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Examining the Regulation of Kv7 K+ Channels in Airway Smooth Muscle Cells and Their Potential as Novel Therapeutic Targets for the Treatment of Asthma

Jennifer Haick
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

EXAMINING THE REGULATION OF Kv7 K^+ CHANNELS IN AIRWAY SMOOTH MUSCLE CELLS AND THEIR POTENTIAL AS NOVEL THERAPEUTIC TARGETS FOR THE TREATMENT OF ASTHMA

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For My Parents and My Fiancé

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

4α-PMA	4α-Phorbol 12-Myristate 13-Acetate
AC	Adenylyl Cyclase
AHR	Airway Hyperresponsiveness
AKAP	A-kinase Anchoring Protein
ASMC	Airway Smooth Muscle Cell
AVP	Arginine^{8}-Vasopressin
BFNC	Benign Familial Neonatal Convulsions
BK_{Ca}	Large Conductance Calcium-Activated Potassium Channel
Ca^{2+}	Calcium
$[Ca^{2+}]_{cyt}$	Cytosolic Calcium Concentration
CaM	Calmodulin
cAMP	Cyclic Adenosine Monophosphate
CCB	Calcium Channel Blocker
CPI-17	PKC-Potentiated Inhibitory Protein for Heterotrimeric MLCP of 17 kDa
DAG	1,2-diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DRG	Dorsal Root Ganglia
EC_{50}	Half-Maximal Effective Concentration
EndoK	Endoproteinase Lys-C
EndoQ	Endoproteinase Glu-C

GFP	Green Fluorescent Protein
hKv7.5	Human Kv7.5 channels
HTSMC	Human Trachealis Smooth Muscle Cell
IgE	Immunoglobulin E
IL-5	Interleukin 5
IL-13	Interleukin 13
IP	Immunoprecipitation
IP_3	1,4,5-trisphosphate
IP_3R	1,4,5-trisphosphate Receptor
i.v.	Intravenous
I-V	Current-Voltage Relationship
K^+	Potassium
K_{ATP}	ATP-Sensitive Potassium Channel
KCNQ	Name of Gene Family that Encodes Kv7 Channel Alpha Subunits
Kv	Voltage-Dependent Delayed Rectifier Potassium Channel
Kv7	Family of Voltage Gated Ion Changes Encoded by KCNQ Genes
LABA	Long Acting β_2-Agonist
mHBSS	Modified Hank's Balanced Salt Solution
mKreb's	Modified Kreb's Buffer
mRNA	Messenger Ribonucleic Acid
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase

MOI	Multiplicity of Infection
Na^+	Sodium
NCX	Sodium/Calcium Exchanger
NH_4HCO_3	Ammonium Bicarbonate
PCLS	Precision Cut Lung Slice
PIP_2	Phosphatidylinositol 4,5-Bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol 12-Myristate 13-Acetate
PSS	Physiological Saline Solution
REST	Repressor Element 1-Silencing Transcription Factor
ROCC	Receptor Operated Calcium Channel
S385	Serine 385
S441	Serine 441
S441A	Serine 441 Mutated to Alanine
S752	Serine 752
S772	Serine 772
SABA	Short Acting β_2-Agonist
SERCA	Sarco/Endoplasmic Reticulum Calcium-ATPase
shControl	Short Hairpin RNA Targeting a Sequence Not Found in the Human Genome
shPKCα	Short Hairpin RNA Targeting Protein Kinase C Alpha

shRNA	Short Hairpin Ribonucleic Acid
SOCC	Store Operated Calcium Channel
Sp1	Specialty Factor 1 Transcription Factor
SR	Sarcoplasmic Reticulum
STIM1	Stromal Interaction Molecule 1
T505	Threonine 505
T505A	Threonine 505 Mutated to Alanine
TRP	Transient Receptor Potential
VSCC	Voltage Sensitive Calcium Channel
WHO	World Health Organization
WT	Wild Type

CHAPTER ONE

INTRODUCTION

Asthma is a chronic, heterogeneous disease of the airways in the lung that affects 235 million people worldwide and is characterized by inflammation of the airways, airway remodeling, and exaggerated narrowing of the airways, termed airway hyperresponsiveness (AHR) (WHO 2013, CDC 2014). The hypercontraction of airways occurs in response to a variety of stimuli, or triggers, such as allergens, cold air, or physical activity. Airway smooth muscle is the effector behind the hypercontraction, however the specific role that it plays in the pathophysiology of asthma and AHR remain unclear despite extensive study (An, Bai et al. 2007). The contractile state of airway smooth muscle is predominantly determined by G_q-coupled bronchoconstrictor agonist signaling. These agonists include acetylcholine, histamine, endothelin, and leukotriene D4. Activation of G_q-coupled receptors culminates in an increase in cytosolic calcium [Ca^{2+}] concentrations ($[Ca^{2+}]_{cyt}$) that leads to contraction of airway smooth muscle cells (ASMCs) and airway constriction (Penn 2008). Bronchodilator therapy is one type of asthma therapy that aims to reverse the airway constriction that results from G_q-coupled receptor signaling, however there are a limited number of therapeutic targets currently available (Pera and Penn 2016). There is also a large population of individuals with asthma who have poor control of their disease or develop a tolerance to currently available treatment options (Peters, Jones et al. 2007,

Chapman, Boulet et al. 2008, Cazzola, Page et al. 2013) indicating the pressing need to explore new therapeutic targets for bronchodilator therapy and additional treatment options.

Our lab recently found that Kv7 potassium (K^+) channels are expressed in airway smooth muscle and that Kv7 currents are inhibited upon treatment with G_q-coupled bronchoconstrictor agonists in ASMCs. Blockade of Kv7 channels resulted in robust constriction of human airways and activation of Kv7 channels attenuated airway constriction induced by the bronchoconstrictor agonist histamine (Brueggemann, Kakad et al. 2012). Kv7 channels are well-known regulators of smooth muscle contractility (Haick and Byron 2016) and are also recognized as being downstream of G_q-coupled receptor signaling in a variety of tissues (Marrion 1997, Delmas and Brown 2005, Mackie, Brueggemann et al. 2008). This project aims to elucidate the mechanism of Kv7 K^+ channel regulation by the bronchoconstrictor histamine in airway smooth muscle and to test the potential of Kv7 channel activators as novel bronchodilatory therapy for the treatment of asthma.

CHAPTER TWO

LITERATURE REVIEW

Airway Smooth Muscle.

Anatomy and physiology of airway smooth muscle. Airway smooth muscle is located along the posterior aspect of the trachea and encircles airways in a helical manner down the bronchial tree. Physiologically, its main purpose is to establish and regulate airway diameter and therefore the rate of and resistance to airflow, particularly in disease states. When the cells that compose this layer of smooth muscle contract, airways constrict, and when the ASMCs relax, airways also relax. The narrowing and opening of airways are often a result of signal transduction initiated by mediators released from nearby nerves or cells of the immune system or epithelium.

Signal transduction in airway smooth muscle cells. *G_q-protein coupled receptor signaling*. The contractile state of airway smooth muscle is largely dictated by the balance of G_s and G_q-coupled receptor signaling, with tone being established primarily by procontractile G_q-coupled signaling, with minimal input from prorelaxant G_s-coupled receptor signaling in a non-disease state. Acetylcholine, a G_q-coupled m3 muscarinic acetylcholine receptor agonist, is released from nearby parasympathetic nerves and is the principal mediator of airway smooth muscle tone (Penn 2008). Other G_q-coupled receptor agonists that

promote contraction of ASMCs include histamine, endothelin, and leukotriene D4 (Penn and Benovic 2008). As depicted in Figure 1, activation of G_q-coupled receptors results in the activation of phospholipase C (PLC) which in turn cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). DAG activates protein kinase C (PKC), whose effects will be discussed later in this chapter. IP_3 binds to the IP_3 receptor (IP_3R) located on the intracellular Ca^{2+} storage organelle called the sarcoplasmic reticulum (SR) and induces Ca^{2+} release into the intracellular space. The newly released Ca^{2+} binds to calmodulin, forming Ca^{2+}-calmodulin complexes that activate myosin light chain (MLC) kinase (MLCK). MLCK phosphorylates MLCs which promotes the interaction between actin and myosin, called cross-bridge formation, and the cycling between an interacting and non-interacting state, called cross-bridge cycling, that causes contraction of the smooth muscle cell (Hall 2000, Penn 2008, Penn and Benovic 2008).

G_s-protein coupled receptor signaling. The counterbalance to bronchoconstrictor signaling, and a common target pathway activated by asthma therapeutics such as β_2-adrenergic agonists, is G_s-coupled receptor signaling. The activation of adenylyl cyclase (AC) following G_s-coupled receptor stimulation results in the generation of cyclic adenosine monophosphate (cAMP) and the subsequent activation of protein kinase A (PKA). PKA phosphorylates IP_3Rs, G_q-coupled receptors, and MLCK resulting in their inhibition, as well as MLC phosphatase (MLCP) to promote decreased phosphorylation of MLCs. These

actions ultimately result in reduced phosphorylation of MLC and therefore reduced actin and myosin cross-bridge cycling and relaxation of the smooth muscle cell. Another PKA target is the Ca^{2+} activated K^+ channel, which, when phosphorylated, has an increased open probability and therefore promotes membrane hyperpolarization and smooth muscle cell relaxation (Hall 2000, Penn 2008, Penn and Benovic 2008).

Protein kinase C in airway smooth muscle. The serine and threonine kinase PKC is widely acknowledged as a mediator of bronchoconstriction. Multiple isoforms have been observed at the mRNA and protein level in human airway smooth muscle. Freshly isolated human trachealis and lung tissues express the Ca^{2+}-dependent conventional isoforms α, β_1, and β_2, Ca^{2+}-independent novel isoforms δ, ε, and η, and the atypical isoforms ζ and ι (Webb, Lindsay et al. 1997, Sakai, Yamamoto et al. 2009). Cultured human airway smooth muscle cells are reported to express all isoforms except γ, although that particular isoform was not examined (Carlin, Yang et al. 1999). The PKC isoforms that mediate bronchoconstriction have not been specified.

As described above, the formation of DAG in ASMCs that follows G_q-coupled receptor activation results in the activation of PKC. PKC acts to phosphorylate a number of substrates to promote cross-bridge cycling and ASMC contraction, such as MLCP (Webb, Hirst et al. 2000), calponin (Pohl, Winder et al. 1997), and the PKC-potentiated inhibitory protein for heterotrimeric MLCP of 17 kDa, CPI-17 (Sakai, Chiba et al. 2005). Despite the recognized effects facilitated

through phosphorylation of ASMC contractile machinery, there are other PKC-dependent aspects of ASMC contraction that cannot be explained by these mechanisms. Some evidence indicates that PKC-mediated contraction of airway smooth muscle is at least partially dependent on extracellular Ca^{2+} and L-type voltage sensitive Ca^{2+} channel (VSCC) activity (Park and Rasmussen 1985, Knox, Baldwin et al. 1993, Rossetti, Savineau et al. 1995, Yang and Black 1996, Mukherjee, Trice et al. 2013), signifying a roll for ionic fluxes from the extracellular to the intracellular space in airway constriction.

Regulation of cytosolic Ca^{2+} in airway smooth muscle. The necessity of Ca^{2+} for ASMC excitation-contraction coupling has been well established and multiple participants have been implicated as contributors to the required increase in cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$). Bronchoconstrictor agonist stimulation of ASMCs results in a biphasic increase in $[Ca^{2+}]_{cyt}$: an initial, transient phase mediated primarily by formation of IP_3 and release of Ca^{2+} from intracellular stores followed by a sustained, plateau phase dependent on the influx of extracellular Ca^{2+} (Figure 1).

Release of Ca^{2+} from intracellular stores in airway smooth muscle cells. The initial IP_3-mediated increase in $[Ca^{2+}]_{cyt}$ happens within seconds (Hall 2000) and stimulates a series of Ca^{2+} oscillations (Hirota, Helli et al. 2007, Jude, Wylam et al. 2008) where the newly released Ca^{2+} activates neighboring IP_3Rs and thus propagates a wave. IP_3Rs are inactivated when Ca^{2+} interacts with a second inhibitory site or when SR stores are depleted and Ca^{2+} is removed from

the intracellular space by sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) located on the SR membrane or efflux of Ca^{2+} via sodium (Na^{+})/Ca^{2+} exchangers (NCXs) and plasma membrane Ca^{2+}-ATPases located on the plasma membrane (Perez-Zoghbi, Karner et al. 2009). Ryanodine receptors have also been implicated in contributing to Ca^{2+} release from internal stores and participating in the Ca^{2+} oscillations (An, Bai et al. 2007, Hirota, Helli et al. 2007).

Mechanisms of Ca^{2+} influx in airway smooth muscle cells. An influx of extracellular Ca^{2+} is necessary to maintain the increase in $[Ca^{2+}]_{cyt}$ and for sustained ASMC contraction. Although there is still disagreement as to the predominant mechanism of this Ca^{2+} influx, largely due to differences observed between species or bronchoconstrictor agonists, evidence suggests that there are voltage-independent and voltage-dependent components. Entry of Ca^{2+} through store operated Ca^{2+} channels (SOCCs), likely involving the Ca^{2+} store depletion sensor stromal interaction molecule 1 (STIM1) (Peel, Liu et al. 2006) and the Ca^{2+} release-activated Ca^{2+} channel protein Orai 1 (Peel, Liu et al. 2008), is one voltage-independent mechanism that has been implicated in Ca^{2+} influx in ASMCs. Receptor operated Ca^{2+} channels (ROCCs) have also been indicated as a mechanism of Ca^{2+} influx. Transient receptor potential (TRP) channels have been indicated as both SOCCs and ROCCs (Gosling, Poll et al. 2005, Perez-Zoghbi, Karner et al. 2009, Wang and Zheng 2011). Lastly, the NCX is thought to switch to reverse mode upon an accumulation of Na^{+} in the intracellular space and/or a change in membrane potential in ASMCs to promote Ca^{2+} influx (Hirota, Pertens

et al. 2007). The voltage-dependent element of Ca^{2+} influx is evidenced to occur through L-type VSCCs.

***L-type voltage sensitive Ca^{2+} channels in airway smooth muscle cells*.** L-type VSCC currents have been identified in ASMCs from multiple species (Hirota, Helli et al. 2007), including human (Marthan, Cecile et al. 1989, Worley and Kotlikoff 1990). Their role in *vascular* smooth muscle cell contraction is well understood, however the debate over their importance in ASMC contraction wages on. L-type VSCCs are activated by membrane depolarization. More specifically, the channels activate when membrane voltages reach -45 to -25 millivolts (mV), are maximally active at 0 to +20 mV, and are blocked by dihydropyridines, phenylalkylamines, or inorganic cations (Hirota, Helli et al. 2007). Bronchoconstrictor agonists induce ASMC membrane depolarization (Janssen and Killian 2006, Hirota, Helli et al. 2007, Byron, Brueggemann et al. 2014, Haick, Brueggemann et al. 2017) and in many instances L-type VSCC blockers have been shown to reverse the effects of bronchoconstrictors, such as Ca^{2+} influx (Flores-Soto, Reyes-Garcia et al. 2013) and ASMC contraction (Kamishima, Nelson et al. 1992, Watts, Cox et al. 1994, Dai, Kuo et al. 2007, Hirota and Janssen 2007). Although there is some evidence of a lesser role for L-type VSCCs in Ca^{2+} influx in ASMCs (Murray and Kotlikoff 1991, Janssen 2002, Janssen and Killian 2006), there is clinical evidence that offers support for their role in the regulation of airway diameter.

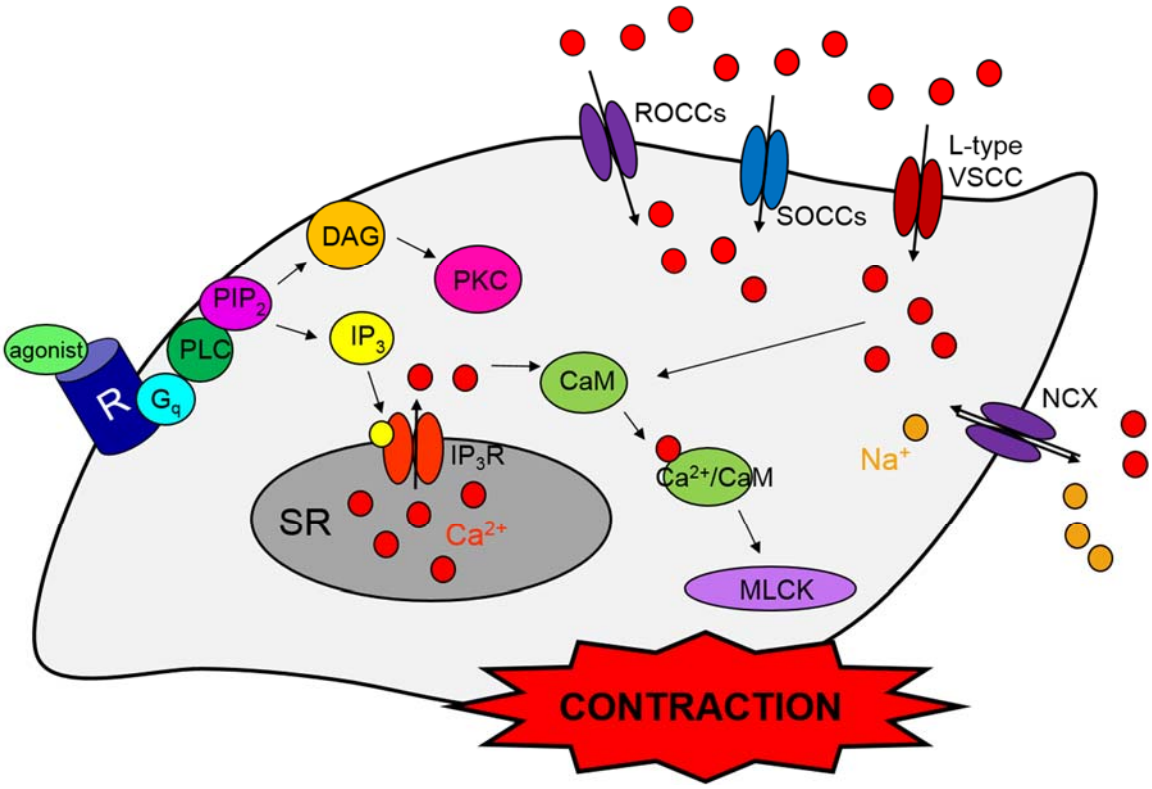

Figure 1. Schematic of G_q-coupled receptor signaling in ASMCs. Activation of G_q-coupled receptors by bronchoconstrictor agonists (such as histamine, acetylcholine, endothelin, leukotriene D4) results in the activation of phospholipase C (PLC), the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 binds to the IP_3 receptor located on the sarcoplasmic reticulum (SR), promoting Ca^{2+} release into the intracellular space. This is the initial transient phase of increased cytosolic Ca^{2+}. Sustained contraction of ASMCs requires an influx of Ca^{2+} from the extracellular space. This occurs through receptor operated Ca^{2+} channels (ROCCs), store operated Ca^{2+} channels (SOCCs), L-type voltage sensitive Ca^{2+} channels (VSCCs) and reversal of the Na^+/Ca^{2+} exchanger. Ca^{2+} binds to calmodulin (CaM), forming the Ca^{2+}/CaM complex and activates myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chains and promotes actin and myosin cross-bridge formation and ASMC contraction.

In the 1980's, the calcium channel blockers (CCBs) nifedipine and verapamil underwent numerous clinical trials for the treatment of asthma with variable amounts of bronchoprotection observed, from no protection to significant

protection. Overall, they have been deemed an ineffective treatment despite the fact that many studies contained at least a subset of asthmatic patients that benefited from CCB treatment (Fish 1984, Barnes 1985, Fanta 1985, Solway and Fanta 1985). Although it is important to acknowledge evidence on both sides of the controversy, there are certain considerations to be made for re-evaluating the potential of L-type VSCCs as therapeutic targets for asthma. Multiple studies have indicated that the effectiveness of CCBs appears to be dependent on bronchoconstrictor agonist concentration or the extent of bronchial provocation. At low concentrations of the bronchoconstrictor methacholine, rat airway constriction was reversed upon treatment with the VSCC blocker verapamil, however this was not the case when airways were exposed to high concentrations of methacholine (Byron, Brueggemann et al. 2014). A similar bronchoconstrictor concentration-dependence was observed for verapamil-induced relaxation of acetylcholine-induced contraction of canine trachealis; verapamil attenuated agonist-induced contraction of the smooth muscle, but only at low acetylcholine doses (Farley and Miles 1978). In human trials, oral nifedipine was only effective at attenuating isocapnic hyperventilation of cold air at lower and intermediate minute ventilation rates (Solway and Fanta 1985). These results suggest that there are different sources of Ca^{2+}, perhaps a voltage-dependent source with submaximal stimulus versus voltage-independent source with maximum stimulus, for ASMC contraction. Other considerations include the fact that cardiovascular side-effects have limited the maximum concentration of CCB that can be used, as has formulation

development (Fish 1984, Barnes 1985, Solway and Fanta 1985). Despite the fact that clinical trials using CCBs were largely deemed unsuccessful, targeting different players in this pathway remains a viable option for the treatment of asthma and should be explored.

K^+ channels in airway smooth muscle. The resting membrane potential of human ASMCs ranges from -45 to -60 mV and oscillates in slow waves, however the oscillations rarely result in the generation of spontaneous action potentials due to the strong outward rectification of K^+ through K^+ channels that promotes membrane hyperpolarization (Thirstrup 2000). Inhibition of the outward K^+ currents results in the induction of action potentials (Marthan, Cecile et al. 1989). One family of K^+ efflux mediators found in ASMCs, and the most important in terms of membrane potential regulation, are voltage-dependent delayed rectifier K^+ (Kv) channels (Kotlikoff 1990, Boyle, Tomasic et al. 1992, Fleischmann, Washabau et al. 1993, Hall 2000, An, Bai et al. 2007, Hirota, Helli et al. 2007). Large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, whose hyperpolarizing currents are involved in β_2-agonist mediated airway relaxation (Kume, Hall et al. 1994), and ATP-sensitive K^+ (K_{ATP}) channels, believed to have little, if any, impact on ASMC contraction or relaxation, are also expressed in ASMCs (Hall 2000, Liu and Xu 2005, Hirota, Helli et al. 2007).

Different subtypes of Kv channels have been found to be expressed in ASMCs. Kv1.2, Kv1.5, and Kv1.1 channels have been detected at the mRNA level in subcultured human trachealis and bronchial myocytes (Adda, Fleischmann et

al. 1996) while Kv7.1, Kv7.4, and Kv7.5 channels have been detected at the mRNA level in freshly isolated human trachealis smooth muscle (Brueggemann, Kakad et al. 2012). Some evidence exists that Kv currents in rat and human ASMCs are inhibited upon activation of PKC (Liu, Xu et al. 2003, Cheng, Xu et al. 2007), and Kv7 currents in freshly isolated guinea pig ASMCs are inhibited following treatment with bronchoconstrictor agonists (Brueggemann, Kakad et al. 2012). However, the specific mechanism(s) behind this current inhibition or how to exploit this knowledge for a therapeutic benefit have yet to be explored.

Asthma.

Pathophysiology of asthma. Asthma is a chronic disease of the airways that affects an estimated 235 million people worldwide (WHO 2013). In the United States specifically, more than 25 million people have asthma, with 7 million of those cases affecting children (CDC 2014). Asthma is characterized by reversible, exaggerated obstruction to airflow that results from a combination of AHR, chronic airway inflammation, increased mucus production, and airway remodeling that ultimately results in the thickening of airway walls (Fanta 2009, Kudo, Ishigatsubo et al. 2013, Olin and Wechsler 2014). To briefly summarize an extensive and complicated aspect of the disease, the chronic airway inflammation results from an increased infiltration of immune cells, such as mast cells, eosinophils, and T cells. These immune cells release mediators, such as cytokines and bronchoconstrictors, that can trigger bronchoconstriction or promote airway

remodeling (Hamid and Tulic 2009). The remodeling is principally an expansion of non-smooth muscle connective tissue and smooth muscle, as well as an increase in the number of mucus glands. The structural changes, such as increased airway smooth muscle mass and fibrosis below the epithelium, associated with airway remodeling are often a consequence of the tissue damage that results from chronic inflammation and inflammatory mediator release (Homer and Elias 2005).

Airway smooth muscle in asthma. Airway smooth muscle is known to have multiple roles in the asthmatic disease state. Hyperplasia and hypertrophy of ASMCs are linked to the increased airway smooth muscle mass associated with airway remodeling (Homer and Elias 2005, Prakash 2013). ASMCs also secrete a variety of factors, including cytokines that affect the inflammatory state of surrounding tissue and molecules that participate in growth and remodeling (Xia, Redhu et al. 2013). Airway smooth muscle is most well known as the contractile effector of the hyperresponsiveness associated with asthma. A hallmark of asthma, AHR is the nonspecific, exaggerated constriction of airway smooth muscle in response to stimuli such as bronchoconstrictor agonists, cold air, or exercise. AHR is attributed to an increase in sensitivity of asthmatic airways, however the extent to which functional, structural, or molecular properties of airway smooth muscle contributes to the increased sensitivity remains unclear (An, Bai et al. 2007, Pascoe, Wang et al. 2012, Lauzon and Martin 2016).

