoforms of PDE in mammals are PDE4, PDE7, and PDE8. PDE5, PDE6, and PDE9 hydrolyze cGMP specifically, and the 5 remaining PDEs (PDE1, 2, 3, 10, and 11) can hydrolyze both cAMP and cGMP [67]. This substrate specificity has been suggested to be due to a glutamine residue within the catalytic site. This residue can form hydrogen bonds with cAMP, cGMP, or both depending on its orientation within the catalytic site and its ability to rotate [68]. Each PDE subtype has

multiple isoforms and splice variants, which increases the difficulty in pinpointing particular isoforms as essential contributors to specific pathological conditions [64].

PDE4

The PDE4 family selectively hydrolyzes cAMP and is encoded by four genes: PDE4A, PDE4B, PDE4C, and PDE4D. These genes have different promoters which give rise to a multitude of proteins via splice variants [64]. More than 25 human isoforms have been identified, and PDE4 is found in several tissues including brain, lung, testis, and immune cells [67]. These PDE4 variants are localized within tissues by protein kinase A anchoring proteins (AKAPs) [69, 70] near PKA to regulate cAMP signaling and functional responses [36]. PDE4 has been targeted in recent years for inflammatory conditions such as asthma and chronic obstructive pulmonary disease (COPD). This exploration was based on the findings that elevating cAMP levels within inflammatory cells inhibited their function and that PDE4 was widely distributed within inflammatory cells and the lung [71, 72]. Therefore, the first PDE4 inhibitors were developed to effectively inhibit a wide range of inflammatory cell functions. This anti-inflammatory action coupled with the ability to induce relaxation in isolated human bronchus [73] showed promise for PDE4 inhibitors as an effective treatment for patients suffering from asthma and COPD.

Klein and colleagues reported celecoxib, and not rofecoxib, to be a specific inhibitor of PDE4 and PDE5. The evidence presented in that study was

more strongly suggested that celecoxib was a cGMP-specific PDE5 inhibitor than a cAMP-specific PDE4 inhibitor [8]. The evidence for PDE5 inhibition by this group included celecoxib's ability to increase coronary flow in a guinea pig Langendorff heart, celecoxib's ability to relax rat aortic ring preparations that were pre-constricted with phenylephrine, an increase in cGMP levels in aortic ring tissue in response to celecoxib treatment (as measured by an immunoassay), and an in vitro measurement of PDE5A1 inhibition using a recombinant human enzyme [8]. The evidence for PDE4 inhibition was not as strong. Klein and colleagues reported that celecoxib increased cAMP levels in rat aortic tissue 1.6-fold but that it never reached statistical significance and the data was not shown [8]. They also reported celecoxib inhibited a recombinant human PDE4 enzyme, but again, the data was not shown [8].

Hypertension

Hypertension is the leading risk factor for cardiovascular disease and is a major cause of mortality [74]. About 1 in 3 American adults have high blood pressure and only about half of all adults suffering from high blood pressure have it under control [74]. Hypertension is characterized by chronic elevation in blood pressure and can be classified into two categories. Primary or essential hypertension is a chronically elevated blood pressure with no identifiable cause while secondary hypertension is a chronically elevated blood pressure that can be attributed to a specific cause, such as kidney disease.

Blood pressure is directly proportional to the product of the blood flow (cardiac output, CO) and the resistance of blood passage through precapillary arterioles (peripheral vascular resistance, PVR) [75].

Hypertension, in general, has been associated with increased vascular tone that results from a more depolarized membrane potential in VSMCs. This evidence makes it clear that understanding the role of the potassium channels that regulate VSMC membrane potential is important. To that end, there have been reports that certain Kv7 potassium channels are downregulated in hypertension. The Greenwood lab reported that of the 5 KCNQ genes, only KCNQ4 was downregulated in hypertensive rats [59]. Since Kv7.4 and Kv7.5 subunits form both homo- and hetero-tetramers in the vasculature, it is possible that the selective downregulation of KCNQ4 could lead to increased formation of Kv7.5 homotetramers in these patients. This means drugs like celecoxib, which activate Kv7.5 potassium channels, could prove to be therapeutically beneficial in patients suffering from this type of hypertension.

Current Treatments

The currently used treatments for hypertension can be categorized into four main categories: diuretics, sympathoplegic agents, direct vasodilators, and agents that block the production/action of angiotensin. Diuretics lower blood pressure by depleting the body of sodium and lowering blood volume. Sympathoplegic agents reduce peripheral vascular resistance (PVR), inhibit cardiac function, and increase venous pooling in capacitance vessels. The latter

two actions effectively decreases cardiac output. Direct vasodilators are true to their name and decrease blood pressure by relaxing VSMCs which causes vasodilation in resistance vessels and could potentially increase capacitance. Agents that block the production and/or action of angiotensin decreases PVR to decrease blood pressure [75].

The fact that direct vasodilators are already used clinically to treat hypertension is promising for pharmaceutical agents such as celecoxib whose ability to activate Kv7 potassium channels directly opposes cell excitability and results in relaxation in VSMCs. Previous research from our laboratory has demonstrated that celecoxib can dilate mesenteric resistance arteries from rats *in vitro* and *in vivo* [76].

CHAPTER 3

RESEARCH AIMS

In this proposal, I hypothesize that activation of the cAMP/PKA pathway is necessary and sufficient to induce the activation of Kv7 potassium channels in vascular smooth muscle cells. [Figure 1] To test this hypothesis I propose the following aims:

Aim 1: Determine if PDE inhibition is sufficient to induce the activation of Kv7 potassium channels in vascular smooth muscle cells.

In this aim, I will utilize PDE inhibitors that are structurally different and have different specificities for cAMP. The PDE4 (cAMP)-specific inhibitor, rolipram will be used as a comparison to celecoxib's effects because a previous doctoral student, Bharath Mani, found that rolipram was able to mimic celecoxib's ion channel modulatory activity in the A7r5 cell line as well as its vasorelaxant effects in pre-constricted vessels. I will also utilize the non-specific PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), in conjunction with the adenylyl cyclase activator, forskolin, to maximally elevate cAMP levels to determine whether or not maximal elevation of cAMP concentration is sufficient to mimic celecoxib's Kv7 channel activation. The electrophysiology experiments will utilize cells exogenously expressing human Kv7.4, Kv7.5 or Kv7.4/7.5 channels, since these are the predominant channel assemblies found in the vasculature. The results of

these experiments, combined with preliminary data collected from other members of the Byron laboratory, will give a clearer answer as to whether or not PDE inhibition is sufficient to induce Kv7 channel activation.

Aim 2: Determine whether celecoxib-induced enhancement of Kv7.5 currents is a consequence of a critical tryptophan residue on the channel.

Celecoxib's ability to activate Kv7.5 potassium channels in vascular smooth muscle cells could be the result of our proposed indirect mechanism or it could be the result of a critical tryptophan residue on the channel. Many pharmacological Kv7 potassium channel activators act by binding directly to the channel. For example, retigabine, the prototypical pharmacological Kv7 channel activator, binds to a homologous site on Kv7.2, Kv7.3, Kv7.4, and Kv7.5 channels. Celecoxib was previously proposed to enhance Kv7.2 currents by binding to the retigabine binding region, based on the loss of this effect in a retigabine-insensitive mutant of Kv7.2, though this was not tested for Kv7.5 [77]. The retigabine binding site on Kv7.5 contains a critical tryptophan at position 235 (W235) [42]. Kv7 channel activators like retigabine bind this tryptophan residue located in the S5 domain and stabilize the channel in an open position, allowing increased efflux of potassium ions and membrane hyperpolarization [78]. Our laboratory has a KCNQ5 construct that is mutated at the critical tryptophan residue (Q5W235L), making it retigabine-insensitive [42]. If celecoxib is working

through an indirect pathway (e.g. PDE4 inhibition or cAMP elevation) to activate Kv7 potassium channels, then celecoxib is expected to enhance the currents of our Q5W235L mutant. If celecoxib utilizes the retigabine binding motif on the Kv7 channels to activate them, then application of celecoxib is not expected to enhance Q5W235L currents.

Aim 3: Determine if celecoxib-induced activation of Kv7 potassium channels is a consequence of cAMP/PKA-dependent regulation in vascular smooth muscle cells.

It has yet to be determined whether activation of the cAMP/PKA-dependent pathway is necessary for celecoxib to induce Kv7 potassium channel activation. There are inhibitors and dominant-negative analogs of the pathway's components that can be utilized to disrupt normal signal transduction to determine the involvement of this pathway. Disrupting normal cAMP/PKA-dependent signaling and observing the effect on celecoxib's ability to activate Kv7 potassium channels could aid in fully elucidating celecoxib's mechanism of channel activation. The inhibitor that will be utilized in this thesis work is (9*R*,10*S*,12*S*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, hexyl ester (KT5720). It is a PKA inhibitor that, when used as a pretreatment, can prevent PKA activation by cAMP [79]. If celecoxib is activating Kv7 channels through a cAMP/PKA dependent pathway, pretreatment with KT5720 should attenuate the celecoxib-induced enhancement of the Kv7 currents. Additionally, using commercially available kits to determine whether or

not celecoxib is elevating cAMP levels, as we predict, will further verify celecoxib's role as a PDE4 inhibitor as well as provide further evidence to help determine whether Kv7 channel activation by celecoxib is the consequence of this cAMP/PKA-dependent indirect signaling mechanism. The kit that will be utilized in this thesis work is the direct cAMP ELISA by Enzo Life Sciences. More details on this kit and the protocol used can be found in the next chapter.

Summary of Specific Aims:

Upon completion of the proposed research, the signaling mechanism involved in celecoxib's ability to activate Kv7 potassium channels will be better elucidated. This will shed light on the potential differential regulation of the different Kv7 channel subunits in the vasculature. This could lead to the repurposing of already available drugs, like rolipram, to promote vasorelaxation. This would also give an alternative signaling pathway to target when developing new drugs for the purpose of promoting vasorelaxation in the vasculature. Of course this thesis work would not be the end to the question of Kv7 channels' sensitivity to a cAMP/PKA-dependent pathway and would, instead, act as the starting point for a host of new scientific questions which are discussed in much greater detail in the discussion/future directions portion of this thesis.

