**LOYOLA UNIVERSITY CHICAGO**

# Kv7 $K^+$ CHANNELS IN AIRWAY SMOOTH MUSCLE CELLS AS TARGET FOR ASTHMA THERAPY

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**BY**

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*Priyanka*

*To my wonderful family for*
*all their love, care and support*

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| | |
|---|---|
| Ach: | Acetycholine |
| AHR: | Airway Hyperresponsiveness |
| AKAP: | A-Kinase Anchoring Protein |
| ASMCs: | Airway Smooth Muscle Cells |
| AVP: | Arginine-(8)-Vasopressin |
| $Ca^{+2}$: | Calcium ion |
| $[Ca^{+2}]_{cyt}$: | Cytosolic calcium concentration |
| CaM | Calmodulin |
| COX-2: | Cyclooxygenase-2 |
| DAG: | Diacylglycerol |
| DMEM: | Dulbecco's Modified Eagle Medium |
| $EC_{50}$: | Half-Maximal Effective Concentration |
| $E_{max}$: | Maximal Effect in dose-response curve |
| $Gd^{3+}$: | Gadolinium |
| Gq: | Class of guanine-nucleotide binding protein that activates phospholipase C |
| GPCR: | G Protein Coupled Receptor |
| HBSS: | Hank's Balanced Salt Solution |
| i.p: | Intra-Peritoneal |

| | |
|---|---|
| $IP_3$: | Inositol-1,4,5-Triphosphate |
| ITS: | Insulin-Transferrin-Selenium |
| $K^+$: | Potassium ions |
| $K_{ATP}$: | ATP sensitive potassium channels |
| KCNQ: | Gene that encodes Kv7 channel proteins |
| Kv: | Voltage sensitive potassium channels |
| Kv7: | Class of voltage-gated potassium channels |
| LTD4: | Leukotriene D4 |
| L-type VSCC: | Long-Lasting Voltage-Sensitive Calcium Channels |
| MLCK: | Myosin Light Chain Kinase |
| mRNA: | Messenger RNA |
| $Na^+$: | Sodium ions |
| NSAID: | Non-Steroidal Anti-Inflammatory Drugs |
| OVA: | Ovalbumin |
| PCLS: | Precision Cut Lung Slices |
| PIP2: | Phosphatidylinositol-4,5-Bisphosphate |
| PKC: | Protein Kinase C |
| PLC: | Phospholipase C |
| PSS: | Physiological Saline Solution |
| ROCC: | Receptor Operated Calcium Channels |
| qRT-PCR: | Quantitative Real Time Polymerase Chain Reaction |
| SOCC: | Store Operated Calcium Channels |
| SR: | Sarcoplasmic Reticulum |

| | |
|---|---|
| TRP: | Transient Receptor Potential Channel Family |
| $E_m$: | Membrane voltage or membrane potential |
| VSCC: | Voltage-Sensitive Calcium Channels |
| VSMCs: | Vascular Smooth Muscle Cells |
| WHO: | World Health Organization |

**ABSTRACT**

Asthma continues to be one of the major global health problems with the current therapies being effective yet inadequate in controlling the disease. Asthma is commonly associated with airway hyperresponsiveness (AHR) involving hypercontraction of airway smooth muscle cells (ASMCs). Despite extensive research, the molecular mechanisms of AHR are poorly understood, thus preventing development of specific and effective therapies in the treatment of asthma. The overarching goal of the research presented in this thesis is to elucidate the mechanisms underlying AHR in asthma. This will not only help us gain a better insight into the pathophysiology of asthma, but may further lead to the discovery of novel and improved therapeutic agents. Our research specifically focuses on investigating the role played by Kv7 voltage-activated potassium channels in the pathophysiology of AHR, and evaluating the significance of Kv7 channel regulation in the treatment of asthma.

Kv7 channel expression in various excitable cells and their functional role in maintaining the cell membrane in a hyperpolarized state have been studied in great detail. However, their expression and function in ASMCs was largely unknown. Through the research presented in this thesis, we provide the first evidence for the expression of multiple Kv7 channel subtypes in guinea pig and human ASMCs using qRT-PCR studies. Our further investigation of expression of Kv7 channels in

allergen-sensitized guinea pig ASMCs reveal a trend indicating a decrease in the expression of all KCNQ (genes encoding Kv7 channels) isoforms in allergen-sensitized guinea pig airways compared to the saline-treated control animals. This decreased Kv7 channel expression would be expected to cause greater membrane depolarization and increased contractility of airways to various stimuli, common feature of AHR. To investigate the function of Kv7 channels in ASMCs we further conducted *ex vivo* studies using precision-cut lung slices (PCLS). Consistent with the existing scientific literature, our PCLS demonstrate that the guinea pig airways become hypersensitive to bronchoconstrictor agonists post-bronchial provocation, with an increase in potency and magnitude of constriction in response to bronchoconstrictor agonist treatment. Importantly, our studies also reveal that this increased, hypersensitive response of allergen-sensitized airways to bronchoconstrictor agonists can be attenuated by treatment with the clinically used Kv7 channel activator, retigabine. This protective effect of a Kv7 channel activator is likely due to an increase in activity of remaining Kv7 channels in allergen-sensitized airways, thereby improving control on membrane polarization.

In summary, our observations reported in this thesis provide new insights into how Kv7 channel expression and function in airways play a vital role in regulating airway diameter. Importantly, the attenuation of bronchoconstrictor agoinst-mediated airway constriction by a clinically used Kv7 channel activator in allergen-sensitized airways as observed in our studies provides a promising new strategy for treatment of AHR in asthma or other airway diseases.

# CHAPTER 1

## PATHOPHYSIOLOGY OF ASTHMA

Asthma continues to be one of the major global health problems. According to a recent update from the World Health Organization (WHO), approximately 235 million people in the world currently suffer from asthma (WHO fact sheet, 2011). A recent national health statistics report indicates that the prevalence of asthma is on the rise in the United States with 24.6 million people (8.2% of American population) currently diagnosed with this disease (Akinbami et al. 2011). Asthma is also the most common chronic disease afflicting children with its prevalence in children being higher than that in adults.

The current therapy for asthma, constituting mainly the inhaled corticosteroids and the long-acting inhaled β2-agonists, is effective and relatively inexpensive, but also has a number of limitations. Primarily, it is a general strategy targeted toward controlling and containing asthma by suppressing its symptoms. A significant variability in patients' responses to the current therapies has been reported, especially in the case of inhaled corticosteroids (Szefler et al. 2010). Severe cases of asthma accounting for approximately 10 % of the patient population are mostly untreatable with the current lines of treatment. Hence the quest for newer, more specific and curative therapies for asthma still continues. These therapies are likely to emerge from novel drug classes that act on biological targets

specific to asthma. Such specific and druggable asthma targets can only be identified *via* in-depth understanding of the pathophysiology of this heterogeneous and complex disease. Tremendous efforts are currently underway to discover complex molecular pathways that underlie asthma symptoms (Holgate et al. 2011), however, the pathophysiology of asthma still remains poorly understood.

According to current understanding, the three fundamental components in pathogenesis of asthma are airway inflammation, airway remodeling and airway hyperresponsiveness (AHR), which together lead to a progressive decline of normal lung function (Bousquet J et al. 2000). AHR is defined as excessive contraction of airway smooth muscle cells (ASMCs) in response to a variety of stimuli. A number of different pharmacological, chemical and physical stimuli lead to such an exaggerated response. However, the molecular mechanisms underlying AHR remain unclear. Elucidating the molecular mechanisms of AHR will undoubtedly provide better insight into this intricate process while simultaneously uncovering novel therapeutic targets for improved treatments in asthma.

**Regulation of calcium signaling in ASMCs**

It is well known that intracellular calcium regulation plays a central role in ASMCs contraction (Armani Y et al. 2002). An increase in the cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) triggers bronchoconstriction. Various bronchoconstrictor agonists like acetylcholine (ACh), leukotriene D4 (LTD4), endothelin and histamine activate the $G_q/_{11}$-coupled receptors on ASMCs leading to an increase in $[Ca^{2+}]_{cyt}$ (Penn et al. 2008). The local concentrations of these bronchoconstrictor agonists have been found to elevate during ASMC hypercontraction (Belmonte 2005; Smith

et al. 2005; Hamid Q et al. 2009). Studies have shown that the exposure of ASMCs to these bronchoconstrictor agonists results in a biphasic elevation of $[Ca^{2+}]_{cyt}$ (Hirota et al. 2007; Jude et al. 2008) (**Figure 1**). The initial rapid or transient phase of $[Ca^{2+}]_{cyt}$ elevation is mediated by release of calcium from inositol-1,4,5-triphosphate ($IP_3$)-sensitive intracellular calcium stores also called sarcoplasmic reticulum (SR). This transient increase in $[Ca^{2+}]_{cyt}$ is usually followed by a sustained elevation of $[Ca^{2+}]_{cyt}$ that is maintained by influx of $Ca^{2+}$ across the plasma membrane from the extracellular space. This sustained elevation of $Ca^{2+}$ plays an important role in prolonging ASMC contraction. Influx of $Ca^{2+}$ is believed to occur *via* several ion channels on the surface of ASMCs. There are differences in opinion about the contribution of various $Ca^{2+}$ ion channels on ASMCs in agonist-mediated $Ca^{2+}$ influx (Janssen et al. 2002). In general, two mechanisms being debated are the voltage-independent calcium influx and voltage-dependent $Ca^{2+}$ influx (Hirota et al. 2007). The voltage-independent calcium influx is proposed to happen *via* store-operated calcium channels (SOCC), receptor-operated calcium channels (ROCC) and/or non-selective cation channels belonging to the Transient Receptor Potential (TRP) channel family. The influx of $Ca^{2+}$ through SOCC can be triggered by the emptying of internal $Ca^{2+}$ stores (SR) (Ay et al. 2004), while the ROCC can be directly activated by the diacylglycerol (DAG) produced as a result of agonist-mediated activation of membrane receptors (Murray et al. 1993). The TRP family of channels have been shown to be involved in $Ca^{2+}$ influx in other types of smooth muscle cells. A similar role was proposed for them in ASMCs after they were found to be expressed in human ASMCs (Ong et al. 2004; Gosling et al. 2005; Perez-Zoghbi et al. 2009).

Voltage-dependent calcium influx in ASMCs is proposed to proceed *via* voltage sensitive calcium channels (VSCC), similar to the process in other smooth muscle cells (Perez-Zoghbi et al. 2009). There is evidence, both in support and against significant contributions of these different channel subtypes to the sustained phase elevation of $Ca^{2+}$.

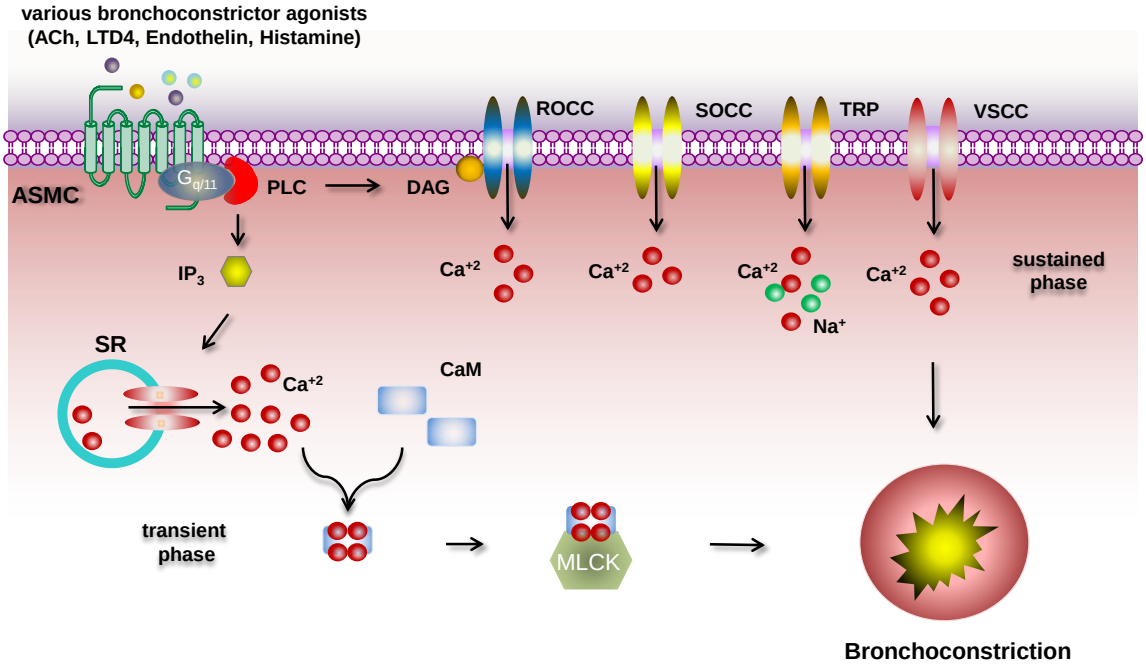


**Figure 1: Regulation of calcium signaling in Airway Smooth Muscle Cells (ASMCs).** Bronchoconstrictor agonists bind to their receptors on ASMCs leading to activation of phospholipase C (PLC) followed by synthesis of inositol-1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG) from membrane lipids. $IP_3$ in turn stimulates the release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR) followed by repetitive release and uptake of $Ca^{2+}$ resulting in $Ca^{2+}$ oscillations. Calcium influx across the plasma membrane (sustained phase) may then occur *via* a number of $Ca^{2+}$ channels like Store Operated Calcium Channels (SOCC), Receptor-operated calcium channels (ROCC), Transient Receptor Potential (TRP) family of channels and voltage sensitive calcium channels (VSCC). Elevated $ca^{2+}$ by these two mechanisms binds to calmodulin (CaM), thereby activating myosin light chain kinase (MLCK), which phosphorylates MLC and initiates actomyosin cross-bridge cycling thus leading to bronchoconstriction. The individual contribution of these channels in $Ca^{2+}$ influx during the sustained phase is still under debate.

**$Ca^{2+}$ influx *via* VSCC on ASMCs**

VSCCs open (activate) in response to membrane depolarization. VSCCs on ASMCs have threshold for steep voltage-dependent activation ranging from -45 to -25 mV, with maximal activation at 0 to +20 mV (**Figure 2**). They are known to be blocked by dihydropyridine based compounds (Hirota et al. 2007). All these properties resemble the characteristic properties of "L-type" or long-lasting voltage-sensitive calcium channels. The resting membrane potential ($E_m$) of ASMCs ranges from -60 to -45 mV (Liu et al. 2005), so it is unlikely for L-type VSCC to be appreciably active at the resting state of the ASMC membrane. However, membrane depolarization is likely to result in activation of L-type VSCC and influx of $Ca^{2+}$. In fact, many bronchoconstrictor agonists depolarize the ASMC membrane to potentials positive to the L-type VSCC activation threshold (Hirota et al. 2007). Therefore, L-type VSCC are likely to be the key contributors toward the influx of $Ca^{2+}$ from the extracellular space into the cytoplasm, eventually leading to bronchoconstriction. There is ample evidence in the literature supporting involvement of L-type VSCC in elevating $[Ca^{2+}]_{cyt}$ (Jude et al. 2008; Perez-Zoghbi et al. 2009), particularly at low agonist concentrations (Barnes et al. 1985). A number of studies have also shown that agonist-induced $Ca^{2+}$ signaling in ASMCs is attenuated by L-type $Ca^{2+}$ channel blockers (Solway et al. 1985; Thirstrup et al. 1997; Hirota et al. 2003) emphasizing the role played by L-type VSCC in the calcium influx during the sustained phase of agonist induced airway constriction.