Asthma therapy. ***Airway inflammation.*** One important goal of asthma therapy is to reduce and prevent, or even eliminate, the inflammation associated with the disease. Corticosteroids are the most frequently used therapeutic option to manage airway inflammation. Briefly, the glucocorticoid binds to its receptor in the cytosol and the newly formed complex translocates to the nucleus where it dimerizes with another glucocorticoid-receptor complex, binds to the glucocorticoid response element on the promoter region of specific DNA sequences, and exerts its effects through regulation of gene transcription. Examples of these inflammatory suppressive effects include decreased cytokine and inflammatory mediator production and increased anti-inflammatory mediator production (Umland, Schleimer et al. 2002).

Although corticosteroids are considered to be the most efficacious form of anti-inflammatory therapy for asthma, they do not suppress all inflammatory responses – including leukotriene production. Leukotrienes are inflammatory mediators that participate in the pathogenesis of asthma by acting as chemoattractants, promoting mucus secretion, and inducing airway smooth muscle proliferation. Cysteinyl conjugate leukotrienes also act as potent bronchoconstrictors. Leukotriene modifiers are another class of anti-inflammatory asthma therapeutics that act to either inhibit the synthesis of leukotrienes or antagonize their actions at leukotriene receptors (Salvi, Krishna et al. 2001).

In cases of severe allergic asthma or corticosteroid-resistant asthma, anti-IgE therapy is one of the newer anti-inflammatory approaches to treatment. The

anti-IgE antibodies bind to serum IgE and prevent IgE from binding to its receptor located on effector cells, such as mast cells and basophils, thus averting the cellular response to allergens that manifests in asthmatic symptoms, such as inflammation and allergen-induced bronchoconstriction (D'Amato, Stanziola et al. 2014). Targeting specific inflammatory agents to modify the immunologic responses associated with cytokine cell signaling is one of the new approaches being undertaken to better control asthmatic symptoms. Antibodies against IL-5 and IL-13 are two examples of potential treatment options that have undergone clinical trials with promising results (Olin and Wechsler 2014, Pera and Penn 2016).

Airway relaxation. Another essential goal of asthma therapy is to prevent or attenuate the exaggerated airway constriction associated with the disease. The most common targets for bronchodilator therapy are the G-protein coupled receptors located on airway smooth muscle that are involved in the regulation of its contractile state. Opposing the downstream effects of G_q-coupled agonists by activating the G_s-coupled receptor pathway is one method to promote airway relaxation (Pera and Penn 2016). The β_2-adrenergic receptor has been the primary target of bronchodilator therapy for decades. The initial β_2-agonists exhibited a short duration of action, now referred to as short acting β_2-agonists (SABAs), and are currently used as “rescue” medications for acute asthma attacks. Long acting β_2-agonists (LABAs) were developed with the purpose of extending the duration of action of β_2-agonist therapy and are currently used as “maintenance” therapy

(Cazzola, Page et al. 2013). Other G_s-coupled receptors that relax airway smooth muscle when activated include bitter taste receptors, which promote ASMC relaxation via activation of BK_{Ca} channels, and prostaglandin E2 receptors, which exert their actions via traditional G_s-protein coupled receptor signaling. The development of therapeutic agonists for these receptors, however, has yet to be completed (Dowell, Lavoie et al. 2014).

Antagonizing G_q-coupled muscarinic receptors is another therapeutic approach currently utilized to relax airways, particularly when corticosteroid and LABA therapy is insufficient for symptom management. By competing with acetylcholine for the muscarinic receptors, muscarinic antagonists promote bronchodilation and also have the potential to attenuate acetylcholine-induced inflammation and mucus secretion that contribute to the pathophysiology of asthma (Busse, Dahl et al. 2016, D'Amato, Vitale et al. 2017).

Combination therapy. Seeing as there are multiple components that contribute to the pathology of asthma, such as inflammation and excessive bronchoconstriction, a combination of therapeutic approaches is necessary to manage the disease symptoms. The most commonly prescribed therapy is the combination of a LABA and an inhaled corticosteroid (Cazzola, Page et al. 2013, Ricciardolo, Blasi et al. 2015). There are also new studies that indicate the beneficial effects of combining a LABA with a long-acting muscarinic receptor antagonist (Ricciardolo, Blasi et al. 2015), introducing the possibility of a more advanced combination therapy that may become available in the future.

Need for new therapy. Mainstream asthma therapy has remained relatively constant over the past few decades. Some enhancements have been made to improve the duration or specificity of current treatment options, however the most common courses of action have not changed. Despite the improvements made and the continued research into new asthma therapeutics, it has been reported in some studies that greater than 50% of the asthmatic population have poor control over their symptoms (Peters, Jones et al. 2007, Chapman, Boulet et al. 2008). Inadequately controlled asthma leads to not only a poorer quality of life for the patient but also an increased financial burden on the patient and healthcare system (Busse, Dahl et al. 2016). One cause of the poor symptom management is the tolerance, or tachyphylaxis, that develops upon extended used of β_2-agonists. This manifests as decreased sensitivity to bronchodilator therapy that is believed to occur as a result of desensitization of the β_2-receptor (Cazzola, Page et al. 2013, Pera and Penn 2016). There is also a portion of the asthmatic population that does not benefit from the standard inhaled corticosteroid therapy. Many of these patients attempt to control their asthma with high doses of oral corticosteroid therapy, however the high dose and delivery method make the patients more prone to the negative side effects of treatment (Ricciardolo, Blasi et al. 2015, Pera and Penn 2016).

It is clear that new therapeutic options must be explored to meet the pressing need for additional asthma treatment options. The prevalence of asthma is expected to continue to rise, leading to potentially even more individuals with

poorly managed disease symptoms. The research presented in this dissertation suggests that targeting Kv7 K^+ channels with channel activators may be a novel and efficacious approach to asthma therapy, potentially meeting the currently unmet need.

Kv7 Potassium Channels.

Kv7 K^+ channels were named by the International Union of Pharmacology based on the phylogenetic relationships established for Kv1-Kv9 channels. There are five subtypes, Kv7.1-Kv7.5, whose five gene names, KCNQ1-KCNQ5, were established by the Human Genome Organization Nomenclature Committee (Gutman, Chandy et al. 2005). The Kv7 and KCNQ nomenclature are used interchangeably when discussing the channel protein.

The outwardly rectifying K^+ currents conducted by Kv7 channels have three distinct biophysical properties (Marrion 1997). (1) The threshold of activation of Kv7 current is approximately -60 mV, which is similar to the resting membrane potential of excitable cells such as neurons, (2) the current produced following membrane depolarization is enhanced in a slow and voltage-dependent manner, and (3) the current does not inactivate. These properties make Kv7 channels uniquely qualified to set and maintain the resting membrane potential of neurons and other excitable cells. Additional channel activation that occurs as a result of membrane depolarization opposes additional depolarization while promoting repolarization, therefore opposing prolonged firing of action potentials. Conversely,

if these channels are no longer able to function, either due to mutation or inhibition, the membrane potential would be expected to trend more positive, leading to increased firing of action potentials.

Discovery of Kv7 channels. The Kv7.1 subtype, the first of the Kv7 channel family to be identified, was initially discovered in the heart (Sanguinetti, Curran et al. 1996). Kv7.1 α-subunits form a homotetramer that, with the aid of two auxiliary subunits (see *structure and composition of functional channels* section below), mediate the slow delayed rectifier current I_{KS}, which contributes to the repolarization of the cardiac action potential (Barhanin, Lesage et al. 1996, Sanguinetti, Curran et al. 1996).

"M-currents" were first isolated in frog sympathetic neurons nearly four decades ago (Brown and Adams 1980). This outwardly rectifying K^+ current, named after their suppression following the activation of muscarinic receptors (Kobayashi and Libet 1968, Weight and Votava 1970, Krnjevic, Pumain et al. 1971), is slow to activate, does so in a voltage-sensitive manner, and exhibits little, if any, inactivation during sustained membrane depolarizations. Due to the negative threshold of activation, approximately -60 mV, these currents promote the stabilization of the negative membrane voltage in neurons and act to suppress neuronal excitability (Brown and Adams 1980). It wasn't until the late 1990's that the gene products responsible for producing the M-current were identified. When messenger RNAs encoding human KCNQ2 (Kv7.2) and rat KCNQ3 (Kv7.3) were co-injected into *Xenopus* oocytes, the resulting current resembled that of the M-

current observed in neurons. Similar to endogenous M-currents in sympathetic neurons, these exogenous currents were sensitive to the Kv7 channel blockers linopirdine and XE991, providing additional evidence for the identification of the K^+ channel family behind the M-current (Wang, Pan et al. 1998).

Structure and composition of functional Kv7 channels. Functional Kv7 channels are formed homo- or heterotetramers of α-subunits. Each α-subunit has six transmembrane domains (S1-S6) that contain a voltage sensor domain (S4) and a single pore loop between the S5-S6 domains that acts as a selectivity filter when the four subunits are assembled (Jentsch 2000). Both the amino and carboxy termini of each Kv7 subunit are intracellular. The amino terminus is evidenced to be involved in the localization of Kv7.1 channels to the membrane and it is believed to play a role in the open probability of Kv7.2/Kv7.3 channels (Choveau and Shapiro 2012). More comprehensive studies have taken place examining the different aspects of the long carboxy termini of Kv7 channels, which are comprised of four helical regions, A-D. Helices A and B are primarily recognized for their critical interaction with calmodulin (CaM) (Yus-Najera, Santana-Castro et al. 2002, Haitin and Attali 2008), while the coiled-coil domains, helices C and D, are believed to be responsible for determining the subunit specificity of channel assembly (Schwake, Jentsch et al. 2003, Schwake, Athanasiadu et al. 2006, Haitin and Attali 2008). There are also regions of the C-terminus that are critical for channel trafficking, assembly, cell surface expression, and gating (Haitin and Attali 2008) as well as interactions with scaffolding proteins, such as A-kinase anchoring

proteins (AKAPs) (Hoshi, Zhang et al. 2003, Haitin and Attali 2008). The C-terminus also contains amino acid residues that are phosphorylated by a variety of kinases, such as PKC (Hoshi, Zhang et al. 2003) and Src tyrosine kinase (Gamper, Stockand et al. 2003), that have the potential to modify channel function.

All five Kv7 subtypes are able to form homotetramers, however heteromization is limited to specific combinations. Kv7.1 does not co-assemble with any other channel subtype. Co-expression studies have revealed that Kv7.3 and Kv7.2 can form functional heteromers (Wang, Pan et al. 1998), Kv7.4 heteromizes with Kv7.3 (Kubisch, Schroeder et al. 1999), and Kv7.5 can co-assemble with Kv7.3 (Schroeder, Hechenberger et al. 2000) and Kv7.4 (Bal, Zhang et al. 2008, Brueggemann, Mackie et al. 2014, Chadha, Jepps et al. 2014).

Auxiliary β-subunits are often important determinants of the expression and function of Kv channels (Brueggemann, Gentile et al. 2013). With regard to Kv7 channels, there is considerable evidence that KCNE1 (also called minK) and KCNE2-5 (or minK-related peptides), can associate with and regulate channel expression and function. KCNE1 is the best studied due to its partnership with Kv7.1 to mediate I_{KS} in cardiac myocytes. Two KCNE1 subunits are thought to associate with a homotetramer of Kv7.1 α-subunits to form functional channels that give rise to the slowly activating I_{Ks} current (Morin and Kobertz 2008). The presence of KCNE1 is responsible for the slow channel activation (Sanguinetti, Curran et al. 1996) and it also increases channel conductance (Pusch 1998). There is limited information on the roles of natively expressed KCNE subunits in

modulating Kv7 channel expression and function in tissues other than the heart, but KCNE subtypes have been found to modulate the function of Kv7 channel subtypes in expression systems. For example, KCNE2 decreases Kv7.1 current density (Tinel, Diochot et al. 2000) and KCNE3 greatly enhances Kv7.1 current density (Bendahhou, Marionneau et al. 2005), while KCNE4 and 5 both suppress Kv7.1 currents (Angelo, Jespersen et al. 2002, Grunnet, Jespersen et al. 2002, Grunnet, Olesen et al. 2005). KCNE1 enhances Kv7.5 currents while slowing their activation (Roura-Ferrer, Etxebarria et al. 2009), KCNE2 accelerates the activation and deactivation kinetics of Kv7.2/3 currents (Tinel, Diochot et al. 2000), KCNE3 suppresses Kv7.4 (Schroeder, Hechenberger et al. 2000) and Kv7.5 (Roura-Ferrer, Etxebarria et al. 2009) currents, whereas KCNE4 increases Kv7.4 current amplitude (Strutz-Seebohm, Seebohm et al. 2006, Jepps, Carr et al. 2015).

Regulation of Kv7 channels. ***Transcriptional regulation***. Although the functional importance of Kv7 channels in a variety of tissues is acknowledged and continues to be an area of intense study, little is known about the mechanisms governing transcriptional regulation of the channels. Expression of KCNQ2 and KCNQ3 genes is activated by the specialty factor 1 (sp1) in dorsal root ganglia (DRG) neurons (Mucha, Ooi et al. 2010), however little else is known in regards to transcriptional activation of KCNQ genes. A slightly more widespread research effort has been put into examining repression of KCNQ gene expression by repressor element 1-silencing transcription factor (REST), a transcriptional repressor whose levels are increased in response to inflammation. Functional

binding sites for REST are present in KCNQ2, KCNQ3, KCNQ5 (Mucha, Ooi et al. 2010), and KCNQ4 (Iannotti, Barrese et al. 2013) genes. Treatment of DRG neurons with inflammatory mediators (Mucha, Ooi et al. 2010) and a neuropathic injury rat model (Rose, Ooi et al. 2011) demonstrated an increase in REST expression that correlated with a decrease in Kv7.2 expression. These observations have led to the hypothesis that REST-induced Kv7.2 downregulation may be a mechanism that contributes to neuronal over-excitability associated with neuropathic pain.

Receptor mediated Kv7 current suppression. The suppression of Kv7 currents following activation of a variety of G_q-coupled receptors has been widely studied ever since the first instance of M-current inhibition was observed in 1980 (Brown and Adams 1980). Stimulation of G_q-coupled receptors found in neurons (Marrion 1997, Delmas and Brown 2005), vascular smooth muscle cells (Mackie, Brueggemann et al. 2008, Mani, O'Dowd et al. 2013), and ASMCs (Brueggemann, Kakad et al. 2012, Evseev, Semenov et al. 2013, Brueggemann, Haick et al. 2014) results in Kv7 current inhibition.

PIP_2 mediated regulation. One consequence of G_q-coupled receptor activation is the activation of PLC and subsequent hydrolysis of PIP_2 to IP_3 and DAG. Once it was established that PIP_2 depletion was the mechanism for M-current inhibition following muscarinic receptor activation (Suh and Hille 2002), numerous studies have examined the necessity of PIP_2 for Kv7 channels to remain open (Zhang, Craciun et al. 2003, Delmas and Brown 2005, Suh and Hille 2008),

including investigations of the correlation between a channel's affinity for PIP_2 being related to its open probability (Li, Gamper et al. 2005).

Ca^{2+}/CaM mediated regulation. Receptor-mediated inhibition of Kv7 currents can also be mediated by Ca^{2+}/CaM, as is the case for M-current suppression by the hormone bradykinin. The mechanism of M-current inhibition by bradykinin differs from that of muscarinic receptor-mediated M-current inhibition in that it is dependent on IP_3-mediated Ca^{2+} release from intracellular stores (Cruzblanca, Koh et al. 1998). Ca^{2+} was found to inhibit M-currents with an IC_{50} of approximately 100 nM (Selyanko and Brown 1996), a sensitivity that is dependent on the presence of CaM acting as a Ca^{2+} sensor (Gamper and Shapiro 2003).

Phosphorylation mediated regulation. Another consequence of G_q-coupled receptor activation is the activation of the serine/threonine kinase PKC. Past experiments investigating the inhibition of M-currents by phorbol esters, which directly activate PKC, and PKC inhibitors have yielded variable results (Marrion 1997). The study completed by Hoshi *et al.* (2003) was pivotal in terms of beginning to clarify the role of PKC in Kv7 current regulation. AKAP interacts with PKC and Kv7 channels, among other proteins, to form a signalosome and facilitate signal transduction. PKC-induced phosphorylation of Kv7.2 channels was attenuated upon expression of a mutated AKAP that was unable to bind PKC (Hoshi, Zhang et al. 2003). Since then, AKAP has been deemed necessary for M-current inhibition following muscarinic receptor stimulation in mice (Tunquist, Hoshi et al. 2008) and has been shown to co-localize with Kv7.2/Kv7.3 heteromers, as well as

all Kv7 subtypes save for Kv7.1. The interaction between AKAP and Kv7 channels acts to “sensitize” the channels to muscarinic inhibition (Bal, Zhang et al. 2010). The role of PKC in Kv7 current regulation is also evident in vascular smooth muscle. The suppression of native Kv7 currents following treatment with the G_q-coupled receptor agonist arginine^8-vasopressin (AVP) was found to be dependent on the activation of PKC in cultured A7r5 rat aortic smooth muscle cells, that express Kv7.5 homomeric channels, and freshly isolated rat mesenteric artery smooth muscle cells, that express Kv7.4/Kv7.5 heteromeric channels (Brueggemann, Moran et al. 2007, Mackie, Brueggemann et al. 2008, Brueggemann, Mackie et al. 2014). Treatment of A7r5 cells with AVP resulted in a PKC-dependent increase in channel phosphorylation of overexpressed Kv7.5, but not Kv7.4, channels (Brueggemann, Mackie et al. 2014).

Members of the Src family of non-receptor tyrosine kinases are known to phosphorylate Kv7 channels, resulting in Kv7 current inhibition. Src phosphorylates Kv7.3, Kv7.4, and Kv7.5 homomeric channels, but not Kv7.1 or Kv7.2 homomers. This phosphorylation correlates with subunit-specific current inhibition and slowing of channel activation kinetics (Gamper, Stockand et al. 2003). Native M-currents, produced by Kv7.2/Kv7.3 heterotetramers, were also suppressed by Src overexpression in rat sympathetic neurons (Gamper, Stockand et al. 2003), implying the functional importance of Kv7.3 phosphorylation by Src. Tyrosine residues 67 and 349, located on the amino and carboxy termini of Kv7.3, respectively, were later found to be necessary for M-current modulation by Src (Li,

Langlais et al. 2004). The physiological relevance of this channel regulation by Src remains to be determined.

PKA is yet another kinase that has been implicated in the regulation of Kv7 currents. Activation of the β-adrenergic receptor/cAMP/PKA pathway increased Kv7.1 current amplitude in cardiomyocytes (Chen and Kass 2011). Kv7.2/Kv7.3 heteromeric currents were enhanced by PKA and Kv7.2 serine 52 was phosphorylated in a PKA dependent manner in overexpression systems, (Schroeder, Kubisch et al. 1998) however no association to endogenous M-current regulation has been established. Application of the PKA catalytic subunit enhanced Kv7.4 currents, (Chambard and Ashmore 2005) although a link between the PKA-dependent regulation of Kv7.4 channels and signaling events that occur in cochlear outer hair cells have yet to be established. The G_s-coupled receptor agonist isoproterenol enhanced Kv7.5 homomeric currents, both endogenous and exogenous, in a PKA-dependent manner in A7r5 rat aortic smooth muscle cells. Isoproterenol enhanced exogenous Kv7.4/Kv7.5 heteromeric currents to a lesser extent and had no effect on exogenous Kv7.4 homomeric currents (Mani, Robakowski et al. 2016). Additional research examining the physiological relevance of PKA-induced enhancement of Kv7 currents is necessary when considering the possibility of exploiting this mechanism for therapeutic benefit in a variety of tissues.

Pharmacology of Kv7 channels. The presence and function of Kv7 channels in various cell and tissue types has been elucidated primarily through the

development of pharmacological agents that selectively block or activate these channels. Linopirdine (1,3-Dihydro-1-phenyl-3,3-bis(4-pyridinylmethyl)-2*H*-indol-2-one) was initially observed to be an enhancer of depolarization-induced neurotransmitter release and ultimately found to do so via its inhibitory actions on M-currents (Aiken, Lampe et al. 1995). Both linopirdine, and it's more potent analog XE991 (10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone), are the most widely used experimental Kv7 channel blockers, effectively block all five Kv7 subtypes, and are largely specific for Kv7 channels at concentrations up to 10 μM (Schnee and Brown 1998, Wang, Pan et al. 1998, Wang, Brown et al. 2000, Wladyka and Kunze 2006). Chromanol 293B (*trans*-N-[6-Cyano-3,4-dihydro-3-hydroxy-2,2-dimethyl-2H-1-benzopyran-4-yl]-N-methyl-ethanesulfonamide) (Busch, Suessbrich et al. 1996, Bleich, Briel et al. 1997) and HMR-1556 (N-[3-Hydroxy-2,2-dimethyl-6-(4,4,4-trifluoro-butoxy)-chroman-4-yl]-N-methyl-methanesulfonamide) (Thomas, Gerlach et al. 2003) are reported to be specific blockers of the Kv7.1 channel subtype.

The Kv7 channel pharmacological toolbox also contains a variety of channel activators. Flupirtine ([2-Amino-6-[[(4-fluorophenyl)methyl]amino]-3-pyridinyl]carbamic acid ethyl ester) and retigabine (N-(2-Amino-4-(4-fluorobenzylamino)phenyl)carbamic acid ethyl ester) are the most frequently used Kv7 channel activators. These drugs activate only Kv7.2-Kv7.5 channels; the Kv7.1 subtype lacks a critical tryptophan residue required for activation by either compound (Tatulian, Delmas et al. 2001, Schenzer, Friedrich et al. 2005). L-

364373 (also known as R-L3; 5-(2-Fluorophenyl)-1,3-dihydro-3-(1*H*-indol-3-ylmethyl)-1-methyl-2*H*-1,4-benzodiazepin-2-one) is reported to activate only Kv7.1 channels (Salata, Jurkiewicz et al. 1998, Seebohm, Pusch et al. 2003). Other Kv7 activators include ICA-27243 (N-(6-chloropyridin-3-yl)-3,4-difluorobenzamide), a more effective activator of Kv7.2/Kv7.3 heterotetramers compared to Kv7.1, Kv7.3 or Kv7.4 homotetramers and Kv7.3/Kv7.5 heterotetramers (Wickenden, Krajewski et al. 2008, Padilla, Wickenden et al. 2009), and its analog, ICA-069673 (*N*-(2-Chloro-5-pyrimidinyl)-3,4-difluorobenzamide), an activator that enhanced Kv7.4 homotetramer currents to a greater extent than Kv7.5 homotetramer currents (Brueggemann, Haick et al. 2014). Diclofenac (2-[2-(2,6-dichloroanilino)phenyl]acetic acid) is unique in that it was found to enhance currents from Kv7.4 homotetramers but block currents from Kv7.5 homotetramers, with only moderate inhibition of currents Kv7.4/Kv7.5 heterotetramers (Brueggemann, Mackie et al. 2011).

Expression of Kv7 channels. Cardiomyocytes express Kv7.1 homotetramers that, with the help of KCNE1 β-subunits, mediate I_{KS} (Barhanin, Lesage et al. 1996, Sanguinetti, Curran et al. 1996). Mutations in Kv7.1 often result in a cardiac condition called long QT syndrome where delayed repolarization of the cardiac action potential can cause potentially fatal cardiac arrhythmias (Chiang and Roden 2000). In neurons, Kv7.2 and Kv7.3 are the predominant Kv7 subtypes expressed and they form heterotetramers responsible for the M-current, although expression of Kv7.5 has also been confirmed (Jentsch 2000). Mutations in Kv7.2

and Kv7.3 channels cause a reduction of M-current and result in a neonatal form of epilepsy called benign familial neonatal convulsions (BFNC) (Biervert, Schroeder et al. 1998, Singh, Charlier et al. 1998, Jentsch 2000). Kv7.4 channels mediate I_{KN}, a Kv current that opposes the excitability of the specialized sensory neurons in the sensory outer hair cells of the cochlea. Mutations in Kv7.4 result in an autosomal dominant progressive deafness that results from eventual degradation of the sensory hair cells (Kubisch, Schroeder et al. 1999, Kharkovets, Hardelin et al. 2000). Multiple Kv7 subtypes are expressed in skeletal muscle cells (Iannotti, Panza et al. 2010) and Kv7.5 in particular has been implicated in the regulation of the proliferation and differentiation of myoblasts (Roura-Ferrer, Sole et al. 2008). Functional Kv7 channels have also been discovered in a wide variety of smooth muscle tissues (see *Kv7 channels in smooth muscle* and *Kv7 channels in airway smooth muscle* sections below).

Kv7 channels in smooth muscle. Kv7 channels are expressed in a variety of different types of smooth muscle (Greenwood and Ohya 2009, Jepps, Olesen et al. 2013, Stott, Jepps et al. 2014, Haick and Byron 2016). Smooth muscle located in the gastrointestinal tract (Ohya, Asakura et al. 2002, Jepps, Greenwood et al. 2009, Ipavec, Martire et al. 2011, Adduci, Martire et al. 2013), bladder (Rode, Svalo et al. 2010, Svalo, Hansen et al. 2012, Afeli, Malysz et al. 2013, Anderson, Carson et al. 2013, Svalo, Bille et al. 2013, Provence, Hristov et al. 2015, Svalo, Sheykhzade et al. 2015), and myometrium (Aaronson, Sarwar et al. 2006, McCallum, Greenwood et al. 2009, McCallum, Pierce et al. 2011) of multiple

species express Kv7 channels that regulate the contractile state of the smooth muscle they are located in; treatment of tissue preparations with Kv7 channel inhibitors induces contraction of visceral smooth muscle while treatment with Kv7 channel activators relaxes visceral smooth muscle.