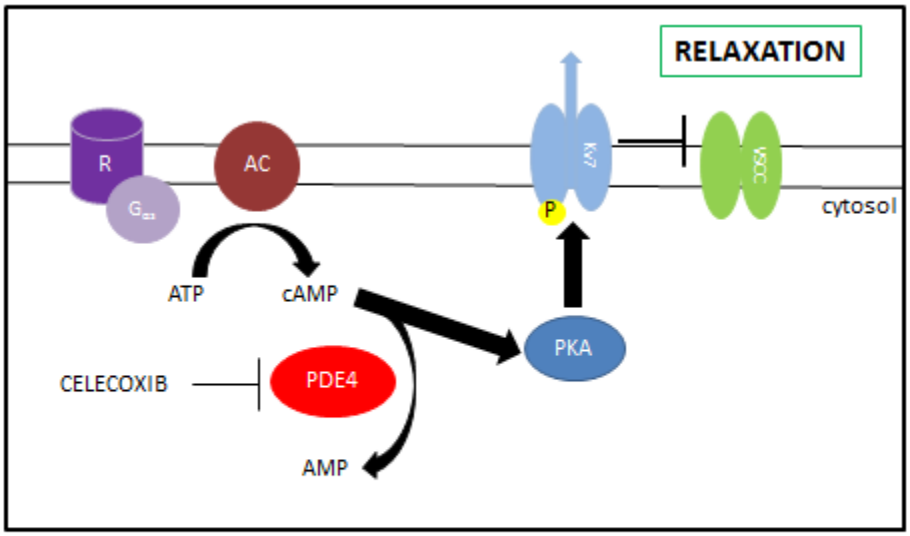

Figure 1: Schematic of potential PDE involvement in Kv7 potassium channel activation and L-type Ca^{2}+ inhibition.
When a stimulatory ($G_{\alpha S}$) G-protein coupled receptor (GPCR) is activated, it goes on to activate adenylyl cyclase (AC), which is responsible for synthesizing cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). Phosphodiesterase 4(PDE4) degrades cAMP to adenosine monophosphate (AMP) and thereby attenuates cAMP-dependent cell signaling. Inhibition of cAMP-specific PDE4 by celecoxib results in an increase in cellular cAMP concentration. This can lead to enhanced downstream signaling of many cAMP effectors, one of which is protein kinase A (PKA). PKA goes on to phosphorylate downstream targets, one of which may be Kv7 potassium channels. Activation of Kv7 channels directly opposes the activation of voltage sensitive calcium channels (VSCC). Both Kv7 channel activation and VSCC inhibition promote relaxation in vascular smooth muscle cells.

CHAPTER 4

MATERIALS AND METHODS

Cell Culture

A7r5 cells were cultured as described previously [61]. For overexpression studies, subcultured A7r5 cells at 50-70% confluence were transfected with a FLAG-tagged human KCNQ5 (or KCNQ4, or KCNQ4 and KCNQ5 in combination) DNA sequence, which was inserted into a pIRES-2-enhanced green fluorescent protein vector, using Lipofectamine (Invitrogen, Carlsbad, CA) transfection reagent according to the manufacturer's protocol. Confluent subcultures of A7r5 cells were trypsinized with 0.25% trypsin +EDTA and replated on glass coverslips. Green fluorescent protein-expressing cells were used for electrophysiological recordings 5-10 days after transfection.

Electrophysiology

Whole cell perforated-patch configurations were used to measure membrane currents under voltage clamp conditions in single A7r5 cells. All experiments were performed at room temperature with continuous perfusion of external solution. It was not necessary to distinguish between endogenous and exogenous Kv7 currents in recordings where Kv7 channels were exogenously

expressed in A7r5 cells. Exogenous Kv7 current amplitude is approximately two orders of magnitude larger than the endogenously expressed Kv7.5 channel currents in the A7r5 cells; therefore, any contribution from endogenous current would be negligible [9].

The external solution for measuring Kv7 K^+ currents contained (in mM): 5 KCl, 130 NaCl, 10 HEPES, 2 $CaCl_2$, and 1.2 $MgCl_2$. Bath solution pH was adjusted to 7.3 using NaOH. The bath solution osmolarity was adjusted to 269-272 mOsm/L using D-glucose. The internal (pipette) solution contained (in mM): 30 KCl, 110 K-gluconate, 5 HEPES, 1 K_2EGTA. The internal solution pH was adjusted to 7.2 using KOH. The internal solution osmolarity was adjusted to 268-272 mOsm/L using D-glucose.

Recordings of native Kv7.5 currents in A7r5 cells required the addition of 100 μM gadolinium (III) chloride ($GdCl_3$) in the external solution to inhibit all non-selective cation channels.

Experiments in whole-cell perforated patch configuration were started with series resistance R_s < 30MΩ; cells with an abrupt decrease in R_s were discarded. Voltage-clamp command potentials were generated using an Axopatch 22B amplifier under the control of PCLAMP10 software.

cAMP Assay

The direct cAMP ELISA kit from Enzo Life Sciences (Cat#:ADI 900-066, Lot#:10071456D) was used. The kit is a competitive immunoassay for the quantitative determination of cyclic AMP in cells treated with hydrochloric acid (HCl). The optional acetylated assay format was used which provides a ~10-fold increase in sensitivity. Standards (cAMP standard; 2000 pmol/mL) and samples were added to wells of the provided 96-well plate per manufacturer instructions. Individual 35mm dishes of A7r5 cells were plated at 50-100,000 cells per mL and were serum deprived at 4pm the day before the assay was to be run. The experiment was run per the manufacturer's protocol with the following modifications: Media was aspirated from each dish and washed with room temperature Kreb's solution instead of PBS. Kreb's solution contained (in mM): 135 NaCl, 5.9 KCl, 1.5 $CaCl_2$, 1.2 $MgCl_2$, 11.6 HEPES, and 11.5 D-glucose. The solution pH was adjusted to 7.3 using NaOH. The protocol was lengthened to accommodate a resting period to minimize any mechanical stimulation washing may have caused, and cell scraping, sonication, and centrifugation steps were added to maximize the lysis of the cells and the amount of cAMP collected. The manufacturer provided 96-well plate was used for all samples and standards and the optical density was read in each well at 405nm on the Synergy HT plate reader located in our laboratory. The optical densities are read in each well after adjusting for the substrate blank, and the plate reader protocol calculates the concentration of cAMP according to a standard curve that is generated from the standards plated using a 4 parameter logistic (4PL) curve fitting program. The

amount of signal read at 405 nm is indirectly proportional to the amount of endogenous cAMP in the sample. Therefore, the more endogenous cAMP in the sample, the more conjugated cAMP will be outcompeted for binding with the antibody and the less yellow color will be produced when substrate is added.

Statistics

SigmaStat was used for all statistical analyses. Paired Student's t-tests were used when comparing any parameters before and after treatment. Comparisons among multiple treatment groups were evaluated by analysis of variance (ANOVA) followed by a Holm-Sidak post hoc test. Differences where $p \leq 0.05$ were considered to be statistically significant.

Materials

AmpB, celecoxib, rolipram, forskolin, IBMX, and KT5720 were purchased from Tocris Research Laboratories (United Kingdom).

CHAPTER 5

RESULTS

PDE Inhibition is Sufficient to Enhance Kv7 channel currents

Considering that celecoxib had been reported to be a PDE inhibitor [8] we first set about determining if this action could explain the observed effect of celecoxib on Kv7 currents. To determine if PDE inhibition is sufficient to enhance the currents through Kv7 channels, rolipram was used in electrophysiological studies. Bharath Mani, PhD, had previously found that rolipram, a PDE4 (cAMP specific) inhibitor mimicked celecoxib in its ability to enhance endogenous Kv7.5 channel currents in A7r5 cells. (Not shown) He also found that rolipram mimicked celecoxib in its ability to inhibit L-type Ca^{2+} channels in A7r5 cells and promote vasodilation in vessels pre-constricted with the hormone arginine vasopressin (AVP) [85].

As stated previously in the literature review, A7r5 cells only express Kv7.5 homo-tetramers while Kv7 channels in the vasculature can assemble as Kv7.4 homotetramers, Kv7.5 homotetramers, and Kv7.4/7.5 heterotetramers. To determine if these channel assemblies are differentially regulated by the PDE/cAMP/PKA pathway, I used A7r5 cells overexpressing human (h)Kv7.4, hKv7.5, or both for electrophysiology experiments. Upon application of the PDE4

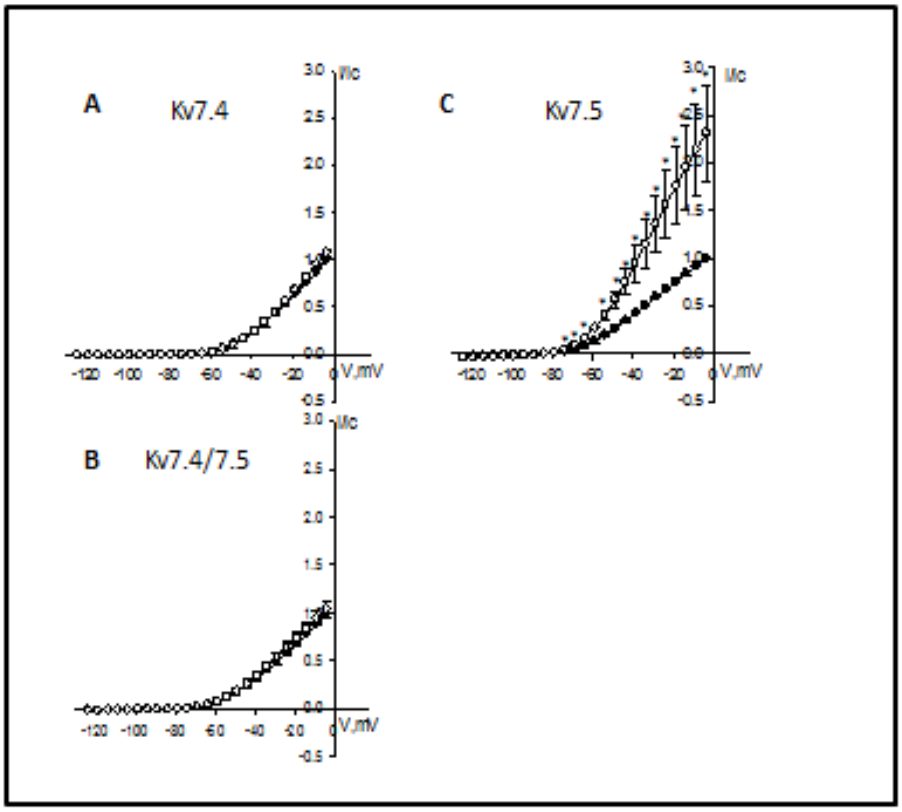

Figure 2: Rolipram enhances Kv7 currents in A7r5 cells expressing human Kv7 channels. Current voltage relationship curves showing A7r5 cells expressing human Kv7 channels before (control, black circles) and after treatment with 100nM of the PDE4 inhibitor, rolipram (white circles) (A) Mean IV curve of hKv7.4 currents showing no effect of rolipram; n=5 (B) Mean IV curve of hKv7.4/7.5 currents showing no effect of rolipram; n=5 (C) Mean IV curve of hKv7.5 currents showing enhancement upon treatment with rolipram; n=5 *Significant difference between control and 100nM rolipram treatments. Paired Student's t-test; $p<0.05$, n=5 (CJ Robakowski, publication under review)

inhibitor, rolipram (100nM), hKv7.5 currents were enhanced but hKv7.4 and hKv7.4/7.5 currents were not affected. [Figure 2]

Furthermore, treatment of A7r5 cells with the adenylyl cyclase activator, forskolin, in combination with the non-specific PDE inhibitor, IBMX, (10μM and 500 μM respectively) also enhanced hKv7.5 currents. Once again, hKv7.4 channel currents were unresponsive to regulation by this pathway even with the more robust combination of an adenylyl cyclase activator with a PDE inhibitor. However, the hetero-tetramer hKv7.4/7.5 did show a modest but significant enhancement with forskolin/IBMX treatment and became sensitive to regulation by the cAMP/PKA-dependent pathway with the more robust treatment combination. [Figure 3] While it is apparent that these channel assemblies are differentially sensitive to enhancement by PDE inhibition, it is unclear why one treatment would affect the hetero-tetrameric channel while the other would not. It is possible that the more robust forskolin/IBMX treatment used here produces a larger increase in the cellular concentration of cAMP than rolipram and this larger increase is required to elicit an effect on the less sensitive hKv7.4/7.5 channel currents. The question regarding the amount of cAMP produced by these different treatments is addressed later in the aim three experiments of this section.