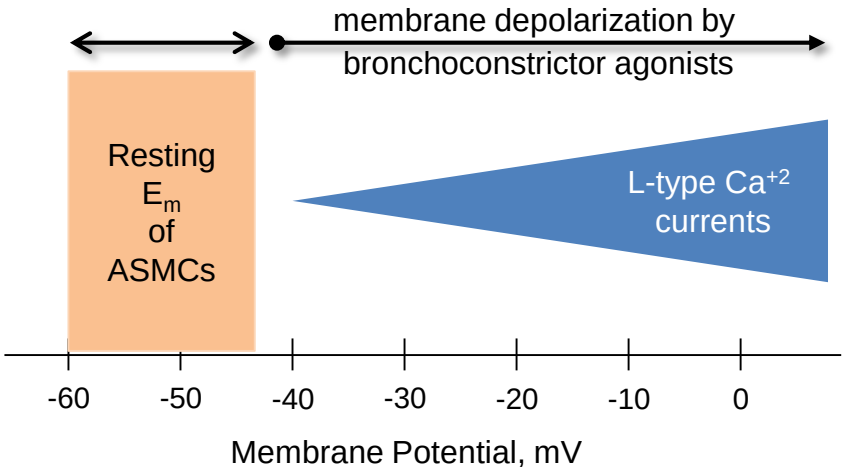


**Figure 2:** Resting membrane potential on ASMCs and threshold for activation of L-type VSCC depicting their role in mediating influx of $Ca^{+2}$ from extracellular space upon membrane depolarization by bronchoconstrictor agonists. The width of the cone reflects fractional magnitude of $Ca^{+2}$ current at a value of membrane potential ($E_m$).

Therapeutic potential of calcium channel blockers has been evaluated previously in a number of clinical trials (Fish et al. 1984). Results from these clinical trials suggested that calcium channel blockers like verapamil and nifedipine were effective in dilating airways, but only in a subset of patients. Administration of these calcium channel blockers showed significant protection against bronchoconstriction induced by exercise, hyperventilation, histamine and methacholine (Barnes et al. 1983). Their therapeutic potential, however, was not fully explored mainly due to their limited potency and systemic adverse effects.

**$K^+$ channels and their role in maintaining the $E_m$ on ASMCs**

$K^+$ channels play an important role in $Ca^{2+}$ signaling through their ability to maintain a negative membrane potential. As indicated before and depicted in **figure 2**, ASMCs have a resting membrane potential of -60 to -45 mV (Liu et al. 2005). In human and guinea pig ASMCs the membrane potential is reported to oscillate in a characteristic manner called slow waves (Small et al. 1982; Ito et al. 1989).

However, these waves are rarely converted in to spiking action potentials as ASMC membrane displays strong outward rectification under resting conditions. This outward rectification is mainly in the form of $K^+$ efflux *via* $K^+$ channels on ASMC membrane, thus maintaining a hyperpolarized state of the membrane (Thirstrup et al. 2000). It therefore stands to reason that inhibition of $K^+$ channels would result in membrane depolarization and consequent activation of other voltage-dependent channels on the ASMC membrane, including VSCC, which would promote $Ca^{2+}$ influx and ASMC contraction.

Based on this principle, our laboratory has previously reported such mechanism for the $G_{q/11}$-coupled receptor pituitary hormone [$Arg^8$]vasopressin (AVP) in its vasoconstrictor actions on vascular smooth muscle cells (VSMCs). It is generally accepted that AVP leads to vasoconstriction by the activation of PLC and consequently the release of $Ca^{+2}$ from SR. The half-maximal activation of PLC requires nano-molar concentrations of AVP, however, the circulating AVP concentrations are usually in the pico-molar range (Doyle et al. 1985). Our laboratory observed that at physiologically relevant (low) concentrations, AVP acts exclusively by activating the L-type VSCC in VSMCs (Henderson et al. 2007). It does so by suppressing the activity of Kv7 channels *via* a protein kinase C (PKC) - dependent pathway (**Figure 3**) (Brueggemann et al. 2007; Mackie et al. 2008a). These results provide strong evidence for Kv7 channels playing a pivotal role in maintaining a negative resting potential in the vascular myocytes and opposing the opening of VSCC. Suppression of Kv7 channel activity by physiologically relevant concentrations of AVP results in membrane depolarization and activation of VSCC.

Through the research presented in this thesis, we propose a similar mechanism for bronchoconstrictor agonist-mediated ASMC contraction (please refer to chapter 2 for further discussion).

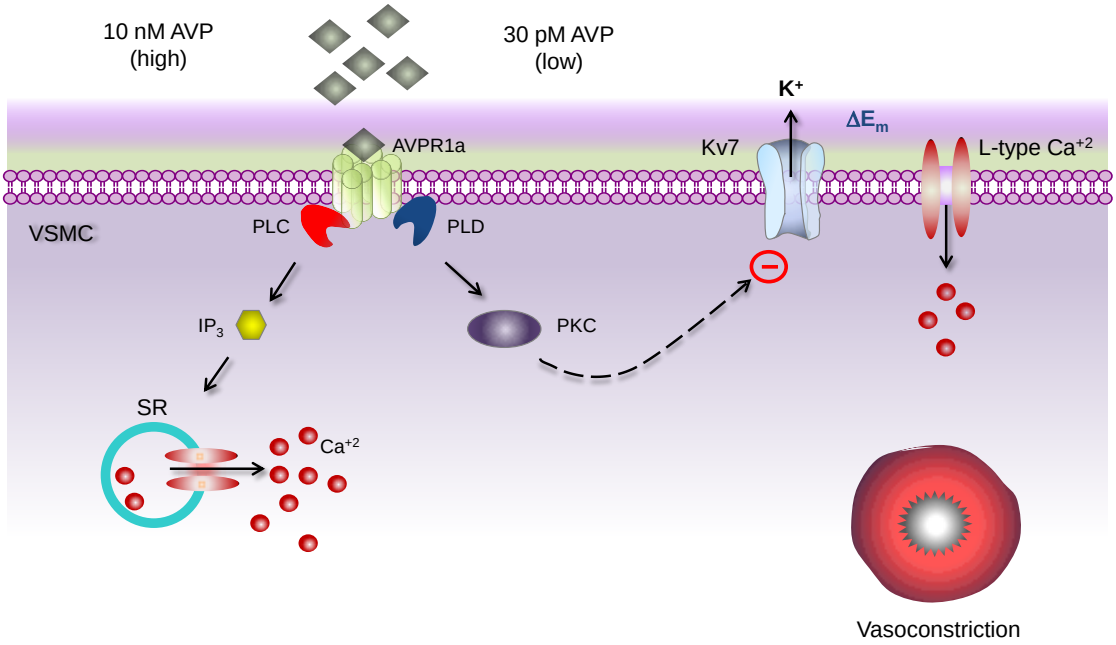


**Figure 3: Two concentration-dependent signaling pathways of [Arg$^8$] vasopressin (AVP) in VSMCs**. Nanomolar (nM) concentrations of AVP acutely activate the well-accepted AVP signaling pathway that involves activation of phospholipase C (PLC) and inositol 1,4,5-trisphosphate ($IP_3$)-mediated calcium ($Ca^{2+}$) release. On the other hand, picomolar (pM) concentrations of AVP induce voltage-sensitive $Ca^{2+}$ entry *via* protein kinase C (PKC) activation and inhibition of voltage-activated $K^+$ channels (Kv7).

Three different types of $K^+$ channels are known to be expressed in ASMCs, $Ca^{+2}$-activated potassium channels ($K_{Ca}^{+2}$), ATP-sensitive potassium channels ($K_{ATP}$) and voltage-gated potassium channels ($K_v$) (**Figure 4**). Previous studies have shown that $K_{Ca}^{+2}$ channels do not contribute significantly toward controlling the $E_m$ on ASMCs (Liu et al. 2003a). $K_{Ca}^{+2}$ channel blocker used in the study had no effect on resting membrane potential in rat ASMCs. Similarly, $K_{ATP}$ channel inhibitor glibenclamide showed no effect on $E_m$ under resting condition in rat and human

ASMCs, indicating that $K_{ATP}$ do not control the $E_m$ in ASMCs (Liu et al. 2003b; Zhao et al. 2005).

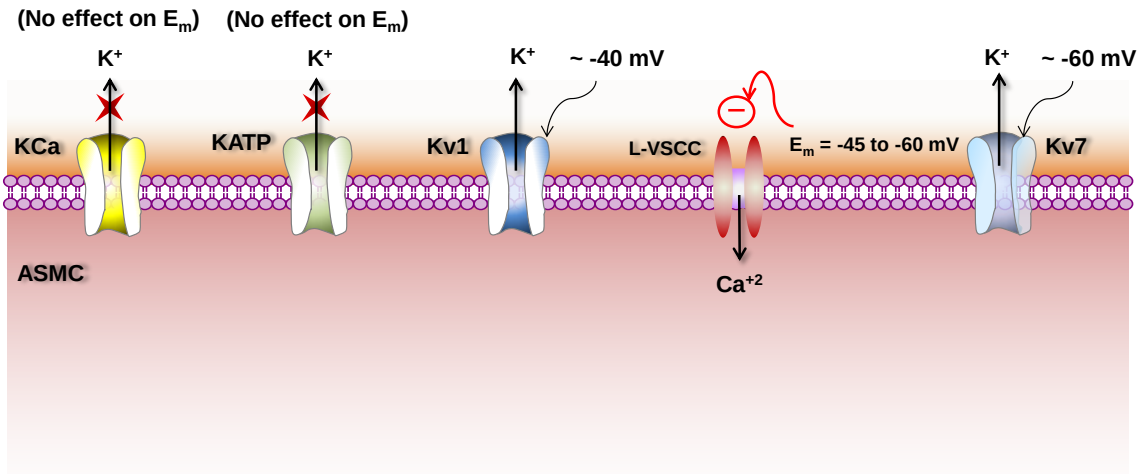


**Figure 4: $K^+$ channels and their role in controlling $E_m$ on ASMCs.** Three different types of $K^+$ channels are known to be expressed in ASMCs: KCa, KATP and Kv. Blocking the activity of KCa and KATP channels has been shown to have no effect on the Em indicating that they do not govern the $E_m$ on ASMCs. Evidences suggest that Kv channels are important determinants of $E_m$, however, the Kv1 channels that were found to be expressed in ASMCs are not likely to contribute significantly to the resting $E_m$ due to their relatively positive threshold of activation (-40 mV). Kv7 channel, on the other hand, have threshold of activation that lies in the range of the resting $E_m$ on ASMCs making them likely candidates governing the $E_m$ on ASMCs.

Kv channels are voltage-sensitive, $Ca^{+2}$-independent $K^+$ channels that are considered the most important class of $K^+$ channels in determining $E_m$ on ASMCs (Hall et al. 2000). A few subtypes of Kv channels (Kv1.1, Kv1.2 and Kv1.5) have been found to be expressed in human ASMCs (Adda et al. 1996). The threshold for voltage-dependent activation of Kv1 channels lies positive to -40 mV. Contradictory to literature reports, it seems unlikely for Kv1 channels with a relatively positive threshold of activation to contribute to the resting membrane potential (-45 to -60 mV) on ASMCs. Kv7 channels, another type of voltage-activated potassium channels, have a threshold for activation at ~ -60 mV. Hence, they seem more likely candidates governing resting membrane potential on ASMCs compared to Kv1

subtypes. However, their expression and function in ASMCs was largely unknown before the start of the investigation presented in this thesis.

**Structure, assembly and function of Kv7 channels**

The Kv7 family of voltage-gated $K^+$ channels is encoded by KCNQ genes. Five subtypes have been identified so far: Kv7.1 – Kv7.5 (KCNQ1-5 as an alternative terminology) (Gutman et al. 2003). Kv7 channels are composed of four subunits, each consisting of six α-helical transmembrane domains (S1-S6) and a single P-loop region (**Figure 5**). Of particular importance is the fourth transmembrane domain (S4) that consists of regularly-spaced, positively-charged amino acids (arginine) (Schroeder et al. 2000a), which plays an essential role in sensing membrane potential and triggering conformational changes. The P-loop consists of potassium signature sequence (TXXTXGYG) that serves as a characteristic feature of voltage-gated $K^+$ channels (Heginbotham et al. 1992).

Functional Kv7 channels require homomeric or heteromeric assemblies of four subunits (tetramers). While the Kv7.1 (or KvLQT1) subtype has not been found to co-assemble with other Kv7 subunits (Schwake et al. 2003), the Kv7.3 subunit has shown the capability to form hetero-tetramers with all other subunits except for Kv7.1 (Jentsch, 2000). Unlike other Kv channels, the tetramerization domain in Kv7 channels lies in the cytosolic C-terminus rather than the transmembrane domain 1 (Wehling et al. 2007). Besides this, the C-terminus also carries binding sites for various regulatory elements such as phosphatidylinositol-4,5-bisphosphate ($PIP_2$) (Zang et al. 2003), calmodulin (CaM) (Yus-Najera et al. 2002), A-kinase anchoring

protein (AKAP) (Hoshi et al. 2003), and phosphorylation sites for protein kinase C (PKC) (Hoshi et al. 2003).

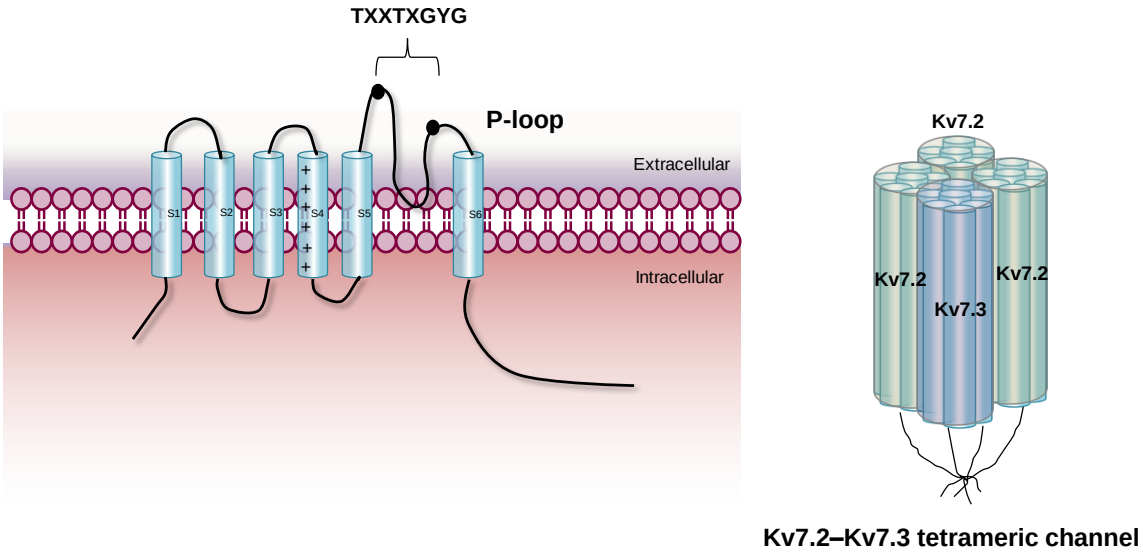


**Figure 5: Structural aspects of Kv7 $K^+$ channels.** (+) indicates the positively charged amino acid arginine in transmembrane domain 4 (S4). TXXTXGYG is the potassium signature sequence in the P-loop region. The picture on the right shows assembly of Kv7.2-Kv7.3 hetero-tetrameric channel.

A particular distribution pattern has been observed for different subtypes of Kv7 channels. Kv7.1 subtype has been found to be expressed predominantly by cardiac myocytes, whereas Kv7.2–7.5 have been considered largely 'neuronal' (Jentsch, 2000; Schroeder et al., 2000a). The Kv7.4 subtype has been found to have specialized cellular location in auditory neurons (Kharkovets et al., 2000). Adult rat aorta and rat mesentric artery has been found to express Kv7.1, Kv7.4 and Kv7.5, whereas only Kv7.5 was shown to be expressed in embroyonic rat aorta derieved (A7r5) smooth muscle cells (Brueggemann et al. 2007; Mackie et al. 2008a). More recently, Kv7.1 and Kv7.5 subtypes were also shown to be expressed in visceral and myometrial smooth muscle (Jepps et al 2009; McCallum et al. 2008). Mutations in the KCNQ genes encoding different Kv7 channel subtypes are known to be the

underlying causes of various diseases. For example, mutations in Kv7.1 channels result in cardiac arrhythmias, mutations in Kv7.2 and Kv7.3 result in neuronal excitability (Jentsch, 2000), whereas mutations in Kv7.4 result in deafness (Robbins et al. 2001). Therefore, different Kv7 channel subtypes have been found to be attractive molecular targets for novel anti-epileptic (Main et al. 2000), anti-migraine (Gribkoff 2003) and neuroprotective (Jensen 2002) agents.