The expression of Kv7 channels has also been observed in a variety of vascular smooth muscle beds in mice (Ohya, Asakura et al. 2002, Ohya, Sergeant et al. 2003, Yeung, Pucovsky et al. 2007, Yeung, Schwake et al. 2008, Lee, Yang et al. 2015), rats (Mackie, Brueggemann et al. 2008, Joshi, Sedivy et al. 2009, Zhong, Harhun et al. 2010, Mani, Brueggemann et al. 2011, Chadha, Zunke et al. 2012, Khanamiri, Soltysinska et al. 2013, Zavaritskaya, Zhuravleva et al. 2013, Jepps, Carr et al. 2015, Morales-Cano, Moreno et al. 2015), pigs (Hedegaard, Nielsen et al. 2014), and humans (Ng, Davis et al. 2011), with many accompanying functional studies that emphasize the importance of Kv7 channels in the regulation vascular tone in these different vessels. Studies cited above emphasize that the regulation of vascular tone occurs due to the role of Kv7 channels in regulating the membrane potential of vascular smooth muscle cells; inhibition of Kv7 channels depolarizes vascular smooth muscle cell membranes and initiates contraction of vascular smooth muscle (Mackie, Brueggemann et al. 2008).

Kv7 channels in airway smooth muscle. Kv7 channels were recently found to be expressed in airway smooth muscle. Guinea pig ASMCs express primarily Kv7.2, Kv7.4 and Kv7.5, (Brueggemann, Kakad et al. 2012), whereas human, mouse and rat airways express Kv7.1, Kv7.4 and Kv7.5, with little to no

expression of Kv7.2 and Kv7.3 (Brueggemann, Kakad et al. 2012, Evseev, Semenov et al. 2013, Brueggemann, Haick et al. 2014). The importance of Kv7 channels as determinants of airway smooth muscle excitability has been emphasized by the electrophysiological and functional studies that have been completed in these tissues. Carbachol, methacholine, and histamine, G_q-coupled receptor agonists, inhibited Kv7 currents in freshly isolated airway myocytes from mouse, rat and guinea pig, respectively (Brueggemann, Kakad et al. 2012, Evseev, Semenov et al. 2013, Brueggemann, Haick et al. 2014). Activation of Kv7 channels was found to reverse airway constriction in rat (Brueggemann, Haick et al. 2014) and human precision cut lung slices (Brueggemann, Kakad et al. 2012), as well as contraction of mouse and rat tracheal rings (Evseev, Semenov et al. 2013). Evidence also suggests that nebulized retigabine may act as a bronchodilator *in vivo.* Evseev and colleagues found that retigabine attenuated the methacholine-induced increase in enhanced pause (an indirect measurement of airway resistance (Lomask 2006, Frazer, Reynolds et al. 2011)), in mice (Evseev, Semenov et al. 2013).

Targeting Kv7 channels in smooth muscle as a therapeutic strategy. The effects of modulating Kv7 channel activity in the smooth muscle beds listed above suggest that Kv7 channels may be novel therapeutic targets for a variety of conditions. Fortunately there are some Kv7 channel activators that are currently in clinical use for other applications. Flupirtine, while only clinically available as an analgesic in Europe, has been granted permission to be used in clinical trials in

the United States for the treatment of fibromyalgia. Retigabine has been clinically available in the United States as an adjuvant treatment for epilepsy in adults since mid-2011 (Harris and Murphy 2011) and although celecoxib's current clinical use is limited to anti-inflammatory pain relief (McCormack 2011), it has been shown to enhance Kv7 currents in vascular and airway smooth muscle cells (Brueggemann, Mackie et al. 2009, Mani, Brueggemann et al. 2011, Brueggemann, Kakad et al. 2012). Repurposing these drugs for the treatment of smooth muscle disorders is an attractive prospect and may be applicable for a number of diseases.

Targeting Kv7 channels in the gastrointestinal tract may be a novel approach to treat functional dyspepsia or irritable bowel syndrome, both conditions associated with gastrointestinal mobility (Leake 2013, Soares 2014). Relaxing the smooth muscle found in the bladder with Kv7 channel activators may help to relieve symptoms associated with overactive bladder. Interestingly, and in support of this theory, urinary retention is one side effect associated with taking retigabine for the treatment of epilepsy (Brickel, Gandhi et al. 2012). Lastly, suppressing myometrial contraction is one therapeutic approach currently used to delay preterm labor. (Haas, Caldwell et al. 2012). Activation of the Kv7 channels in human myometrial smooth muscle at full term attenuated spontaneous myometrial contractions (McCallum, Pierce et al. 2011), suggesting that Kv7 channel activators may also represent a novel therapeutic approach to treat preterm labor. The ability of Kv7 channel activators to relax vascular smooth muscle and Kv7 channel blockers to constrict vascular smooth muscle has made targeting Kv7

channels located in the vasculature a rapidly expanding area of interest (Mackie and Byron 2008, Jepps, Olesen et al. 2013, Stott, Jepps et al. 2014). These channels may be considered novel targets for the treatment of a variety of vascular disorders, including hypertension, pulmonary arterial hypertension, preeclampsia, cerebral vasospasm and subarachnoid hemorrhage, coronary artery disease, and hypotension (Haick and Byron 2016).

As detailed previously, asthma is a disease that is partially characterized by reversible airway obstruction associated with exaggerated ASMC contraction and airway narrowing in response to bronchoconstrictor stimuli. Although there are a variety of treatments available, a surprisingly large portion of the asthmatic population has poor control over their disease (Peters, Jones et al. 2007, Chapman, Boulet et al. 2008). Despite the extensive efforts of researchers to elucidate the role of airway smooth muscle in the pathophysiology of asthma and identify new targets to treat the disease, no new pharmacological agents directed towards targets in ASMCs have become available in recent years. Those that have are often associated with unwanted side effects or poor selectivity for their targets (Janssen 2009, Siddiqui, Redhu et al. 2013, Dowell, Lavoie et al. 2014). Kv7 channels are expressed in ASMCs across multiple species, their currents are inhibited by bronchoconstrictor agonists, and activation of Kv7 channels relaxes airways (Brueggemann, Kakad et al. 2012, Evseev, Semenov et al. 2013, Brueggemann, Haick et al. 2014). The specific mechanism(s) responsible for bronchoconstrictor agonist-induced inhibition of Kv7 channels are undefined and

the potential of Kv7 channel activators to act as a novel treatment option for asthma have yet to be fully explored, however the work in this dissertation aims to address these knowledge gaps.

CHAPTER THREE

HYPOTHESIS AND AIMS

Main hypothesis: This project aims to test the hypothesis that Kv7 K^+ channels in airway smooth muscle are regulated by the bronchoconstrictor agonist histamine in a PKC-dependent manner and that targeting these channels with clinically available Kv7 channel activators represents a novel strategy for bronchodilatory asthma therapy.

Specific Aim 1: To elucidate the mechanism by which the bronchoconstrictor agonist histamine regulates Kv7 channels in airway smooth muscle.

Previous studies have revealed that Kv7 currents in guinea pig ASMCs are inhibited upon treatment with the G_q–coupled receptor bronchoconstrictor agonists histamine and methacholine (Brueggemann, Kakad et al. 2012). Silencing Kv7.5 expression in freshly isolated rat ASMCs abolished detectable Kv7 currents, suggesting the functional importance of this particular subtype in mediating the Kv7 currents in airway smooth muscle (data not shown). Treatment of freshly isolated human trachealis smooth muscle with histamine or the PKC activator, phorbol 12-myristate 13-acetate (PMA), resulted in the increased presence of endogenous Kv7.5 channels in immunoprecipitates isolated using an antibody that recognizes phosphorylated serine residues that fall within the PKC consensus motif (Haick, Brueggemann et al. 2017), further highlighting the potential

significance of this particular channel subtype. In *vascular* smooth muscle cells, G_q-coupled receptor stimulation results in PKC-dependent phosphorylation of Kv7.5 channels and inhibition of Kv7.5 currents (Brueggemann, Moran et al. 2007, Mackie, Brueggemann et al. 2008, Brueggemann, Mackie et al. 2014). We hypothesize that the G_q-coupled receptor agonist histamine phosphorylates Kv7.5 channels and inhibits Kv7.5 currents in a PKC-dependent manner in *airway* smooth muscle as well.

ASMCs were isolated from human trachealis smooth muscle and cultured at low passages (HTSMCs). Wild type (WT) or mutant FLAG-tagged human Kv7.5 (hKv7.5) channels were overexpressed using an adenoviral vector. Immunoprecipitation and western blotting approaches were used to examine the phosphorylation status of WT and mutant hKv7.5 channels and whole cell perforated patch clamp electrophysiology was used to measure WT and mutant hKv7.5 currents in response to treatment with histamine or direct activation of PKC with PMA.

Specific Aim 2: To determine the efficacy of Kv7 channel activators in attenuating agonist-induced bronchoconstriction and explore their therapeutic potential for the treatment of asthma.

In earlier studies, the Kv7 channel activator flupirtine attenuated histamine-induced constriction of human airways and the Kv7 channel inhibitor XE991 robustly constricted human airways (Brueggemann, Kakad et al. 2012). Precision

cut lung slices (PCLS) prepared from Sprague-Dawley rats and discarded human donor tissue were used to test the ability of the Kv7 channel activator retigabine to attenuate agonist-induced airway constriction. PCLS prepared from tissue of a guinea pig ovalbumin (OVA)-sensitization model of allergic asthma was used to compare the ability of retigabine to reverse bronchoconstriction in normal and asthmatic tissues. The ability of retigabine to alleviate the symptoms observed in sensitized animals upon exposure to OVA was tested *in vivo*, as was the ability of retigabine to attenuate bronchoconstrictor agonist-induced increases in respiratory resistance.

CHAPTER FOUR

MATERIALS AND METHODS

Cell Culture.

HTSMCs were isolated as described previously (Janssen and Sims 1992, Brueggemann, Kakad et al. 2012) from de-identified donor tissues (approved/exempted by the Loyola University Chicago Institutional Review Board for the Protection of Human Subjects). Briefly, trachealis muscles were dissected in ice-cold dissecting solution, containing (in mM): 145 NaCl, 4.7 KCl, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 2 $CaCl_2$_$2H_2O$, 2 pyruvic acid, 0.02 EDTA dihydrate, 3 MOPS, 5 D-glucose, and 1% fatty acid-free BSA (pH adjusted to 7.4 with NaOH on ice), cut into approximately 2 mm diameter strips, and immediately transferred to ice-cold physiological saline solution (PSS), containing (in mM): 140 NaCl, 5.36 KCl, 0.34 Na_2HPO_4, 0.44 KH_2PO_4, 1.2 $MgCl_2$, 0.05 $CaCl_2$, 10 HEPES, and 10 D-glucose (pH adjusted to 7.2 with NaOH on ice, 298 mOsm/l). Strips were then transferred into 37°C PSS (pH adjusted to 7.2 with NaOH at 37°C) supplemented with BSA (1 mg/ml; fraction V, Roche Diagnostics USA), collagenase Type VIII (950 units/ml; Sigma-Aldrich), papain (38 units/ml; Worthington), and DL-dithiothreitol (1 mM; Sigma-Aldrich) for 45 min. Following enzymatic digestion, the tissue strips were washed three to five times with ice-cold PSS and gently triturated to release individual myocytes. Myocytes were cultured at low passages (2-9) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine

serum (Atlanta Biologicals), antibiotics and antimycotics (Sigma Aldrich) at 37°C under 5% CO_2.

Adenoviral infection. For overexpression of WT or mutant (S441A or T505A) human Kv7.5 (hKv7.5) channels, HTSMCs were infected at confluence with an adenoviral expression vector (Adv-hKCNQ5-FLAG-WT, Adv-hKCNQ5-FLAG-S441A, or Adv-hKCNQ5-FLAG-T505A) at a multiplicity of infection (MOI) of 100 for a minimum of 6 days prior to experimentation. Expression was confirmed by detection of green fluorescent protein (GFP) fluorescence (GFP is co-expressed via the same hKCNQ5-FLAG adenoviral vector) and by western blot analysis.

Retroviral infection. For knockdown of PKCα expression, a retroviral vector driving expression of a PKCα short hairpin RNA (shRNA) was employed essentially as described previously (Denning, Wang et al. 2002). HTSMCs were plated in 6 well plates at 10^5 cells per well. Approximately 24 hr later, shControl or shPKCα retroviral supernatants supplemented with 4 μg/ml hexadimethrine bromide (Sigma-Aldrich) were added to the appropriate wells and plates were spun at 300 x g for 1 hr at 32°C. Viral supernatants were immediately replaced with fresh culture medium. For some experiments this process was repeated after approximately 6 hr to assure adequate infection. The following day, cells were washed twice with phosphate-buffered saline and then incubated with media containing 0.5 μg/ml puromycin dihydrochloride (EMD Millipore) for selection

purposes. Effective knockdown of shPKCα expression was confirmed by western blot analysis.

Constructs used for infections. An adenoviral vector to express human KCNQ5-WT (Adv-hKCNQ5-FLAG-WT, with a FLAG epitope on the amino terminus), human KCNQ5-S441A (Adv-hKCNQ5-FLAG-S441A, with a FLAG epitope on the amino terminus), or human KCNQ5-T505A (Adv-hKCNQ5-FLAG-T505A, with a FLAG epitope on the amino terminus) were created previously using the AdEasy Adenoviral Vector System (Stratagene). Retroviral vectors to express shRNA targeting human PKCα (shPKCα, AGGCUGAGGUUGCUGAUGA) or shRNA targeting a sequence not found in the human genome (shControl, GCGCGCTTTGTAGGATTCG) were created previously using the pSUPER.retro.puro vector system (OligoEngine) (Jerome-Morais, Rahn et al. 2009).

Electrophysiology.

Whole-cell membrane currents in single HTSMCs overexpressing hKv7.5 channels (infected with Adv-hKCNQ5-FLAG WT, S441A or T505A for a minimum of 6 days) were measured using perforated patch configurations under voltage-clamp conditions. Cells were trypsinized and allowed to attach to glass coverslips for 15-20 min at 37°C. Experiments were performed at room temperature while being continuously perfused with an external bath solution containing (in mM): 5 KCl, 130 NaCl, 10 HEPES, 2 $CaCl_2$, 1.2 $MgCl_2$, 5 D-glucose (pH 7.3, 298 mOsm).

Standard internal (pipette) solution contained (in mM): 110 potassium gluconate, 30 KCl, 5 HEPES, 1 K_2EGTA (pH 7.2, 298 mOsm) and was supplemented with 120 μg/ml Amphotericin B immediately prior to experimentation for patch perforation.

Voltage-clamp command voltages were generated using an Axopatch 200B amplifier (Molecular Devices) under control of pCLAMP 10 (Molecular Devices). Series resistances after amphotericin perforation were 20-35 MΩ and were compensated by 40%. Whole-cell currents were digitized at 2 kHz and filtered at 1 kHz.

Exogenous hKv7.5 currents were recorded using a 5 sec voltage step protocol from a -74mV holding potential to test voltages ranging from -124 mV to -4 mV, followed by a 1 sec step to -120 mV. The currents recorded during the last 1 sec of recording time for each voltage step were averaged and normalized by cell capacitance to obtain end-pulse steady-state hKv7.5 currents. The time course of drug application was recorded with repetitive 5 sec steps from -74 to -20 mV every 5 sec.

Immunoprecipitation and Western Blot Analysis.

HTSMCs were infected with Adv-hKCNQ5-FLAG-WT or Adv-hKCNQ5-FLAG-S441A at 100 MOI for 6 days and serum deprived overnight prior to experimentation. On the day of the experiment, HTSMCs were incubated for 3 hr at room temperature in modified Kreb's buffer (mKreb's) containing (in mM): 5.9

KCl, 135 NaCl, 11.6 HEPES, 1.5 $CaCl_2$, 1.2 $MgCl_2$, 11.5 glucose (pH 7.3) prior to treatment. Immediately following treatment, cells were lysed with ice-cold lysis buffer containing 100 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 30 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 20 mM HEPES (pH 7.4) and supplemented with 10 µg/ml leupeptin (Bachem), 0.5 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich) or Pefabloc (Acros Organics), 10 µg/ml aprotinin (Sigma-Aldrich) and 500 µM Na_3VO_4. Equal protein concentrations of cell lysates (350-500 µg) were incubated overnight at 4°C with 4 µg of anti-FLAG antibody (Sigma-Aldrich, F7425). 20 µl of Dynabeads® Protein G (Invitrogen, Life Technologies) were added to antibody-containing lysates and incubated for 2 hr at 4°C. The beads were washed and immune complexes were eluted with 1X Laemmli sample buffer (5% SDS, 10% glycerol, 50 mM Tris, pH 6.8, 0.01% bromophenol blue, 10% β-mercaptoethanol, 100 mM dithiothreitol), heated for 5 min at 97°C and separated by SDS-PAGE on 10% acrylamide gels. Proteins were transferred to polyvinylidene fluoride membranes, blocked in 5% nonfat milk, and probed with anti-phosphoserine PKC substrate antibody (rabbit polyclonal 1:1000, Cell Signaling Technology, 2261) overnight at 4°C. Membranes were washed and then probed with anti-rabbit IgG HRP-conjugated secondary antibody (1:10,000, Thermo Scientific, 31460) for 1 hr at room temperature. For immunoprecipitation (IP) loading controls, 25 µg of protein from lysates for each IP sample were run on separate 10% acrylamide gels or original membranes were quenched with 1%

sodium azide and then re-probed with anti-FLAG M2 peroxidase conjugate (mouse monoclonal 1:10,000, Sigma-Aldrich, A8592) for 1 hr at room temperature.

To confirm PKCα knockdown, 25 μg of protein from lysates for each experiment were run on separate 10% acrylamide gels, probed with anti-PKCα (mouse monoclonal 1:1000, BD Biosciences, 610108) overnight at 4°C, washed, and then probed with anti-mouse IgG HRP-conjugated secondary antibody (1:10,000, Thermo Scientific, 31430) for 1 hr at room temperature. For loading controls, membranes were incubated with anti-GAPDH (mouse monoclonal 1:1000, Novus Biologicals, NB300-221) for 1 hr at room temperature, washed, and then probed with anti-mouse IgG HRP-conjugated secondary antibody for 1 hr at room temperature.

Western blot results were visualized using enhanced chemiluminescence (Optiblot ECL Detect Kit, abcam or Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare Life Sciences). ImageJ software was used for densitometry analysis.

$[Ca^{2+}]_{cyt}$ Measurements with Fura-2.

Confluent monolayers of cultured HTSMCs in six-well plates were serum deprived overnight prior to experimentation. On the day of experiment, cells were washed twice with mKreb's buffer at room temperature and background fluorescence was recorded using a Biotek Synergy HT plate reader (340 and 380 nm excitation, 510 nm emission) and Gen5 software (BioTek Instruments,

Winooski, VT). Cells were then incubated in mKreb's plus 1μM fura-2 acetoxymethyl ester (Invitrogen), 0.1% bovine serum albumin, and 0.02% Pluronic F127 detergent for 90 min at room temperature in the dark. After this fura-2 loading period, cells were washed twice with mKreb's and incubated for an additional 60 min in mKreb's at room temperature in the dark. Changes in $[Ca^{2+}]_{cyt}$ in response to treatments were estimated based on fura-2 fluorescence measured at 510 nm with alternating excitation at 340 and 380 nm wavelengths. Background values were subtracted from each wavelength prior to calculating the 340/380 fluorescence ratio. Values were normalized to the average fluorescence ratio recorded during the 2-min period prior to treatment. Sustained elevation of $[Ca^{2+}]_{cyt}$ in response to treatment was evaluated based on the average fluorescence ratio recorded during the 5-min period beginning 10 min after initiation of treatment. Each "n" is the average of 3-6 wells.

Immunofluorescence.

Freshly isolated HTSMCs and HTSMC at passages 2, 4, 6, and 8 were plated on 12 mm coverslips 1 day prior to fixing. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline and permeabilized with 0.5% Triton X 100 in phosphate-buffered saline. Coverslips were incubated in Image-iT signal enhancer (Thermo Fischer Scientific) prior to blocking. A coverslip from each passage was incubated with polyclonal goat anti-SM22α (abcam, ab10135) or polyclonal rabbit anti-KCNQ5 (abcam, ab96707) antibodies at 1:100 dilution at

4°C overnight. Alexa Fluor® 488 rabbit anti-goat IgG or Alexa Fluor® 594 goat anti-rabbit at 1:400 dilution were used as secondary antibodies. Each coverslip was treated with SlowFade Antifade Equilibration Buffer (Molecular Probes, Eugene, OR) and ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific) prior to mounting on a glass slide. Cell images were acquired with an Olympus 1X71 inverted epifluorescence microscope, a Hamamatsu Orca 12-bit digital camera, and Simple PCI software. SM22α images were captured with a 0.1 second exposure using a 505 nm LP dichroic filter (480/40 nm excitation filter, 535/50 nm emission). Kv7.5 images were captured with a 0.5 second exposure using a 564 nm LP dichroic filter (535/50 nm excitation filter, 620/60 nm emission). DAPI images were captured with a 0.2 second exposure using a 400 nm LP dichroic filter (340 & 380 nm filters in Sutter DG-4). Coverslips incubated without primary antibody had no detectable fluorescence at the wavelength, gain, or exposure settings used.

Mass Spectrometry.

HTSMCs were grown to confluence on 150 mm dishes and infected at 100 MOI with Adv-hKCNQ5-FLAG-WT or Adv-hKCNQ5-FLAG-S441A or at 500 MOI with Adv-hKCNQ5-FLAG-T505A for 6 days and serum deprived overnight prior to experimentation. On the day of the experiment, HTSMCs were incubated for 3 hr at room temperature in mKreb's prior to treatment. Immediately following treatment, cells were lysed with ice-cold lysis buffer. Equal protein concentrations

of cell lysates (1.1 mg) were incubated overnight at 4°C with 4 µg of anti-FLAG antibody. 20 µl of Dynabeads® Protein G were added to antibody-containing lysates and incubated for 2 hr at 4°C. The beads were washed and immune complexes were eluted with 1X Laemmli sample buffer, heated for 5 min at 97°C, and stored at -20°C until further use by Dr. Kolla Kirstjansdottir at Midwestern University, Downers Grove, IL.

Enzymatic digestion. Samples were separated by SDS-PAGE on 10% acrylamide gels. Gel was stained with Coomassie Brilliant Blue to identify the bands for analysis. These bands were excised using a razor blade and cut into approximately 1 mm^3 pieces. Each piece was washed in water and destained using 100 mM ammonium bicarbonate (NH_4HCO_3, pH 7.5) in 50% acetronitrile. The reduction step was performed by the addition of 100 µl 50 mM NH_4HCO_3 (pH 7.5) and 10 µl 10 mM Tris(2-carboxyethyl)phosphine HCl for 30 min at 37°C. Proteins were alkylated by adding 100 µl 50 mM iodoacetamide for 30 min at 20°C in the dark. The gel sections were then washed in water, then acetonitrile, and dried in a SpeedVac.

Samples were digested with either endoproteinase Glu-C (EndoQ) or endoproteinase Lys-C (EndoK). EndoQ digestion was carried out with 160 ng Mass Spectrometry Grade Glu-C enzyme (Thermo #90054) in 50 mM NH_4HCO_3 (pH 7.5) overnight at 37°C. EndoK digestion was carried out with 160 ng Mass Spectrometry Grade Lys-C enzyme (Thermo #Q12057892) in 50 mM NH_4HCO_3

(pH 7.5) overnight at 37°C. Peptides were extracted with 5% formic acid, vacuum dried, and sent to Don Wolfgeher at the University of Chicago (Chicago, IL).

High Performance Liquid Chromatography for mass spectrometry. Samples were then re-suspended Burdick & Jackson HPLC-grade water containing 0.2% formic acid (Fluka), 0.1% TFA (Pierce), and 0.002% Zwittergent 3–16 (Calbiochem), a sulfobetaine detergent (MH^+ at 392, and in-source dimer [2 M + H^+] at 783, and some minor impurities of Zwittergent 3–12 seen as MH^+ at 336; peaks that will be at the end of the chromatograms). Peptide samples were loaded to a 0.25 µl C_8 OptiPak trapping cartridge custom-packed with Michrom Magic (Optimize Technologies) C8, washed, then switched in-line with a 20 cm by 75 µm C_{18} packed spray tip nano column packed with Michrom Magic C18AQ, for a 2-step gradient. Mobile phase A was water/acetonitrile/formic acid (98/2/0.2) and mobile phase B was acetonitrile/isopropanol/water/formic acid (80/10/10/0.2). A flow rate of 350 nl/min was used during a 90 min, 2-step LC gradient: 60 min from 5% B to 50% B, 10 min 50%–95% B, 10 min hold at 95% B, and then back to starting conditions and re-equilibrated.