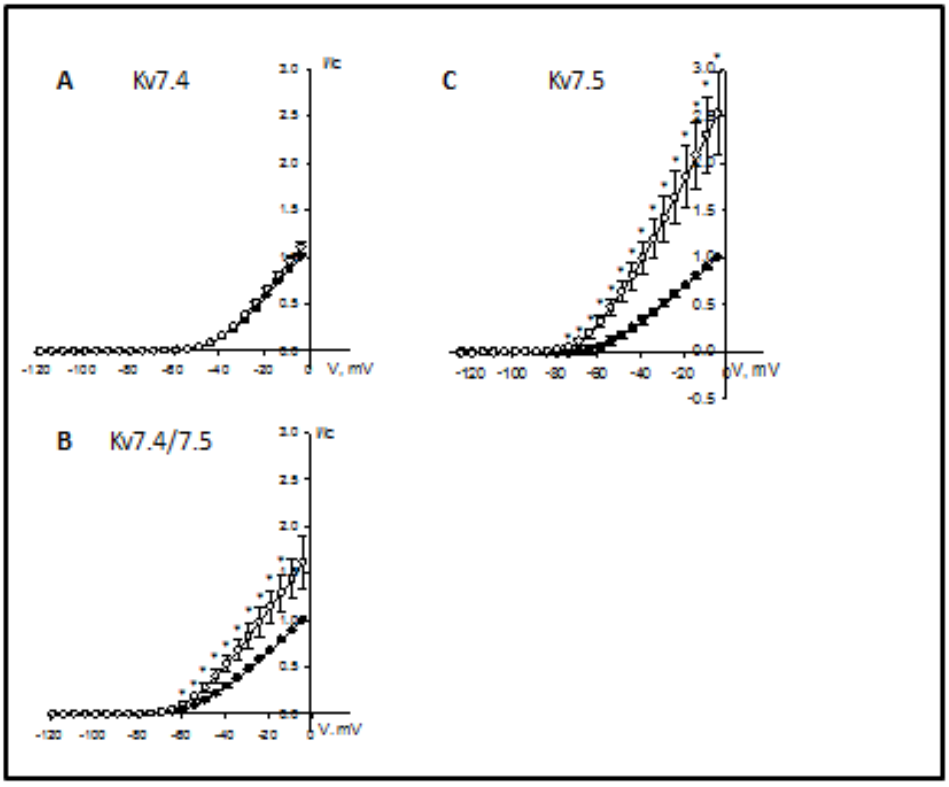

Figure 3: IBMX/Fsk treatment mimics celecoxib and rolipram in Kv7.5 current enhancement. Current voltage curves of human Kv7 currents in A7r5 cells before (control, black circles) and after treatment of 500 μM of non-specific PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), and 10 μM of adenylyl cyclase activator, forskolin (Fsk), (white circles) (A) Mean IV curve of hKv7.4 currents showing no effect of IBMX/Fsk treatment. (B) Mean IV curve of hKv7.4/7.5 currents showing little enhancement with IBMX/Fsk treatment. (C) Mean IV curve of hKv7.5 currents showing significant enhancement comparable to that seen with celecoxib and rolipram upon IBMX/Fsk treatment *Significant difference between control and IBMX/Fsk treatments. Paired Student's t-test; $p<0.05$, n=4-6. (CJ Robakowski and LI Brueggemann, publication under review)

While rolipram and the forskolin/IBMX combination were the only treatments I tested myself for this aim, other members of our laboratory have collected additional data to support the hypothesis that PDE inhibition and cAMP elevation are sufficient to enhance Kv7.5 channel currents. Bharath Mani previously found that another non-specific PDE inhibitor, papaverine, also dose-dependently (10 μM and 100 μM) enhanced endogenous Kv7.5 currents in A7r5 cells. [Figure 4] Dr. Lyubov Brueggemann found that increasing cAMP levels by addition of 1mM Br-cAMP, a membrane-permeant cAMP analogue, enhanced Kv7.5 channel currents in A7r5 cells. She also found that the adenylyl cyclase activator forskolin (1 μM) was sufficient by itself to enhance Kv7.5 channel currents. [Figure 4]

Altogether, these experiments suggest that PDE inhibition and cAMP elevation are sufficient to activate human Kv7.5 channels, as well as rat Kv7.5 channels endogenously expressed in A7r5 cells, and this Kv7.5 channel activation mimics the celecoxib-induced activation observed previously.

Celecoxib Requires a Critical Tryptophan Residue for Kv7.5 Channel Activation

There are clinically available Kv7 channel activators currently on the market. Retigabine is one such Kv7 channel activator and it is currently marketed as an anticonvulsant. Retigabine and many other pharmacological agents developed to activate Kv7 channels, share a common mechanism of action.

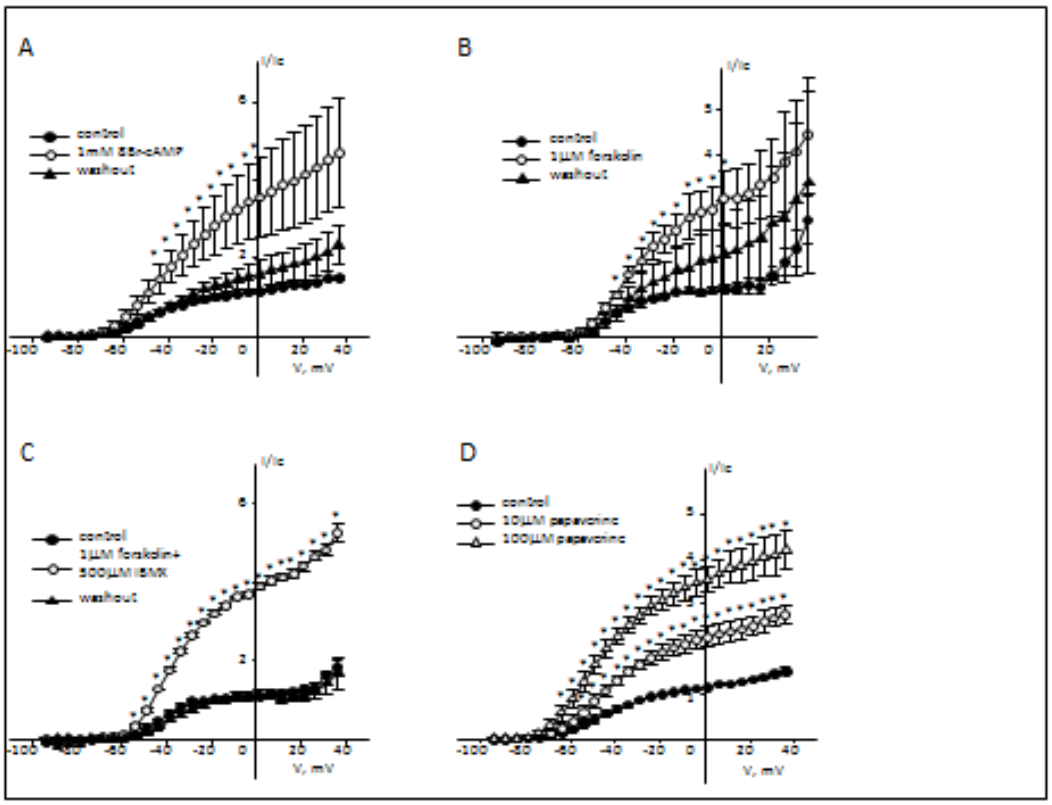

Figure 4. Elevation of cAMP enhances endogenous Kv7.5 currents in A7r5 cells. A. I-V relationships of endogenous Kv7.5 currents recorded in A7r5 cells before (control, filled circles), after 15 min treatment with 1 mM 8Br-cAMP (open circles) and after 10 min of washout (filled triangles). Current were normalized to currents recorded at -20 mV before application of 8Br-cAMP (n= 4, * indicates significant difference from control, $P < 0.05$, One Way Repeated Measures ANOVA). B. I-V relationships of endogenous Kv7.5 currents recorded in A7r5 cells before (control, filled circles), after 5 min treatment with 1 µM forskolin (open circles) and after 10 min of washout (filled triangles). Current were normalized to currents recorded at -20 mV before application of forskolin (n= 4, * indicates significant difference from control, $P < 0.05$, One Way Repeated Measures ANOVA). C. I-V relationships of endogenous Kv7.5 currents recorded in A7r5 cells before (control, filled circles), after 5 min treatment with 1 µM forskolin in the presence of 500 µM IBMX (open circles) and after 10 min of washout (filled triangles). Current were normalized to currents recorded at -20 mV before application of forskolin/IBMX (n= 4, * indicates significant difference from control, $P < 0.05$, One Way Repeated Measures ANOVA). D. I-V relationships of endogenous Kv7.5 currents recorded in A7r5 cells before (control, filled circles) and after treatment with papaverine (10 µM open circles, 100 µM open triangles). Current were normalized to currents recorded at -20 mV before application of papaverine (n= 4, * indicates significant difference from control, $P < 0.01$, One Way Repeated Measures ANOVA). (LI Brueggemann, publication under review)

These agents activate Kv7 channels by binding to a critical tryptophan residue located on the S5 pore-forming domain of the channel, thereby stabilizing its open conformation. This critical tryptophan residue is conserved in Kv7.2 – Kv7.5 channels. On the Kv7.5 channel, the tryptophan is located at position 235.

When the tryptophan at residue 235 is mutated to a leucine (Q5W235L), the channel is rendered retigabine-insensitive, i.e., the channel is still functional but can no longer be regulated by retigabine or other typical pharmacological Kv7 channel activators. Our laboratory possesses this Q5W235L mutant and I used it for the electrophysiology studies in aim 2.