As far as pharmacological regulation of Kv7 channels with synthetic small molecules is concerned, a number of relatively selective Kv7 channel blockers and activators have been developed over the years (Xiong et al. 2008). Chromanol 293B is known as a selective Kv7.1 channel blocker (Busch et al. 1997; Schroeder et al. 2000b), although it also partially inhibits Kv7.5 currents at concentrations higher than 100 μM (Lerche et al. 2000). Linopirdine (DuP 996, 1-phenyl-3,3-bis(pyridin-4-ylmethyl)-1,3-dihydro-2H-indol-2-one) and its more potent analog XE991 (10,10-bis(4-Pyridinylmethyl)-9(10H)-anthracenone) (Zaczek et al. 1998) are widely used, relatively selective blockers of all Kv7 channels at concentrations below 10 μM (Mackie et al. 2008b). At higher concentrations these compounds can potentially block other types of $K^+$ channels (Schnee et al. 1998). Among the Kv7 channel activators, flupirtine (ethyl-N-[2-amino-6-(4-fluorophenylmethylamino)pyridin-3-yl]carbamate) and retigabine (ethyl N-[2-amino-4-[(4-fluorophenyl)methylamino]-phenyl]carbamate) have found wide usage. Flupirtine is used clinically as a non-opioid analgesic with muscle relaxant properties (Devulder 2010), while retigabine is used as an anticonvulsant in the treatment of epilepsy (Harden 2012). Retigabine selectively activates Kv7.2–7.5 at concentrations up to 100 μM, beyond which it also

activates Kv7.1 channels (Tatulian et al. 2001; Schenzer et al. 2005; Wuttke et al. 2005). Our laboratory has recently shown that the cyclooxygenase-2 (COX-2) enzyme inhibitor celecoxib (Celebrex®), and its COX-2 independent structural analog 2,5-dimethylcelecoxib (DMC) (Schönthal et al. 2008), dramatically enhanced Kv7 $K^+$ currents and suppressed L-type VSCC channels in A7r5 rat aortic smooth muscle cells (Brueggemann et al. 2009). But another popular COX-2 inhibitor, rofecoxib (Vioxx®, withdrawn from the market), which is known to have adverse cardiovascular effects upon long-term treatment, did not show enhancement of Kv7 $K^+$ currents. These findings provides a likely mechanistic basis for differential cardiovascular risk profiles of COX-2 enzyme inhibitors used as non-steroidal anti-inflammatory drugs (NSAID).

**Biophysical and pharmacological characterization of $K^+$ currents in ASMCs**

In parallel to the investigation presented in this thesis on expression and function of Kv7 channels in ASMCs, our laboratory has carried out electrophysiological characterization of $K^+$ currents in ASMCs with an objective to detect Kv7 currents in ASMCs, and determine their contribution to the total currents from all voltage-dependent potassium channels ($Kv_{total}$) in ASMCs. Among the typical characteristic features that differentiate Kv7 currents from other Kv currents is that they display slow activation and deactivation kinetics, Kv7 channels typically display very little or no deactivation even during sustained depolarization, and some exibit a relatively negative threshold of activation (Jensen et al. 2007; Mackie et al. 2008b). The non-inactivating nature of Kv7 channels is considered

fundamental to their role in stabilizing the resting membrane potential in excitable cells like neuronal cells.

In order to detect and measure the contribution of Kv7 currents in ASMCs, our laboratory recorded $K^+$ currents in freshly dissociated guinea pig and human ASMCs. The whole cell perforated patch configuration was used to measure membrane currents under voltage-clamp conditions. Amphotericin B was added to the internal solution for membrane patch perforation. To isolate Kv7 currents, if present, 100 μM gadolinium(III) chloride ($GdCl_3$) was added to external solutions based on the methods already developed in our laboratory (Brueggemann et al. 2007; Mackie et al. 2008a). Use of 100 μM $GdCl_3$ reversibly shifted the $V_{0.5}$ activation of 4-aminopyridine sensitive Kv currents to more positive voltages, thus enabling measurements of purely Kv7 currents at voltages negative or equal to -20 mV. The currents recorded from guinea pig ASMCs (at voltages ≤ -20 mV) showed all the characteristic features of Kv7 currents: (i) slow kinetics of activation with no apparent inactivation during a 5s voltage step (**Figure 6A**); (ii) voltage-dependent activation with a very negative threshold (~ -60mV) and a half-maximal potential of activation ($V_{0.5}$) of ~-31 mV (**Figure 6B; Figure 7C & 7E**); (iii) reversible enhancement by selective activators of Kv7.2-7.5 channels, flupirtine and retigabine, accompanied by a shift of the activation curve to more negative voltages (**Figure 7**); (iv) complete and irrever-sible suppression by the Kv7 channel blockers linopirdine (**Figure 7B & 7D**) and XE991 (Brueggemann et al. 2012). As discussed earlier, neuronal and vascular Kv7 channels are known to be inhibited upon activation of $G_{q/11}$-coupled receptors (Delmas et al. 2005; Mackie et al. 2008a). After biophysical

and pharmacological characterization of Kv7 currents in guinea pig ASMCs, our laboratory was interested in testing the ability of $G_{q/11}$-coupled bronchoconstrictor agonists (methacholine and histamine) to suppress the Kv7 currents in guinea pig ASMCs. A rapid and near complete inhibition of Kv7 currents was observed upon application of 100 nM methacholine. However, the currents were partially restored within 1-2 min to about 40% of the control levels while methacholine was still present (**Figure 8A**). Approximately 60% reduction of Kv7 currents was observed at all voltages positive to -24mV as a sustained effect of methacholine in 100 nM concentration (**Figure 8B & 8E**). Most importantly, treatment with Kv7 channel activator flupirtine (10 μM) was found to restore the amplitude of Kv7 currents post-methacholine suppression to 64% of control (**Figure 8B & E**). Repeating these studies with histamine, another

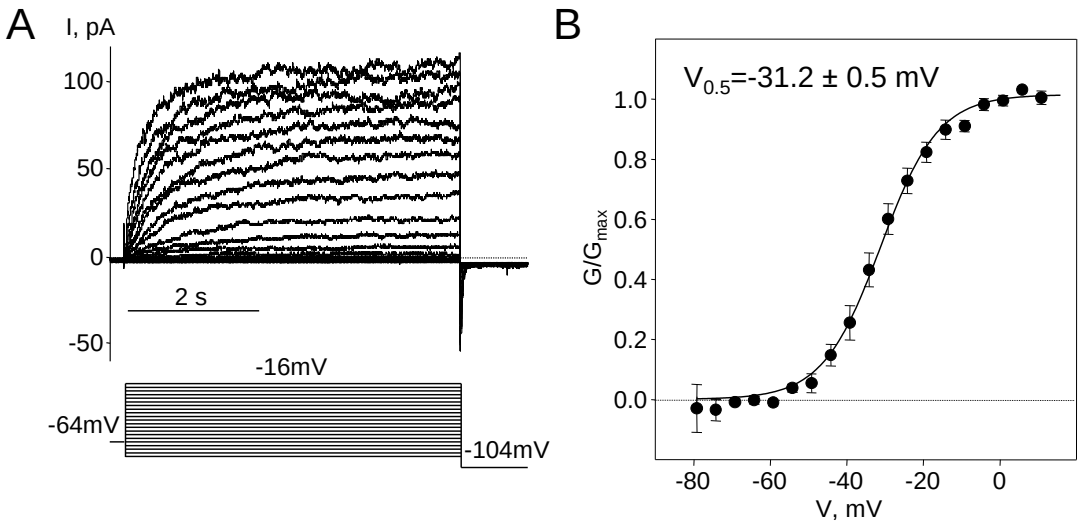


**Figure 6: Biophysical characterization of $K^+$ currents in guinea pig ASMCs.** ***A.*** Representative traces of the current recorded using a 5s voltage step protocol depicted below (from -64 mV holding voltage, cell capacitance (C) = 15 pF). ***B.*** Averaged fractional conductance plots calculated from steady-state Kv7 currents fitted to a Boltzmann distribution ($V_{0.5}$ = -31.0 ± 1.5 mV, n=13). (Adapted from: Brueggemann LI, Kakad PP, et al. (2012) *Am J Physiol Lung Cell Mol Physiol* **302**(1):L120-132).

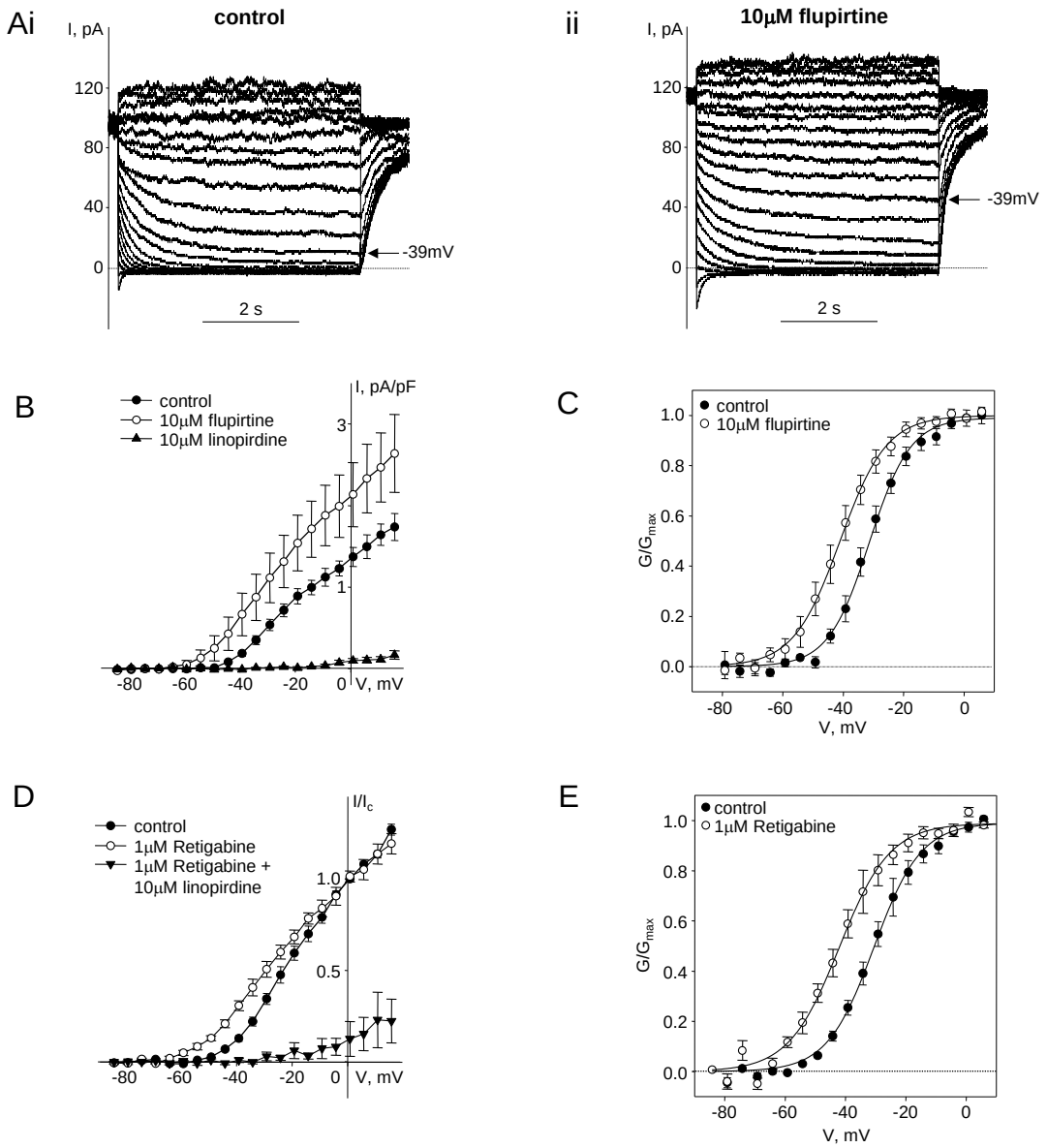


**Figure 7: Pharmacology of Kv7 currents in guinea pig ASMCs.** ***A***. Representative traces of Kv7 currents recorded in control (***i***) and in the presence of 10 μM flupirtine (***ii***) with a voltage step protocol from -4 mV holding voltage. ***B***. I-V relationships of Kv7 currents recorded in ASMCs before (control, filled circles, n=5), after 5 min treatment with 10 μM flupirtine (open circles, n=5) and after 5 min treatment with linopirdine (10 μM, filled triangles, n=5). ***C.*** Averaged fractional conductance plots calculated from steady-state Kv7 currents measured in ASMCs in control (filled circles) and in the presence of 10 μM flupirtine (open circles) (n= 10) fitted to a Boltzmann distribution. ***D.*** I-V relationships of Kv7 currents normalized to control currents measured at +1 mV before (control, filled circles, n=4), after 5 min treatment with 1 μM retigabine (open circles, n=4) and after 5 min treatment with 10 μM linopirdine in the presence of 1 μM retigabine (filled triangles, n=5). ***E.*** Averaged fractional conductance plots calculated from steady-state Kv7 currents measured in ASMCs in control (filled circles,) and in the presence of 1 μM retigabine (open circles) fitted to a Boltzmann distribution. (Adapted from: Brueggemann LI, Kakad PP, et al. (2012) *Am J Physiol Lung Cell Mol Physiol* **302**(1):L120-132).

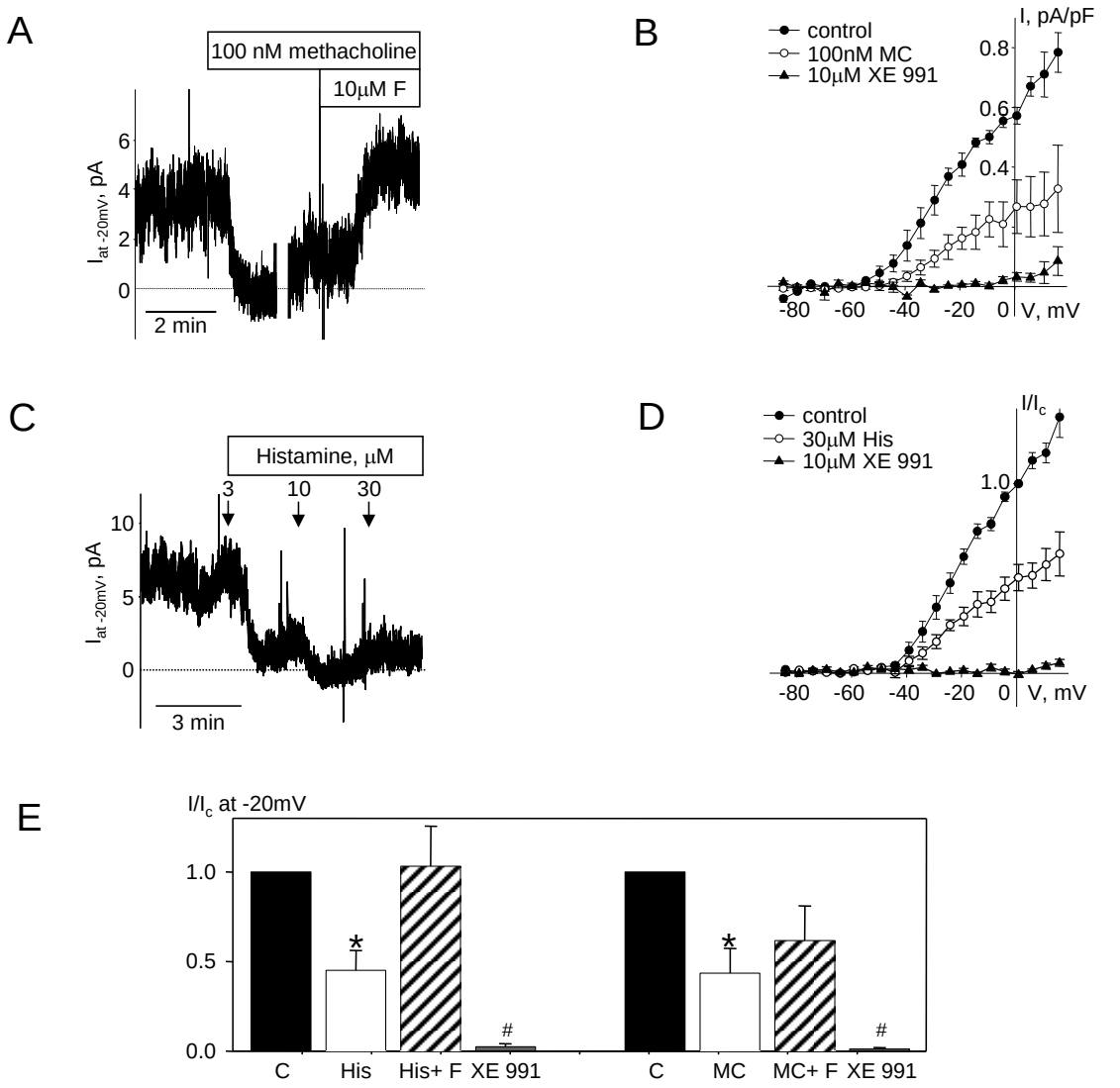


**Figure 8: Suppression of Kv7 currents by bronchoconstrictor agonists in guinea pig ASMCs and their restoration by the Kv7 channel activator flupirtine (F).** ***A.*** The time course of Kv7 current inhibition was recorded in ASMCs, at -20 mV holding voltage in control (2 min) following by methacholine application (100 nM for 3 min). After an additional 1 min recording flupirtine (10 μM) was applied in the presence of methacholine (C= 21 pF, representative of 4 similar experiments). ***B.*** Average I-V relationships of Kv7 currents recorded from -4 mV holding voltage in ASMCs before (control, filled circles, n=4), during treatment with 100 nM methacholine (open circles, n=4) and in the presence of XE991 (10 μM, filled triangles, n=3) applied in the end of each experiment. ***C.*** Dose-dependent suppression of Kv7 currents recorded at -20 mV holding voltage was observed upon application of histamine (3-30 μM, indicated by arrows). ***D.*** Average I-V relationships of Kv7 currents recorded from -4 mV holding voltage and normalized to control currents recorded at +1mV in control (filled circles, n=4), in the presence of 30 μM histamine (open circles, n=4) and in the presence of XE991 (10 μM, filled triangles, n=4). ***E.*** Normalized currents recorded at -20mV in control (black bars), in the presence of agonists (open bars: His- 30 μM histamine, n=4; MC- 100nM methacholine, n=4); in the presence of agonists plus 10 μM flupirtine (striped bars: His+F - 10 μM flupirtine in the presence of 30 μM histamine, n=4; MC+F - 10 μM flupirtine in the presence of 100nM methacholine, n=4) and in the presence of 10 μM XE991 (n=4). * significant difference from control (P< 0.05, one way ANOVA). # significant difference from all other treatment (P< 0.05, one way ANOVA). (Adapted from: Brueggemann LI, Kakad PP, et al. (2012) *Am J Physiol Lung Cell Mol Physiol* **302**(1):L120-132).