Liquid Chromatography-MS/MS analysis. Samples were analyzed via electrospray tandem mass spectrometry (LC–MS/MS) on a Thermo Q-Exactive Orbitrap mass spectrometer. A 70,000 RP survey scan in profile mode, m/z 360–2000 Da, with lockmasses was used, followed by 20 MSMS HCD fragmentation scans at 17,500 resolution on doubly and triply charged precursors. Single charged

ions were excluded, and ions selected for MS/MS were placed on an exclusion list for 60 sec.

Tandem mass spectra were extracted, charge state deconvoluted and deisotoped by Proteowizard MSconvert version 3.0.9134. Mascot (Matrix Science, London, UK; version 2.3.02) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)) were set up to search the 160107_SPROT_Human_Iso_UP000005640 database (1-07-2016, 92013 entries) assuming the digestion enzymes Lys-C of Glu-C (V8). Fragment search specifications include an ion mass tolerance of 0.40 Da and a parent ion tolerance of 10.0 PPM. Fixed modification settings included Carbamidomethyl of cysteine. Variable modification settings included de-amidated of asparagine and glutamine, oxidation of methionine, formyl of the n-terminus and phospho of serine, threonine and tyrosine in Mascot and Glu->pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln->pyro-Glu of the n-terminus, de-amidated of asparagine and glutamine, oxidation of methionine, formyl of the n-terminus and phospho of serine, threonine and tyrosine in X! Tandem.

Criteria for protein identification. Validation of MS/MS based peptide and protein identifications was made using Scaffold (version Scaffold_4.7.5, Proteome Software Inc., Portland, OR). Peptide identifications were accepted if they could be established at greater than 87.0% probability to achieve a false discovery rate less than 1.0%. The Mascot peptide probabilities were assigned by the Scaffold Local false discovery rate algorithm while the X! Tandem peptide probabilities were

assigned by the Peptide Prophet algorithm (Keller, Nesvizhskii et al. 2002) with Scaffold delta-mass correction. The criteria for acceptable protein identifications included containing at least 2 identified peptides and a greater than 6.0% probability to achieve a false discovery rate less than 1.0%, with the probabilities being assigned by the Protein Prophet algorithm (Nesvizhskii, Keller et al. 2003). Proteins that could not be differentiated by MS/MS analysis due to similar peptides were grouped to satisfy the principles of parsimony and proteins that shared significant peptide evidence were grouped into clusters. Proteins were annotated with gene ontology terms from NCBI (downloaded Mar 10, 2016 (Ashburner, Ball et al. 2000)).

Post-translational modification site localization. Post-translational modification sites were annotated using Scaffold Post-Translational Modification (version Scaffold_4.4.6, Proteome Software, Portland, Oregon, USA). The site localization algorithm developed by Beausoleil *et al.* (Beausoleil, Villen et al. 2006) was used to re-analyze MS/MS spectra identified as modified peptides and develop a score that is calculated to assess the level of confidence in each PTM localization. Localization probabilities for all peptides containing each identified post-translational modification site are combined to obtain the best estimated probability that a post-modified is present at that particular site.

Animal Welfare and Housing.

Male Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN). Animals were housed in pairs in standard cages and fed with standard chow in a 12 hr dark-light cycle. All procedures were approved by the Institutional Animal Care and Use Committee, Loyola University Medical Center, Maywood, IL and were executed in accordance with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals.

Male Hartley guinea pigs were purchased from Harlan Laboratories or Charles River Laboratories (Skokie, IL). Animals were housed in pairs in standard cages and fed with standard chow in a 12 hr dark-light cycle. All procedures were approved by the Institutional Animal Care and Use Committee (Loyola University Medical Center, Maywood, IL or Edward Hines, Jr. Veterans Affairs Hospital, Hines, IL) and were executed in accordance with the National Academy of Science's Guide for the Care and Use of Laboratory Animals.

Guinea Pig Sensitization Model.

Male Hartley guinea pigs, 250-350g, were given 3 intraperitoneal injections of OVA (100 μg OVA and 100 mg aluminum hydroxide in 1 ml saline) or saline alone (control) on days 1, 3 and 5. Following a 2 week sensitization period, on day 19 the animals were exposed to a fine mist of OVA (5 mg/ml) in a cylindrical plexiglass chamber for 30 min. Control guinea pigs did not display any symptoms. OVA-sensitized guinea pigs displayed symptoms such as chewing, rapid and

labored breathing, and gasping within 5-10 min, followed by more severe symptoms, including coughing, cyanosis, and convulsions, at approximately 15 min into the challenge prompting their removal from the chamber and cessation of the challenge. This procedure was repeated on days 20 and 21. Tissues were collected post-euthanasia for study on day 22.

Tissue collection. Guinea pigs were euthanized by intraperitoneal injection of sodium pentobarbital or Euthasol®. The trachea was cut and cannulated with a 3 mm outer-diameter polypropylene-barbed tubing connector connected to 3-way stopcock and two 10 mL syringes. The lungs and heart were excised and placed into a modified Hank's balanced salt solution (mHBSS) containing (in mM): NaCl 137.9, KCl 5.33, $CaCl_2$ 1.26, $MgCl_2$ 0.49, $MgSO_4$ 0.41, HEPES 20, KH_2PO4 0.44, Na_2HPO_4 0.34, D-glucose 5 (pH 7.4 at 37°C, 298–300 mOsm) that had been preheated to 37°C.

Precision cut lung slices. Approximately 5 mL of air was withdrawn from the lungs to deflate them followed by inflation of the lungs with 20-25 mL of 2% low-melting temperature agarose in mHBSS 37°C supplemented with 1 μM isoproterenol. 5 mL of air was injected back into the lungs following inflation with agarose and tissue was cooled in mHBSS 4°C (pH 7.4 at 4°C, 298-300 mOsm) for 30 min to allow for agarose solidification. Lobes were then separated, trimmed perpendicular to the bronchus, and sliced 500 μm thick using a Leica VT 1200S vibratome (Leica Microsystems, Buffalo Grove, IL). Lung slices were incubated in serum-free tissue culture serum-free F-12/DMEM medium supplemented with

insulin-transferrin-selenium (Sigma-Aldrich), antibiotics and antimycotics (Sigma-Aldrich), and 1 μM isoproterenol for 4-6 hours and then changed to medium with no isoproterenol overnight. The slices were kept at 37°C in 5% CO_2 and used for up to 4 days after preparation.

If guinea pig lung slices are taken out of media immediately prior to experimentation, airways often go through a slow contraction and relaxation cycle that lasts approximately 4-6 hours in which the airways will be nonresponsive to bronchoconstrictor agonist treatment. Therefore, slices that contained round, open airways with beating cilia, and an intact airway wall and surrounding parenchyma attachments were selected and incubated overnight at room temperature in control medium containing (in mM) 140 NaCl, 5.36 KCl, 1.2 MgCl2, 2 CaCl2, 10 HEPES, 10 D-glucose (pH 7.3, 298 mOsm/l) so as to avoid the contraction-relaxation cycle and to promote a more stable airway diameter for experimentation.

To measure airway area, a lung slice was mounted in a perfusion chamber located on the stage of an Olympus IX-71 inverted microscope and a single airway was visualized using a 5X or 10X objective. All experiments were completed at room temperature. The lung slice was perfused with control medium (~1 drop/sec) for 20 minutes, or until a stable baseline area was achieved for 20 minutes, prior to perfusion of control medium containing drug. Images were captured every 5 sec with a Hamamatsu Orca 12-bit digital camera and luminal airway area was determined using Simple PCI software.

Human Precision Cut Lung Slices.

Human lung tissue not suitable for transplantation was obtained from Dr. Jeffrey Schwartz of the Department of Thoracic and Cardiovascular Surgery at Loyola University Chicago or from Dr. Julian Solway at the University of Chicago. Tissue was infused with injections of 2% low-melting temperature agarose in mHBSS at 37°C and cooled in mHBSS 4°C to allow for solidification of agarose. Once solidified, tissue was sliced 0.5-1 mm thick perpendicular to airways using a Leica VT 1200S vibratome. Lung slices were incubated in serum-free tissue culture F-12/DMEM medium supplemented with insulin-transferrin-selenium (Sigma-Aldrich) and antibiotics and antimycotics (Sigma-Aldrich). The slices were kept at 37°C in 5% CO_2 and used for up to 4 days after preparation.

To measure airway area, a lung slice was mounted in a perfusion chamber located on the stage of an Olympus IX-71 inverted microscope and a single airway was visualized using a 5X or 10X objective. All experiments were completed at room temperature. The lung slice was perfused with control medium (~1 drop/sec) for 20 minutes, or until a stable baseline area was achieved for 20 minutes, prior to perfusion of control medium containing drug. Images were captured every 5 sec with a Hamamatsu Orca 12-bit digital camera and luminal airway area was determined using Simple PCI software.

Guinea Pig flexiVent Optimization Experiments.

Naïve male Hartley guinea pigs (250-550 g) were weighed and anesthetized with intraperitoneal injection of Inactin® (thiobutabarbital, 300-400 mg/kg, given 100 mg/kg initially followed by 50 mg/kg increments every 15 minutes until the animal is fully anesthetized). The trachea was cannulated the animal was ventilated using a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montréal, QC, Canada) at a rate of 60 breaths/minute and a tidal volume of 5 ml/kg. Respiratory resistance was measured using the flexiVent "snapshot" maneuver (1 second duration, 1 Hz frequency) and Newtonian resistance, tissue dampening, and tissue elastance were measured using the flexiVent "quick prime-6" maneuver (6 second duration, 1-20.5 Hz frequency) using flexiWare software (flexiVent, SCIREQ, Montréal, QC, Canada). Once a stable baseline resistance value was obtained, the animals were given doses of histamine or methacholine using either an intravenous (i.v.) catheter or an in-line computer-controlled Aeroneb Lab nebulizer at approximately 5-minute intervals. For i.v. administration, 100 µl of each concentration in saline was injected followed by a 50 µl heparinized saline flush (50 U/ml). For nebulized administration, 200 µl of each concentration in water was pipetted onto the nebulizer. After each administration of bronchoconstrictor, the peak resistance value was recorded using a series of "snapshot" and "quick prime-6" maneuvers for 5 minutes prior to the next administration. At the end of each experiment, animals were euthanized via intraperitoneal injection of Euthasol® Euthanasia Solution.

Statistics.

Data are expressed as mean ± standard error of the mean (S.E.). SigmaStat (Systat Software, Inc.) was used for all statistical analyses. Independent *t*-test or the *t*-test followed by the Mann-Whitney Rank Sum test was used for comparisons between two independent groups. Paired Student's *t*-test was used for comparisons of parameters measured before and after treatments. Comparisons between more than two independent treatment groups were evaluated by analysis of variance (ANOVA) followed by the Holm-Sidak post hoc test. Comparisons among multiple treatments of the same subject (cell or airway) were evaluated by repeated measures ANOVA followed by the Holm-Sidak post hoc test. Differences associated with p values ≤ 0.05 were considered statistically significant.

CHAPTER FIVE

RESULTS

Cultured HTSMCs Retain SM22α Expression.

Culturing ASMCs to study the regulation of airway smooth muscle responses at the cellular level, including cell signaling and regulation of protein expression, is a method that has been in place for decades (Hall and Kotlikoff 1995). We considered it necessary to confirm that the smooth muscle phenotypes persisted upon the culturing of HTSMCs by using immunofluorescence to confirm the expression of SM22α, a protein that is largely specific for smooth muscle (Lees-Miller, Heeley et al. 1987), in freshly isolated HTSMCs cultured to passage 8 (Figure 2).

Histamine Increases $[Ca^{2+}]_{cyt}$ in Cultured HTSMCs.

As described in detail in chapter 2, bronchoconstrictor agonists induce a biphasic increase in $[Ca^{2+}]_{cyt}$ that is necessary for ASMC contraction. To confirm that the cultured HTSMCs retain this Ca^{2+} response to the bronchoconstrictor histamine, fura-2 fluorescence techniques were used to monitor changes in $[Ca^{2+}]_{cyt}$. Treatment of cultured HTMSCs with 1 μM histamine resulted in a 1.445-fold increase in sustained $[Ca^{2+}]_{cyt}$ over initial control levels (Figure 3B).

To examine the involvement of L-type VSCCs in the sustained increase in $[Ca^{2+}]_{cyt}$, we simultaneously treated cells with histamine (1 μM) and the L-type

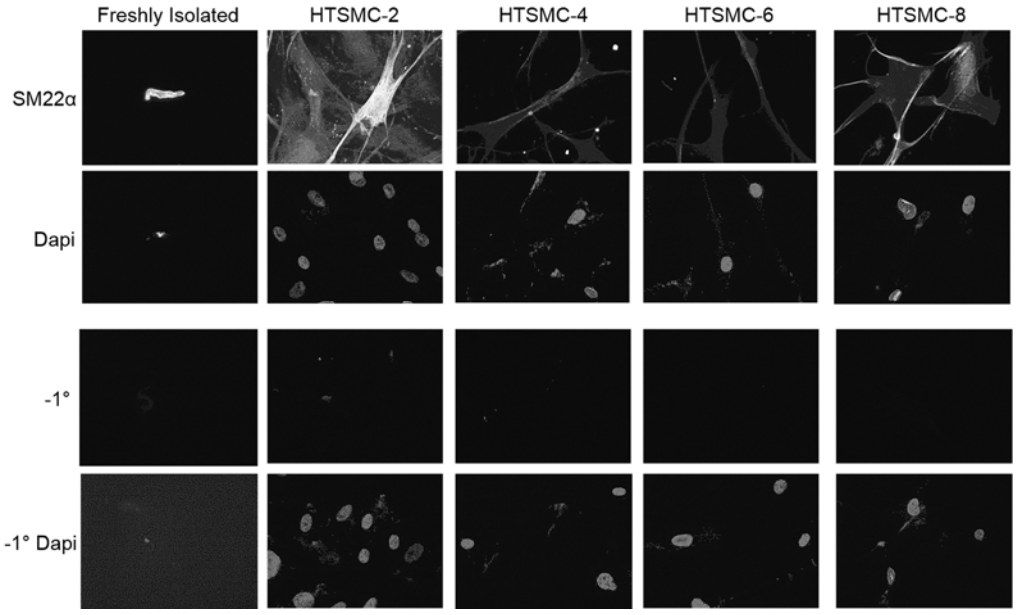

Figure 2. Cultured HTSMCs express SM22α through passage 8. The top row of panels are representative images of freshly isolated (far left) or cultured HTSMCs (passages 2, 4, 6, and 8) expression of SM22α. The second row of panels are the same field, but showing DAPI stained nuclei of the cells imaged in the top row. The third row of panels are representative images of the minus primary SM22α antibody control. The fourth row of panels are the same field, but showing DAPI stained nuclei of the cells shown in the third row.

VSCC blocker verapamil (10 μM). The increase in sustained $[Ca^{2+}]_{cyt}$ was significantly smaller (1.209-fold over initial control levels, Figure 3B) than observed with histamine treatment alone, confirming a role for L-type VSCC in Ca^{2+} influx following bronchoconstrictor agonist treatment.

It is also worth noting that the Kv7 channel blocker XE991 (10 μM) induced a small but significant increase in sustained $[Ca^{2+}]_{cyt}$ in cultured HTSMCs (Figure 3C).

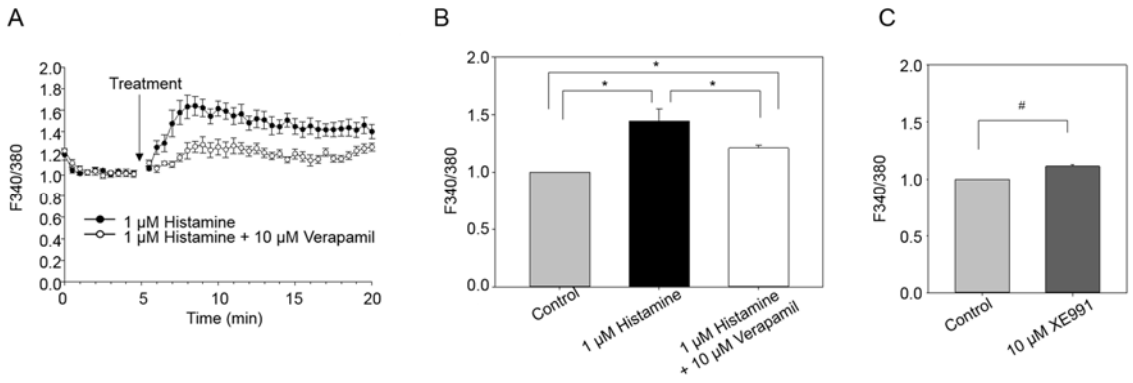

Figure 3. Histamine induces L-type VSCC-dependent increases in $[Ca^{2+}]_{cyt}$ in cultured HTSMCs. (A) Averaged time courses of $[Ca^{2+}]_{cyt}$ following treatment of cultured HTSMCs with histamine (1 μM, closed circles, n=15) or histamine plus verapamil (10 μM, open circles, n=14). (B) Summary of 5 experiments (triplicate wells). Data shows average $[Ca^{2+}]_{cyt}$ for the final 10-15 minutes of treatment ("sustained" $[Ca^{2+}]_{cyt}$ increases) with histamine (black bar) or histamine plus verapamil (white bar). (C) Summary of 3 experiments (triplicate wells). Data shows average $[Ca^{2+}]_{cyt}$ for the final 10-15 minutes of treatment ("sustained" $[Ca^{2+}]_{cyt}$ increases) with Kv7 channel blocker XE991 (10 μM, dark grey bar). NOTE: All values were normalized to the average F340/380 values recorded during the 2 minutes prior to treatment. *, significant difference from indicated groups, $p < 0.05$, one way ANOVA followed by Holm-Sidak post hoc test multiple comparisons among all groups. #, significantly different from control, $p < 0.05$, paired t-test.

Activation of PKC Suppresses hKv7.5 Currents in Cultured HTSMCs.

HTSMCs were cultured at low passages and used as an expression system to study the regulation of WT or mutant hKv7.5 channels. Similar to previous observations our laboratory has made regarding endogenous Kv7 currents (Brueggemann, Kakad et al. 2012, Haick, Brueggemann et al. 2017), in cultured HTSMCs overexpressing WT hKv7.5 channels, the application of histamine (1 μM) significantly suppressed hKv7.5 currents (by 34.4 ± 6.2% at -20 mV, Figure 4C) approximately 1-2 minutes following the start of histamine treatment (Figure 4B). If cells were pretreated with the H1 histamine receptor antagonist

chlorpheniramine (30 μM) before being treated with histamine, no hKv7.5 current inhibition was observed (Figure 4D). The vehicle for chlorpheniramine did not have an effect on hKv7.5 currents (data not shown). Pretreatment with the PKC inhibitor Ro-31-8220 (3 μM) for 1 hour prior to histamine treatment significantly attenuated the amount of histamine-induced hKv7.5 suppression that was observed (9.66 ± 3% at -20 mV, Figure 5) compared to treatment with histamine alone (by 34.4 ± 6.2% at -20 mV, Figure 4C). The direct activation of PKC with PMA (1 nM, Figure 6A), also induced significant suppression of hKv7.5 currents (by 75.5 ± 7.6% and 10.2 ± 4.9%, respectively, at -20 mV). No current suppression was observed following treatment with PMA's inactive analog, 4α-PMA (1 nM, Figure 6B).

Activation of PKC Induces Phosphorylation of hKv7.5 Channels in Cultured HTSMCs.

We next wanted to test the effects of PKC activation with either histamine or PMA on hKv7.5 channel phosphorylation. A FLAG antibody was used to immunoprecipitate FLAG-tagged hKv7.5 channels following no treatment, treatment with histamine (1 μM) for 10 minutes, or treatment with PMA (1 nM) for 30 minutes. An antibody that recognizes phosphorylated serine residues that fall within a specific PKC consensus motif (anti-phosphoserine PKC substrate) was used to examine hKv7.5 channel phosphorylation. This antibody recognizes phosphorylated serine residues that fall within the conventional PKC consensus motif: arginine or lysine is found at positions -2 and +2, while a hydrophobic amino

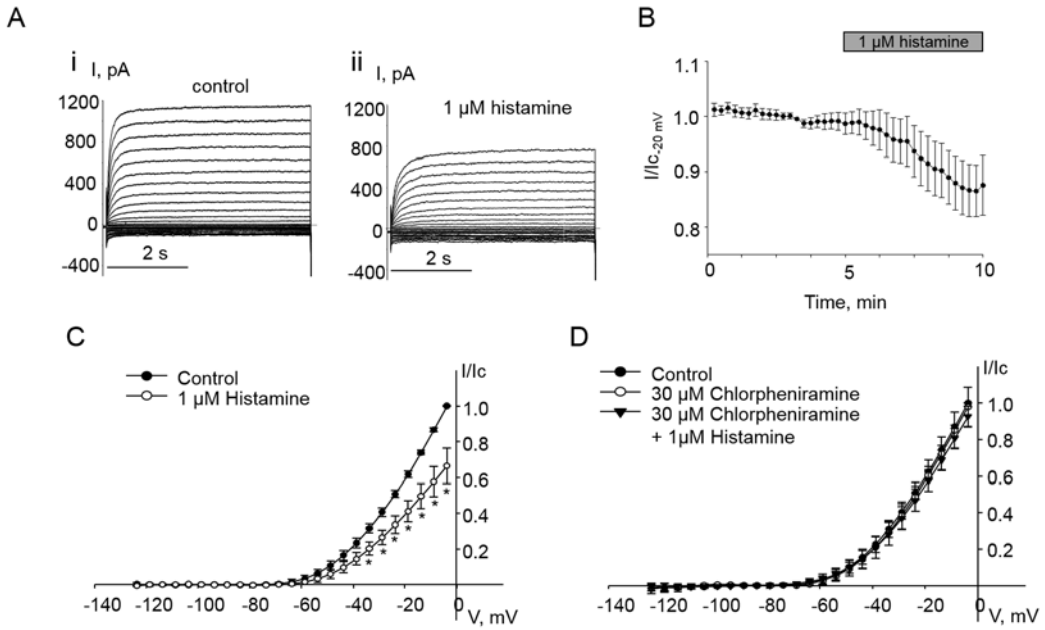

Figure 4. Histamine inhibits hKv7.5 currents in cultured HTSMCs. (A) Representative traces of hKv7.5 currents recorded with a voltage-step protocol (see *materials and methods*) in one cultured HTSMC, *i*, before and, *ii*, after treatment with 1 μM histamine (capacitance = 100 pF). (B) Averaged time course of histamine-induced hKv7.5 current inhibition at -20 mV in cultured HTSMCs (n=5). Normalized to average current 5 minutes prior to treatment with histamine. (C) Current-voltage (I-V) curves of steady state currents through hKv7.5 channels recorded in cultured HTSMCs before (filled circles) and after treatment with histamine (1 μM, open circles, n=6). (D) I-V curves of steady state currents through hKv7.5 channels recorded in cultured HTSMCs before (filled circles) treatment with chlorpheniramine (30 μM, open circles) and then histamine (1 μM) in the continued presence of chlorpheniramine (filled triangles, n = 4). *, significantly different from control, $p < 0.05$, paired t-test.

acid (phenylalanine, isoleucine, leucine, valine, or tyrosine) is commonly found at the +1 position.

Indirect activation of PKC with histamine and direct activation of PKC with PMA both resulted in a significant increase in hKv7.5 phosphorylation compared to untreated control samples (1.82 ± 0.28 -fold and 1.73 ± 0.3 -fold greater than

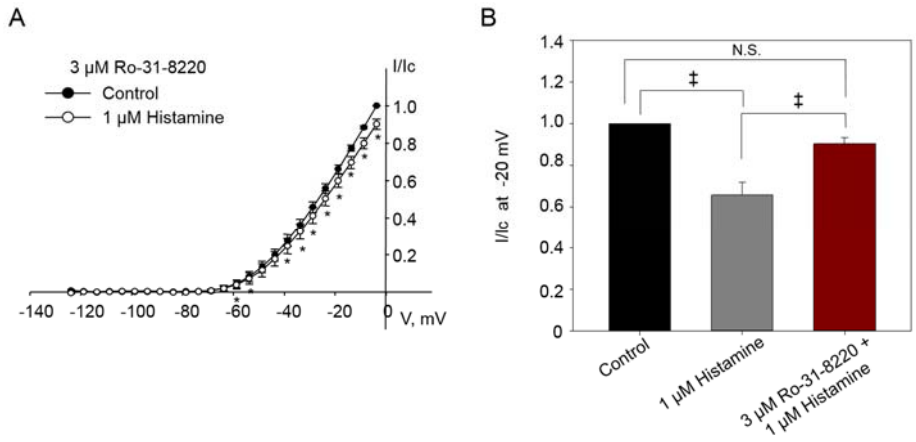

Figure 5. Histamine-induced inhibition of hKv7.5 currents in cultured HTSMCs is dependent on PKC. (A) I-V curves of stead state currents through hKv7.5 channels recorded in cultured HTSMCs pretreated with for 1 hour with Ro-31-8220 (3 μM) before (filled circles) and after histamine (1 μM, open circles) treatment (n=5). (B) Summarized data shows average hKv7.5 current at -20 mV under control conditions (black bar), following treatment with histamine (1 μM, grey bar, n=6), or after 1 hour Ro-31-8220 (3 μM) pretreatment prior to histamine treatment (red bar, n=5). *, significantly different from control, $p < 0.05$, paired t-test. ‡, significant difference from indicated groups, $p < 0.05$, one way ANOVA followed by Holm-Sidak post hoc test, with multiple comparisons among all groups.

untreated control, respectively, Figure 7C). Pretreatment with the PKC inhibitor Ro-31-8220 (1 hour, 1, 3, and 10 μM) significantly attenuated histamine-induced phosphorylation of hKv7.5 (Figure 7D).