First, rolipram was used as a positive control. Since it is known that rolipram is a PDE4 inhibitor and works through the indirect cAMP/PKA-dependent pathway we are trying to study, it is expected to still be able to enhance the mutant Q5W235L channel currents. That is, in fact, what was observed. Application of rolipram (100nM) to the retigabine-insensitive mutant Kv7.5 channels resulted in enhancement of the currents as had been observed in A7r5 cells expressing the wild-type Q5 channels. [Figure 5]

If celecoxib is also enhancing Kv7.5 currents through the indirect cAMP/PKA-dependent mechanism, then it too should enhance the currents through the Q5W235L mutant channels. However, the currents were not enhanced upon application of celecoxib (10μM). [Figure 6] There are two possible explanations for this result. First, it is possible that celecoxib enhances

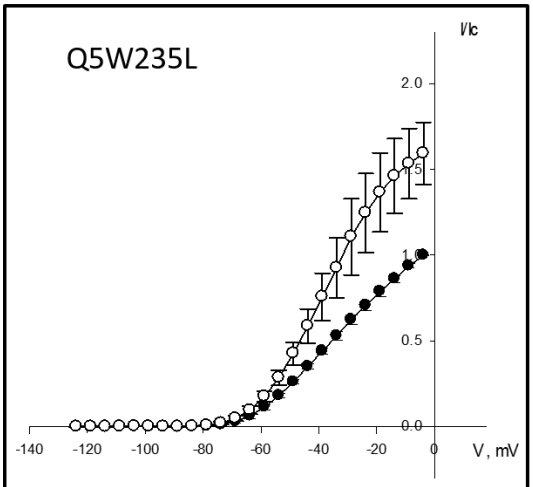

Figure 5: Rolipram enhances mutant Q5W235L potassium currents in A7r5 cells. Current-voltage curves showing mutant Q5W235L currents before (control, black cirlces) and after treatment with 100nM rolipram, PDE4 inhibitor (white circles). Rolipram is still able to enhance the Kv7 currents in this mutant channel suggesting an indirect mechanism of channel activation. n=5 (LI Brueggemann, unpublished)

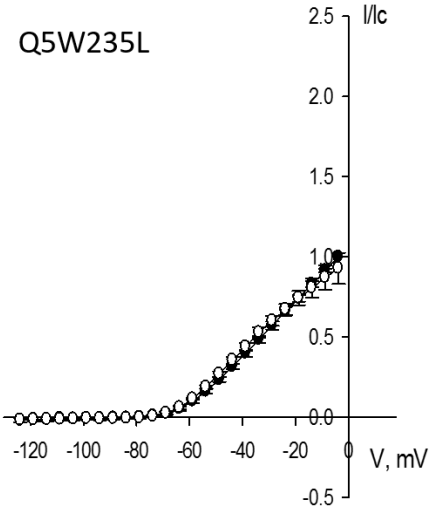

Figure 6: Celecoxib does not enhance mutant Q5W235L potassium currents in A7r5 cells. Current-voltage curves showing mutant Q5W235L currents before (control, black cirlces) and after treatment with 10μM celecoxib (white circles). Celecoxib is unable to enhance the Kv7 currents in this mutant channel suggesting a direct mechanism of channel activation. n=5 (CJ Robakowski, unpublished)

Kv7.5 channel currents through the critical tryptophan residue as other pharmacological Kv7 activators do. It is also possible that celecoxib does not produce as much of an increase in cellular cAMP levels around the channel as rolipram does. In this case, the amount of cAMP produced in the cell after treatment with celecoxib may be insufficient to enhance the currents of the mutated, and therefore less sensitive, channel. This fundamental question is addressed by experiments in aim 3.

Cellular concentrations of cAMP vary with different treatments

Troubleshooting

The cAMP ELISA proved to be a learning process. There were several factors to consider and alter before finding a protocol that we felt produced the most accurate results for the question we sought to address. The original protocol, which was graciously taught to me by Dr. Abishek Tripathi in the Majetschak lab, required our A7r5 cells to be treated in suspension and included many spin and vortex steps. This was not ideal as A7r5 cells are normally adherent and are mechanically sensitive. This meant that those steps where the cells were detached and excessively handled could alter the cells' signaling and response to treatments. Therefore, data collected in the initial run of the assay were disregarded. The protocol was then altered to reflect the way we handle the cells before biochemistry experiments such as western blots and immunoprecipitations. This prolonged the protocol by adding a 3 hour wait time

between washing the cells and treating them so as to minimize any mechanical stimulation before treatment. The cells were then lysed with the HCl provided in the kit and scraping, sonicating, and centrifugation steps were added to remove and lyse our adherent cells from their plates to collect the samples for the assay.

When troubleshooting the high variability that I experienced when I first started running the assay, Dr. Patel brought up the excellent point HCl might not be sufficient to lyse our cells. After reading the troubleshooting literature available on Enzo Life Science's website, I concluded that there are some cell lines that are hardier than others and require longer lysing times. To determine if the 10 minute lysis time suggested in the protocol was sufficient, I used a plate of A7r5 cells and washed it as I would in my normal assay protocol. I left the cells untreated, added the 0.1M HCl, and started a timer. I had the dish under a microscope so I could observe lysing of the cells and any other morphological changes in real time. This experiment revealed that the 10 minute lysis time suggested in the protocol was sufficient for our cell line. Additionally, the cell scraping, sonication, and centrifugation steps that we added to the protocol should have all but guaranteed that the cells were completely lysed and cellular contents were available for the assay.

Another issue that presented itself when using this assay was that a 15-minute treatment with isoproterenol, a beta-adrenergic agonist that was expected to stimulate adenylyl cyclase and produce cAMP to increase the cellular concentration of cAMP, did not yield cAMP concentrations above control. To

determine if the original treatment time (15 minutes) was not optimal, I ran a time course using the same concentration (1 µM) of isoproterenol for 30 seconds, 1 minute, 2 minutes, 5 minutes, or 10 minutes. [Figure 7] A significant elevation of cAMP levels were observed, with the maximal levels of cAMP produced at the 1-minute time point which then steadily declined at later time points. By the 10-minunte time point, cAMP levels were back to baseline, presumably due, at least in part, to the activation of PDEs. The maximal 1-minute treatment time was used for the remainder of the experiments unless otherwise stated.

Assay Results

To determine whether the different treatments used throughout this thesis work produce different amounts of cAMP to elicit the differential activation of the vascular Kv7 channels showed previously (hKv7.4, hKv7.5, and hKv7.4/7.5) the direct cAMP ELISA from Enzo Life Sciences was used. For this set of experiments, A7r5 cells were treated with vehicle, forskolin with IBMX, rolipram alone, celecoxib alone, or isoproterenol alone. As expected, the robust combination treatment of forskolin (adenylyl cyclase activator; 10 µM) and IBMX (non-specific PDE inhibitor; 500 µM) induced a significant increase in the cAMP concentration in A7r5 cells compared with cells treated with a DMSO vehicle control. [Figure 8] Both rolipram alone and celecoxib alone failed to produce a detectable change in cAMP levels compared to control. This is a reasonable

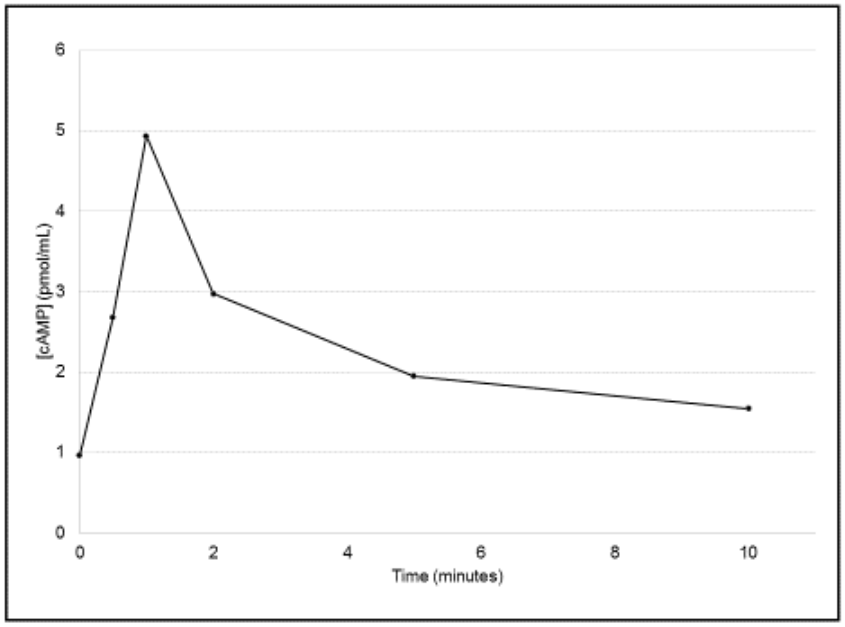

Figure 7: Isoproterenol Time Course. Each sample of A7r5 cells (run in duplicate) was treated with 1μM isoproterenol for various lengths of time. The peak concentration of cAMP upon isoproterenol treatment was reached at 1 minute with the cellular levels of cAMP returning to baseline by the 10 minute time point. To elicit the maximal effect of isoproterenol in subsequent experiments, the 1 minute treatment time was used. n=1. (CJ Robakowski, unpublished)

result to obtain because there was no stimulatory step involved in those treatments. The only cAMP to be detected in those samples would have been the basal levels that were present in the cell at the time of treatment that were prevented from degradation. This is consistent with the results of Klein and colleagues, who reported that celecoxib only weakly increased cAMP levels in the assay they used; the increase they observed never reached statistical significance though their data was not shown [8]. In A7r5 cells, isoproterenol (1 µM) alone induced a significant increase in cAMP levels compared to control. [Figure 8] This was expected, as isoproterenol is a beta-adrenergic agonist and should stimulate adenylyl cyclase activity to produce cAMP. Since neither celecoxib or rolipram alone produced a detectable change in cAMP levels, isoproterenol (1 µM) was administered simultaneously with either celecoxib or rolipram (10 µM or 100 nM respectively) with the rationale that stimulating cAMP production while inhibiting its degradation by PDE4 is likely to result in a higher overall concentration of cAMP than with isoproterenol alone. However, results were variable and even after repeating the experiment six times, isoproterenol in combination with either rolipram or celecoxib was not statistically different from isoproterenol alone. [Figure 9] It is possible that the short (1 minute), simultaneous treatment of the cells with isoproterenol along with celecoxib or rolipram did not provide sufficient time for celecoxib or rolipram to inhibit PDE4 and cAMP degradation before the treatment was stopped and the cells were lysed. To determine if the lack of change observed between the treatments was