bronchoconstrictor agonist, showed suppression of Kv7 currents in airway myocytes in a dose-dependent manner (**Figure 8C**). Histamine-induced Kv7 current inhibition was transient at agonist concentrations from 1-10 μM, but application of 30 μM histamine induced sustained current inhibition at all voltages positive to -29 mV (**Figure 8C-E**). Subsequent addition of flupirtine (10 μM) in the continued presence of 30 μM histamine completely restored remaining Kv7 current amplitude to the values measured prior to histamine treatment (**Figure 8E**).

The electrophysiological studies discussed above from our laboratory confirm the activity of Kv7 currents in ASMCs. They also showed that Kv7 currents can be suppressed by $G_{q/11}$-coupled receptor agonists similar to other smooth muscle cells. These results suggest that common signal transduction intermediates are likely to be involved in the mechanism of suppression of Kv7 currents by $G_{q/11}$-coupled receptor agonists in ASMCs. Following histamine and or methacholine-induced suppression of Kv7 currents, the current amplitude was restored to near control values by flupirtine, indicating that flupirtine may either act by reversing the blockage of Kv7 currents by bronchoconstrictor agonists, or by simply enhancing the unblocked Kv7 currents. In either case, Kv7 channel activators show tremendous potential in antagonizing the effect of bronchoconstrictor agonists on airways in this preliminary study.

**Summary**

Asthma is a disease characterized by constriction of ASMCs with AHR being an integral component of its complex pathophysiology. Regulation of calcium signaling is considered to be the key in understanding ASMCs contraction and

relaxation. Influx of calcium from extracellular space is an important event that leads to contraction of ASMCs. Several bronchoconstrictor agonists are known to cause membrane depolarization that can lead to the opening of L-type VSCC, which are considered to be the major contributors to bronchoconstrictor-agonist mediated influx of calcium. Our laboratory has previously shown that $G_{q/11}$-coupled receptor vasoconstrictor hormone AVP, in low, physiologically relevant concentration acts by suppressing the activity of Kv7 channels, thereby leading to membrane depolarization and activation of L-type VSCC. In other words, Kv7 channels are determinants of resting membrane potential in VSMCs and their suppression can lead to membrane depolarization. We envision a similar role for Kv7 channels in ASMCs, wherein their activity is suppressed by bronchoconstrictor agonists to mediate bronchoconstriction. However, expression and function of Kv7 channels in ASMCs was largely unknown. The research presented in this thesis focuses on detecting expression of various Kv7 channel subtypes in ASMCs, comparing their expression pattern in normal and diseased airways, and determining the functional role they play in airway modulation. The electrophysiological studies that were conducted in parallel to this investigation have detected Kv7 currents in guinea pig and human ASMCs. The Kv7 currents in guinea pig ASMCs are suppressed by bronchoconstrictor agonists and were shown to be restored to near control levels upon treatment with Kv7 channel activators. The expression and *ex-vivo* functional studies presented in this thesis evaluate the potential of targeting Kv7 channels as novel molecular targets for asthma therapy.

# CHAPTER 2

# PROJECT OVERVIEW

## Objective

Airway Hyperresponsiveness (AHR) is the characteristic feature of asthma pathophysiology, the molecular mechanisms of which are poorly understood. *The overarching goal of the research presented in this thesis is to elucidate the mechanisms underlying AHR in asthma.* This will not only help us gain a better insight into the pathophysiology of asthma, but may further lead to the discovery of novel and improved therapeutic agents. The *primary objectives of this research are to investigate the role played by a Kv7 $K^+$ channels in pathophysiology of AHR, and to explore the regulation of Kv7 channels as a novel therapeutic strategy in treatment of asthma.*

## Rationale

Although Kv7 channels were considered to be primarily expressed in neuronal cells, recent findings have revealed their expression in different excitable cells like cardiac myocytes and vascular smooth muscle cells (VSMCs) (Joshi et al. 2006; Yeung et al. 2007; Brueggemann et al. 2007; Greenwood et al. 2009). The key role attributed to Kv7 channels is the prevention of membrane depolarization by stabilizing the negative membrane potentials on these cells (Mackie et al. 2008b). Mutations in KCNQ genes encoding Kv7 channels have been shown to be linked to

various high-impact diseases like cardiac arrhythmias, epilepsy and deafness. As discussed in chapter one, our laboratory has discovered that $G_q/_{11}$-coupled hormone [$Arg^8$]vasopressin (AVP) elicits its vasoconstrictor action by inhibiting the activity of Kv7.5 channels in VSMCs *via* a PKC-dependent pathway (Brueggemann et al. 2007). Findings from our laboratory have further suggested that the Kv7 channel activators that are used clinically in management of neuronal disorders are effective in reversing the vasoconstrictor effect of AVP in VSMCs (Brueggemann et al. 2007; Mackie et al. 2008a). The crucial role played by Kv7 channels in VSMCs and other excitable cells has motivated us to explore the significance of these channels in airway smooth muscle cells (ASMCs) excitability.

**Hypothesis**

Our main hypothesis is that Kv7 channels play a critical role in maintaining negative resting membrane voltages in ASMCs similar to other excitable cells, and that bronchoconstrictor agonists promote ASMC contraction and airway narrowing by suppressing the activity of Kv7 channels **(Figure 9)**. We further hypothesize that Kv7 channel activators will reverse the bronchoconstrictor agonist-mediated ASMC contraction by restoring the activity of Kv7 channels.

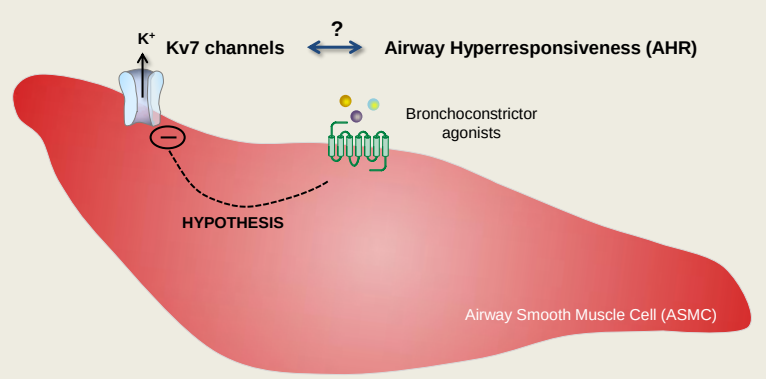


**Figure 9:** Hypothesis for the research presented in this thesis.

These hypotheses provide a pathophysiological basis for AHR in asthma and will be tested by studies proposed in following two specific aims.

**Specific Aim 1**

Identify the Kv7 channel-subtypes expressed and compare their expression profiles in normal and allergen-sensitized guinea pig ASMCs.

**Specific Aim 2**

Evaluate the efficacy of Kv7 channel activators in relaxation of allergen-sensitized guinea pig airways pre-constricted with bronchoconstrictor agonists using precision-cut lung slices (PCLS).

**Significance**

Successful completion of this research will identify the subtypes of Kv7 channels that are predominantly expressed in normal and allergen-sensitized guinea pig airways. The differences, if any, in the expression pattern of various subtypes in normal and asthmatic ASMCs will provide us with important leads in determining the role played by Kv7 channels in pathophysiology of AHR. The PCLS studies will provide us with information on effectiveness of Kv7 channel activators in relaxation of bronchoconstrictor agonist-mediated airway constriction. Overall, the proposed research will not only probe into the unknown mechanisms for AHR in asthma, but it is also likely to lead in to identification and pharmacological characterization of Kv7 channels as novel targets for treatment of asthma.

# CHAPTER 3

## GUINEA PIG MODEL OF AIRWAY HYPERRESPONSIVENESS

Asthma is a prime example of a complex and multifactorial disease. It is also a disease that is unique to humans as none of the commonly employed laboratory animals naturally acquire the asthma-like syndrome that is endemic in humans. Asthma, therefore, poses a considerable challenge for development of animal models with symptoms that can accurately mimic those observed in human asthma. Despite inherent challenges, various animal models of asthma have been developed over the years that mimic several important symptoms observed in human asthma. So far, asthma-like symptoms have been reproduced in animals like mice, rats, guinea pigs, ferrets, dogs, sheep, monkeys and horses. However, each animal model has its own advantages and disadvantages (Canning 2003).

Mice offer several advantages in the study of asthma compared to other animals. Firstly, numerous inbred strains are available for mice compared to other species. A detailed understanding of mouse genetics arguably improves its suitability for employment of transgenic technology (Elias et al. 2003). Moreover, numerous commercially available mouse-specific probes, immunological reagents, growth factors, and cell surface markers are available for mice. Importantly, mice have IgE as the primary allergic immunoglobulin similar to that found in humans. However, there are considerable physiological differences between mice

and humans that do not favor a straightforward extrapolation of asthma related findings to humans. For example, unlike humans, mice do not exhibit spontaneous AHR and have limited airway musculature (Shin et al. 2009). Additionally, human airways have a larger diameter compared to mouse airways and different airway diameter respond differently. Therefore, transposing data from mice to human could be misleading (Kips et al. 2003). In fact it has been shown that human airway responses to agonists are not similar to the responses in mouse airways (Held et al 1999) .These factors limit the use of mice as an animal model of asthma.

Guinea pigs are the animals most frequently used to model anaphylactic (early, immediate type) bronchoconstriction. Guinea-pigs are readily sensitized to ovalbumin (OVA) (Sundstrom et al. 2003) and it is easy to elicit a response to a challenge that is similar to an asthmatic phenotype involving increased airway responsiveness (Pretolani et al. 1996). To evaluate if they are a good model for human airways, the response of isolated guinea-pig airways to pharmacological agonists has been compared directly with humans (Muccitelli et al. 1987). This study revealed that histamine and methacholine had comparable $EC_{50}$ values and showed similar maximal responses in both guinea pig trachea and human bronchus. Notably, allergen-induced bronchoconstriction in guinea pigs and human bronchial asthma have several features in common, and hence many important findings in human asthma pathophysiology have emerged from the guinea pig model of asthma (Szeleny I. 2000). Additionally, the potencies and efficacies of agonists and antagonists have been found to be very similar in human and guinea pig airways

(Canning et al. 2008). However, the guinea pig model also suffers from a few disadvantages. The major anaphylactic immunoglobulin in guinea pigs is IgG1 as compared to IgE in humans. Furthermore, there is a lack of guinea pig-specific immunological and biochemical reagents, and there is also a shortage of inbred strains. Therefore, genetic influences on susceptibility to sensitization and development of airway disease are difficult to investigate in guinea pigs (Shin et al. 2009). However, weighing the successes of the guinea pig model in studying asthma and asthma-related diseases, as well as the similarity to human airways in responses to various bronchoconstrictor agonists, justifies our use of this animal model for the studies presented in this thesis.

**Animal Welfare and Housing**

Male Hartley guinea pigs (250-350gm) were purchased from Elm Hill/Cady Ridge Farms, and housed in a plexiglass cage (2 animals per cage) and fed ad libitum in a regular 12-h dark/light cycle. All procedures described in this study were approved by the Institutional Animal Care and Use Committee, Loyola University Medical Center, Maywood, IL and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (1996, National Academy of Sciences, Washington D.C.)

**Induction of AHR in guinea pigs**

The stepwise protocol for induction of AHR in guinea pigs is as depicted in **Figure 10**. Young male guinea pigs (350-550 g) were given three intraperitoneal (i.p.)

injections of ovalbumin (OVA) (100 µg OVA and 100 mg aluminium hydroxide in 1 mL saline) or saline alone (control) on days 1, 3 and 5 (Mazzone SB et al. 2002).

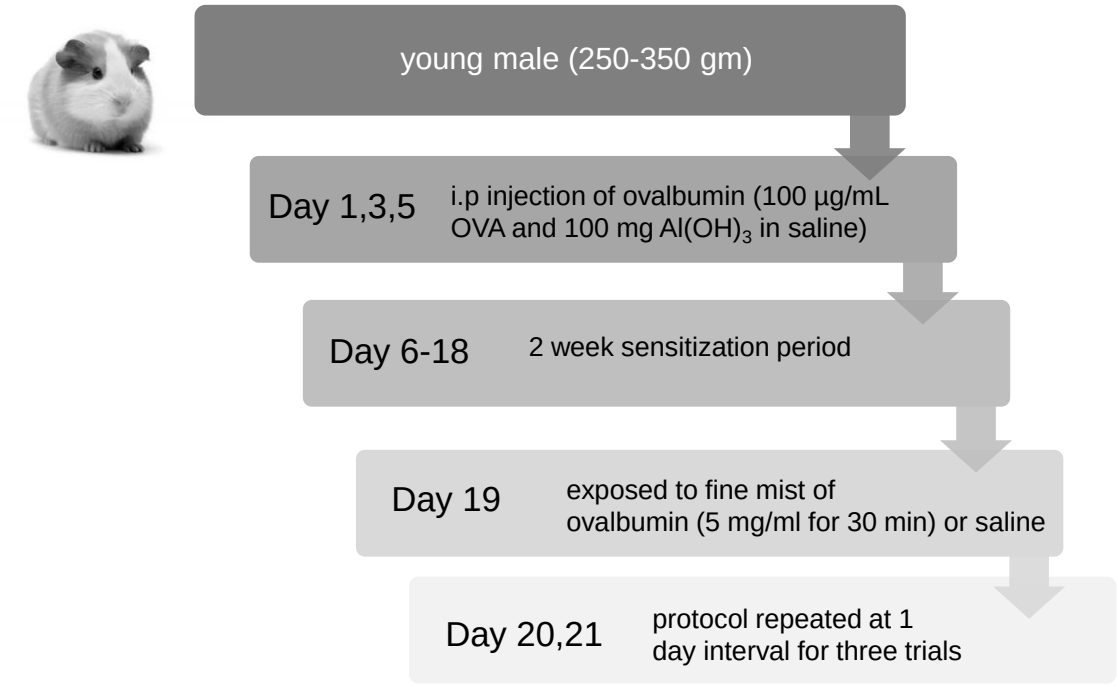


**Figure 10:** Flow chart depicting various treatment stages in the development of guinea pig model of airway disease.

After a subsequent 2-week sensitization period, the animals were placed in a cylindrical Plexiglas chamber where they were observed for at least 1 minute and then exposed to a nebulized solution of OVA. This protocol was subjected to optimization with different concentrations of OVA and duration of its exposure (see troubleshooting section below). Finally, the animals were exposed to an optimized concentration (5 mg/ml) of OVA in saline for 30 min using a commercial DeVilbiss nebulizer with a constant pressure at ~0.3 ml/min (Smith et al. 2005). This protocol was repeated at one day intervals for a total of 3 trials. Two days prior to the exposure of aerosolized ovalbumin, the animals were acclimatized to fine mist of saline under the same conditions. According to the published studies, this protocol

should induce asthma attacks in a fairly uniform fashion. Ottenberg and coworkers list the sequence of respiratory signs observed in this model as follows: “rapid breathing, restlessness, chewing, ruffling of the fur, then labored breathing with the use of accessory muscles of respiration, prolonged expiration, gasping, coughing, dilation of alae nasi, and finally cyanosis and convulsion” (Ottenberg et al. 1958). The allergen-sensitized guinea pigs were observed for measurement of the time of onset of respiratory attack. The attack was characterized by the use of accessory muscles for respiration, gasping, coughing, and pronounced respiratory distress. Animals were closely monitored and any symptoms observed were recorded.