In retrospect, a time course examining the phosphorylation status of hKv7.5 following histamine treatment should have been completed in order to determine the optimal time point for experimentation. Before completing the additional experiments to examine the PKC isoform or the amino acid residues involved in histamine-induced phosphorylation of hKv7.5, we examined the level of hKv7.5 phosphorylation following 30 seconds, and 1, 2, 5, 10, or 30 minutes of histamine, or vehicle, treatment (Figure 8). Maximal channel phosphorylation occurred within

the first two minutes of treatment (3.36 ± 1.52 -fold, 2.54 ± 0.97 -fold, and 2.47 ± 0.58 -fold greater than untreated control, at 30 seconds, 1 minute, and 2 minutes, respectively) and eventually declined to near control levels by 10 and 30 minutes (1.27 ± 0.19 –fold and 1.39 ± 0.39 –fold greater than untreated control, respectively, Figure 8). The initial increase in hKv7.5 channel phosphorylation corresponds with the time course observed for histamine-induced hKv7.5 current inhibition (Figure 4B). The 1 minute time point was selected for the remaining experiments.

PKCα is Necessary for the Regulation of hKv7.5 Currents by Histamine, but not for Channel Phosphorylation.

PKCα is known to be activated following the activation of the H1 histamine receptor (Eto, Kitazawa et al. 2001, Megson, Walker et al. 2001, Rimessi, Rizzuto et al. 2007) and studies completed in A7r5 cells and rat mesenteric artery smooth muscle cells have suggested that the activation of PKCα is sufficient to suppress Kv7 currents in vascular smooth muscle (Brueggemann, Mackie et al. 2014). In order to determine if histamine-induced suppression of hKv7.5 currents in ASMCs is mediated by PKCα, we infected cultured HTSMCs with a retroviral vector that drives the expression of shRNA against PKCα (shPKCα), or control vector (shControl), before overexpressing hKv7.5 channels as we had done for the

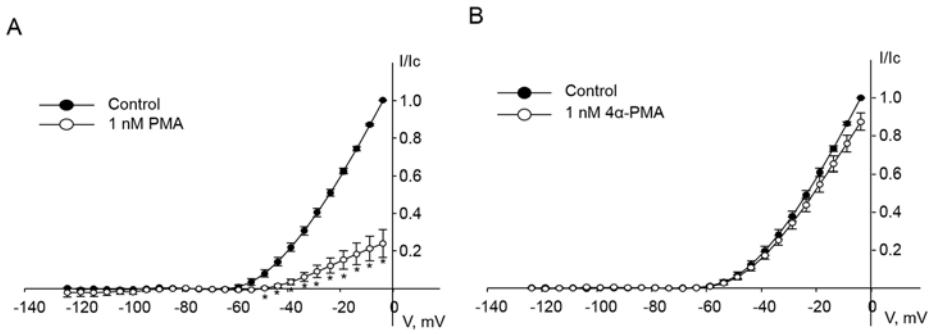

Figure 6. PMA, but not 4α-PMA, inhibits hKv7.5 currents in cultured HTSMCs. (A) I-V curves of steady state currents through hKv7.5 channels recorded in cultured HTSMCs before (filled circles) and after treatment with PMA (1 nM, open circles, n=4). (B) I-V curves of steady state currents through hKv7.5 channels recorded in cultured HTSMCs before (filled circles) and after treatment with 4α-PMA (1 nM, open circles, n=3). *, significantly different from control, $p < 0.05$, paired t-test.

studies described above. PKCα knockdown was confirmed by western blotting (Figure 10A). There was no histamine-induced suppression of hKv7.5 currents in cells that lacked PKCα (Figure 9A). Cells that lacked PKCα also had a significantly smaller histamine-induced increase in sustained $[Ca^{2+}]_{cyt}$ compared to control cells (Figure 9D). Unexpectedly, knocking down PKCα did not prevent the significant increase in histamine-induced phosphorylation of hKv7.5 channels on serine residues located in the PKC consensus motif (1.48 ± 0.23 fold above untreated control, Figure 10).

Threonine Residue 505 may be Necessary for the Regulation of hKv7.5 Currents by Histamine.

Serine residue 541 in rat Kv7.2 is phosphorylated by PKC and required for channel inhibition by acetylcholine (Hoshi, Zhang et al. 2003). This residue

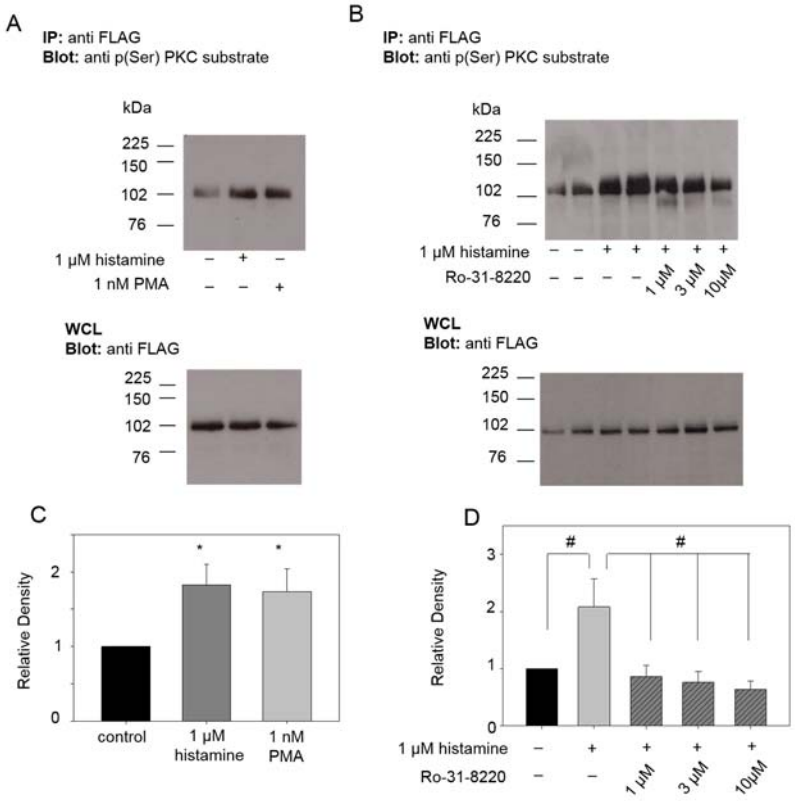

Figure 7. PKC activation promotes hKv7.5 channel phosphorylation in HTSMCs. (A) Representative western blot of FLAG immunoprecipitates from cultured HTSMCs overexpressing FLAG-hKv7.5 channels that were left untreated, treated with histamine (1 μM, 10 minutes), or treated with PMA (1 nM, 30 minutes) probed with an anti-phosphoserine PKC substrate antibody (top). Whole cell lysates (50 μg) were run separately and probed with an anti-FLAG antibody to serve as a loading control (bottom). (B) Representative western blot of immunoprecipiates from cultured HTSMCs overexpressing FLAG-hKv7.5 channels that were left untreated, treated with histamine (1 μM, 10 minutes), or pretreated with Ro-31-8220 (1, 3, or 10 μM, 1 hour) prior to histamine treatment probed with an anti-phosphoserine PKC substrate antibody (top). Whole cell lysates (50 μg) were run separately and probed with an anti-FLAG antibody to serve as a loading control (bottom). (C) Densitometry summarized from 11 experiments depicted in *A*. (D) Densitometry summarized from 5 different experiments depicted in *B*. *, significantly different from control, $p < 0.05$, one-way ANOVA followed by Holm-Sidak post hoc test multiple comparisons versus control group. #, significantly different from histamine group, $p < 0.05$, one-way ANOVA followed by Holm-Sidak post hoc test multiple comparisons versus histamine group.

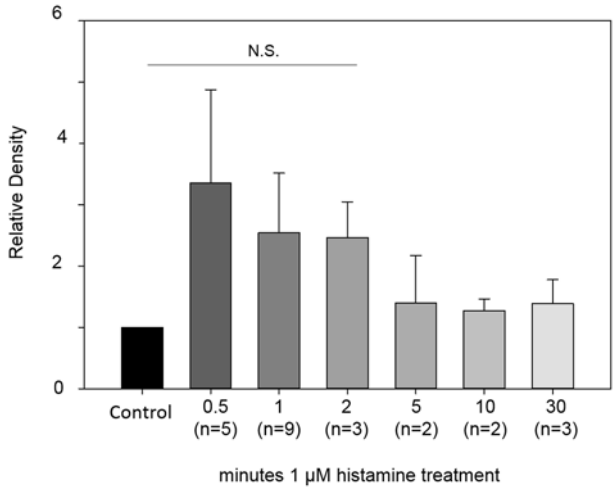

Figure 8. Time course of histamine-induced phosphorylation of hKv7.5 channels in cultured HTSMCs. Summarized densitometry results from immunoprecipitation experiments examining the phosphorylation of hKv7.5 channels in HTSMCs following treatment with vehicle (mKreb's buffer) or 1 μM histamine for 0.5, 1, 2, 5, 10 or 30 minutes. hKv7.5 channels were immunoprecipitated with an anti-FLAG antibody and western blots were probed with an anti-phosphoserine PKC substrate antibody. Whole cell lysates (25 μg) were run separately and western blots were probed using an anti-FLAG antibody to serve as a loading control. Anti-phosphoserine PKC substrate signals were normalized to anti-FLAG signals from whole cell lysates and vehicle and histamine treatments were then normalized to untreated control. Each histamine treatment time point was then normalized to its own vehicle control.

corresponds to threonine 505 (T505) in hKv7.5. In order to determine if phosphorylation of T505 is necessary for histamine-induced hKv7.5 current inhibition, a vector was previously created to drive expression of a mutant hKv7.5 in which T505 was replaced by alanine (T505A). Treatment with histamine (1 μM) and PMA (1 nM) still resulted in significant inhibition of hKv7.5 currents (17.5 ± 6.7% and 52.6 ± 7.6%, respectively, Figure 11A, C) at -20mV. Although it appeared as if histamine suppressed mutant T505A currents to a lesser extent compared to WT current suppression, a statistical difference was not reached (Figure 11B).

There was also no significant difference between WT and T505A channels in the amount of current inhibition at -20 mV for PMA (Figure 11D). These results indicate that this particular amino acid residue may be necessary for the regulation of hKv7.5 currents by histamine, however current results are inconclusive.

Serine Residue 441 is Necessary for Regulation of hKv7.5 Current by Histamine, but not for Channel Phosphorylation.

MIT NEW Scansite 3 software predicted that hKv7.5 serine residue 441 (S441) to be a potential PKCα phosphorylation site, a residue that also happens to fall within the recognition sequence for the anti-phosphoserine antibody used in our previous studies examining hKv7.5 channel phosphorylation following histamine treatment. In order to determine if S441 phosphorylation is required for hKv7.5 current inhibition and channel phosphorylation following treatment with histamine and PMA, a vector was constructed that would drive the expression of a nonphosphorylatable mutant hKv7.5 in which S441 was replaced by alanine (S441A). PMA still significantly inhibited hKv7.5-S441A currents (63.0 ± 7.5% at -20 mV, Figure 12C, D), however histamine was unable to suppress hKv7.5-S441A currents (Figure 12A, B). Despite the lack of histamine-induced hKv7.5-S441A current suppression, a significant increase in hKv7.5-S441A channel phosphorylation was observed upon treatment with either histamine or PMA (1.72 ± 0.23 fold and 1.66 ± 0.13 fold, respectively, Figure 13B).

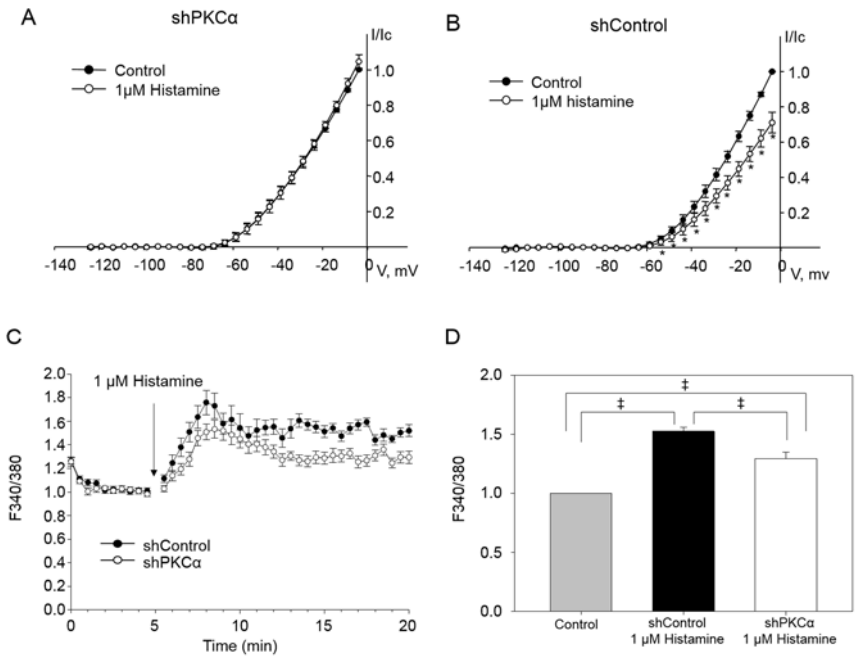

Figure 9. PKCα is necessary for histamine-induced inhibition of hKv7.5 currents and histamine-induced sustained increases in $[Ca^{2+}]_{cyt}$ in cultured HTSMCs. (A) I-V curves of steady state currents through hKv7.5 channels recorded in cultured HTSMCs lacking PKCα before (filled circles) and after treatment with histamine (1 μM, open circles, n=5). (B) I-V curves of steady state currents through hKv7.5 channels recorded in cultured HTSMCs expressing scrambled control shRNA before (filled circles) and after treatment with histamine (1 μM, open circles, n=4). (C) Averaged time courses of $[Ca^{2+}]_{cyt}$ following treatment of cultured HTSMCs lacking shPKCα (open circles) or expressing scrambled control shRNA (closed circles) with histamine (1 μM, n=12). (B) Summary of 3 experiments (triplicate wells). Data shows average $[Ca^{2+}]_{cyt}$ for the final 10-15 minutes of treatment ("sustained" $[Ca^{2+}]_{cyt}$ increases) with histamine in cultured HTSMCs lacking shPKCα (white bar) or expressing scrambled control shRNA (black bar). *, significant difference from control, $p < 0.05$, paired t-test. ‡, significant difference from indicated groups, $p < 0.05$, one way ANOVA followed by Holm-Sidak post hoc test, with multiple comparisons among all groups.

Preliminary Mass Spectrometry Results are Inconclusive.

Results thus far implicate S441 as necessary for histamine-induced hKv7.5 current suppression, though immunoprecipitation studies suggest that there are additional serine residues that are phosphorylated on hKv7.5 channels following

treatment with histamine. Serine residue 385 (S385) is a putative PKCα phosphorylation site and also falls within the recognition motif for the anti-phosphoserine antibody (-K/R-*X*-S-FILVY-K/R-) and might contribute to the remaining signal detected when S441 was mutated to alanine. MIT NEW Scansite 3 also identifies serine residue 772 (S772) as a putative PKC phosphorylation site, however this particular amino acid does not fall within the recognition motif for the antibody used.

Our initial mass spectrometry analysis was inconclusive due to low sample concentration and digestion with trypsin not producing fragments with the amino acid residues of interest. Our second preliminary mass spectrometry results indicated that in the EndoQ digested samples for hKv7.5-WT and hKv7.5-S441A channels, treatment with histamine (1 μM) induced phosphorylation of S385 and this phosphorylation was not present in untreated samples or samples that had been pretreated with the PKC inhibitor Ro-31-8220 (1 μM). No S385 phosphorylation was observed in hKv7.5-T505A expressing HTSMCs. Interestingly, in the same samples digested with EndoK, all samples showed phosphorylation at S385.

S772 phosphorylation was also observed in nearly all samples from both EndoQ and EndoK digestions, except for the EndoQ hKv7.5-WT untreated sample and the hKv7.5-S441A Ro-31-8220 pretreated sample.

A new site not predicted by MIT NEW Scansite 3 software, serine residue 752 (S752), was also phosphorylated in the EndoQ digested hKv7.5-WT histamine

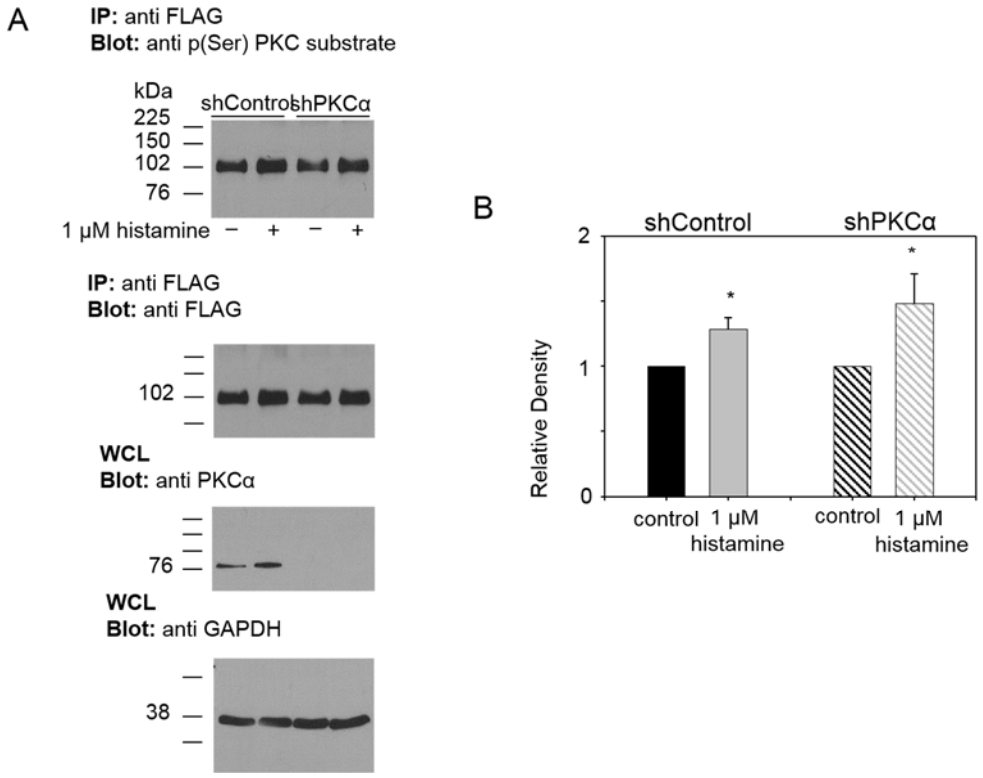

Figure 10. PKCα is not necessary for histamine-induced increases in hKv7.5 channel phosphorylation in cultured HTSMCs. (A) Representative western blot of FLAG immunoprecipitates from cultured HTSMCs overexpressing FLAG-hKv7.5 channels that expressed scrambled control shRNA or shRNA against PKCα and were left untreated or treated with histamine (1 μM, 1 minute) probed with an anti-phosphoserine PKC substrate antibody (top). The same membrane was probed with an anti-FLAG antibody to serve as a loading control (second from top). Whole cell lysates (50 μg) were run separately and probed with an anti-PKCα antibody to confirm PKCα knockdown (second from bottom) and an anti-GAPDH antibody to serve as a loading control (bottom). (B) Densitometry summarized from 5 experiments. *, significant difference from control, $p < 0.05$, independent *t*-test followed by Mann-Whitney U rank sum test.

treated sample and hKv7.5-S441A histamine and Ro-31-8220 plus histamine treated sample. No phosphorylation was observed at this site with EndoK digested samples.

No phosphorylation at T505 was observed in any samples that contained an observable fragment, and no fragment containing S441 was analyzed despite our enzyme selection.

Due to the discrepancies between EndoQ and EndoK samples and the different phosphorylation patterns that were observed (Table 1), it is important to repeat these studies before any conclusions can be made.

Retigabine Dose-dependently Relaxes Rat Airways Preconstricted with Methacholine.

We have demonstrated on multiple occasions that bronchoconstrictor agonists suppress Kv7 currents in ASMCs (Brueggemann, Kakad et al. 2012, Haick, Brueggemann et al. 2017). We also observed that the Kv7 channel activator flupirtine can attenuate histamine-induced bronchoconstriction and that inhibiting Kv7 channels with XE991 is sufficient to induce robust constriction of human airways (Brueggemann, Kakad et al. 2012). These observations led us to hypothesize that Kv7 channel activators represent a novel class of bronchodilators that may be used in the treatment of asthma.

PCLS were used as an experimental model to further assess the functional consequences of Kv7 channel manipulation in airway smooth muscle. This *in vitro* system allows the user to visualize and monitor the responses of individual airways to various treatments while allowing for the preservation of cellular processes within the tissue system and can be used with essentially any mammalian species, permitting for inter-species comparison and parallel human tissue experimentation

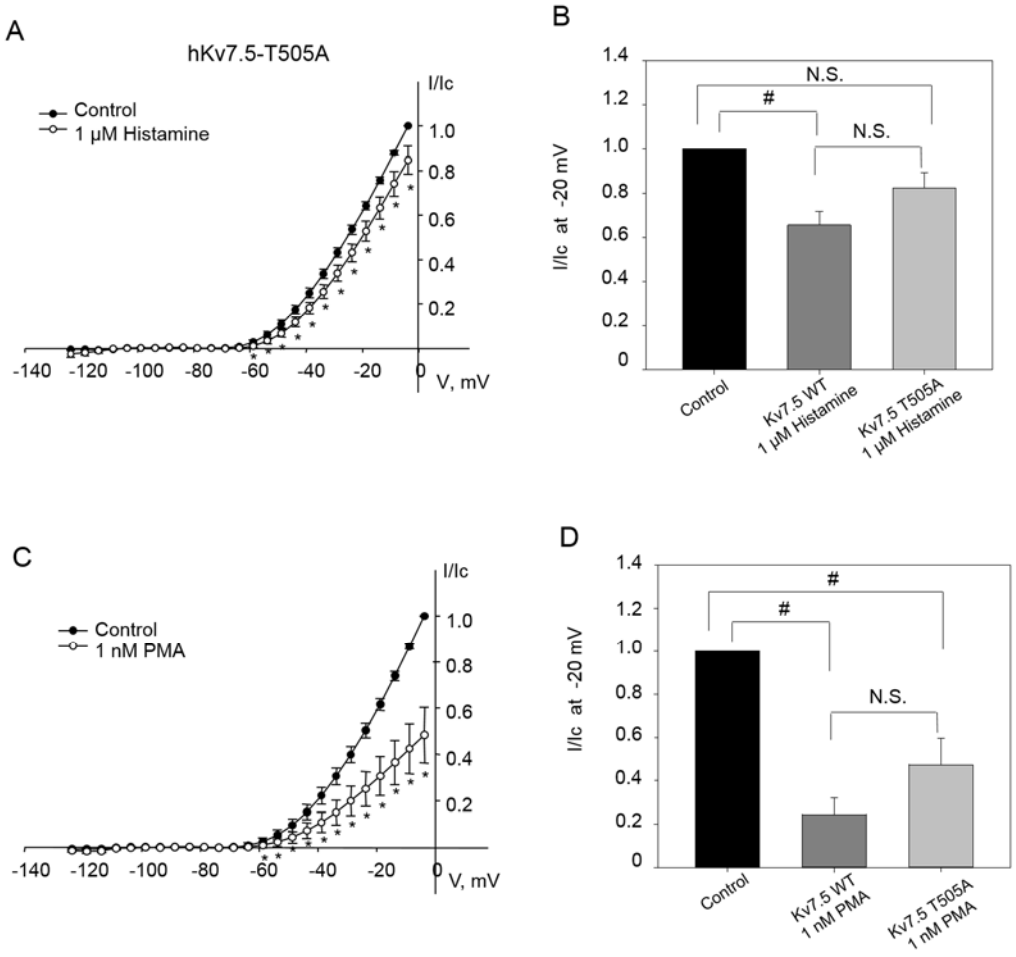

Figure 11. Threonine 505 may be necessary for histamine-induced suppression of hKv7.5 currents in cultured HTSMCs. (A) I-V curves of steady state currents through hKv7.5-T505A channels recorded in cultured HTSMCs before (filled circles) and after treatment with histamine (1 μM, open circles, n=7). (B) Summarized data shows average hKv7.5 current at -20 mV under control conditions (black bar), following treatment of hKv7.5-WT expressing HTSMCs with histamine (1 μM, dark grey bar, n=6), or following treatment of hKv7.5-T505A expressing HTSMCs with histamine (1 μM, light great bar, n=7). (C) I-V curves of steady state currents through hKv7.5-T505A channels recorded in cultured HTSMCs before (filled circles) and after treatment with PMA (1 nM, open circles, n=7). (D) Summarized data shows average hKv7.5 current at -20 mV under control conditions (black bar), following treatment of hKv7.5-WT expressing HTSMCs with PMA (1 nM, dark grey bar, n=4), or following treatment of hKv7.5-T505A expressing HTSMCs with PMA (1 nM, light great bar, n=7). *, significantly different from control, $p < 0.05$, paired t-test. #, significant difference from indicated groups, $p < 0.05$, one way ANOVA on RANKS followed by Dunn's post hoc analysis, with multiple comparisons among all groups.