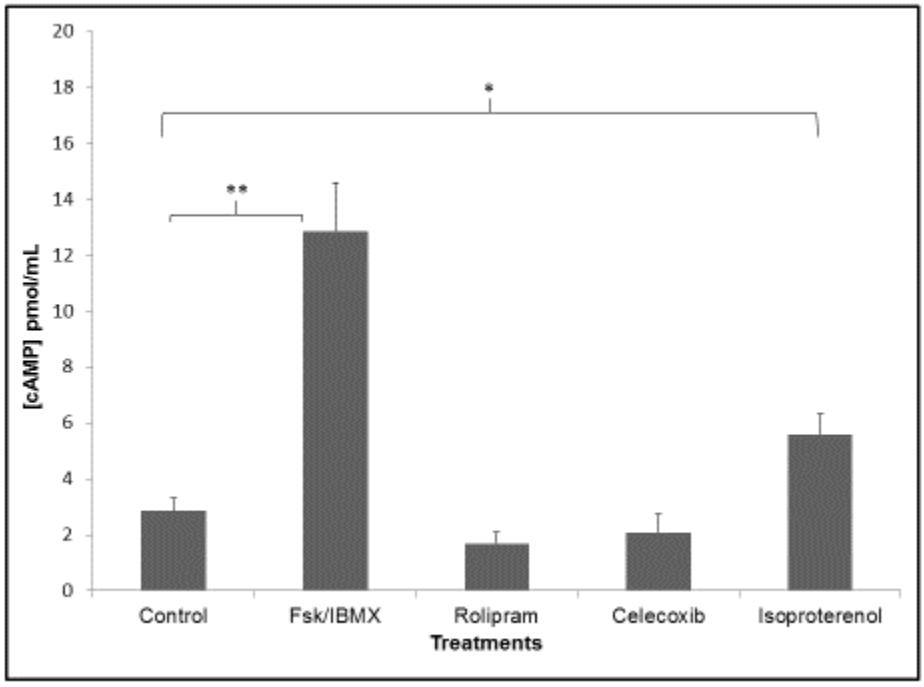

Figure 8: cAMP is elevated to different concentrations with different treatments. cAMP levels are very significantly and consistently increased upon combination treatment of forskolin with IBMX. PDE4 inhibitors, rolipram and celecoxib, do not significantly elevate cAMP concentrations alone. Isoproterenol (1μM) is able to significantly increase cAMP levels. *Significant difference by independent t-test compared to control; $p<0.05$, n=3-10. **Significant difference between control and Fsk/IBMX treatments; independent t-test, $p<0.01$, n=5-10. (CJ Robakowski, unpublished)

due to insufficient time, the assay was repeated and the treatment times were altered. Cells were treated with celecoxib or rolipram (10 µM or 100 nM respectively) five minutes prior to isoproterenol treatment. Isoproterenol (1 µM) was then added to the cells for 15 minutes. Previously, 15-minute isoproterenol treatments yielded cAMP levels that were comparable to baseline. [Figure 9] This is assumed to be due to the activation of PDEs. The rationale behind this new time course was that celecoxib and rolipram would have more time to elicit their inhibitory effects on PDE4 and prevent cAMP degradation before stimulation of cAMP production by an agonist occurred. Ideally, the addition of celecoxib or rolipram as a pretreatment would prevent the time-dependent degradation of cAMP observed with isoproterenol alone and would reveal differences in the effectiveness of celecoxib and rolipram to prevent cAMP degradation. The results of the celecoxib or rolipram pretreatment experiments were inconclusive. With only an n of 3 for both celecoxib and rolipram as pretreatments, the results were highly variable. Rolipram pretreatment before isoproterenol stimulation trended toward preventing the cAMP degradation that is observed between the 1- and 15-minute time points with isoproterenol alone. [Figure 9] Celecoxib pretreatment before isoproterenol stimulation did not suggest a prevention of cAMP degradation based on its trend.

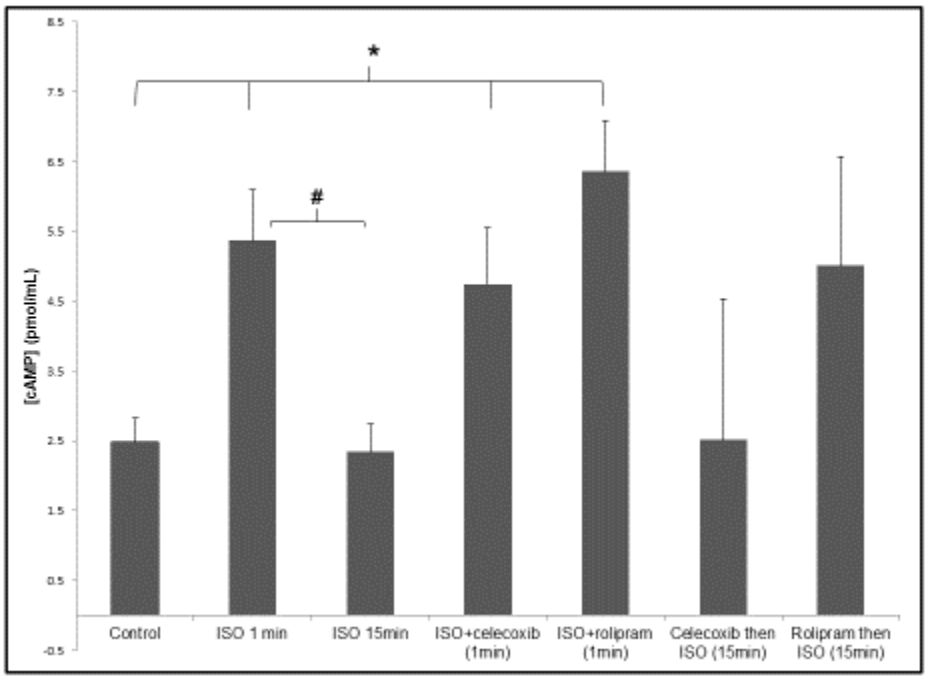

Figure 9: Celecoxib does not prevent cAMP degradation. cAMP levels return to baseline after treatment with 1μM isoproterenol (ISO,15min) . Simultaneous treatment with a PDE4 inhibitor (rolipram, 100nM or celecoxib, 10μM) did not significantly increase cAMP levels with isoproterenol compared to isoproterenol alone. Only pretreatment with rolipram (100nM) prevented degradation of cAMP after isoproterenol treatment though it did not reach statistical significance. Celecoxib (10μM) treatment suggested an inability to prevent cAMP degradation when used as a pretreatment in cells later treated with isoproterenol. *Significant difference by independent t-test compared to control; $p<0.05$, n=3-10. #Significant difference between ISO alone at 1 minute and ISO alone at 15 minutes; independent t-test, $p<0.05$, n=4-6. (CJ Robakowski, unpublished)

In summary, the results of this assay demonstrate that there is indeed a difference in the cellular concentrations of cAMP present after different treatments are administered. It is especially clear for the forskolin (10 μM) and IBMX (500 μM) combination treatment as it consistently produced a very significant ($p<0.005$) increase in cAMP concentration compared to the control. [Figure 8] These differences in the amount of cAMP generated with each treatment can provide a possible explanation as to why the forskolin/IBMX combination treatment enhanced the typically less sensitive hetero-tetrameric hKv7.4/7.5 channel currents while rolipram treatment did not.

PKA activation is involved in celecoxib-induced Kv7 channel activation

In order to determine if PKA activation is involved in celecoxib-induced Kv7 channel activation, I utilized a PKA inhibitor, KT5720. This drug, when used as a pretreatment, should inhibit the activation of PKA [79] thus attenuating any downstream signaling, such as PKA-dependent channel activation. As a control, rolipram (100nM) was used. When cells were pretreated with KT5720 for 30 minutes, the endogenous Kv7.5 channel current enhancement normally elicited by rolipram was completely attenuated compared to vehicle control. [Figure 10]

If celecoxib is activating endogenous Kv7.5 channel currents in a PKA-dependent manner, then KT5720 pretreatment should also attenuate any celecoxib-induced enhancement of Kv7.5 currents. Interestingly, KT5720 pretreatment (30minutes) did attenuate the celecoxib-induced enhancement of

Kv7.5 current relative to vehicle controls. [Figure 11] This suggests that celecoxib could, at least partially, require PKA activation to elicit its Kv7 channel activation. Further experiments will have to be completed to definitively implicate PKA activation in celecoxib-induced Kv7 channel enhancement and will be discussed in the future directions of this thesis.

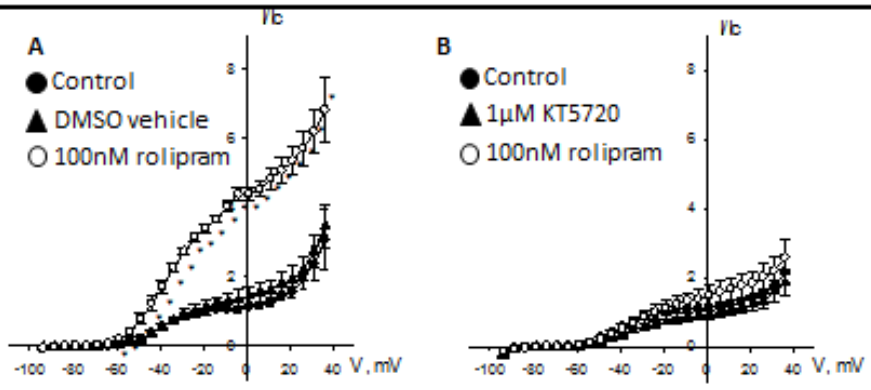

Figure 10: PKA inhibitor, KT5720, inhibited the enhancement of Kv7.5 currents by rolipram. (A) Kv7.5 currents in A7r5 cells were enhanced by 100 nM rolipram (white circles) as compared to control (black circles) and vehicle (black triangles) as seen previously. (B) A 30-minute pretreatment of the cells with 1 µM PKA inhibitor, KT5720, resulted in an inhibition of the rolipram-induced Kv7 current activation. *Significant difference between controls and 100 nM rolipram treatments. One-way ANOVA and Holm-Sidak post hoc test; $p<0.001$, n=4. (LI Brueggemann, publication under review)

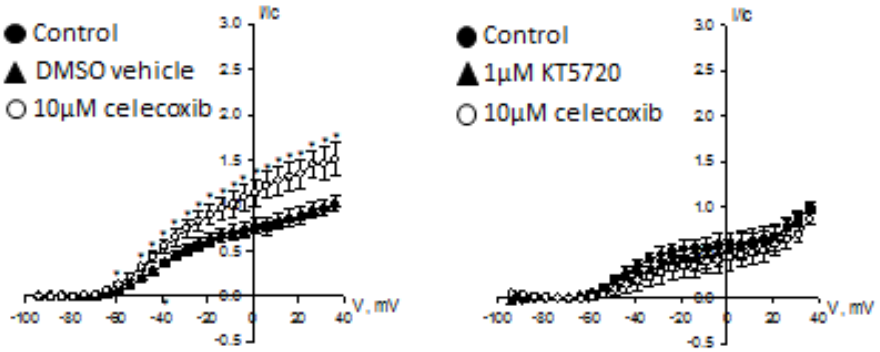

Figure 11: PKA inhibitor, KT5720, inhibited the enhancement of Kv7.5 currents by celecoxib. (A) Kv7.5 currents in A7r5 cells were enhanced by 10µM celecoxib (white circles) as compared to control (black circles) and vehicle (black triangles) as seen previously. (B) A 30-minute pretreatment of the cells with 1 µM PKA inhibitor, KT5720, resulted in an inhibition of the celecoxib-induced Kv7 current activation. *Significant difference between controls and 10 µM celecoxib treatments. One-way ANOVA and Holm-Sidak post hoc test; $p<0.05$, n=4. (CJ Robakowski, unpublished)

CHAPTER 6

DISCUSSION

This thesis work sought to uncover the mechanism through which celecoxib enhances Kv7 currents, which was proposed to be the reason behind its safer cardiovascular risk profile. To determine if phosphodiesterase inhibition was the mechanism by which celecoxib elicits Kv7 channel activation, I sought to answer three main questions: 1.) Is PDE inhibition sufficient to enhance Kv7 currents? 2.) Is PKA activation necessary for Kv7 current enhancement? 3.) Does celecoxib enhance Kv7 currents through a critical residue that is typical of other pharmacological Kv7 channel activators?