The animals were staggered in such a way that one animal was injected every week starting with either saline control or allergen sensitized animal in the first week and reversing the order with the next set of animals. The treatments and schedules involved three i.p injections (saline or OVA) on Monday (Day 1), Wednesday (Day 3) and Friday (Day 5) followed by exposure to aerosolized OVA on Friday (Day 19), Saturday (Day 20) and Sunday (Day 21), i.e two weeks following the last injection . The animals were then euthanized on the following Monday (Day 22) and the airway smooth muscle cells were isolated (see chapter 4 for detailed procedures) from the upper part of the trachea for the expression studies. One half of the surgically excised lung was inflated with agarose for preparation of slices (see chapter 5 for detailed procedures). The other half of the lung was reserved to isolate cells for the electrophysiological studies (not covered under this thesis).

**Troubleshooting**

Our initial use of 10 μg/ml ovalbumin concentration to challenge guinea pig following the 2 week sensitization period did not show the expected symptoms of airway disease. A previous study using different concentration of ovalbumin (10 μg/ml, 100 μg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml) on five successive days reported that 1mg/ml OVA induced symptoms like labored breathing, coughing and rubbing of nose (Boichot et al. 1991). Based on this report, we modified our protocol for OVA challenge, by increasing the aerosolized OVA concentration from 10 μg/ml to 5 mg/ml. This concentration developed some of the symptoms like labored breathing and gasping through the end of 15 minutes trial in allergen-sensitized guinea pigs, but not in the saline control animals as expected. However, the most severe symptoms (like prolonged expiration, coughing, dilation of alae nasi, sneezing cyanosis and convulsion) were not observed in these animals. Based on these observations and some additional published studies, we decided to prolong the exposure time to 30 minutes (Lai et al. 2003; Ram et al. 2003).

**Summary**

In total, 4 saline control and 4 allergen treated guinea pigs were monitored in a staggered fashion. The protocol for induction of AHR was revised (change in the concentration of OVA challenge and exposure time) based on the observations made during these studies. Optimization in the protocol led to the observation of some important symptoms like labored breathing and gasping in the allergen sensitized animals but not in the saline control animals.

## CHAPTER 4

## EXPRESSION OF Kv7 CHANNELS IN NORMAL AND ASTHAMATIC AIRWAYS

As discussed in chapter 1, Kv7 channel subtypes are found to be expressed in a variety of excitable cells such as neuronal cells and vascular smooth muscle cells (VSMCs), and play a pivotal role in controlling membrane excitability in these cells. However, to our knowledge the expression of Kv7 channels in airway smooth muscle cells (ASMCs) was previously unknown. Therefore, in order to investigate the role of Kv7 channels in airway smooth muscle cells (ASMCs), it was essential to know whether these channels are expressed in airways in the first place. Secondly, it is known that particular subtypes of Kv7 channels are predominantly expressed in specific tissues, for example, Kv7.1 subtype has been found to be expressed largely in cardiac myocytes, whereas Kv7.2–7.5 have been found to be expressed mainly in neuronal cells (Jentsch 2000; Schroeder et al. 2000a). So if Kv7 channels are expressed in ASMCs, then we were also interested in measuring expression of different subtypes of Kv7 channels in ASMCs. Finally, to achieve our objective of exploring the role played by Kv7 channels in AHR, it is important to study and compare the expression levels of Kv7 channels in normal and asthmatic airways. Therefore, quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) studies were conducted in ASMCs obtained from normal, saline-control and allergen-sensitized guinea pigs using primers specific for guinea pig KCNQ1-5.

The availability of human tissues also led us to identify and measure the expression levels of different Kv7 subtypes in human ASMCs (studies not a part of proposed research). The use of human tissues for this study was reviewed and approved by the Loyola University Chicago Institutional Review Board for the Protection of Human Subjects.

**Step 1: Isolation of Airway Smooth Muscle Cells (ASMCs)**

The ASMCs from normal, saline-control and allergen-sensitized guinea pigs were isolated using a method reported by Janssen and Sims (**Figure 11**) (Janssen et al. 1992). In short, connective tissue, vasculature and innervation were removed from primary and secondary bronchi. Bronchial segments 3-5 mm in length were then 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, 10 D-Glucose, pH adjusted to 7.2 with NaOH on ice, 298 mOsm/L. The segments were then cut open and the epithelium was removed using a cotton-tipped applicator. Each bronchial segment was cut into strips approximately 1 mm wide. The bronchial strips were then transferred into PSS (pH adjusted to 7.2 with NaOH at 37 °C) supplemented with BSA (1 mg/ml), collagenase Type VIII (400 units/ml), papain (30 units/ml), and DL-dithiothreitol (1 mM). The strips were then incubated for 45-60 min at 37 °C. After enzymatic digestion, the tissue was washed 3-5 times with ice cold PSS and gently triturated with a fire-polished Pasteur pipette to release individual myocytes. After trituration of the tissue, remaining undigested pieces were removed and ASMCs were pelleted by centrifugation (5 min at 2000 g). The

cells were then stored at -20 °C in a Qiagen RNAeasy plus™ lysis buffer (350 µL lysis buffer with 1 % β-mercaptoethanol) and used as required for qRT-PCR studies.

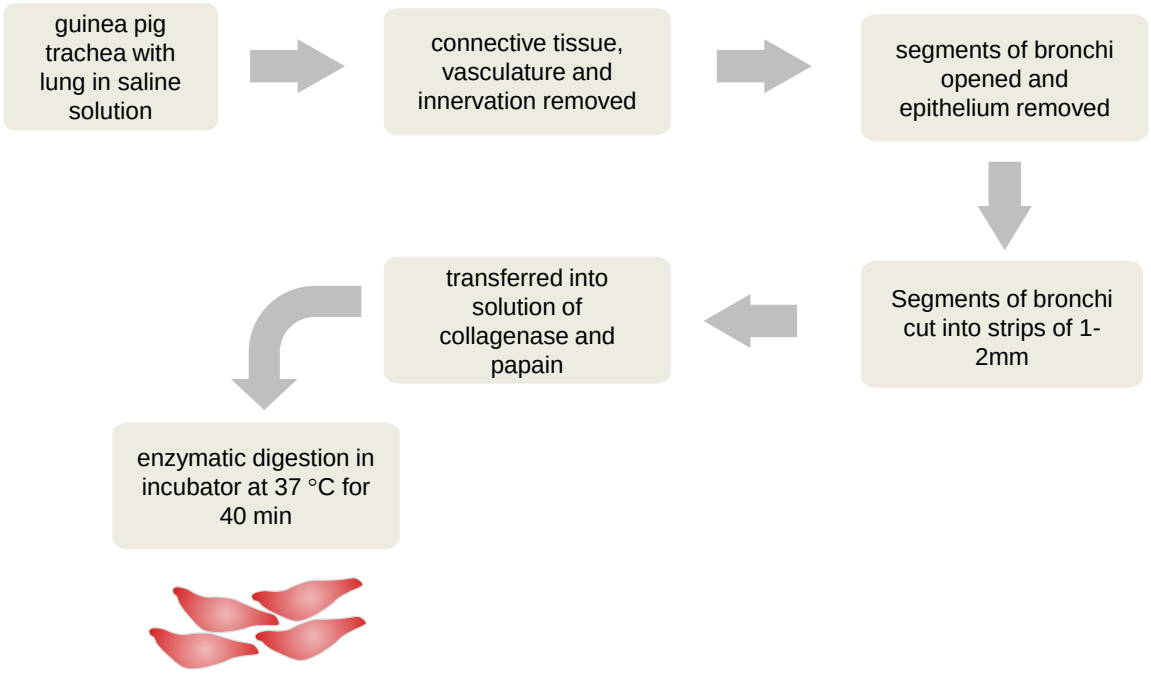


**Figure 11:** stepwise procedure for isolation of guinea pig ASMCs.

Human tracheal tissue was obtained from discarded lung transplant donor tissue and trachealis muscles were dissected from the trachea in ice cold dissecting solution and cut into strips approximately 2 mm in diameter. Strips of muscle were then transferred to ice cold PSS and then into PSS at 37 °C (pH adjusted to 7.2 with NaOH at 37 °C) supplemented with BSA (1mg/ml; fraction V, Roche Diagnostics USA), collagenase Type VIII (950 units/ml; Sigma), papain (38 units/ml; Worthington), and DL-dithiothreitol (1 mM; Sigma). The strips were then incubated for 45 min at 37 °C. After enzymatic digestion, the tissue was washed 3-5 times with ice cold PSS and gently triturated to release individual myocytes.

**Step 2: qRT-PCR studies in (normal, saline-control and allergen-sensitized) guinea pig ASMCs and normal human trachealis muscles**

qRT-PCR was used to determine the expression of KCNQ1–5 in guinea pig ASMCs and human trachealis muscle. ASMCs were isolated as described above from smooth muscle dissected free from the entire trachea. The cells (or trachealis muscles) so obtained were disrupted and homogenized using a Polytron homogenizer for 30 Seconds. Total RNA was extracted from ASMCs by use of RNeasy Plus Mini Kit (Qiagen, Valencia, CA). The RNA purified with a RNeasy silica-membrane method, is expected to remove most of the genomic DNA contamination efficiently. The RNA so obtained was not further treated with DNAase. RNA was quantified using a NanoDrop spectrophotometer, and the mRNA was reverse transcribed with a Bio-Rad iScript™ cDNA synthesis kit. The cDNA thus obtained was amplified in a qRT-PCR reaction by using primers specific for guinea pig KCNQ1–3, 5 (Navarro-López et al. 2009) and KCNQ4 (Liang et al. 2006) (**Table 1**). Quantitative expression was performed with Applied Biosystems 7300 Gene Expression System in 25 µl reactions consisting of 12.5 µl Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas, Burlington, Ontario, Canada), cDNA derived from 25–250 ng mRNA, and 5 pmol each primer. Cycle parameters were typically 95°C for 15 s, followed by 60°C for 30 s (40 cycles), and followed by a dissociation step to confirm a single PCR product. Specificity of the PCR primers was also verified by DNA sequencing. Standard curves were plotted by 10-fold serial dilution of known amounts of cDNA for each target. Primer efficiencies were

determined from the slope of the standard curve dilution series for each of the KCNQ targets, by using the formula Efficiency = 1+10(1/slope). The correlation coefficients for all standard curves were >0.99, and slope values gave efficiencies ≥90% in all cases. The target copy number in a sample was estimated by relating the PCR threshold cycle to the known target value on the standard curve. The mRNA levels of KCNQ1–5 were averaged from four male guinea pigs (250–350 g) with each reaction performed in duplicate. RNA extracted from one guinea pig ASMC sample was reverse transcribed as described above for end point PCR to confirm PCR product sizes for each primer pair. cDNA was amplified by using Platinum Taq PCR Mix (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and conventional PCR cycle parameters of 95°C for 1 min, 50°C for 1 min (30 cycles), and final extension at 72°C for 5 min. The PCR products were separated by agarose gel electrophoresis and the gel was visualized and imaged by use of a BioImaging System (UVP, Upland, CA).

Human trachealis RNA was extracted from human donor lung trachealis muscle (cut into 2 mm - 5 mm pieces; 4–5 pieces per human sample) by the acid-guanidinium-phenol extraction method (Chomczynski et al. 1987). Reverse transcription was carried out as above, and qRT-PCR was performed with primer pairs specific for human KCNQ genes (Ng et al. 2011) (**Table 2**). To determine if the RNA sample was contaminated with genomic DNA we included a no reverse transcriptase control in the real time PCR. The PCR end products (plus/minus RT) were than allowed to run on an agarose gel. All the minus RT samples except KCNQ4

showed absence of band confirming that the RNA samples were free of genomic DNA. However, the RNA samples were not treated with DNase for further purification. Each reaction was performed in triplicate for at least four individuals, and KCNQ1–5 transcript copy numbers were estimated as described above for guinea pig, by comparing to standard curves constructed for each of the human KCNQ gene targets.

**Table 1: Guinea Pig Primers**

| Gene | Primer Sequence | Product size (bp) |
|---|---|---|
| KCNQ 1 | F:5'-ATTGTCCTGGTGGTGTTCTTTG-3'<br>R:5'-CCCCTGATGGCTGATGTGG-3' | 206 |
| KCNQ 2 | F:5'-TCTACGCCACCAACCTGTC-3'<br>R:5'-TACATGGGCACCGTGACC-3' | 79 |
| KCNQ 3 | F:5'-CTTGAAAACCGTCATCTGC-3'<br>R:5'-CAAGTTCACAGGGTCGTG-3' | 124 |
| KCNQ 4 | F:5'-GGGCCTCTCTAAGACTCAAG-3'<br>R:5'-AGGTGTCCTGCTGAATACTG-3' | 235 |
| KCNQ 5 | F:5'-CGTCCGCACTCAGAAGTC-3'<br>R:5'-TCCAATGTACCAGGCTGTG-3' | 137 |

**Table 2: Human Primers**

| Gene | Primer Sequence | Product size (bp) |
|---|---|---|
| KCNQ 1 | F:5'-AACCTCATGGTGCGCATCA-3'<br>R: 5'-CCGCGATCCTTGCTCTTTT-3' | 100 |
| KCNQ 2 | F: 5'-GGAAACCGTTCTGTGTGATTGAC-3'<br>R:5'-TCCGCAGAATCTGCAGGAA-3' | 128 |
| KCNQ 3 | F:5'-CCACGCCAAAACACAAGAAGT-3'<br>R: 5'-TGATGTGGATGGTCTGGCTACA-3' | 100 |
| KCNQ 4 | F: 5'-GCGACCGTACGACGTGAAG-3'<br>R: 5'-CAATTTGGTCCACCCGAGTT-3' | 100 |
| KCNQ 5 | F: 5'-TCCCTGAGCACACAAAATTGG-3'<br>R: 5'-CCCGCAGACCAGATTCGA-3' | 102 |

F=Forward; R= Reverse

## Results

*Expression of multiple Kv7 channel subtypes in normal guinea pig and human ASMCs:*

The qRT-PCR expression studies using primers specific for guinea pig and human KCNQ1-5 revealed expression of multiple subtypes of Kv7 channels in normal guinea pig and human ASMCs. The expression pattern in normal guinea pig ASMCs was as follows: KCNQ2>KCNQ5>KCNQ4>KCNQ3>>KCNQ1 (**Figure 12A**). The ASMCs from human trachealis muscles revealed a more variable pattern of expression, with abundant expression of KCNQ1, no detectable expression of KCNQ2 and KCNQ3, and modest expression of KCNQ4 and KCNQ5 (**Figure 12B**). The products were also confirmed by sequencing.