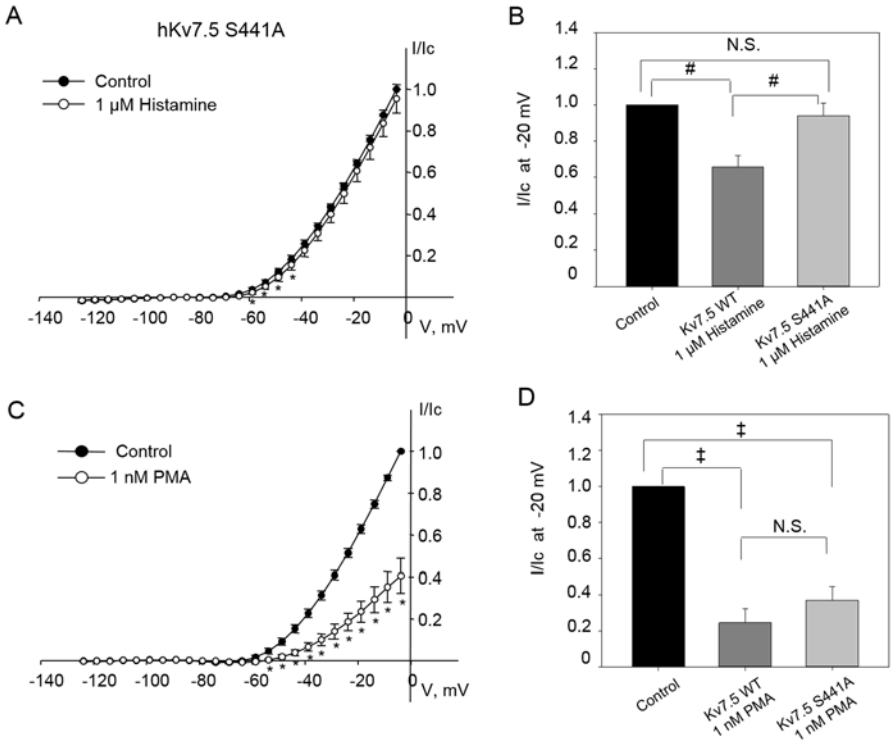

Figure 12. Serine 441 is necessary for histamine- but not PMA-induced inhibition of hKv7.5 currents in cultured HTSMCs. (A) I-V curves of steady state currents through hKv7.5-S441A channels recorded in cultured HTSMCs before (filled circles) and after treatment with histamine (1 μM, open circles, n=9). (B) Summarized data shows average hKv7.5 current at -20 mV under control conditions (black bar), following treatment of hKv7.5-WT expressing HTSMCs with histamine (1 μM, dark grey bar, n=6), or following treatment of hKv7.5-S441A expressing HTSMCs with histamine (1 μM, light great bar, n=9). (C) I-V curves of steady state currents through hKv7.5-S441A channels recorded in cultured HTSMCs before (filled circles) and after treatment with PMA (1 nM, open circles, n=6). (D) Summarized data shows average hKv7.5 current at -20 mV under control conditions (black bar), following treatment of hKv7.5-WT expressing HTSMCs with PMA (1 nM, dark grey bar, n=4), or following treatment of hKv7.5-T505A expressing HTSMCs with PMA (1 nM, light great bar, n=6). *, significantly different from control, $p < 0.05$, paired t-test. #, significant difference from indicated groups, $p < 0.05$, one way ANOVA on RANKS followed by Dunn's post hoc analysis, with multiple comparisons among all groups. ‡, significant difference from indicated groups, $p < 0.05$, one way ANOVA followed by Holm-Sidak post hoc test, with multiple comparisons among all groups.

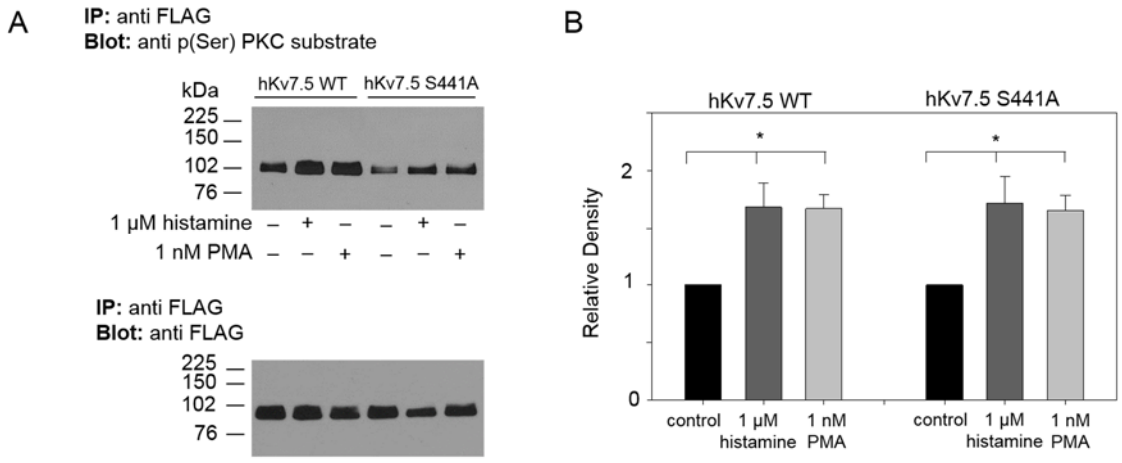

Figure 13. Serine 441 is not necessary for histamine- or PMA- induced phosphorylation of hKv7.5 channels in cultured HTSMCs. (A) Representative western blot of FLAG immunoprecipitates from cultured HTSMCs overexpressing FLAG-hKv7.5-WT or FLAG-hKv7.5-S441A channels that were left untreated, treated with histamine (1 μM, 1 minute), or treated with PMA (1 nM, 30 minutes) probed with an anti-phosphoserine PKC substrate antibody (top). The same membrane was probed with an anti-FLAG antibody to serve as a loading control (bottom). (B) Densitometry summarized from 5 experiments. *, significant difference from indicated columns, $p < 0.05$, one-way ANOVA followed by Holm-Sidak post hoc test, with multiple comparisons versus control group.

(Liberati, Randle et al. 2010, Sanderson 2011). Retigabine dose-dependently relaxed rat airways preconstricted with 230 nM methacholine, the approximate EC_{50} for methacholine in rat airways (Brueggemann, Haick et al. 2014) (EC_{50}=3.6 ± 0.3 μM; maximum relaxation 61 ± 9%, Figure 14B). Retigabine-induced relaxation was rapid and sustained through the entire treatment period (Figure 14A).

Channel	Treatment	S385	S441	T505	S752	S772
hKv7.5-WT	untreated	K	-	-	-	K
	1 μM histamine	Q/K	-	-	Q	Q/K
	1 μM Ro-31-8220 + 1 μM histamine	K	-	-	-	Q/K
hKv7.5-S441A	untreated	K	-	-	-	Q/K
	1 μM histamine	Q/K	-	-	Q	Q/K
	1 μM Ro-31-8220 + 1 μM histamine	K	-	-	Q	K
hKv7.5-T505A	untreated	K	-	-	-	Q/K
	1 μM histamine	K	-	-	-	Q/K
	1 μM Ro-31-8220 + 1 μM histamine	K	-	-	-	Q/K

Table 1. Preliminary Mass Spectrometry Results. Phosphorylation sites of interest on hKv7.5 channels. HTSMCs expressing FLAG-hKv7.5-WT, FLAG-hKv7.5-S441A, or FLAG-hKv7.5-T505A channels were left untreated, treated with 1 μM histamine for 1 minute, or pretreated with 1 μM Ro-30-8220 for 1 hour prior to treatment with 1 μM histamine prior to immunoprecipitation with an anti-FLAG antibody. Each sample was digested with EndoK ("K") and EndoQ ("Q) and subjected to mass spectrometry analysis. K or Q identifies residues where phosphorylation was detected for each enzymatic digest.

Formoterol Dose-dependently Relaxes Rat Airways Preconstricted with Methacholine.

Formoterol is a commonly prescribed LABA that is typically used for "maintenance" therapy, but also produces rapid and effective bronchodilation during acute asthma attacks (Rodrigo, Neffen et al. 2010, Ricciardolo, Blasi et al. 2015). Similar to retigabine, formoterol dose-dependently relaxed rat airways preconstricted with 230 nM methacholine (EC_{50}=2.4 ± 0.3 nM; maximum relaxation 72 ± 5%, Figure 15B), however there was a substantial time-dependent decline in relaxation by the end of the treatment period (Figure 15A).

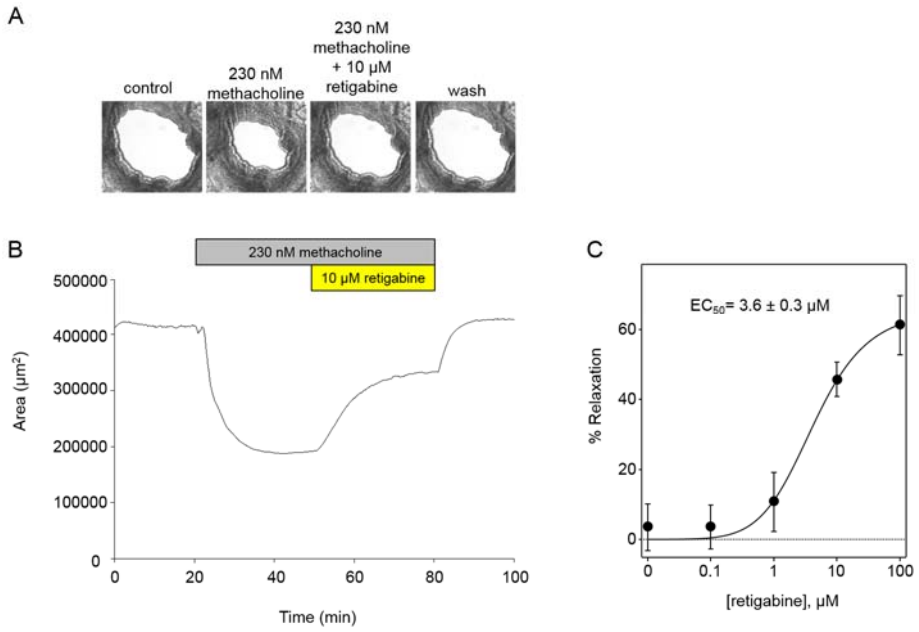

Figure 14. Retigabine dose-dependently relaxes rat airways preconstricted with methacholine. (B) Representative time course depicting the changes in area of a single rat airway (pictured in (A)) treated with methacholine (230 nM, 30 minutes), followed by retigabine (10 µM) in the continued presence of methacholine (30 minutes), and then washed. (C) Averaged dose-response curve of retigabine-induced relaxation of rat airways preconstricted with methacholine (230 nM) fitted to the Hill equation (n=6).

Retigabine Enhances Formoterol-induced Relaxation of Rat Airways Preconstricted with Methacholine.

Airways were constricted with 230 nM methacholine and then a submaximal concentration of retigabine (10 µM) was included with the different increasing concentrations of formoterol (Figure 16A). The addition of retigabine increased both the maximal and the sustained airway relaxation compared to formoterol alone (Figure 16B) and also significantly decreased the time-dependent decline in airway relaxation initially observed with formoterol treatment alone (Figure 16C).

The time-dependent decline in airway relaxation, or “desensitization”, to formoterol treatment was of particular interest and warranted further investigation. There is considerable evidence that regular use of long-acting β_2-agonists, such as salmeterol or formoterol, for the treatment of asthma, results in the development of tolerance and reduced efficacy. When formoterol (10 nM) was used repetitively as a bronchodilator on a single airway, a time-dependent decline in relaxation was observed during each consecutive application and there was also an overall decrease in the final extent of relaxation achieved following successive formoterol applications (Figure 17A). The initial maximum relaxation peaked at 68 ± 10% and tapered significantly to a final relaxation of 46 ± 14% after 30 minutes. The second application of formoterol reached a maximum relaxation of only 48 ± 8% and declined to a final relaxation of 32 ± 6% after 30 minutes (Figure 17C). Adding retigabine (10 μM) to the second application of formoterol resulted in significantly greater relaxation (72 ± 8%, maximum and final relaxation, Figure 17B, D) and there was no time-dependent decline in relaxation after 30 minutes.

Retigabine Appears to Enhance Formoterol-induced Relaxation of Human Airways Preconstricted with Histamine.

Formoterol (10 pM) was applied repetitively to human airways constricted with 50 nM histamine. Although a time-dependent decline in formoterol-induced relaxation was not observed, the second consecutive application of formoterol results on average in a smaller amount of relaxation (29 ± 10.5% and 8 ± 11.9%

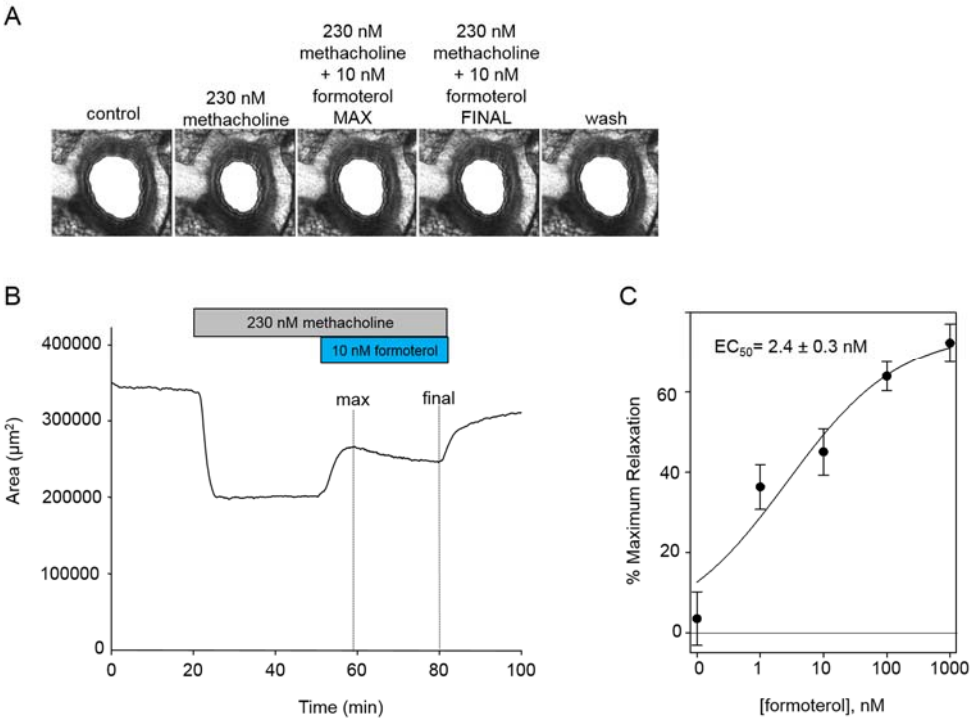

Figure 15. Formoterol dose-dependently relaxes rat airways preconstricted with methacholine. (B) Representative time course depicting the changes in area of a single rat airway (pictured in (A)) treated with methacholine (230 nM, 30 minutes), followed by formoterol (10 nM) in the continued presence of methacholine (30 minutes), and then washed. (C) Averaged dose-response curve of *maximum* formoterol-induced relaxation of rat airways preconstricted with methacholine (230 nM) fitted to the Hill equation (n=6).

relaxation, respectively, Figure 18A, C). When retigabine (10 μM) is included with the second application of formoterol, airways relax to a greater extent than the initial formoterol treatment (28 ± 6.7% and 58 ± 16.9%, respectively, Figure 18B, C). While retigabine's ability to enhance formoterol-induced airway relaxation is approaching significance, additional studies are required before any conclusions can be drawn.

Retigabine Relaxes OVA-sensitized Guinea Pig Airways Preconstricted with Methacholine.

Results thus far indicate that retigabine is an effective bronchodilator in normal rat and human airways, however it is necessary to determine if retigabine retains its bronchodilatory effects in asthmatic airways. Seeing as the availability of human tissue, particularly asthmatic tissue, can be limited, it is beneficial to have an animal model system that is comparable to human. The guinea pig model of allergic asthma is commonly used to observe and test airway hyperresponsiveness (Meurs, Gosens et al. 2008, McGovern and Mazzone 2014). PCLS prepared from guinea pigs are considered to be a more appropriate model than rat or mouse (Ressmeyer, Larsson et al. 2006) and the late asthmatic responses and immediate hypersensitivity to irritants following sensitization in guinea pigs are comparable to the human allergic response (Meurs, Gosens et al. 2008).

Our laboratory has adopted a guinea pig OVA-sensitization protocol that consistently induces asthma-like symptoms upon exposure to nebulized OVA. Briefly, male Hartley guinea pigs are given 3 intraperitoneal injections of OVA or saline (control) on days 1, 3 and 5. Following a 2 week sensitization period, the animals are exposed on 3 successive days to a fine mist of OVA for 30 minutes while being monitored for symptoms such as chewing, labored breathing, coughing, and gasping. Tissues are collected post-euthanasia for PCLS. OVA produces robust constriction of airways in PCLS from sensitized but not control

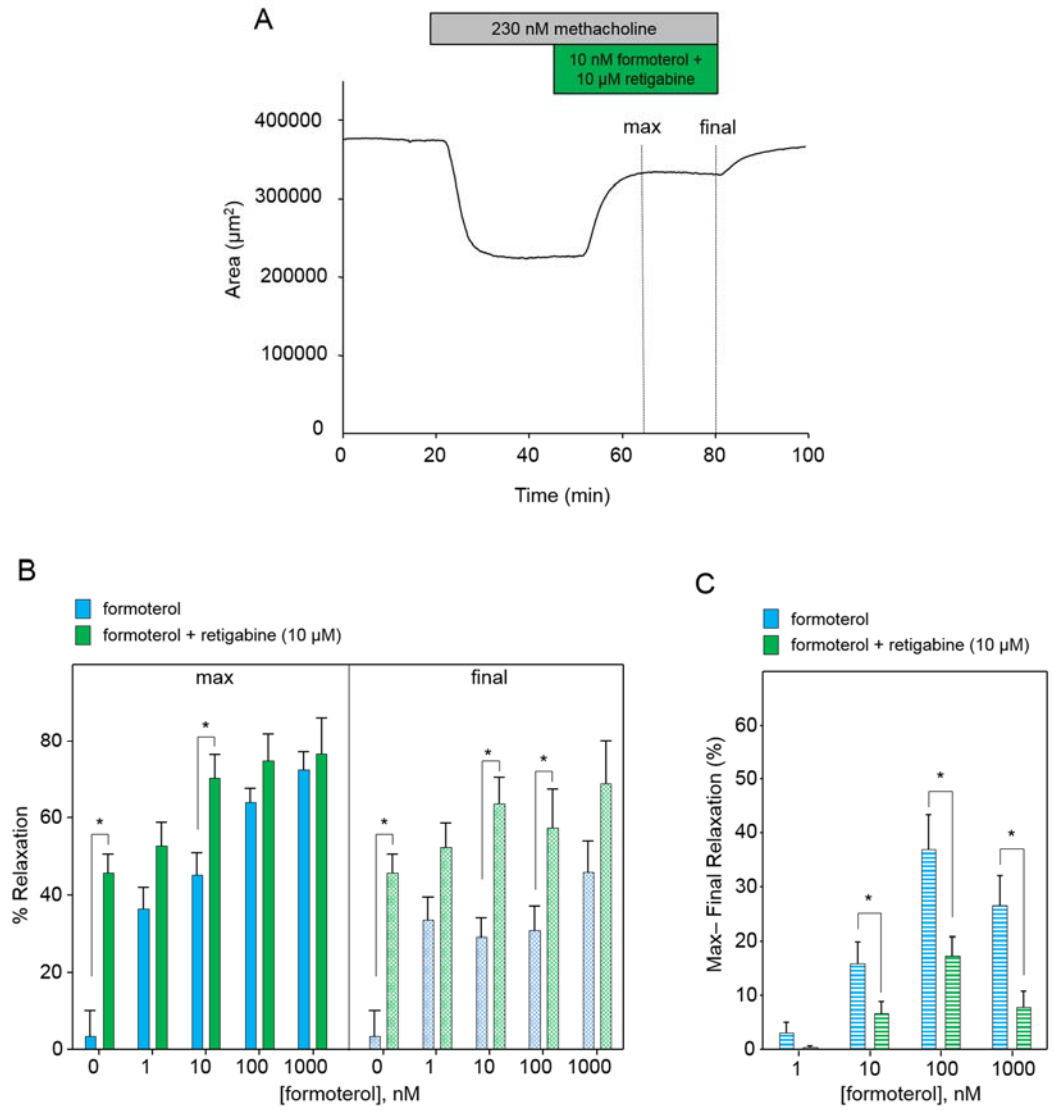

Figure 16. Retigabine enhances formoterol-induced relaxation of rat airways preconstricted with methacholine. (A) Representative time course depicting the changes in area of a single rat airway treated with methacholine (230 nM, 30 minutes), followed by formoterol (10 nM) plus retigabine (10 µM) in the continued presence of methacholine (30 minutes), and then washed. (B) Summarized bar graph depicting the maximum (solid bars) and final (checkered bars) amount of formoterol (blue bars) versus formoterol plus retigabine (10 µM, green bars) induced relaxation of rat airways preconstricted with methacholine (230 nM) (n=6). (C) Summarized bar graph depicting formoterol (blue striped bars) versus formoterol plus retigabine (10 µM, green striped bars) maximum minus final relaxation, or the total time-dependent decline in relaxation during the 30 minute treatment period. *, significant difference between indicated group, $p < 0.05$, student's t-test.

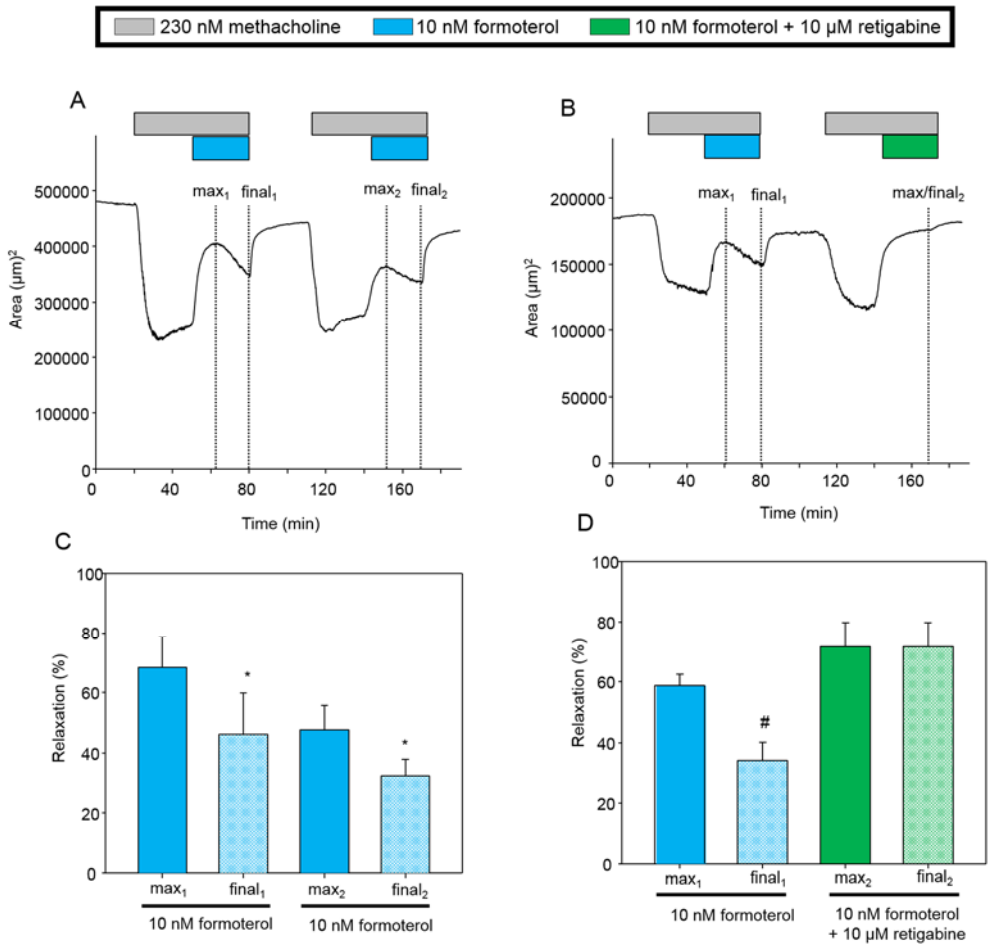

Figure 17. Retigabine attenuates the time-dependent decline in formoterol-induced relaxation of rat airways preconstricted with methacholine observed upon repetitive administration. (A) Representative time course depicting the changes in area of a single rat airway treated with methacholine (230 nM, 30 minutes), followed by formoterol (10 nM) in the continued presence of methacholine (30 minutes), with a 45 minute washout period between methacholine-induced constrictions. (B) Representative time course depicting the changes in area of a single rat airway treated with methacholine (230 nM, 30 minutes), followed by formoterol (10 nM) or formoterol (10 nM) plus retigabine (10 µM) in the continued presence of methacholine (30 minutes), with a 45 minute washout period between methacholine-induced constrictions. (C) Summarized bar graph depicting the average maximum and final relaxation of airways for each application of formoterol, from experiments depicted in *A* (n=6). (D) Summarized bar graph depicting the average maximum and final relaxation of airways for formoterol and formoterol plus retigabine, from experiments depicted in *B* (n=6). *, significant difference from max1, $p < 0.05$, one way repeated measures ANOVA. #, significant difference from max1, max2, and final2, $p < 0.05$ one way repeated measures ANOVA.