The data reported in chapter 5 suggest that PDE inhibition is in fact sufficient to enhance Kv7.5 channel currents. More importantly, the data revealed that different Kv7 channel subunit assemblies are differentially sensitive to regulation by the cAMP/PKA-dependent pathway being studied. This is important from a physiological standpoint because it provides insight into whether or not certain drugs would be efficacious in the vasculature. By using structurally different PDE inhibitors with different specificities for cAMP, I was able to mimic celecoxib's ability to increase Kv7.5 currents as well as determine a treatment that was able to enhance the physiologically relevant and typically less sensitive

hKv7.4/7.5 hetero-tetrameric channel currents (10 µM forskolin/500 µM IBMX combination).

Utilizing the direct cAMP ELISA, I was able to gain insight into the differential regulation of different channel assemblies by the cAMP/PKA-dependent pathway. The results from this assay revealed that there is a difference in the cellular concentration of cAMP following treatment of A7r5 cells. Forskolin and IBMX most significantly increased cAMP levels compared to control, and there was no detectable change in cAMP levels upon treatment with rolipram or celecoxib. This suggests that the larger increase in cAMP levels produced by the combination treatment of forskolin and IBMX could be responsible for the enhancement of the less sensitive heteromeric hKv7.4/7.5 channel currents that is not mimicked with rolipram treatment.

The ELISA data also brought to light the fact that celecoxib may be a weak PDE4 inhibitor at best. The hypothesis that celecoxib was a PDE4 inhibitor and was utilizing a cAMP/PKA-dependent pathway to activate Kv7 channels was developed after Klein et al reported celecoxib to increase both cGMP and cAMP levels in isolated rat aorta tissue. [8] However, Klein and colleagues never actually show the cAMP-specific data and simply state that celecoxib produced a weak, 1.6-fold increase in cAMP levels and that it did not reach statistical significance. [8] So while it was interesting to find the Kv7.4 and Kv7.5 channel subunits were differentially sensitive to regulation by a cAMP/PKA dependent pathway, it may not be the mechanism through which celecoxib is eliciting its Kv7

channel activation. It could turn out that celecoxib only activates the channel through its critical tryptophan residue just like retigabine and other Kv7 channel modulators.

Electrophysiology experiments that utilized the PKA inhibitor, KT5720, offered perspective on the involvement of the cAMP/PKA pathway on Kv7.5 channel current enhancement. As expected, rolipram-induced Kv7.5 current enhancement was attenuated when cells were pretreated with KT5720, which inhibits activation of PKA. Interestingly, pretreatment of A7r5 cells with KT5720 also prevented celecoxib-induced Kv7.5 current enhancement. This was interesting considering the data in which celecoxib was unable to enhance the mutant Kv7.5 channel currents suggested requirement of the critical tryptophan residue for celecoxib-induced enhancement of the channel. This was also interesting considering the preliminary cAMP ELISA data suggested celecoxib was unable to prevent the degradation of cAMP produced after isoproterenol treatment and did not show any detectable elevation in cAMP levels when used as a treatment on its own. There have been reports that PKA inhibitors KT5720 and H89 have many non-specific targets that effect many different signaling pathways and are not a reliable indication of PKA involvement on their own [80]. A more accurate way to implicate PKA involvement in a specific signaling event, as we are attempting to do, would require the combination use of KT5720 with a protein kinase inhibitor peptide or a more specific molecular method such as RNA interference (RNAi) [81]. Directly measuring PKA activity in response to

celecoxib treatment would also better implicate the necessity of PKA activation for celecoxib-induced current enhancement. This further validates the need for future exploration into the exact mechanism of celecoxib.

While I originally sought to determine whether or not PDE inhibition/cAMP elevation is necessary for celecoxib-induced Kv7 channel activation, with the data discussed above, I cannot draw that conclusion. This is due to the fact that PKA activation, the assumed downstream effect of PDE inhibition/cAMP elevation, was only shown to be potentially involved in celecoxib-induced Kv7 channel activation and in fact, data from the mutant channel experiments lends more evidence to celecoxib requiring the critical tryptophan residue to elicit its effect. Additionally, the cAMP ELISA data was inconclusive as to whether or not celecoxib elevated cAMP levels either on its own or in combination with a stimulus such as isoproterenol. Given the inconclusive nature of the data discussed, further work must be completed before PDE inhibition/cAMP elevation's necessity in celecoxib-induced Kv7 channel activation can be concluded. As it stands, the data suggest multiple possible mechanisms by which celecoxib could be eliciting its Kv7 channel activation with better evidence suggesting requirement of the critical residue for channel activation than an indirect PDE4/cAMP/PKA-dependent pathway.

Protein kinase A anchoring proteins add another layer of complexity to celecoxib's mechanism of Kv7 channel modulation. It is worth noting that PKA anchoring proteins offer cAMP signaling specificity by placing PKA in proximity to

specific substrates and effectors [82]. AKAP 79/150, in particular, has been reported to be an important component of PKA and PKC-mediated phosphorylation of other ion channels [83]. AKAPs position PKA in close spatial proximity to specific substrates that allow a localized increase in cAMP to confine PKA phosphorylation to that specific subset of substrates. It is still unknown, however, if localized or global increases in cAMP are responsible for the cAMP-dependent enhancement of Kv7.5 currents that we have observed in this study, and we have yet to explore any role of AKAP in our present findings. It could be worth exploring this role as detailed in the future directions portion of this thesis.

Recently, the Greenwood lab published an article reporting Kv7.4 channel activation by the heterotrimeric G-protein βγ subunits [84]. In that report, Greenwood and colleagues reported that β-AR-Gβγ subunit signaling activates Kv7.4 channels in HEK cells heterologously expressing Kv7.4 and in rat renal artery myocytes. They reported that G-protein βγ subunits enhanced the open probability of Kv7.4 channels and gallein, an inhibitor of Gβγ subunits, prevented stimulatory effects. Only Kv7.4 channel activity was explored, however [84]. And as stated previously, Kv7.4 and Kv7.5 predominantly form heterotetramers in the vasculature. Since effects on Kv7.5 and Kv7.4/7.5 channels activities were not explored in that report, it is unclear whether β-AR-Gβγ subunit signaling is relevant physiologically. Results from our laboratory (Mani *et al.*, manuscript submitted) do not reproduce the enhancement of Kv7.4 currents by isoproterenol

reported by Stott *et al.*, though our results are based on expression of Kv7.4 in A7r5 cells rather than HEK cells. However, it's possible that the β-AR activation could mediate vasodilatory responses by activating different Kv7 channel subunits through diverse signaling molecules.

Lastly, it is unclear what, if any, structural differences between celecoxib and rofecoxib are responsible for the difference in their ability to activate Kv7 channels. The sulfone group of rofecoxib (which is a sulfonamide in celecoxib) is what allows rofecoxib to be more specific for COX-2 than celecoxib [2]. But since we concluded that the Kv7 channel activation by celecoxib is independent of its COX-2 inhibitory activity, it is unclear of those different groups play a role in Kv7 channel activation. There are a few other noteworthy difference between rofecoxib and celecoxib. Rofecoxib contains a furanone heterocycle and contains no fluorine or additional groups on its aromatic ring [2]. Celecoxib contains a pyrazole heterocycle with a tri-fluorinated carbon as well as a methyl group on its aromatic ring [2]. Any of these structural differences could be responsible for the difference in celecoxib and rofecoxib's abilities to activate the Kv7 channels but that has not yet been explored.

CHAPTER 7

FUTURE DIRECTIONS

PKA Phosphorylation Studies

From the data collected, it is clear that at least the Kv7.5 subunits are sensitive to a cAMP/PKA-dependent pathway. First, it would be beneficial to determine if treatment with celecoxib affects PKA activation. This could be done using cell lysates with fluorescence based assays like those detailed by Shults and colleagues in *Nature Methods* [86]. Normally when PKA is activated, it goes on to phosphorylate and activate many downstream effectors. It would also be helpful to investigate whether or not there is a phosphorylation of the Kv7.5 channel associated with its activation by PKA. Using MIT scansite software, 11 putative PKA phosphorylation sites exist within the Kv7.5 amino acid sequence. These sites exist both on the N-terminus and C-terminus of the channel. Our laboratory has attempted to create mutant channels with either the C or N-terminus truncated to determine which residues are important for PKA regulation, but those mutants never resulted in functional channels. A strategy that could be employed to answer the question as to which, if any, residue(s) is necessary for PKA regulation would be to construct point mutations of candidate phosphorylation sites to render the protein unphosphorylatable at these sites. Each potential residue could be point mutated and electrophysiology experiments

could be run to determine the functional necessity of each of the potential PKA residues. Once this residue is determined, further studies can be done to assess whether that phosphorylation site can be exploited therapeutically. This is also important information to have because we reported that different Kv7 subunits are differentially regulated by this PKA pathway and some, like Kv7.4, are seemingly insensitive to PKA regulation. Using the same MIT scansite software, only three putative PKA phosphorylation sites were identified in the Kv7.4 sequence. Of those three sites, only two corresponded to analogous amino acid sequences in the Kv7.5 channel. The fact that there are so many more potential PKA phosphorylation sites on the Kv7.5 channel and so few on the Kv7.4 channel could explain the differences in sensitivity to PKA regulation between the two subunits.

Revisit cGMP Pathway

The Klein paper that reported celecoxib to be a PDE inhibitor reported that celecoxib, and not rofecoxib, was both a PDE4 (cAMP-specific phosphodiesterase) and a PDE5 (cGMP-specific phosphodiesterase) inhibitor and their evidence for PDE5 inhibition was much stronger than for PDE4 inhibition [8]. Inhibition of PDE5 would result in higher levels of cGMP and the cGMP-NO pathway is a well-defined vasodilatory pathway so it may be worth revisiting. Electrophysiology experiments could be repeated with a PDE5 inhibitor, such as tadalafil or sildenafil, to see if they, too, mimic celecoxib's ion channel modulatory activity. There is also a chance that there is significant cross-

talk between the cAMP and cGMP pathways that could further complicate the mechanism of Kv7 channel current enhancement that we report here. If it turns out that PDE5 inhibition mimics the Kv7.5 current enhancement seen with PDE4 inhibitors, there also exist PKG inhibitors that could be used as a pretreatment in the A7r5 cells similar to the experiment I showed previously using PKA inhibitor, KT5720. The PKG inhibitor, KT5823, works in much the same way and would attenuate any cGMP-induced Kv7 current enhancement when used as a pretreatment in the cells. Electrophysiology experiments utilizing KT5823 would give insight into the necessity of the cGMP pathway in celecoxib's Kv7 channel activation. It would also be interesting to replicate Klein et al.'s reported celecoxib-induced increase in cGMP levels using an ELISA kit akin to the direct cAMP ELISA kit used in this thesis work. Klein et al stated that PDE5 inhibition was more important than PDE4 inhibition to the vasodilatory effect exhibited by celecoxib in their experiments and it would be interesting to see if the same trend was present in our hands.