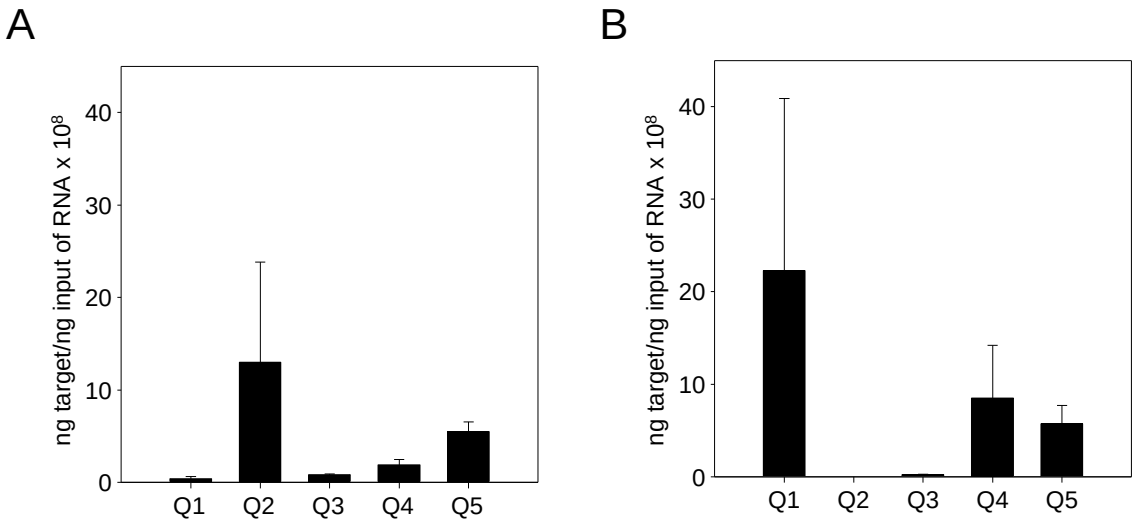


**Figure 12: Multiple KCNQ subtypes are expressed in guinea pig and human airway smooth muscle cells (ASMCs).** Expression levels of mRNAs for KCNQ1-5 were estimated using quantitative real time RT-PCR in guinea pig airway myocytes (***A***) and human trachealis muscle (***B***). Average ng target (normalized to ng input of RNA) mRNA ± SEM from n=4 guinea pigs and from n=4 human trachealis muscle samples. (Brueggemann LI, Kakad PP, et al. (2012) *Am J Physiol Lung Cell Mol Physiol* **302**(1):L120-132).

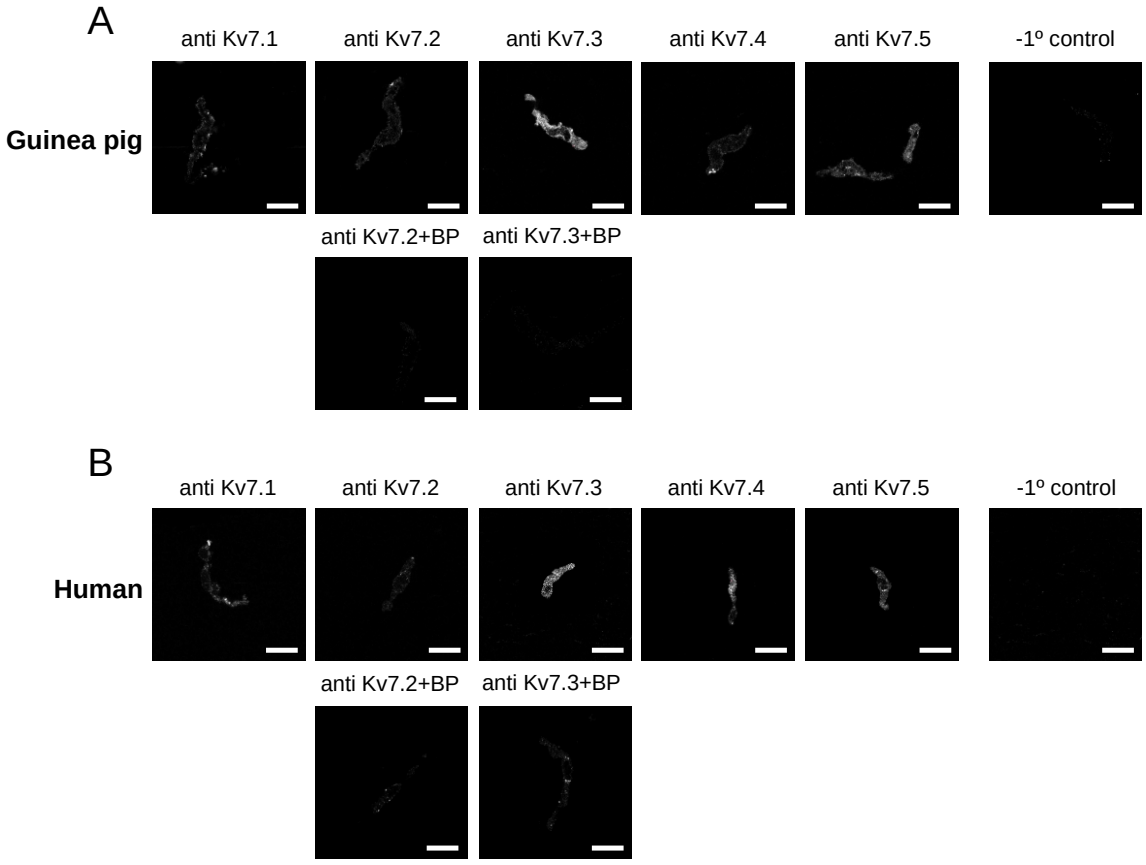


**Figure 13: Immunohistochemistry studies supporting expression of Kv7 channels in airways.** Immunostaining of guinea pig trachealis myocytes (C) and human trachealis myocytes (D) stained with anti-Kv7.1–7.5 antibodies (*top rows*); and stained with Kv7.2 and Kv7.3 antibodies preincubated with the corresponding blocking peptide (anti-Kv7.2 BP; anti-Kv7.3 BP) (*bottom rows*). Cells processed for imaging without primary antibody (1° control) show no detectible fluorescence in ASMCs at the same gain and intensity settings (right). (Brueggemann LI, Kakad PP, et al. (2012) *Am J Physiol Lung Cell Mol Physiol* **302**(1):L120-132).

The expression of multiple Kv7 channel subtypes in guinea pig and human ASMCs was also supported by immunohistochemistry studies (outside the scope of proposed studies; carried out by Dr. L. Brueggemann) (**Figure 13**). The immunohistochemistry studies are not quantitative and hence we did not attempt to compare the expression levels in guinea pig and human ASMCs. Our sole objective was to confirm the expression of translational gene products. Our studies thus provide the first evidence of expression of Kv7 channels in guinea pig and human

airways, thus paving the path for further exploration of their role in AHR. Our next step was to compare the expression pattern of different Kv7 channel subtypes in asthmatic guinea pig ASMCs.

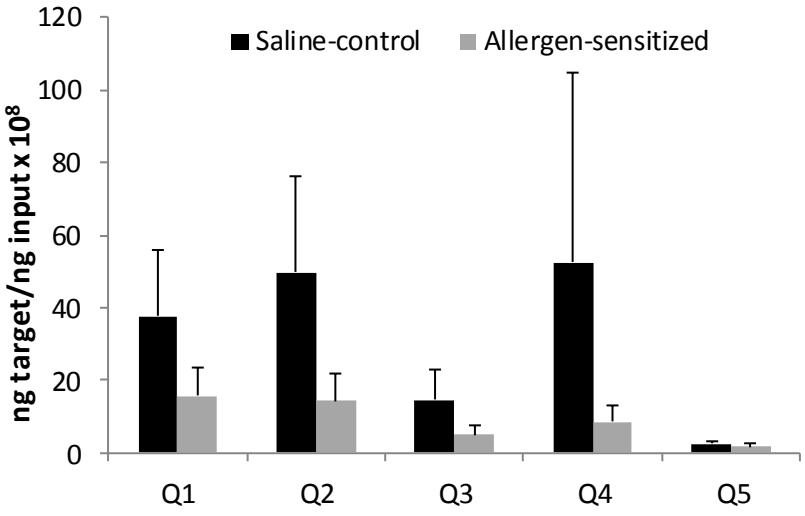


**Figure 14: Comparing expression pattern of different Kv7 channel subtypes in saline-control and allergen-sensitized guinea pig ASMCs.** Expression levels of mRNAs for KCNQ1-5 were estimated using quantitative real time RT-PCR in saline-control guinea pig ASMCs (■), and allergen-sensitized guinea pig ASMCs (■). Average ng target mRNA ± SEM from n=4 saline-control guinea pig ASMCs and n=4 allergen-sensitized guinea pig ASMCs.

*Differences in expression pattern of Kv7 channel subtypes in saline-control and allergen-sensitized guinea pig ASMCs:* In these preliminary studies comparing expression of different Kv7 channel subtypes in saline-control and allergen-sensitized ASMCs (**Figure 14**), an interesting trend was observed: expression of all KCNQ channel subtypes was found to be decreased in allergen-sensitized guinea pig ASMCs. In these initial studies, ASMCs from four saline-control and allergen-sensitized animals were used. The decrease in expression of Kv7 channel subtypes however, was not statistically significant, due to a variety of reasons. Firstly, the number of animals used in the study was not sufficient (power of comparison below

0.80) to obtain significant results due to high variability inherent to animal studies. Secondly, the four allergen-sensitized guinea pigs used for isolation of ASMCs received different amount of allergen treatment as these animals were mostly from the optimization studies we performed in the animal model preparation. Therefore, these studies need to be repeated with ASMCs from allergen-sensitized guinea pigs receiving now-optimized allergen treatment. However, if a decreased expression of KCNQ subtypes in allergen-sensitized guinea pigs is reproducibly observed, this will have important implication for our understanding of the etiology of AHR. Also, the reason for differences in the expression pattern of Kv7 channel subtypes between saline-treated and normal guinea pigs is unclear. This could be possibly attributed to the inherent inter-animal variability. We expect to see diminished variability as the number of animals is increased in our future studies.

## CHAPTER 5

## FUNCTIONAL ROLE OF Kv7 CHANNELS IN AIRWAYS

Our qRT-PCR studies not only revealed the expression of different Kv7 channel subtypes in guinea pig and human ASMCs for the first time, but also provided preliminary evidence that the expression of different subtypes of Kv7 channels is decreased in allergen-sensitized guinea pig airways. These results support our hypothesis that Kv7 channels have an important role to play in normal airway function, which is affected in asthmatic airways. Therefore, our next goal was to determine the functional relevance of these channels in normal and diseased airways. There are several *in vitro* models such as the tracheal or bronchial rings and parenchymal smooth muscle strips, that have been used to study the airway function. However, precision-cut lung slices or PCLS offer many advantages (Henjakovic et al. 2008; Liberati et al. 2010). Using PCLS, functional airway narrowing responses and the concentration-dependent responses to bronchoconstrictors and bronchodilators can be directly observed and recorded with a high degree of accuracy. PCLS preparations maintain cell–cell and cell–matrix relationships reproducing many properties and functions of the whole organs, therefore serving as an excellent *ex vivo* alternative for *in vivo* studies. Moreover, more than 30 lung slices can be prepared from one guinea pig lung and each animal can serve as its own internal control. Therefore, this method provides a

greater throughput for evaluating multiple drugs using fewer animals. However, a major challenge for PCLS studies specific to guinea pig preparations is *post mortem* airway constriction (Lai et al. 1984a). The cause of this *post mortem* constriction is not fully understood but it is attributed to the release of substance P from sensory neurons (Lai et al. 1984b). Ressmeyer and coworkers have reported a solution for *post mortem* constriction by introducing isoproterenol in the media used for PCLS preparation. It is suggested that once the PCLS preparation is complete, isoproterenol use be discontinued. The isoproterenol effect is not sustained after discontinuation and the slices respond normally to various treatments (Ressmeyer et al. 2006).

**Preparation of Precision-Cut Lung Slices (PCLS)**

*Chemicals:* Histamine, methacholine(acetyl-β-methylcholine chloride), ovalbumin and aluminium hydroxide required for PCLS studies were purchased from Sigma-Aldrich (St. Louis, MO). Retigabine dihydrochloride was purchased from LGM Pharma (Boca Raton, FL). XE-991 dihydrochloride was from Ascent Scientific (Princeton, NJ). Low melting point agarose was from GIBCO® (Invitrogen Corp, Carlsbad, CA). Drugs were diluted as needed in the control vehicle (2 mM $Ca^{+2}$ Physiological Saline Solution (PSS)) used for the precision-cut lung slices. Drugs were used in a concentrations ranging from 1 nM to 10 μM.

*Protocol:* Post euthanasia (sodium pentobarbital overdose), a thoracotomy was performed and the heart was excised to expose the bronchi. The lower half of the trachea was cut and cannulated with a 1/16" polyethylene tube connector attached to a 3-way stop-cock. One of the bronchi was tied off and that was reserved for

electrophysiological studies. The cannulated tissue was then placed into a glass beaker with mHBSS (modified-Hank's Balanced Salt Solution (mM): NaCl 137.9, KCl 5.33, $CaCl_2$ 1.26, $MgCl_2$ 0.49, $MgSO_4$ 0.41, HEPES 20, $KH_2PO_4$ 0.44, $Na_2HPO_4$ 0.34, D-glucose 5, pH 7.4-NaOH at 37°C, 298-300 mOsm) preheated to 37°C in a water bath. With a 10 mL syringe attached to one inlet, the lung was deflated. On the another inlet of the stopcock there was a 10 mL syringe filled with low-temperature-melting Type IX-A agarose (2 % in mHBSS supplemented with 20 mM HEPES). The agarose solution also contained isoproterenol in varying doses (see troubleshooting section) at 37 °C. The lung was inflated by injecting the warm agarose solution. After inflating the lungs with warm agarose, 5 ml of air was injected using a second syringe to push agarose into the alveoli from the airways. The inflated lungs were then placed into a glass beaker containing ice cold mHBSS (pH 7.4 at 4°C) and allowed to cool on ice for 30 minutes to solidify the agarose. The non-inflated lobe was cut and used for isolation of airway smooth muscle cells (as discussed in chapter 3). Precision-cut lung slices were made by use of a Leica VT 1200S vibratome, while maintaining the lung at 4 °C. Lung slices (350 μm thick) containing cross sections of 0.05– 0.5 mm in diameter, were incubated for 3 h in serum-free F-12/ DMEM (Dulbecco's Modified Eagle Medium) tissue culture medium containing 1 μM isoproterenol, supplemented with ITS (Insulin-Transferrin-Selenium, Mediatech, Manassas, VA) and antibiotics at 37°C under 5% $CO_2$. Medium was changed after every 30 minutes for 3 h. After 3 h, the slices were transferred to the same medium but without isoproterenol. Lung slices generally remained viable for at least 4 days. Slices containing airways were used only if 1) the airway was approximately

circular; 2) beating cilia were observed (indicating an intact epithelium) and; 3) the airway wall and all parenchymal attachments were intact. The selected lung slice was then transferred into a 35 mm tissue culture dish onto a 25 mm diameter round glass coverslip (sterilized by dipping in 100% ethanol and flame dried). The dish contained 1 ml of 2 mM $Ca^{2+}$ PSS (control medium (mM): 140 NaCl, 5.36 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 D-glucose, pH 7.3, 298 mosmol/l).

*Measurement of airway diameter:* Vacuum grease was applied to the groove of a customized recording chamber. A coverslip with a PCLS was positioned evenly into the groove of the chamber. A silicon ring was gently placed on the coverslip in order to avoid any leak. Finally, a platinum weight was placed on the slice so as to hold the slice in position without blocking the view of the airway. The chamber was then filled with 1 ml of the control medium. The glass bottom of the chamber was cleaned with an alcohol soaked Kim-wipe®. The lung slice, mounted in the perfusion chamber was then placed on the stage of an inverted microscope (Olympus IX-71) and visualized via a 10X objective. A cross section of a small bronchiole (0.05–1.5 mm in diameter) was positioned in the center of the microscopic field and the lung slice was then equilibrated in control medium at room temperature for at least 30 min. Control medium, plus or minus drugs, was continuously superfused over the lung slice *via* a gravity-fed perfusion system at a rate of ~5 ml/min, controlled by a common flow regulator. Experiments were conducted at room temperature. The focus and light intensity was adjusted to see the clear image. The region of interest (ROI) for the measurement was selected (red outline). The threshold range that identifies the object in green for measurement was then selected (**Figure 15**).

Images were captured with a 12-bit digital camera (Hamamatsu Orca) at 5-s intervals. Luminal area was measured by image analysis using Simple-PCI software (Hamamatsu, Sewickley, PA). Summarized measurements of luminal area of the airway in each experiment represent the average for the last 1 minute of drug application or washout (total 12 measurements in each case).