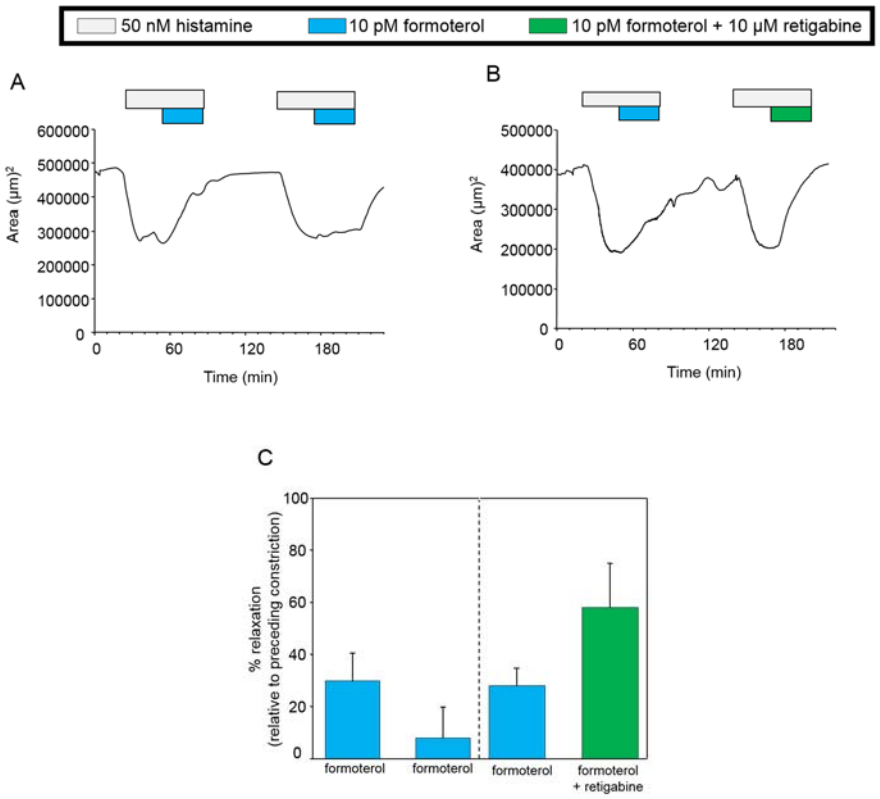

Figure 18. Retigabine appears to attenuate the time-dependent decline in formoterol-induced relaxation of human airways preconstricted with histamine observed upon repetitive administration. (A) Representative time course depicting the changes in area of a single human airway treated with histamine (50 nM, 30 minutes), followed by formoterol (10 pM) in the continued presence of histamine (30 minutes), with a 45 minute washout period between histamine-induced constrictions. (B) Representative time course depicting the changes in area of a single human airway treated with histamine (50 nM, 30 minutes), followed by formoterol (10 pM) or formoterol (10 pM) plus retigabine (10 µM) in the continued presence of histamine (30 minutes), with a 45 minute washout period between histamine-induced constrictions. (C) Summarized bar graph depicting the average repetitive formoterol induced relaxation or formoterol then formoterol plus retigabine induced relaxation of human airways (n=5).

animals and PCLS from OVA-sensitized animals show increased sensitivity to the bronchoconstrictor agonists methacholine and histamine (Kakad 2012).

100 nM methacholine (the approximate EC_{50} in control guinea pig airways, (Kakad 2012)) was used to constrict control guinea pig airways prior to treatment

with 30 μM retigabine (the concentration used in our asthmatic human PCLS experiment) in the presence of methacholine. Treatment with retigabine produced significant airway relaxation (32 ± 8.3%, Figure 19A, C). Retigabine vehicle did not significantly relax airways (Figure 19B, D). Similar results were observed for OVA-sensitized guinea pig airways (29 ± 5.8% relaxation, Figure 20).

Retigabine Attenuates OVA-induced Allergic Asthma Symptoms in OVA-Sensitized Guinea Pigs.

A set of pilot studies were completed to determine if retigabine was capable of attenuating the allergic asthma symptoms displayed by OVA-sensitized guinea pigs during the nebulized OVA challenges. When the unanaesthetized guinea pig was exposed to nebulized OVA alone, symptoms such as coughing and rapid, labored breathing, occurred within minutes. The animal was rescued from the chamber when the symptoms become too severe, approximately 15 minutes into the 30 minute challenge. Once removed from the chamber, the guinea pig was weak and lethargic, requiring a monitored recovery period. When retigabine (1 mM) was included with the nebulized OVA, the same guinea pig did not develop symptoms until approximately 18-22 minutes into the challenge and the symptoms were not nearly as severe as those observed with nebulized OVA alone. The animal withstood the entire 30 minute challenge and was alert once removed from the chamber. A parallel study was completed with control guinea pigs that showed no observable effects of either nebulized OVA or nebulized OVA plus retigabine.

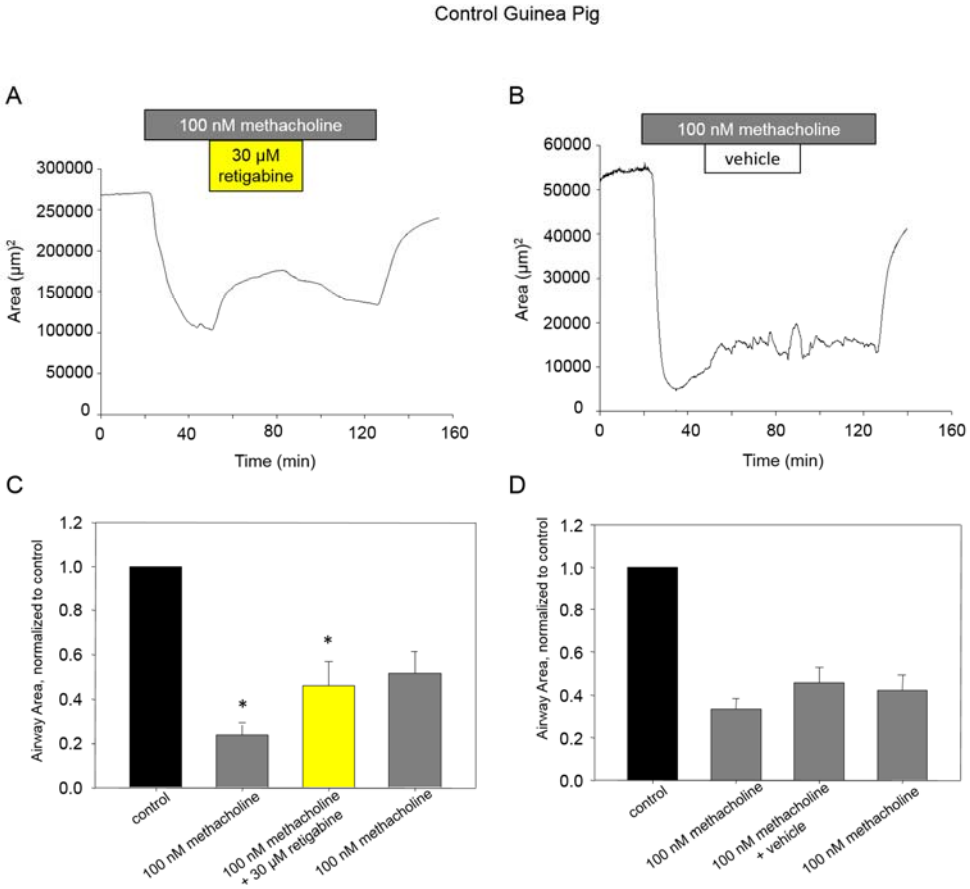

Figure 19. Retigabine relaxes control guinea pig airways preconstricted with methacholine. (A) Representative time course depicting the changes in area of a single guinea pig airway treated with methacholine (100 nM, 30 minutes), then retigabine (30 µM) in the continued presence of methacholine (45 minutes), followed by removal of retigabine (30 minutes) and washout. (B) Representative time course depicting the changes in area of a single guinea pig airway treated with methacholine (100 nM, 30 minutes), then retigabine vehicle in the continued presence of methacholine (45 minutes), followed by removal of retigabine vehicle (30 minutes) and washout. (C) Summarized bar graph depicting average airway area relative to control (black bar, average of 5 minutes before methacholine treatment) upon treatment with methacholine (100 nM, grey bar), methacholine and retigabine (30 µM, yellow bar), and removal of retigabine (grey bar) from experiments depicted in *A*. (D) Summarized bar graph depicting average airway area relative to control (black bar, average of 5 minutes before methacholine treatment) upon treatment with methacholine (100 nM, grey bar), methacholine and retigabine vehicle (grey bar), and removal of retigabine vehicle (grey bar) from experiments depicted in *B*. *, significant difference, $p < 0.05$, one way ANOVA followed by Holm-Sidak post hoc test, with multiple comparisons.

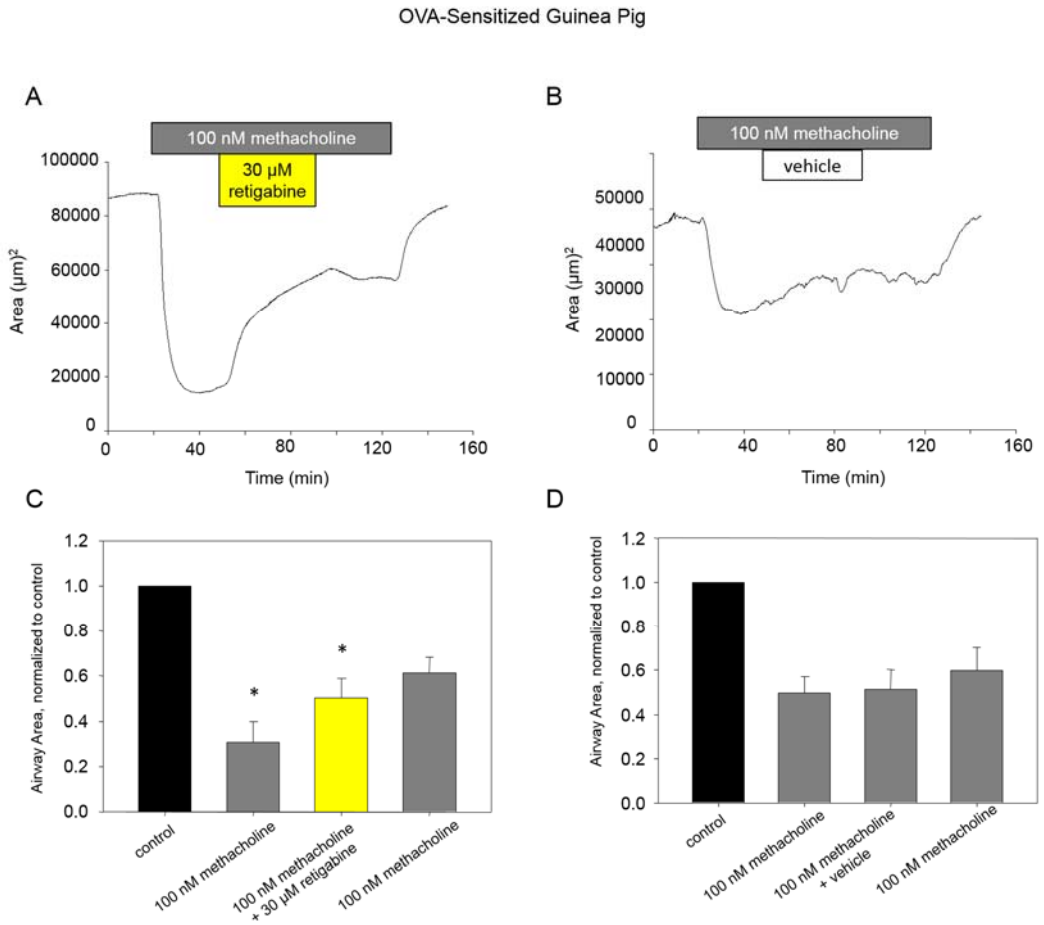

Figure 20. Retigabine relaxes OVA-sensitized guinea pig airways preconstricted with methacholine. (A) Representative time course depicting the changes in area of a single guinea pig airway treated with methacholine (100 nM, 30 minutes), then retigabine (30 µM) in the continued presence of methacholine (45 minutes), followed by removal of retigabine (30 minutes) and washout. (B) Representative time course depicting the changes in area of a single guinea pig airway treated with methacholine (100 nM, 30 minutes), then retigabine vehicle in the continued presence of methacholine (45 minutes), followed by removal of retigabine vehicle (30 minutes) and washout. (C) Summarized bar graph depicting average airway area relative to control (black bar, average of 5 minutes before methacholine treatment) upon treatment with methacholine (100 nM, grey bar), methacholine and retigabine (30 µM, yellow bar), and removal of retigabine (grey bar) from experiments depicted in *A*. (D) Summarized bar graph depicting average airway area relative to control (black bar, average of 5 minutes before methacholine treatment) upon treatment with methacholine (100 nM, grey bar), methacholine and retigabine vehicle (grey bar), and removal of retigabine vehicle (grey bar) from experiments depicted in *B*. *, significant difference, $p < 0.05$, one way ANOVA followed by Holm-Sidak post hoc test, with multiple comparisons.

Guinea Pig flexiVent Optimization Experiments and Troubleshooting.

The results from our PCLS experiments and the nebulized retigabine pilot studies suggest that activating Kv7 channels represents a novel approach to asthma therapy. Our next goal was to quantitatively examine the bronchodilatory effects of retigabine using an *in vivo* approach. Naïve guinea pigs were mechanically ventilated while under anesthesia and a variety of respiratory parameters, such as respiratory resistance, Newtonian resistance, tissue dampening, and tissue elastance, were measured using a small animal ventilator called a flexiVent controlled by flexiWare software. Once a stable baseline respiratory resistance value was obtained, the animals were given i.v. or nebulized histamine or methacholine at approximately 5 minute intervals. Following each administration of bronchoconstrictor, respiratory parameters were recorded using the series of “snapshot” and “quick prime-6” maneuvers during the 5 minute interval.

A variety of experimental approaches were attempted during this optimization study on naïve guinea pigs with the hopes to establish a protocol that could be used to determine if nebulized Kv7 channel activators were capable of attenuating the increase in respiratory resistance observed upon bronchoconstrictor agonist treatment. Each experiment typically began with establishing a dose-response for the i.v. or nebulized bronchoconstrictor. In some instances we would repeat the dose-response to examine the reproducibility in the same animal. The dose-responses were reproducible, however we occasionally

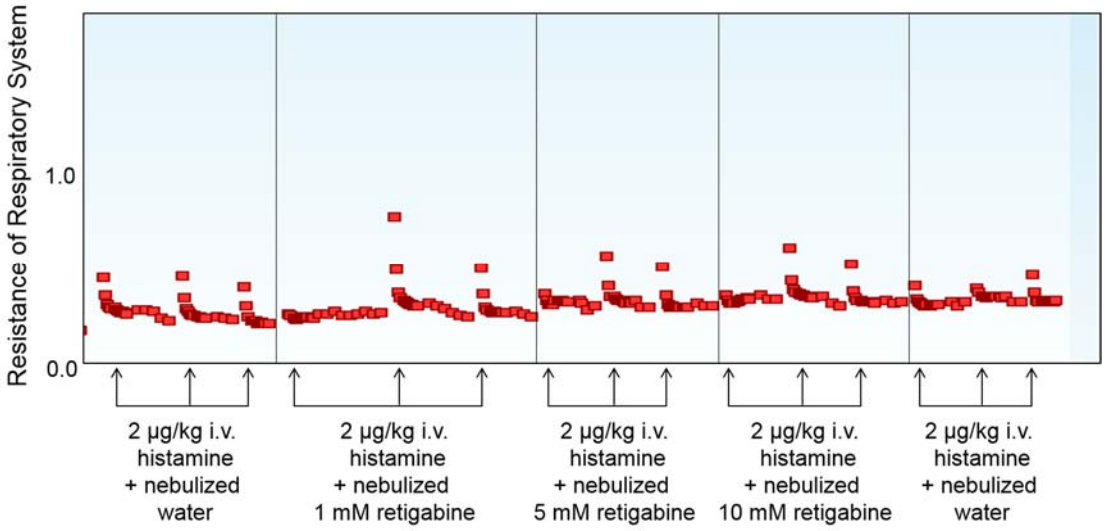

Figure 21. Repetitive intravenous histamine with increasing concentrations of nebulized retigabine. Representative experiment depicting respiratory resistance measured in an anesthetized and mechanically ventilated following the indicated treatments. Vehicle (water) or increasing concentrations of retigabine (1-10 mM) were nebulized prior to each intravenous injection of histamine (2 µg/kg). Respiratory resistance was determined using the flexiWare "snapshot" maneuver following histamine injection.

observed increased sensitivity to the bronchoconstrictor agonist following multiple applications. After establishing a dose-response, we selected an approximate EC_{50} dose and tested for reproducibility upon multiple administrations. The multiple i.v. approximate EC_{50} doses tended to be more reproducible than multiple nebulized approximate EC_{50} doses. The addition of nebulized Kv7 channel activator (1, 5, or 10 mM retigabine, 10 mM flupirtine, 1 or 10 mM ML213) with either i.v. or nebulized bronchoconstrictor did not have an appreciable effect on the increases in respiratory resistance. There was one instance where nebulized 1, 5, and 10 mM retigabine attenuated the increase in respiratory resistance observed during the first of three i.v. histamine approximate EC_{50} doses (Figure

21), however the second and third doses resulted in an increase in respiratory resistance comparable to histamine alone despite nebulizing retigabine prior to each i.v. administration. We were able to establish a positive control, 28 μM nebulized formoterol, which consistently attenuated the increased respiratory resistance observed following bronchoconstrictor agonist treatment.

We decided to try a different approach for the remainder of our approved optimization experiments. First, we would limit the nebulized bronchoconstrictor dose-response so that respiratory resistance only increased to 2-3 times over the initial baseline. This way we were not working with too high of a bronchoconstrictor concentration to observe any protective effects offered by the Kv7 channel activators. We also decided to add a nebulized Kv7 channel activator pretreatment period that attempted to mimic the extended exposure to retigabine during our pilot studies (see *Retigabine attenuates OVA-induced allergic asthma symptoms in guinea pigs*). Following the establishment of a dose-response, retigabine would be nebulized approximately every minute for an extended period of time (initially 30 minutes, but we decreased the time to 18 minutes to avoid potential fluid/obstruction buildup associated with prolonged nebulization), and this pretreatment period would also include deep breaths to ensure that retigabine was reaching the airways. A second dose-response would then be completed to determine if the Kv7 channel activator attenuated the observed increases in respiratory resistance. When the vehicle for retigabine, water, was nebulized during the pretreatment period, there was no effect observed on the respiratory

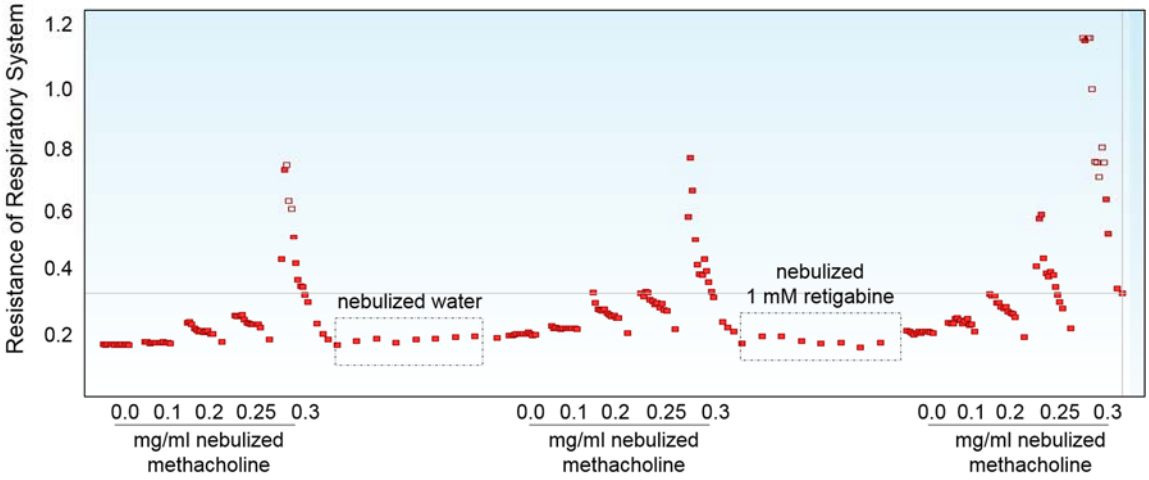

Figure 22. Repetitive nebulized methacholine dose response with nebulized retigabine pretreatment. Representative experiment depicting respiratory resistance measured in an anesthetized and mechanically ventilated following the indicated treatments. Vehicle (water) or retigabine (1 mM) was nebulized approximately every minute for 18 minutes in between treatment with increasing doses of nebulized methacholine (0.0-0.3 mg/ml). Respiratory resistance was determined using the flexiWare "snapshot" maneuver.

resistance dose-response. Unfortunately, we also did not observe any protective effects of retigabine following pretreatment (Figure 22). There was a single instance where pretreating with 10 mM retigabine and including 10 mM retigabine in the second methacholine dose-response appeared to attenuate the increase in respiratory resistance, however we were unable to repeat this result by the conclusion of our allotted optimization experiments. An interesting and consistent result was the lowering of respiratory resistance compared to the initial baseline respiratory resistance following the retigabine pretreatment period (Figure 23). Although these optimization experiments indicate that retigabine is not offering protection against bronchoconstrictor agonist-induced increases in respiratory resistance in these instances, there is some potentially beneficial effect of

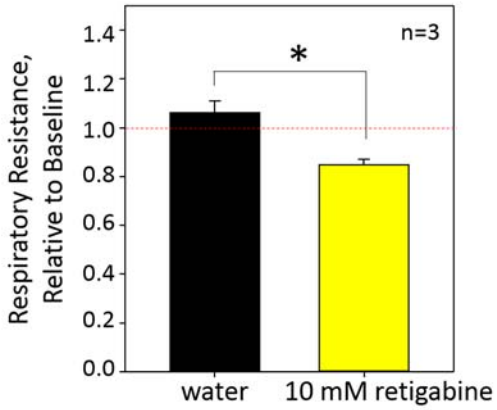

Figure 23. Nebulized retigabine lowers baseline respiratory resistance. Depiction of the average respiratory resistance values measured using the flexiWare "snapshot" maneuver during the vehicle (water) or retigabine (10 mM) "pretreatment" nebulizing period. Values were normalized to the initial baseline resistance for each animal. *, significant difference, $p < 0.05$, student's t-test.

nebulized retigabine. Additional studies to establish a protocol examining the therapeutic potential of Kv7 channel activators *in vivo* would need to be completed before any conclusions can be drawn.

CHAPTER SIX

DISCUSSION

Our previous examination of ASMCs revealed the presence of Kv7 K^+ currents that were inhibited upon treatment of the cells with bronchoconstrictor agonists. Inhibition of Kv7 channels with the specific blocker XE991 was sufficient to induce constriction of human airways, and this inhibition was reversed upon blocking L-type VSCCs with verapamil. Lastly, activation of Kv7 channels attenuated histamine-induced constriction of human airways (Brueggemann, Kakad et al. 2012). These results highlighted the importance of Kv7 channels in the regulation of airway smooth muscle tone, however additional studies would be necessary to clarify the specifics of this role and to examine the therapeutic potential of targeting Kv7 channels for bronchodilator therapy.

The work presented in this dissertation aimed to (1) elucidate the mechanism by which the bronchoconstrictor agonist histamine regulates Kv7 channels in airway smooth muscle and (2) determine the efficacy of Kv7 channel activators in attenuating agonist-induced bronchoconstriction and explore their therapeutic potential for the treatment of asthma. Experiments completed in parallel with this project outlined in this dissertation revealed that the bronchoconstrictor agonist histamine enhanced phosphorylation of endogenous Kv7.5 channels in freshly isolated human trachealis smooth muscle and inhibited endogenous Kv7 currents in cultured HTSMCs (Haick, Brueggemann et al. 2017).

Using low-passage cultured HTSMCs as an expression system, we determined that activation of PKC, both directly with PMA or indirectly with histamine, increased phosphorylation of hKv7.5 channels (Figure 7) and inhibited hKv7.5 currents (Figure 4). The effects of histamine on the phosphorylation status of the channels and on the inhibition of hKv7.5 currents occurred in a PKC-dependent manner, evidenced by an attenuation of hKv7.5 phosphorylation and current inhibition when PKC was inhibited prior to histamine treatment (Figure 7, 5). The regulation of hKv7.5 currents by histamine appears to be dependent on PKCα specifically (Figure 9), as well as a serine residue located at position 441 (Figure 12). Surprisingly the loss of PKCα or the mutation of serine 441 to alanine did not attenuate histamine-induced phosphorylation of hKv7.5 channels (Figure 10, 13) even though it prevented histamine-induced regulation of hKv7.5 currents. We also provide evidence that illustrated the ability of Kv7 channel activation to relax rat, guinea pig, and human airways that had been constricted with bronchoconstrictor agonists and highlight a promising and novel therapeutic approach to treat asthma that should continue to be investigated.