DMC as a PDE inhibitor

Our laboratory previously reported that celecoxib's ion channel modulatory activity was independent of its COX-2 inhibitory activity. This conclusion was drawn after dimethyl celecoxib (DMC) a structural analogue of celecoxib that lacks any COX-2 inhibitory activity, was able to mimic celecoxib in its ability to enhance Kv7 channel currents as well as inhibit L-type calcium channel currents.

Furthermore, rofecoxib, which has an even higher affinity for COX-2 than celecoxib, was unable to mimic either of these ion channel modulatory effects.

It would be interesting to explore whether DMC also mimicked celecoxib's ability to inhibit PDE4 or PDE5. Literature searches yielded no studies in which this specific effect was studied for DMC. If it turns out that DMC also inhibits PDE4 and/or PDE5 activity, it could lead to insight regarding celecoxib's mechanism of Kv7 current enhancement. For instance, if DMC is found to be a PDE4 inhibitor but not a PDE5 inhibitor, it could lend more support to the cAMP/PKA mechanism for Kv7 channel regulation. If DMC is a PDE5 inhibitor as well, it would support the need to revisit the cGMP pathway and its potential effect on the Kv7 enhancement we have observed previously.

AKAP

As discussed in the previous chapter, anchoring proteins are important for bringing signaling components in close proximity to the substrates they target. AKAPs allow for PKA signaling to be determined by the specific pools of cAMP within their vicinity, thus distinguishing between different pools of cAMP within the cell. AKAPs have been reported to bring PKA, PDEs, and substrates such as ion channels together to promote targeted signaling. It has yet to be determined if any anchoring proteins play a role in celecoxib's mechanism of Kv7 channel activation. It would be worth exploring if there exists a signaling complex that brought together the Kv7 channels of interest with PKA and PDE4. To explore this, experiments would have to be run to determine if any of those signaling

components are in close proximity which could provide insight into the possibility of a signaling complex being involved in celecoxib's mechanism of Kv7 channel activation. Our laboratory found previously that AKAP150 co-immunoprecipitates with the Kv7.5 channel in A7r5 cells [unpublished]. It has yet to be explored whether PDE4 or PDE5 are also present in those immunoprecipitates. Proximity ligation assays might be a better method for exploring our direct protein-protein interactions of interest.

BIBLIOGRAPHY

1. Fosslien, E., *Review: Cardiovascular Complications of Non-Steroidal Anti-Inflammatory Drugs.* Annals of Clinical and Laboratory Science, 2005. **35**(4): p. 347-385.

2. Marnett, L.J., *The COXIB Experience: A Look in the Rear-View Mirror.* Annual Review of Pharmacology and Toxicology, 2009. **49**(1).

3. McGettigan, P. and D. Henry, *Cardiovascular Risk and Inhibition of Cyclooxygenase: A Systematic Review of the Observational Studies of Selective and Nonselective Inhibitors of Cyclooxygenase 2.* JAMA, 2006. **296**(13): p. 1633-1644.

4. Aw, T.J., et al., *Meta-analysis of cyclooxygenase-2 inhibitors and their effects on blood pressure.* Arch Intern Med., 2005. **165**(5): p. 490-496.

5. Cho, J., C.E. Cooke, and W. Proveaux, *A retrospective review of the effect of COX-2 inhibitors on blood pressure change.* Am J Ther., 2003. **10**(5): p. 311-7.

6. White, W.B., et al., *Risk of Cardiovascular Events in Patients Receiving Celecoxib: A Meta-Analysis of Randomized Clinical Trials.* The American Journal of Cardiology, 2007. **99**(1): p. 91-98.

7. Hermann, M., et al., *Differential Effects of Selective Cyclooxygenase-2 Inhibitors on Endothelial Function in Salt-Induced Hypertension.* Circulation, 2003. **108**(19): p. 2308-2311.

8. Klein, T., et al., *Celecoxib dilates guinea-pig coronaries and rat aortic rings and amplifies NO/cGMP signaling by PDE5 inhibition.* Cardiovascular Research, 2007. **75**(2): p. 390-397.

9. Brueggemann, L.I. and K.L. Byron, *Cycloxygenase-2 Inhibitor Celecoxib Is A Potent Activator Of Vascular KCNQ K+ Channels And An Inhibitor Of L-type Ca2+ Channels.* Biophysical Journal, 2009. **96**(3, Supplement 1): p. 165a-165a.

10. Gutman, G.A., et al., *International Union of Pharmacology. LIII. Nomenclature and molecular relationships of voltage-gated potassium channels.* Pharmacological Reviews, 2005. **57**(4): p. 473-508.
11. White JA, M.P., Antonarakis S, Cann H, Eppig JT, Frazer K, Frezal J, Lancet D, Nahmias J, Pearson P, Peters J, Scott A, Scott H, Spurr N, C. T, Jr, Povey S, *Guidelines for human genome nomenclature.* Genomics, 1997. **45**: p. 468-71.

12. Sanguinetti, M.C., et al., *Coassembly of K(V)LQT1 and minK (IsK) proteins to form cardiac I(Ks) potassium channel.* Nature, 1996. **384**(6604): p. 80-3.

13. Wang, H.S., et al., *KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel.* Science., 1998. **282**(5395): p. 1890-3.

14. Jentsch, T.J., *Neuronal KCNQ potassium channels: physiology and role in disease.* Nat Rev Neurosci, 2000. **1**(1): p. 21-30.

15. Delmas, P. and D.A. Brown, *Pathways modulating neural KCNQ/M (Kv7) potassium channels.* Nat Rev Neurosci, 2005. **6**(11): p. 850-62.

16. Schwake, M., T.J. Jentsch, and T. Friedrich, *A carboxy-terminal domain determines the subunit specificity of KCNQ K^+ channel assembly.* EMBO Rep., 2003. **4**(1): p. 76-81.

17. Haitin, Y. and B. Attali, *The C-terminus of Kv7 channels: a multifunctional module.* J Physiol., 2008. **586**(7): p. 1803-10. Epub 2008 Jan 24.

18. Abbott, G.W., Xu, X., Roepke, T.K., *Impact of Ancillary Subunits on ventricular repolarization.* Journal of Electrocardiology, 2007. **40**(6): p. S42-S46.

19. Bal, M., et al., *Homomeric and Heteromeric Assembly of KCNQ (Kv7) K^+ Channels Assayed by Total Internal Reflection Fluorescence/Fluorescence Resonance Energy Transfer and Patch Clamp Analysis.* J Biol Chem, 2008. **283**(45): p. 30668-30676.

20. Kubisch, C., et al., *KCNQ4, a novel potassium channel expressed in sensory outer hair cells, is mutated in dominant deafness.* Cell, 1999. **96**(3): p. 437-46.

21. Schroeder, B.C., et al., *KCNQ5, a Novel Potassium Channel Broadly Expressed in Brain, Mediates M-type Currents.* J Biol Chem, 2000. **275**(31): p. 24089-24095.

22. Bal, M., Zhang J, Zaika O, Shapiro, MS, *Ca2+/calmodulin disrupts AKAP79/150 interactions with KCNQ (M-type) K+ channels.* Journal of Neuroscience, 2010. **30**: p. 2311-23.

23. Mackie, A.R. and K.L. Byron, *Cardiovascular KCNQ (Kv7) Potassium Channels: Physiological Regulators and New Targets for Therapeutic Intervention.* Mol Pharmacology, 2008. **74**: p. 1171-1179.

24. Brown, D.A., *Kv7 (KCNQ) potassium channels that are mutated in human diseases.* J Physiol, 2008. **586**(7): p. 1781-1783.

25. Oliveras, A., et al., *Functional assembly of Kv7.1/Kv7.5 channels with emerging properties on vascular muscle physiology.* Arterioscler Thromb Vasc Biol, 2014. **34**(7): p. 1522-30.

26. Hernandez, C.C., et al., *Regulation of neural KCNQ channels: signalling pathways, structural motifs and functional implications.* J Physiol, 2008. **586**(7): p. 1811-1821.

27. Marrion, N.V., *Control of M-current.* Annu Rev Physiol, 1997. **59**: p. 483-504.

28. Zaydman, M.A. and J. Cui, *PIP2 regulation of KCNQ channels: biophysical and molecular mechanisms for lipid modulation of voltage-dependent gating.* Front Physiol, 2014. **5**: p. 195.

29. Horowitz, L.F., et al., *Phospholipase C in living cells: activation, inhibition, Ca^{2+} requirement, and regulation of M current.* Journal of General Physiology, 2005. **126**(3): p. 243-62.

30. Zhang, H., et al., *PIP(2) activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents.* Neuron., 2003. **37**(6): p. 963-75.

31. Zhang, H., et al., *PIP2 Activates KCNQ Channels, and Its Hydrolysis Underlies Receptor-Mediated Inhibition of M Currents.* Neuron, 2003. **37**(6): p. 963-975.

32. Li, Y., et al., *Regulation of Kv7 (KCNQ) K+ channel open probability by phosphatidylinositol 4,5-bisphosphate.* Journal of Neuroscience, 2005. **25**(43): p. 9825-35.

33. Negro A, D.-K.K., Kapiloff MS, *Signalosomes as therapeutic targets.* Progress in pediatric cardiology, 2008. **25**: p. 51-56.

34. Colledge M, S.J., *AKAPs: from structure to function.* Trends in cell biology, 1999. **9**: p. 216-221.

35. Klauck, T.M., et al., *Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein.* Science., 1996. **271**(5255): p. 1589-92.

36. Dodge KL, K.S., Kapiloff MS, Mouton R, Hill EV, Houslay MD, Langeberg LK, Scott JD, *mAKAP assamebles a protein kinase A/PDE4 phosphodiesterase cAMP signaling molecule.* The EMBO journal, 2001. **20**: p. 1921-30.

37. Gamper, N., J.D. Stockand, and M.S. Shapiro, *Subunit-specific modulation of KCNQ potassium channels by Src tyrosine kinase.* Journal of Neuroscience, 2003. **23**(1): p. 84-95.

38. Li, Y., et al., *Dual phosphorylations underlie modulation of unitary KCNQ K(+) channels by Src tyrosine kinase.* Journal of Biological Chemistry, 2004. **279**(44): p. 45399-407.

39. Rose, K., et al., *Transcriptional repression of the M channel subunit Kv7.2 in chronic nerve injury.* Pain, 2011. **152**(4): p. 742-754.