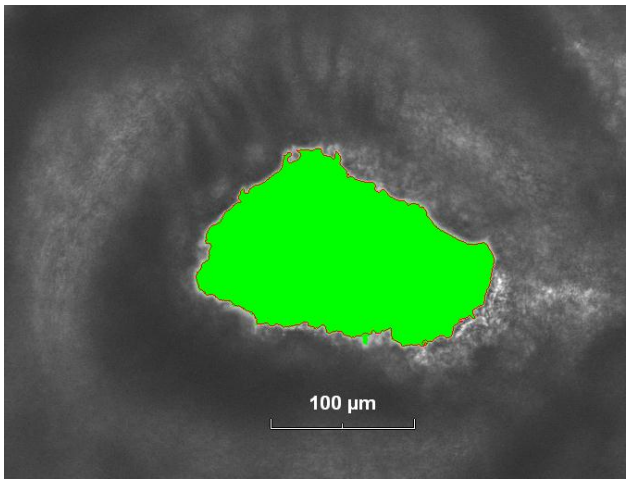


**Figure 15: Image demonstrating luminal area measurement of PCLS preparation**. A cross section of a small bronchiole (0.05–1.5 mm in diameter) is positioned in the center of the microscopic field. The threshold range that identifies the object for measurement in green is selected. Images are captured at 5-s intervals.

To obtain dose-response curves for bronchoconstrictor agoinsts (histamine and methacholine) in saline-control and allergen-sensitized airways, the PCLS preparations from these animals were treated with agonist concentrations ranging from 1 nM to 30 µM. The dose-response studies were repeated in PCLS from multiple animals and the data was then plotted as normalized area *versus* logarithm of agonist concentration using Graph Pad Prism software. The half-maximal effective concentration ($EC_{50}$) was determined by fitting the data to five-parameter, variable-hill slope equation.

**Troubleshooting**

Following the procedure reported by Ressemeyer and coworkers to prevent *post mortem* constriction, 1 μM isoproterenol in agarose solution was used (Ressemeyer et al. 2006). However, the slices using this protocol did not respond to any of the agonists tested and instead constricted or dilated spontaneously in the control medium independently of the drug treatment. After a few repetitions with similar outcome, we decided to omit isoproterenol from our experiments. This, however, did not solve the problem. The slices obtained from these lungs did not respond to any concentration of agonist. Instead a very stable baseline recording was obtained independently of the treatment. A lower concentration of isoproterenol (100 nM) in agarose was also tested. However, the problem persisted despite use of the lower isoproterenol concentration.

Finally, we went back to the original protocol, using 1 μM isoproterenol in 2 % agarose solution to inflate the lungs. However, this time the slices were maintained in medium containing 1 μM isoproterenol after preparation. This solved the problem of post mortem constriction as well as the unusual behavior exhibited by the guinea pig lung slices in the earlier studies.

**Results**

The physiological relevance of functional Kv7 channels in guinea pig airways was investigated using PCLS studies. Before beginning our planned pharmacological studies, we wanted to confirm the hypersensitivity of actively sensitized airways obtained from the allergen-sensitized guinea pig model. This was done by challenging these actively sensitized airways with ovalbumin (passive challenge).

Application of 1 μg/ml ovalbumin alone completely closed the airways, providing strong evidence that the airways of OVA-treated animals were indeed hypersensitized. As observed from the time course (**Figure 15**), the airways started to relax while still in ovalbumin, this could be due to the depletion of the agonist stores (methacoline, histamine), which cannot be resynthesized in the *ex vivo* model of PCLS.

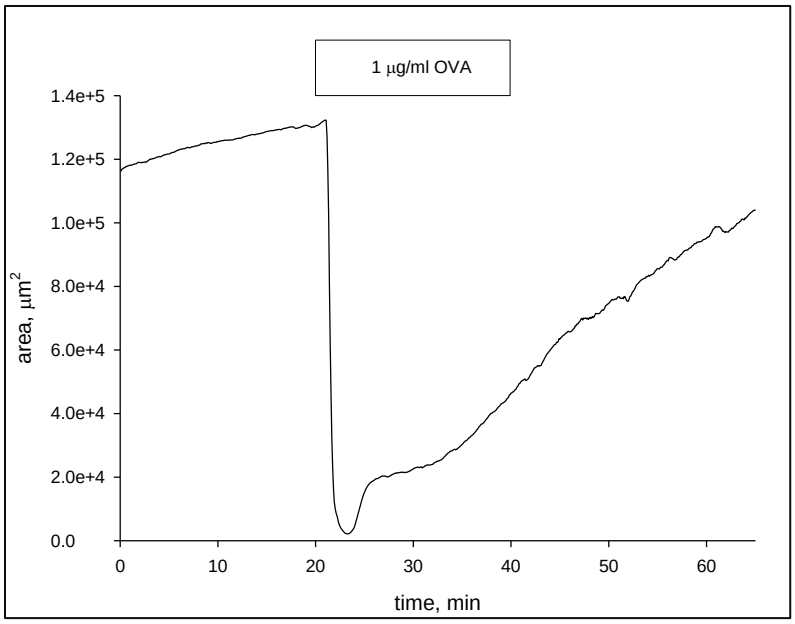


**Figure 16: Passive sensitization of airway from guinea pig model of AHR using ovalbumin (OVA).** Representative time course after treatment with 1 ug/mL OVA to PCLS preparations obtained from actively sensitized guinea pig airways.

***Increased sensitivity of allergen-sensitized airways to bronchoconstrictor agonists:*** Our next goal was to evaluate the differences in response of saline-control and allergen-sensitized airways to the bronchoconstrictor agonists, histamine and methacholine. We expected increased sensitivity of allergen-sensitized airways to bronchoconstrictor agonists. As expected, the allergen-sensitive airways showed clear and distinct hyperresponsiveness to both histamine and methacholine. Our

next step was to determine the half-maximal effective concentrations ($EC_{50}$) for these agonists in both saline-control and allergen-sensitized airways. The dose-response curves for histamine (**Figure 16**) and methacholine (**Figure 17**), were determined in saline-control and allergen-sensitized airways. As expected there was shift in the dose-response curve toward left for allergen-sensitized airways indicating increased sensitivity to bronchoconstrictor agonists. The observed $EC_{50}$ value of 204.5 nM obtained from the dose-response curve of histamine in saline-control guinea pig airways was in a very good agreement with literature reported value of 217 nM in normal guinea pig airways (Ressemeyer et al. 2006). Importantly, there was ~4.5 fold decrease in the $EC_{50}$ value (45.9 nM) observed in allergen-sensitized airways thus providing clear evidence that AHR has been induced in the guinea pig model. Dose-response curve for methacholine showed ~2.5 fold decrease in the $EC_{50}$ value in allergen-sensitized airways (28.7 nM) compared to saline-control guinea pigs airways (72.0 nM). Along with an increase in sensitivity to histamine and methacholine in allergen-sensitized airways, a considerable increase in $E_{max}$ (maximal effect) was observed with both bronchoconstrictor agonists tested (**Figure 16C & 17C**).

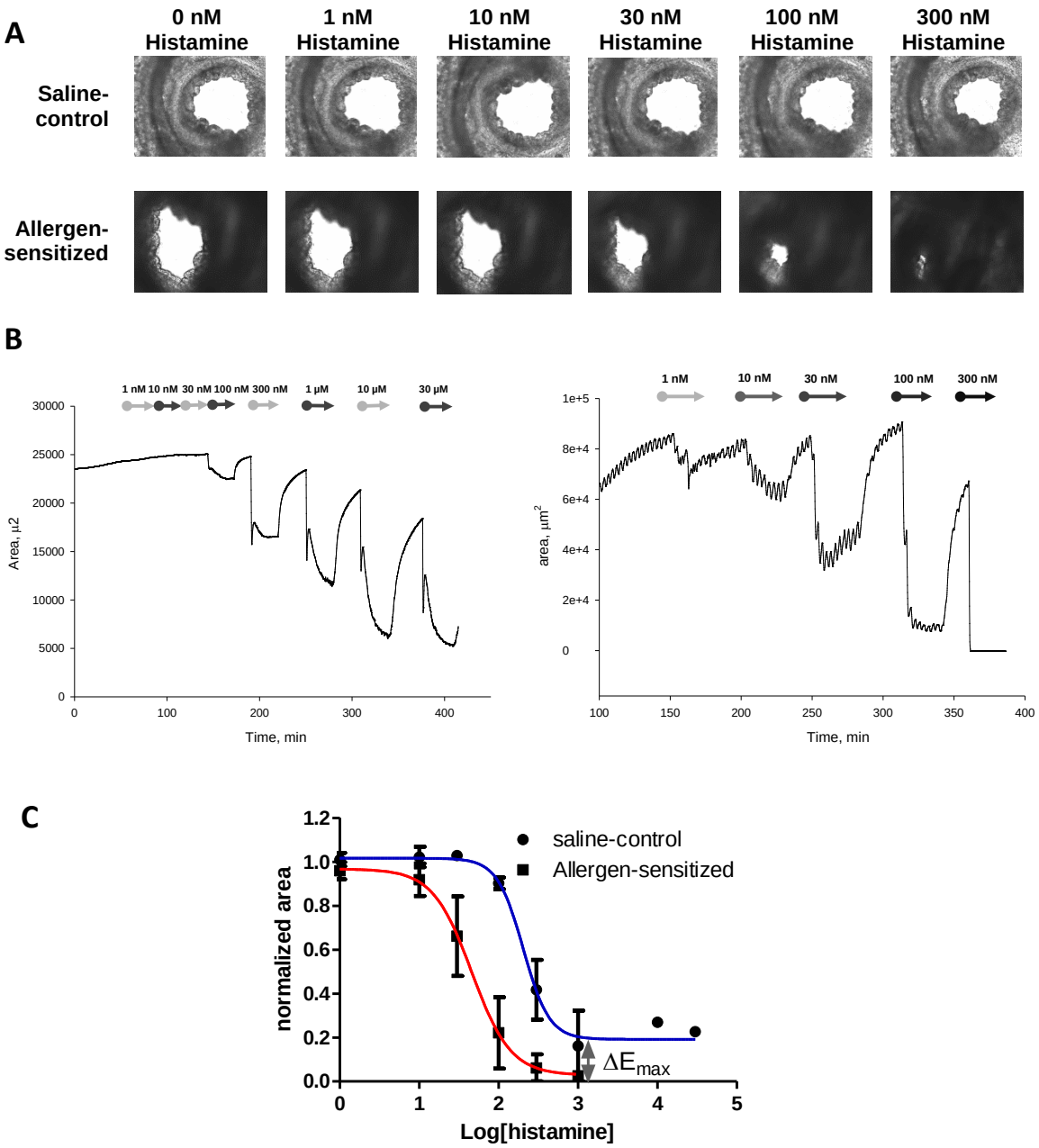


**Figure 17: Dose-response curves for histamine in PCLS preparations from saline-control and allergen-sensitized guinea pig airways.** ***A****:* representative images showing change in luminal area of PCLS preparations after treatment with increasing concentrations of histamine (H) in saline-control (*top*) and allergen-sensitized (*bottom*) airways. ***B****:* corresponding representative time course of changes in luminal area of the same PCLS preparations in saline-control (*left*) and allergen-sensitized (*right*) airways. ***C****:* Dose-response curve obtained by plotting the normalized luminal area *versus* logarithm of histamine concentration in saline-control (●, n = 3) and allergen-sensitized (■, n = 2) airways. Half-maximal effective concentration ($EC_{50}$) values were obtained by fitting the data to the five-parameter, variable slope equation using GraphPad® Prism software (La Jolla, CA). Dose-response curve for histamine in saline-control guinea pigs gave an $EC_{50}$ = 204.5 nM that is in very good agreement with literature reported value of 217 nM (Ressemeyer et al. 2006). The $EC_{50}$ of histamine in allergen-sensitized guinea pigs was calculated to be 45.9 nM.

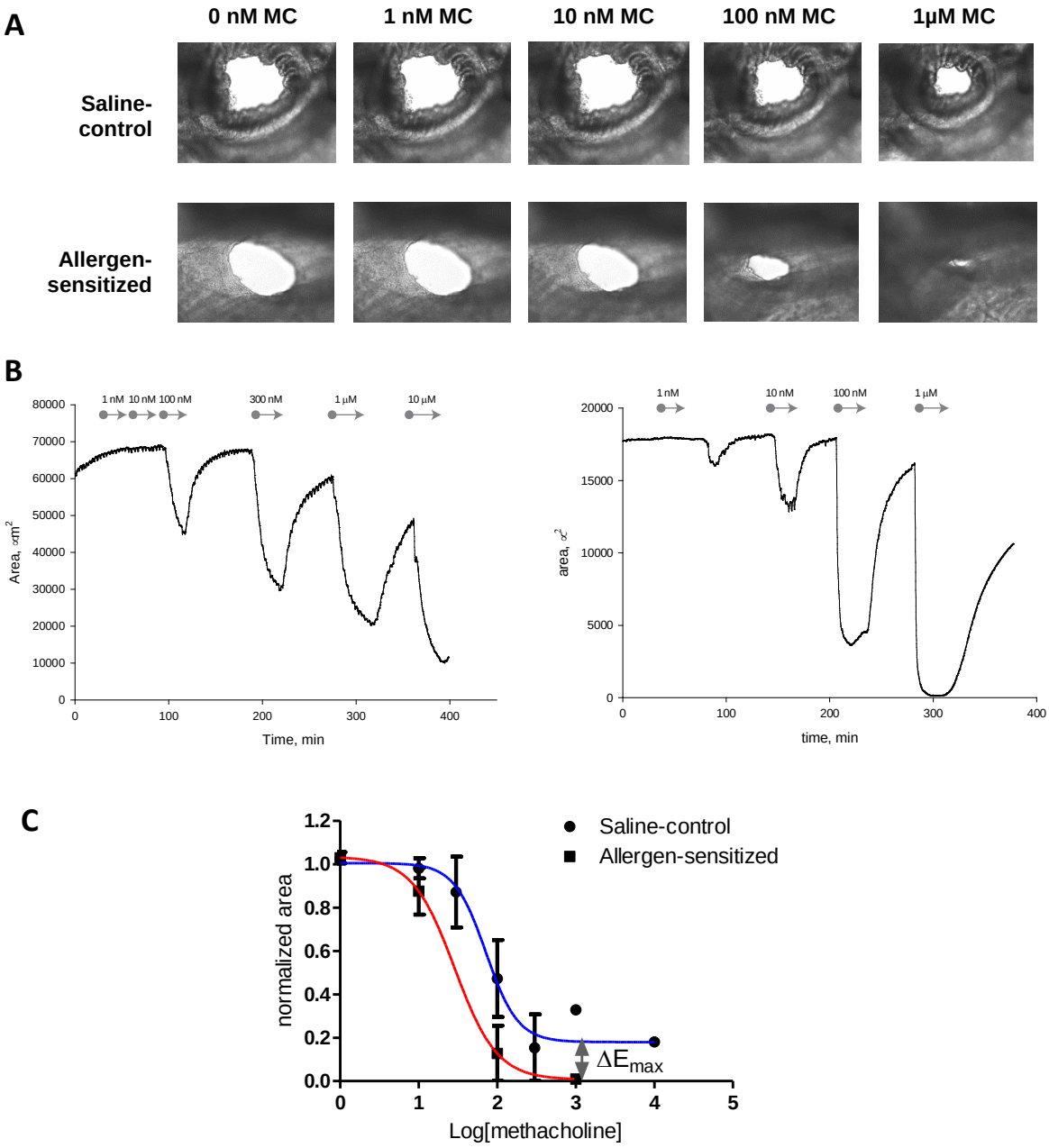


**Figure 18: Dose-response curves for methacholine (MC) in PCLS preparations from saline-control and allergen-sensitized guinea pig airways.** ***A:*** representative images showing change in luminal area of PCLS preparations after treatment with increasing concentrations of MC in saline-control (*top*) and allergen-sensitized (*bottom*) airways. ***B:*** corresponding representative time course of changes in luminal area of the same PCLS preparations in saline-control (*left*) and allergen-sensitized (*right*) airways after MC treatment. ***C:*** Dose-response curve obtained by plotting the normalized luminal area *versus* logarithm of MC concentration in saline-control (●, n = 3) and allergen-sensitized (■, n = 2) airways. $EC_{50}$ values were obtained by fitting the data to the five-parameter, variable-slope equation using GraphPad® Prism software (La Jolla, CA). Dose-response curve for MC in saline-control guinea pigs gave an $EC_{50}$ = 72.0 nM, while the $EC_{50}$ of MC in allergen-sensitized guinea pigs was calculated to be 28.7 nM.