The suppression of Kv7 currents following G_q-coupled receptor activation has been extensively examined in neurons (Marrion 1997, Delmas and Brown 2005) and more recently in vascular smooth muscle (Mackie, Brueggemann et al. 2008, Mani, O'Dowd et al. 2013, Brueggemann, Mackie et al. 2014). As mentioned in chapter 2, PKC is one common effector of G_q-coupled agonist-induced inhibition of Kv7 currents, however the PKC-dependent nature of agonist-induced

suppression of Kv7 currents in airway smooth muscle has not been investigated until now. PKC is also an established regulator of ASMC contraction and airway constriction (Webb, Hirst et al. 2000). Evidence suggests that phosphorylation of a variety of substrates, particularly those involved in the regulation of a cell's contractile machinery (Pohl, Winder et al. 1997, Webb, Hirst et al. 2000, Sakai, Chiba et al. 2005), are responsible for the contractile effects of PKC activation. However, there is also a component of PKC-induced Ca^{2+} signaling in ASMCs and airway smooth muscle constriction that is dependent on extracellular Ca^{2+} and L-type VSCCs (Park and Rasmussen 1985, Knox, Baldwin et al. 1993, Rossetti, Savineau et al. 1995, Yang and Black 1996, Mukherjee, Trice et al. 2013) that cannot be explained by phosphorylation of the previously identified targets. Results presented in this dissertation have also highlighted the importance of L-type VSCCs in the sustained $[Ca^{2+}]_{cyt}$ elevation observed following treatment of HTSMCs with histamine (Figure 3). Evidence suggests that inhibition of Kv7 channels in vascular smooth muscle results in membrane depolarization that promotes the activation of L-type VSCCs (Mackie, Brueggemann et al. 2008). We have established that histamine inhibits Kv7 channels in ASMCs (Brueggemann, Kakad et al. 2012) and upon completion of this project have determined that this occurs in a PKC-dependent manner. We have also demonstrated that inhibition of Kv7 currents is sufficient to depolarize ASMC membranes (Haick, Brueggemann et al. 2017). Therefore, it is reasonable to predict that the L-type VSCC-dependent effects of PKC activation (increases in $[Ca^{2+}]_{cyt}$ and contraction of airway smooth

muscle may be occurring, at least in part, as a result of inhibition of Kv7 channels (Figure 24).

Multiple PKC isoforms have been found to be expressed in ASMCs (Webb, Lindsay et al. 1997, Sakai, Yamamoto et al. 2009), however the specific isoform(s) behind the PKC-dependent effects observed in airway smooth muscle have yet to be identified (Webb, Hirst et al. 2000). PKCα was a particularly promising candidate for the histamine-induced phosphorylation of hKv7.5 channels and inhibition of their currents. In vascular smooth muscle, histamine treatment resulted in redistribution of PKC activity from the cytosol to the plasma membrane (Haller, Smallwood et al. 1990) and PKCα specifically has been implicated in the phosphorylation of vascular smooth muscle contractile proteins and phorbol ester-induced constriction of rabbit femoral arteries (Eto, Kitazawa et al. 2001). Histamine treatment has also been demonstrated to induce PKCα translocation from cytosol to membranes in a HeLa cell expression system (Rimessi, Rizzuto et al. 2007) and in CHO-H1 cells (Megson, Walker et al. 2001). Indeed, PKCα was found to be necessary for histamine-induced inhibition of hKv7.5 currents in cultured HTSMCs (Figure 9). However, when we measured total phosphorylation with an anti-phosphoserine PKC substrate antibody, we still observed a significant increase in histamine-induced phosphorylation of hKv7.5 channels (Figure 10). Although this result was unexpected, all PKC isoforms except γ have been detected in cultured human ASMCs (Carlin, Yang et al. 1999) and it is possible that treatment of HTMSCs with histamine results in the activation of additional PKC

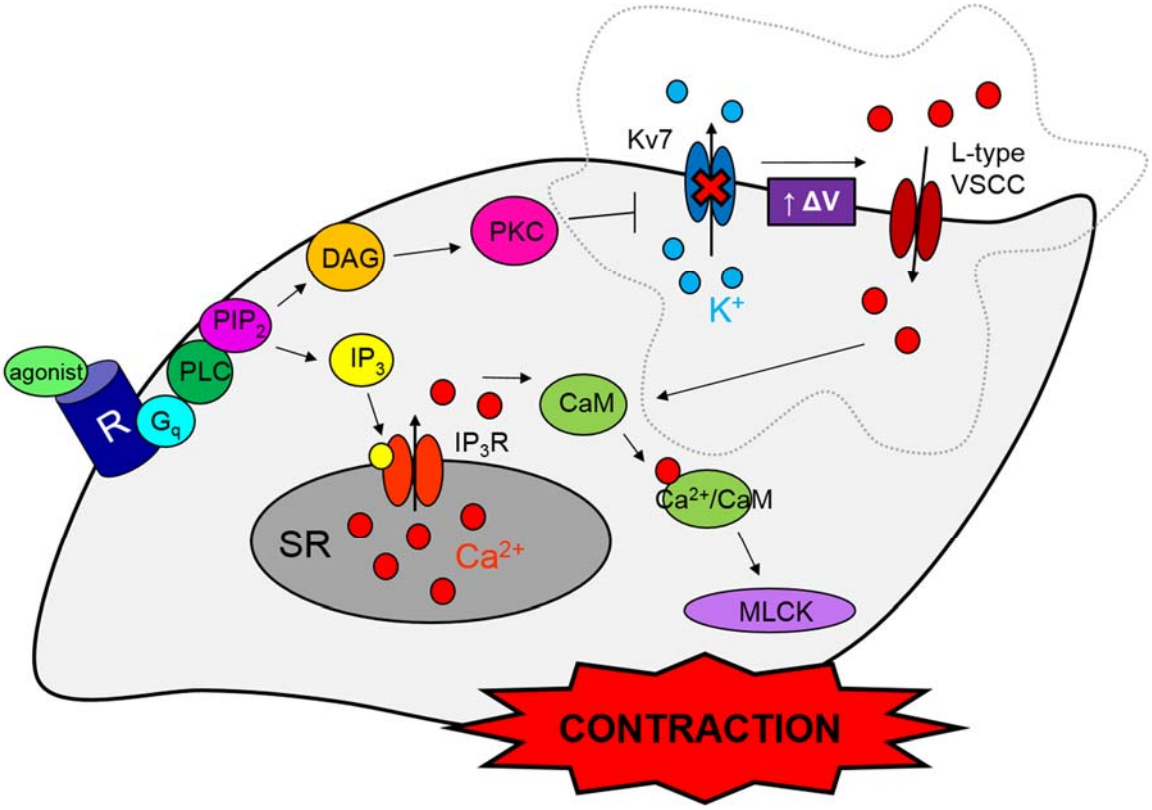

Figure 24. Proposed role of Kv7 channels and L-type VSCCs following G_q-coupled receptor signaling in ASMCs. ASMC contraction has a voltage-dependent component of Ca^{2+} influx necessary for sustained contraction evidenced to occur through L-type voltage sensitive Ca^{2+} channels (VSCC). We hypothesize that the stimulus for the membrane depolarization necessary for L-type VSCC activation occurs via PKC-dependent suppression of Kv7 currents, highlighted in grey. We hypothesize that the stimulus for the membrane depolarization necessary for L-type VSCC activation occurs via PKC-dependent suppression of Kv7 currents, highlighted in grey.

isoforms that phosphorylate hKv7.5 channels. It is important to note that PKCα specifically is necessary for histamine-induced suppression of hKv7.5 currents in HTSMCs and that any additional phosphorylation by other PKC isoforms is likely unrelated to *histamine*-induced effects on hKv7.5 currents.

Repeating the immunoprecipitation experiments with other PKC isoforms knocked down, either individually or in combination with PKCα, is one potential

area of exploration that may elucidate additional PKC isoforms that phosphorylate hKv7.5 channels following histamine treatment. Examining the residues on hKv7.5 that are phosphorylated in response histamine treatment of HTSMCs under normal conditions and comparing them to the same samples that lack PKCα, or other PKC isoforms, using mass spectrometry would also give important insight into not only which PKC isoforms phosphorylate hKv7.5 channels but also which amino acid residues are important in histamine-induced hKv7.5 regulation.

The work completed in this dissertation indicates that threonine residue 505, a residue that corresponds with rat Kv7.2 serine 534 that has been identified as a PKC phosphorylation site on that channel (Hoshi, Zhang et al. 2003), may also be required for histamine-induced suppression of hKv7.5 currents in ASMCs (Figure 11). Results suggest that histamine was still able to significantly suppress the mutant hKv7.5 currents. It appears that histamine suppresses hKv7.5 T505A channels to a lesser extent, however a significant difference was not observed. Our laboratory has observed decreased sensitivity of hKv7.5 T505A mutant channels to AVP-induced current suppression in A7r5 cells (LI Brueggemann, unpublished), therefore it is possible that mutation of this residue results in the decreased sensitivity of hKv7.5 currents to histamine as well.

Although the effect of T505 mutation was inconclusive, our results indicate that hKv7.5 residue serine 441 is absolutely necessary for histamine-induced suppression of hKv7.5 currents (Figure 12). This residue was selected due to its position in the recognition motif for the anti-phosphoserine antibody used in our

immunoprecipitation studies (-K/R-*X*-S-FILVY-K/R-). Mutation of S441 to non-phosphorylatable alanine abolished histamine's ability to inhibit hKv7.5 currents (Figure 12), but interestingly did not attenuate histamine-induced phosphorylation of hKv7.5 (Figure 13). This indicates that there are likely other hKv7.5 amino acid residues that are phosphorylated in response to treatment with histamine, however they do not appear to be necessary for histamine-induced regulation of hKv7.5 currents.

Serine 385 also matches the consensus motif recognized by the antibody and may contribute to the remaining signal detected in our immunoprecipitation studies when S441 was mutated to alanine. EndoQ digested mass spectrometry studies suggest that this may indeed be the case (Table 1), however the experiment must be repeated before any conclusions can be made considering the observation of S385 phosphorylation in all EndoK digested samples. One potentially interesting observation was the lack of S385 phosphorylation following histamine treatment of the EndoQ digested hKv7.5-T505A expressing HTSMCs. It would also be of interest to examine the levels of S441 phosphorylation in the hKv7.5-T505A mutant samples. Seeing as the T505A mutant channels may be less sensitive to regulation by histamine, perhaps sequential phosphorylation of amino acid residues (T505 then S385 and/or S441) is necessary for normal regulation of the channel by histamine.

Although the electrophysiology studies completed in this dissertation suggest that phosphorylation of S441 is necessary for histamine-induced Kv7.5

current suppression, confirmation of S441 phosphorylation, as well as identification of other amino acids that are phosphorylated following treatment with histamine, should be assessed by completing additional mass spectrometry studies. It is essential to find a protocol that will allow for segments containing S441 to be analyzed, considering that the EndoQ and EndoK sample digestions proved unsuccessful in that regard. Re-examining S385 is also of particular interest due to the preliminary mass spectrometry results and the fact that this residue is recognized by the antibody used in all of our previous studies.

The framework for elucidating the mechanism of histamine-induced regulation of hKv7.5 channels has been completed in the work presented in this dissertation. Confirming what we have learned thus far, as well as answering the questions that remain with the proposed experiments, will aid in establishing a clearer pathway of bronchoconstrictor agonist-induced regulation of hKv7.5 channels in HTSMCs.

The second portion of this project aimed to examine the ability of Kv7 channel activators to attenuate agonist-induced bronchoconstriction and begin to determine their therapeutic potential for the treatment of asthma. First we used PCLS to examine the bronchodilatory capabilities of the clinically available Kv7 channel activator retigabine and compared it to that of the LABA formoterol. Retigabine dose-dependently relaxed rat airways that were preconstricted with methacholine (Figure 14). Prolonged use of β_2-agonists has been reported to result in tachyphylaxis, or tolerance; it is believed to result from β_2-adrenergic receptor

desensitization and leads to decreased sensitivity to bronchodilator therapy and ultimately poor symptom management (Cazzola, Page et al. 2013). This phenomenon was exhibited in our rat PCLS model: while formoterol was capable of relaxing preconstricted rat airways, there was a time-dependent decline in relaxation observed during the 30 minute treatment period (Figure 15) that had not been observed with retigabine treatment alone. Enhanced relaxation was observed for some concentrations of formoterol upon inclusion of a submaximal dose of retigabine and the time-dependent decline observed over the 30 minute treatment period was significantly reduced when compared to formoterol treatment alone (Figure 16). We decided to take advantage of the time-dependent desensitization observed upon formoterol treatment in our rat PCLS model and found that consecutive applications of formoterol to airways that had been reproducibly constricted with methacholine resulted in a similar occurrence (Figure 17). When a submaximal concentration of retigabine was added to the second application of formoterol, there was no time-dependent decline in airway relaxation at the end of the 30 minute treatment period and the final airway relaxation was significantly greater than that achieved during the first application of formoterol alone (Figure 17).

The effects observed combining retigabine and formoterol were not additive. One mechanism of airway relaxation following G_s-coupled receptor stimulation with LABAs occurs through the activation of BK channels and subsequent hyperpolarization of ASMC membranes. Activation of Kv7 channels is

also expected to induce airway relaxation via membrane hyperpolarization. These two mechanisms may be somewhat redundant under normal conditions, however Kv7-channel induced membrane depolarization appears to substitute for the loss of BK-channel induced membrane hyperpolarization following the G_s-coupled receptor desensitization that occurs following prolonged β_2-agonist treatment. Combination bronchodilator therapies, specifically a LABA combined with a muscarinic antagonist, have been associated with several benefits (Cazzola, Page et al. 2013). The results observed in rat and human airways highlight the potential of Kv7 channel activators, retigabine in particular, to act as a bronchodilator, either alone or in combination with currently available bronchodilator therapy.

Aside from a single experiment examining the ability of retigabine to enhance formoterol induced bronchodilation in a manner similar to figure 17 using PCLS prepared from asthmatic tissue, little has been done to confirm the ability of Kv7 channel activators to relax airways in as asthmatic state. The availability of human tissue, especially asthmatic human tissue, is quite limited, therefore it was important for us to adopt an animal model of allergic asthma. The guinea pig model of allergic asthma is commonly used to observe and test AHR (Meurs, Gosens et al. 2008, McGovern and Mazzone 2014). PCLS prepared from guinea pigs are often considered to be a more appropriate model than rat or mouse (Ressmeyer, Larsson et al. 2006) and the late asthmatic responses and immediate hypersensitivity to irritants following sensitization in guinea pigs are comparable to the human allergic response (Meurs, Gosens et al. 2008).

The OVA-sensitization guinea pig model we adopted yields PCLS from OVA-sensitized animals that are more sensitive to the bronchoconstrictor agonists methacholine and histamine, a classic manifestation of AHR, when compared to PCLS from control animals (Kakad 2012). It is also interesting to note a downregulation of Kv7 channel expression that was observed when comparing sensitized to control guinea pig ASMCs (Kakad 2012). Our initial studies using guinea pig PCLS were largely successful, however recent attempts to examine the ability of retigabine to relax airways preconstricted with methacholine presented more of a technical challenge. Despite the presence of the β_2-agonist isoproterenol in many of the isolation solutions and media, airways often constricted following PCLS preparation, likely due to the substance P-induced postmortem constriction that occurs in guinea pig airways (Lai, Lamm et al. 1984, Lai and Cornett 1987). We also observed considerable variation in the sensitivity of the guinea pig airways to methacholine. Some airways would not respond while other airways from the same preparation, or even the same lobe, would completely occlude. Our previous experiences with PCLS preparations from rat or human tissues were more consistent and predictable.

Despite these difficulties, a set of experiments comparing the ability of retigabine to relax guinea pig airways preconstricted with methacholine in control and OVA-sensitized tissues were completed (Figure 19, 20). Retigabine relaxed airways to a similar extent (approximately 30%) in both control and sensitized tissues. This occurred despite the decrease in Kv7 channel expression in

sensitized tissues (Kakad 2012), implying that targeting the remaining Kv7 channels with retigabine is sufficient to induce airway relaxation. These results, combined with the study completed in asthmatic human tissue, are indicative of the ability of retigabine to relax diseased airways and provide additional support for Kv7 channels to act as therapeutic targets for the treatment of asthma.

Our *in vitro* results were promising and warranted further investigation. The guinea pig OVA-model allows us to test the effectiveness of retigabine as a bronchodilator *in vivo* as well. An initial pilot study examined the ability of retigabine to attenuate the asthma-like symptoms observed during nebulized OVA challenges with OVA-sensitized guinea pigs. The results provided the first evidence of the *in vivo* potential of Kv7 channel activators as novel asthma therapeutics. The presence of retigabine along with the nebulized OVA offered such substantial protection that only minor symptoms were observed towards the end of the 30 minute challenge period instead of the standard 7-8 minutes into the challenge observed with nebulized OVA alone. The OVA-sensitized guinea pig also lasted the entire 30 minutes of the challenge without having to be rescued from the chamber, as opposed to approximately 15 minutes typically observed with nebulized OVA alone. Upon removal from the chamber, the guinea pig was alert and active and did not require any monitored recovery time.

These results were encouraging, but qualitative in nature. In order to quantitatively measure the bronchodilatory capabilities of retigabine *in vivo*, we sought to develop a protocol using a flexiVent computer-controlled small animal

ventilator. Our initial plan was to test if nebulized retigabine offered protection against the increases in respiratory resistance observed following bronchoconstrictor agonist treatment. In our first flexiVent optimization experiments, retigabine was nebulized directly prior to either nebulized or i.v. administration of bronchoconstrictor. There was only one instance where nebulized retigabine appeared to offer protection from i.v. histamine-induced increases in respiratory resistance (Figure 21), however this result was not reproducible, even in the same experiment. Similarly, retigabine did not offer protection from nebulized bronchoconstrictor challenge when nebulized prior to or in combination with the bronchoconstrictor.

After our initial inconclusive results observed during the first portion of our optimization studies, we decided to adjust our protocol. First, we limited our bronchoconstrictor challenges to concentrations that induced an increase in respiratory resistance of only 2-3 times the initial baseline value. This would prevent us from using an agonist concentration that is too high and prevent unnecessary stress on the animal and its respiratory system. We also wanted to determine if keeping the bronchoconstrictor doses low would offer an improved chance of observing an attenuation of respiratory resistance increases following retigabine treatment. We also attempted to mimic the pilot studies as closely as possible. Our pilot study delivered a continuous mist of 1 mM retigabine in the presence of OVA to a consciously breathing animal. Unfortunately, the flexiVent is limited in that a continuous mist of retigabine is not possible. However, a

“pretreatment” regimen was developed in an attempt to deliver more retigabine to the airways. This “pretreatment” consisted of nebulizing vehicle (water) or retigabine approximately every minute for 18 minutes in between the nebulized bronchoconstrictor dose-response protocols. We also included deep breathes following each nebulization of retigabine in order to ensure that it was being delivered to the airways.

Despite the changes made to the protocol, we did not observe protection against bronchoconstrictor-induced increases in respiratory resistance provided by nebulizing retigabine (Figure 22). In some instances nebulizing retigabine appeared to increase the sensitivity to bronchoconstrictor challenge, however this may be a result of the repetitive administration of the bronchoconstrictor and not necessarily due to the retigabine treatment. Another consideration is that both methacholine and histamine result in an increase in mucus secretion (Boat and Kleinerman 1975, Webber and Widdicombe 1987). This mucus secretion may result in airflow obstruction that is not always detectable using our current methods. In some instances the sinusoidal waveform produced during the “snapshot” maneuver may indicate the presence of an obstruction, however little can be done to remove that obstruction once it occurs. A different approach minimalizing bronchoconstrictor treatment may be considered to avoid this potential side effect, but we intended to use each animal as its own control for consecutive dose-response challenges for these particular optimization experiments.

Although we did not observe consistent retigabine-induced protection during bronchoconstrictor challenges, nebulizing retigabine during the "pretreatment" period did lower respiratory resistance levels below the initial baseline levels recorded prior to the first bronchoconstrictor challenge (Figure 23). This suggests that retigabine is being delivered to the airways and is having some bronchodilatory effect, however the therapeutic relevance of this effect remains to be determined.

Overall, our results indicate that activation of Kv7 channels represents a novel approach to bronchodilation *in vitro*, however developing an *in vivo* model to test the effectiveness of retigabine at relieving bronchoconstriction, particularly in asthmatic airways, is essential for the continuation of this project. One thing that may be important to note is that the initial optimization flexiVent studies utilized naïve guinea pigs; no OVA-sensitized guinea pigs were tested. It would be interesting to asses if retigabine offers protection to OVA-sensitized guinea pigs, either from their hypersensitivity to bronchoconstrictor agonists or from OVA-induced bronchoconstriction. It is possible that retigabine may be effective at attenuating *exaggerated* responses to bronchoconstrictor agonists that are typically associated with this animal model (Kakad 2012). Our *in vivo* pilot study demonstrated the ability of retigabine to attenuate OVA-induced allergic responses typically observed in the unanesthetized OVA-sensitized guinea pigs. Perhaps retigabine would be effective at relieving the bronchoconstriction associated with an asthmatic response that is associated with a specific allergen, as our pilot study

suggests. The anesthetic used may also be interfering with our flexiVent studies. However, our pilot studies completed in conscious animals are more representative of the desired delivery method for retigabine (conscious, inhalation) and suggest the effectiveness of this drug at alleviating the symptoms associated with allergic asthma.

The difficulties experienced with the guinea pig model imply that it may be beneficial to adopt a new model of allergic asthma to determine if Kv7 channel activators are efficacious bronchodilators. There are a number of different rat models that can be used to study asthma (Kucharewicz, Bodzenta-Lukaszyk et al. 2008). Although Sprague Dawley rats, the species of rat used in our laboratory for PCLS experiments, do not develop an allergic reaction to OVA (Olivenstein, Renzi et al. 1993, Sirois and Bissonnette 2001), they can be used as a control group for Brown Norway OVA-sensitized rats (Careau and Bissonnette 2004). This would allow for a comparison of the bronchodilator effects of Kv7 channel activators to be compared more thoroughly in sensitized and non-sensitized rat airways. The difficulty we recently experienced using guinea pigs and guinea pig tissue, even in non-sensitized animals, only served to highlight the ease and reproducibility we observed when using rat tissue for previous studies. It would be interesting to see if this ease of use extends to sensitized rats and their tissue. There are also a variety of well-established mouse models (Nials and Uddin 2008, Shin, Takeda et al. 2009) that can be used to examine the therapeutic potential of Kv7 channel

activators that should be considered for addressing the potential of moving retigabine to clinical trials.

In summary, the bronchoconstrictor agonist histamine induces PKC-dependent hKv7.5 channel phosphorylation and subsequent inhibition of hKv7.5 currents in cultured human trachealis smooth muscle cells. PKCα appears to be necessary for current suppression by the bronchoconstrictor as well as partially necessary for sustained elevation of $[Ca^{2+}]_{cyt}$ following treatment of HTSMCs with histamine. However, our biochemical analysis of phosphorylation of hKv7.5 by PKC indicates that additional PKC isoforms may also be phosphorylating the channel. Similarly, serine residue 441 appears to be necessary for histamine-induced hKv7.5 current inhibition but our phosphorylation studies indicate that additional amino acid residues are also phosphorylated following histamine treatment, though these residues may not have an impact on the channel's function. These findings, along with the other work presented in this dissertation, help to shed light on the ionic mechanisms involved in the regulation of airway smooth muscle contractility.

The bronchodilatory capacity of Kv7 channel activators was also explored. The clinically available Kv7 channel activator retigabine relaxed rat, guinea pig, and human airways *in vitro*, however an *in vivo* allergic asthma animal model that can be used to examine the therapeutic potential of retigabine to relax airway smooth muscle has yet to be developed. The work presented in this dissertation

has created a solid foundation for future studies that explore the potential of Kv7 channel activators as novel asthma therapeutics.

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VITA

Jennifer Haick was born in Elmhurst, Illinois on January 25, 1988 to Craig Haick and Patti Morson. She attended Benedictine University where she earned a Bachelor's of Science, *cum laude*, in Biology in May 2011. After graduation, Jennifer matriculated into the Loyola University Chicago Stritch School of Medicine Molecular Pharmacology and Therapeutics Graduate Program and began her graduate education under the mentorship of Dr. Kenneth Byron.

While at Loyola, Jennifer served on the Graduate Student Council from 2012-2015, including as the Graduate Student Council-Health Science Division Representative on the executive board from 2014-2015. She also served on the Graduate Student Advisory Council and as the Pharmacology Department Student-Faculty Representative from 2014-2015.

Jennifer's dissertation work examining Kv7 potassium channels in airway smooth muscle was supported in part by the Arthur J. Schmitt Dissertation Fellowship. Ms. Haick has also received travel awards from the American Physiological Society and the American Society for Pharmacology and Experimental Therapeutics to present her research findings at national and international scientific symposia.

Following graduation, Jennifer will begin a postdoctoral research position at Loyola University Chicago with Dr. Saverio Gentile where she will continue to study ion channels, but in the context of ovarian and breast cancers.