40. Tam, S.W., D. Rominger, and V.J. Nickolson, *Novel receptor site involved in enhancement of stimulus-induced acetylcholine, dopamine, and serotonin release.* Mol Pharmacol, 1991. **40**(1): p. 16-21.

41. Czuczwar P, W.A., Cioczek-Czuczwar A, Parada-Turska J, Maciejewski R, Czuczwar SJ, *Retigabine: the newer potential antiepileptic drug.* Pharmacol Rep, 2010. **62**: p. 211-219.

42. Schenzer, A., et al., *Molecular determinants of KCNQ (K_v7) K^+ channel sensitivity to the anticonvulsant retigabine.* J Neurosci., 2005. **25**(20): p. 5051-60.

43. Brueggemann, L.I., et al., *Diclofenac distinguishes among homomeric and heteromeric potassium channels composed of KCNQ4 and KCNQ5 subunits.* Mol Pharmacol, 2011. **79**(1): p. 10-23.

44. Yu, H., et al., *Discovery, Synthesis, and Structure Activity Relationship of a Series of N-Aryl- bicyclo[2.2.1]heptane-2-carboxamides: Characterization of ML213 as a Novel KCNQ2 and KCNQ4 Potassium Channel Opener.* ACS Chem Neurosci, 2011. **2**(10): p. 572-577.

45. Brueggemann, L.I., et al., *Differential activation of vascular smooth muscle Kv7.4, Kv7.5, and Kv7.4/7.5 channels by ML213 and ICA-069673.* Mol Pharmacol, 2014. **86**(3): p. 330-41.

46. Korsgaard, M.P.G., et al., *Anxiolytic Effects of Maxipost (BMS-204352) and Retigabine via Activation of Neuronal Kv7 Channels.* Journal of Pharmacology & Experimental Therapeutics, 2005. **314**(1): p. 282-292.

47. Ohya, S., et al., *Molecular variants of KCNQ channels expressed in murine portal vein myocytes: a role in delayed rectifier current.* Circ Res., 2003. **92**(9): p. 1016-23.

48. Yeung, S.Y. and I.A. Greenwood, *Electrophysiological and functional effects of the KCNQ channel blocker XE991 on murine portal vein smooth muscle cells.* British Journal of Pharmacology, 2005. **146**(4): p. 585-95.

49. Brueggemann, L.I., et al., *Vasopressin stimulates action potential firing by protein kinase C-dependent inhibition of KCNQ5 in A7r5 rat aortic smooth muscle cells.* Am J Physiol Heart Circ Physiol, 2007. **292**(3): p. H1352-H1363.

50. Yeung, S.Y., et al., *Molecular expression and pharmacological identification of a role for K(v)7 channels in murine vascular reactivity.* Br J Pharmacol, 2007. **151**(6): p. 758-770.

51. Zhong, X.Z., et al., *Participation of KCNQ (Kv7) potassium channels in myogenic control of cerebral arterial diameter.* J Physiol, 2010. **588**(Pt 17): p. 3277-3293.

52. Joshi, S., et al., *KCNQ Modulators Reveal a Key Role for KCNQ Potassium Channels in Regulating the Tone of Rat Pulmonary Artery Smooth Muscle.* J Pharmacol Exp Ther, 2009. **329**(1): p. 368-376.

53. Mackie, A.R., et al., *Vascular KCNQ potassium channels as novel targets for the control of mesenteric artery constriction by vasopressin, based on studies in single cells, pressurized arteries, and in vivo measurements of mesenteric vascular resistance.* J Pharmacol Exp Ther, 2008. **325**(2): p. 475-83.

54. Ng, F.L., et al., *Expression and function of the K^+ channel KCNQ genes in human arteries.* Br J Pharmacol, 2011. **162**: p. 42-53.

55. Chadha, P.S., et al., *Pharmacological dissection of K(v)7.1 channels in systemic and pulmonary arteries.* Br J Pharmacol, 2012. **166**(4): p. 1377-87.

56. Jepps, T.A., et al., *Downregulation of Kv7.4 Channel Activity in Primary and Secondary Hypertension.* Circulation, 2011. **124**(5): p. 602-11.

57. Khanamiri, S., et al., *Contribution of Kv7 Channels to Basal Coronary Flow and Active Response to Ischemia.* Hypertension, 2013. **62**(6): p. 1090-1097.

58. Zavaritskaya, O., et al., *Role of KCNQ Channels in Skeletal Muscle Arteries and Periadventitial Vascular Dysfunction.* Hypertension, 2013. **61**(1): p. 151-159.

59. Chadha, P.S., et al., *Reduced KCNQ4-Encoded Voltage-Dependent Potassium Channel Activity Underlies Impaired β-Adrenoceptor-Mediated*

Relaxation of Renal Arteries in Hypertension. Hypertension, 2012. **59**(4): p. 877-84.

60. Mani, B.K., et al., *Opposite regulation of KCNQ5 and TRPC6 channels contributes to vasopressin-stimulated calcium spiking responses in A7r5 vascular smooth muscle cells.* Cell Calcium, 2009. **45**(4): p. 400-11.

61. Byron, K.L. and C.W. Taylor, *Spontaneous Ca^{2+} spiking in a vascular smooth muscle cell line is independent of the release of intracellular Ca^{2+} stores.* J Biol Chem, 1993. **268**(10): p. 6945-6952.

62. Schroeder, B.C., et al., *Moderate loss of function of cyclic-AMP-modulated KCNQ2/KCNQ3 K+ channels causes epilepsy.* Nature, 1998. **396**(6712): p. 687-690.

63. Chambard, J.M. and J.F. Ashmore, *Regulation of the voltage-gated potassium channel KCNQ4 in the auditory pathway.* Pflugers Archiv, 2005. **450**(1): p. 34-44.

64. Keravis, T. and C. Lugnier, *Cyclic nucleotide phosphodiesterase (PDE) isozymes as targets of the intracellular signalling network: benefits of PDE inhibitors in various diseases and perspectives for future therapeutic developments.* Br J Pharmacol, 2012. **165**(5): p. 1288-305.

65. Pierce KL, P.R., Lefkowitz RJ, *Seven Transmembrane Receptors.* Nature Reviews Molecular Cell Biology, 2002. **3**: p. 639-650.

66. Haynes, J., Jr., et al., *Role of cAMP-dependent protein kinase in cAMP-mediated vasodilation.* Am J Physiol, 1992. **262**(2 Pt 2): p. H511-6.

67. Ahmad, F., et al., *Cyclic nucleotide phosphodiesterases: important signaling modulators and therapeutic targets.* Oral Dis, 2015. **21**(1): p. e25-50.

68. Maurice, D.H., et al., *Advances in targeting cyclic nucleotide phosphodiesterases.* Nat Rev Drug Discov, 2014. **13**(4): p. 290-314.

69. Skroblin, P., et al., *Mechanisms of protein kinase A anchoring.* Int Rev Cell Mol Biol, 2010. **283**: p. 235-330.

70. Welch, E.J., B.W. Jones, and J.D. Scott, *Networking with AKAPs: context-dependent regulation of anchored enzymes.* Mol Interv, 2010. **10**(2): p. 86-97.

71. Murray, K.J., et al., *Potential use of selective phosphodiesterase inhibitors in the treatment of asthma.* Agents Actions Suppl, 1991. **34**: p. 27-46.

72. Torphy, T.J., et al., *Identification, characterization and functional role of phosphodiesterase isozymes in human airway smooth muscle.* J Pharmacol Exp Ther, 1993. **265**(3): p. 1213-23.

73. Cortijo, J., et al., *Bronchodilator and anti-inflammatory activities of glaucine: In vitro studies in human airway smooth muscle and polymorphonuclear leukocytes.* Br J Pharmacol, 1999. **127**(7): p. 1641-51.

74. CDC, *Vital signs: awareness and treatment of uncontrolled hypertension among adults—United States, 2003–2010.* MMWR, 2012. **61**(35): p. 703-9.

75. Katzung BG, M.S., Trevor AJ, *Basic and Clinical Pharmacology*. 12 ed. 2012: McGraw Hill.

76. Brueggemann, L.I. and K.L. Byron, *Use of Patch Clamp Electrophysiology to Identify Off-target Effects of Clinically Used Drugs.*, in *Patch Clamp Technique*, F.S. Kaneez, Editor. 2012, Intech: Rijeka, Croatia.

77. Du, X.N., et al., *Characteristics and molecular basis of celecoxib modulation on K_V7 potassium channels.* Br J Pharmacol, 2011. **164**(6): p. 1722-1737.

78. Brueggemann, L.I., et al., *KCNQ (Kv7) potassium channel activators as bronchodilators: combination with a β2-adrenergic agonist enhances relaxation of rat airways.* Am J Physiol Lung Cell Mol Physiol, 2014. **306**(6): p. L476-86.

79. Kase, H., et al., *K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases.* Biochem Biophys Res Commun, 1987. **142**(2): p. 436-40.

80. Davies, S.P., et al., *Specificity and mechanism of action of some commonly used protein kinase inhibitors.* Biochem J, 2000. **351**(Pt 1): p. 95-105.

81. Murray, A.J., *Pharmacological PKA inhibition: all may not be what it seems.* Sci Signal, 2008. **1**(22): p. re4.

82. Wong, W. and J.D. Scott, *AKAP signalling complexes: focal points in space and time.* Nat Rev Mol Cell Biol, 2004. **5**(12): p. 959-70.

83. Jeske, N.A., et al., *A-kinase anchoring protein 150 controls protein kinase C-mediated phosphorylation and sensitization of TRPV1.* Pain, 2009. **146**(3): p. 301-7.

84. Stott, J.B., et al., *G-protein betagamma subunits are positive regulators of Kv7.4 and native vascular Kv7 channel activity.* Proc Natl Acad Sci U S A, 2015. **112**(20): p. 6497-502.

85. Mani, B.K., *Vascular Kv7(KCNQ) channels as therapeutic targets in cerebral vasospasm.* Loyola University Chicago, 2012. Dissertation

86. Shults, M.D., et al., *A multiplexed homogenous fluorescence-based assay for protein kinas activity in cell lysates.* Nature Methods, 2005. 2: p. 277-84

VITA

The author, Christina J. Robakowski, was born on July 1, 1988 in South Bend, IN. She majored in chemistry and minored in mathematics at Saint Mary's College in Notre Dame, IN and was awarded her Bachelor of Science degree in May 2011.

After taking some time after graduation to work retail full-time and take care of her father, she knew that her goal to obtain a higher degree would need to be realized. Christina began her graduate studies at Loyola University Chicago in August of 2013 in the Department of Molecular Pharmacology and Therapeutics.

In February of 2014, Christina joined the laboratory of Dr. Kenneth Byron and began studying celecoxib's mechanism of ion channel modulation, specifically focusing on its ability to modulate Kv7 potassium channels.

Immediately following graduation, Christina will be moving to Maui. Her ultimate career aspirations include industrial research and development and/or mentoring/advocating for young people to choose STEM careers.