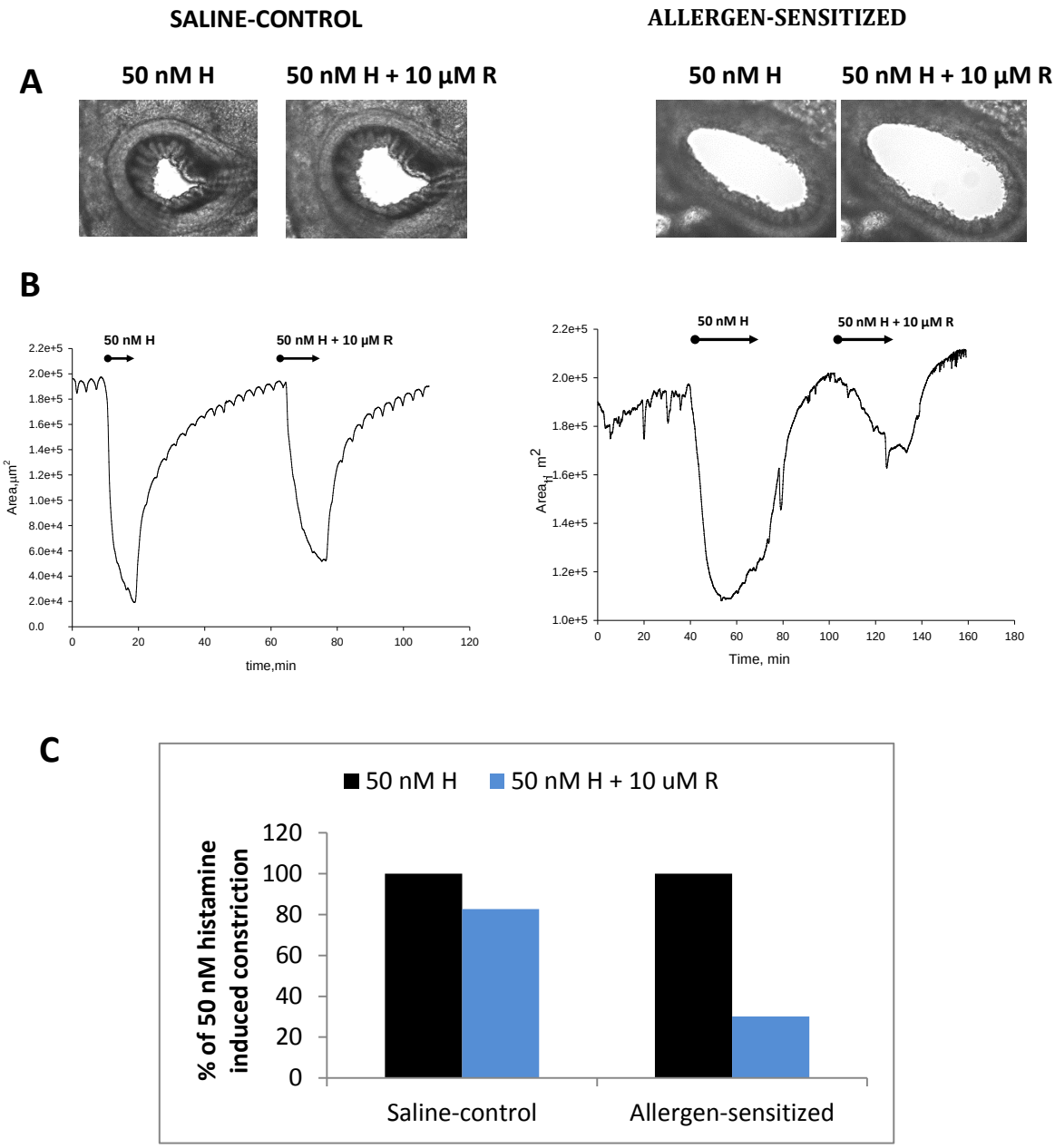


**Figure19. Kv7 Channel activator retigabine (R) attenuated histamine (H)-induced constriction of saline-control and allergen-sensitized airways**. ***A***: Representative images of a small airway from saline-control and allergen-sensitized airways in the presence of 50 nM histamine (50 nM H) alone, and 50 nM histamine applied with 10 µM retigabine after complete recovery from the first treatment (50 nM H + 10 µM R). ***B***: corresponding representative time course of changes in luminal area of the same small airways. ***C***: summarized bar graph of the percentage of 50 nM histamine induced constriction after histamine + retigabine treatment, data normalized to 50 nM histamine treatment. Luminal area was measured during the last min of treatment with 50 nM H and 50 nM H + 10 µM R.

***Kv7 channel activator attenuates histamine-induced bronchoconstriction in guinea pig airways:*** After determining the $EC_{50}$ values of histamine and methacholine in saline-control and allergen-sensitized airways, we wanted to study if the bronchoconstriction effect on allergen-sensitized airways can be attenuated with Kv7 channel activators. Histamine was selected for these studies because it showed a distinctly potentiated effect in allergen-sensitized airways. We treated PCLS preparations from saline-control and allergen-sensitized airways with a near $EC_{50}$ concentration of histamine (50 nM) as measured in allergen-sensitized airways. The 50 nM histamine treatment was repeated (after complete relaxation of the airway) in the presence of 10 μM retigabine (Kv7.2-Kv7.5 channel activator) (**Figure 18A & B**). Importantly, retigabine was able to attenuate the histamine-mediated bronchoconstriction by ~20 % in saline-control airways and ~ 70 % in allergen-sensitized airways (**Figure 18C**). This result is significant as it supports our hypothesis that Kv7 channel activators can attenuate the effect of bronchoconstrictor agonists in allergen-sensitized airways. These studies will be repeated for reproducibility using PCLS preparations from a larger number of animals. Vehicle control for retigabine will also be included.

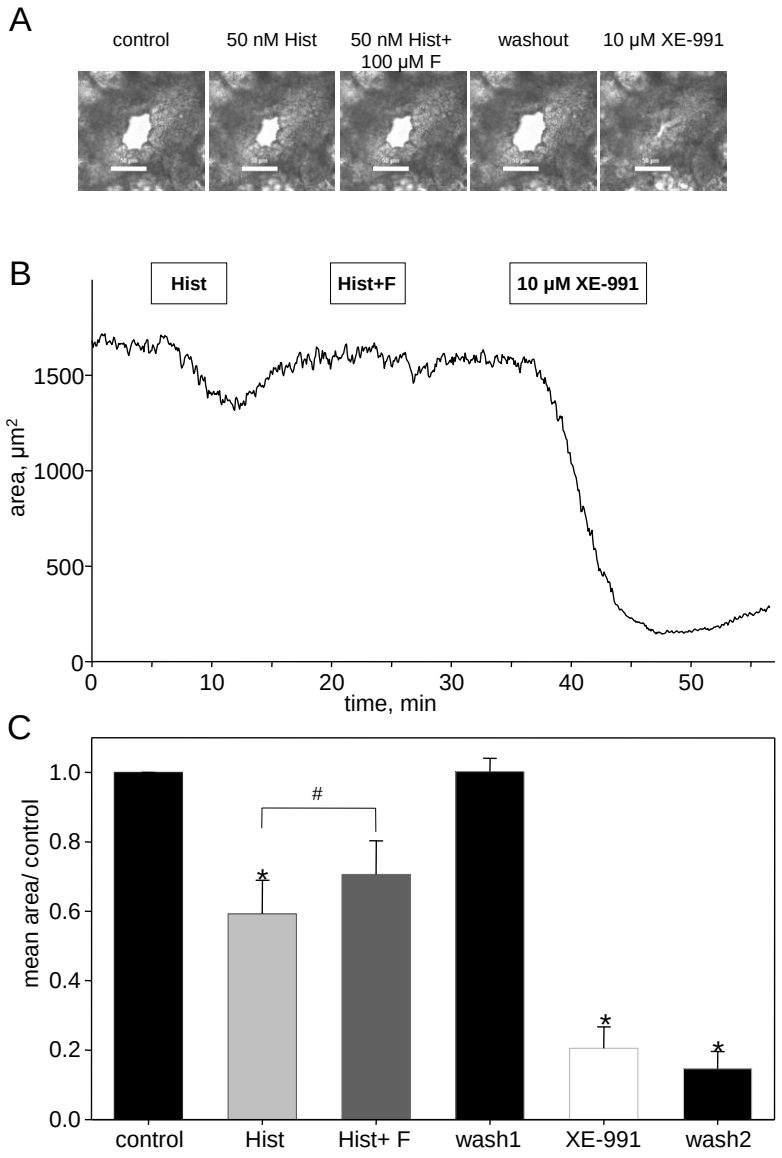


**Figure 20. Functional Kv7 channels are required for maintaining resting diameter of small airways in human lung slices.** ***A***: representative images of a small airway before treatment (control), in the presence of 50 nM histamine applied for 5 min (50 nM Hist), in the presence of 50 nM histamine applied with 100 µM flupirtine for 5 min (50 nM Hist + 100 µM F), following a 10-min washout of histamine and flupirtine (washout), and in the presence of 10 µM XE991 applied for 10 min. ***B***: corresponding representative time course of changes in luminal area of the same small airway. Both 50 nM histamine (Hist) and 50 nM histamine with 100 µM flupirtine (Hist + F) were applied for 5 min followed by a 10-min washout in each case; 10 µM XE-991 was applied for 10 min. ***C***: summarized bar graph of average airway luminal area measured in multiple lung slices from 2 human subjects. Luminal area measured during the last min of treatment with 50 nM histamine (Hist), 50 nM histamine with 100 µM flupirtine (Hist + F), or 10 µM XE991 was normalized to control area measured before treatments; *significant difference from control (1-way ANOVA, $P < 0.001$, n = 6–8); #significant difference between histamine alone and histamine plus flupirtine treatment groups (paired Student's t-test, $P < 0.05$, n = 6). (Adapted from: Brueggemann LI, Kakad PP, et al. (2012) *Am J Physiol Lung Cell Mol Physiol* **302**(1):L120-132).

***Kv7 channel activator attenuates histamine-induced bronchoconstriction in human airways:*** The availability of human lung tissues enabled us to evaluate the physiological relevance of functional Kv7 channels in human airways using PCLS (work carried out by Dr. L. Brueggemann). Application of 50 nM histamine induced a 41 ± 10% constriction of human airways (as estimated by reduction of luminal area). The histamine-induced constriction was slightly but significantly attenuated (29 ± 10%, P = 0.014, paired Student's t-test) in the presence of 100 μM flupirtine, an activator of Kv7.2–7.5 channels (**Figure 19C**). Application of 10 μM of XE991, a potent and selective inhibitor of Kv7 channels, consistently induced near total constriction of small airways (46–89 μm in diameter) in human lung slices (79 ± 6% constriction, n = 6; **Figure 19**).

# CHAPTER 6

## DISCUSSION AND CONCLUSIONS

Airway hyperresponsiveness (AHR), a characteristic feature of asthma, is marked by increased sensitivity of airways to inhaled bronchoconstrictor agonists. The mechanisms underlying AHR are, however, poorly understood. Airway smooth muscle cells (ASMCs) are the main effector cells of airway narrowing. The Kv7 voltage-activated $K^+$ channels are known to play an important role in regulating resting membrane potential of many excitable cells, however, their expression and function in ASMCs has never been investigated before. The research presented in this thesis not only studies the expression and function of Kv7 channels in ASMCs but also reveals a link between modulation of Kv7 channel function and AHR in asthmatic airways. Our results present the first evidence of Kv7 channels expression in guinea pig and human ASMCs. In a parallel investigation to these studies, our laboratory has measured $K^+$ currents in ASMCs with the expected electrophysiological and pharmacological characteristics of Kv7 currents. The $G_{q/11}$-coupled bronchoconstrictor agonists were observed to suppress Kv7 currents, but the currents could be restored by drugs that are selective Kv7 channel activators. These results motivated us to explore the functional relevance of Kv7 channels in further detail using *ex vivo* precision-cut lung slices (PCLS). Our initial PCLS studies revealed that Kv7 channel activators are capable of attenuating

bronchoconstrictor agonist induced airway constriction, highlighting an important contribution of these channels in maintaining the airway diameter. These findings also provide the first evidence in support of our key hypothesis that Kv7 channels have an important role to play in the pathophysiology of AHR. A series of studies were then performed comparing the expression of Kv7 channel subtypes in asthmatic airways to normal airways using a suitable model of asthma and examining the modulation of Kv7 channel function in diseased airways. Some of the key findings from our studies and their implications for our current understanding of AHR are discussed below.

***Kv7 channels in ASMCs***

Our findings reported in this thesis indicate that Kv7 channels are expressed in ASMCs. Multiple Kv7 channel subtypes were found to be expressed in both guinea pig and human ASMCs, although their expression pattern differed in the two species. Guinea pig ASMCs expressed KCNQ2>KCNQ5>KCNQ4>KCNQ3>>KCNQ1, while human ASMCs expressed KCNQ1>KCNQ4>KCNQ5>KCNQ3. The expression pattern in human ASMCs was similar to that reported in vascular smooth muscle cells (VSMCs) in mouse and rat arteries (expression of KCNQ1, KCNQ4, and KCNQ5, with little or no detectable KCNQ2 or KCNQ3) (Brueggemann et al. 2012; Joshi et al. 2009; Yeung et al. 2007). In human arteries, only KCNQ2 was undetectable (Ng et al. 2011). The expression pattern for KCNQ1 and KCNQ2 mRNAs was different between normal guinea pig and human ASMCs. KCNQ2 was the most abundant KCNQ transcript in guinea pig ASMCs, but was undetectable in human ASMCs,

whereas KCNQ1 was most abundant in human and barely detectable in guinea pig. The reason for these inter-species differences in Kv7 channel expression pattern and their functional impact on airway function still remains to be determined. The results from the expression studies were confirmed using immunofluorescence detection of Kv7 channel proteins in normal guinea pig and human ASMCs. These studies also indicated the presence of multiple Kv7 channel subtypes in both human and guinea pig ASMCs, but with similar expression patterns between the species. However, this method is not quantitative and the findings are highly dependent on the quality of available antibodies. Therefore, caution should be exercised while interpreting the immunofluorescence data.

While evaluating the findings from electrophysiological studies (as discussed in Chapter 1) in context with the expression studies (as presented in Chapter 4), it is important to take into consideration that any of the expressed Kv7 channel protein subtypes can potentially contribute to the $K^+$ currents measured in guinea pig or human ASMCs. Therefore, robust currents were detected in guinea pig ASMCs where KCNQ1 expression was not detected. Selective activators of Kv7.2–7.5 subtypes, flupirtine and retigabine, robustly enhanced the currents in both guinea pig and human ASMCs. Combined together, the data indicates that there is a likelihood of some combination of Kv7.2–7.5 subtypes forming functional channels in both guinea pig and human ASMCs that contribute to the regulation of airway diameter.

When we extended the expression studies to allergen-sensitized airways obtained from a guinea pig model of AHR, our preliminary results (n=4) showed a

trend indicating a decrease in the expression of all KCNQ isoforms in allergen-sensitized guinea pig airways compared to the saline-controls. We plan to repeat these experiments to increase the sample size. However, conclusions from these data will be drawn with caution as reduced expression of genes does not necessarily mean reduced presence of functional protein products, hence confirming the decrease in translational gene product would further strengthen our conclusions from the expression studies. If the expression of KCNQ genes is consistently found to be low, then this observation will have important implications on our understanding of the etiology of AHR. It will suggest to us that AHR in asthma is likely to be caused by bronchoconstrictor agonist-mediated suppression of genes encoding Kv7 channel. This will initiate efforts on our part to investigate the effectors downstream of $G_{q/11}$ coupled receptors that are responsible for reduction in KCNQ mRNA levels in allergen-sensitized airways. The mRNA levels in cells can be regulated at the transcriptional and post-transcriptional stages (Latchman 2005). A number of mechanisms can potentially be involved in regulating mRNA levels at these stages. For example, the transcriptional regulation can result from alteration of various transcriptional factors, while the post-transcriptional regulation can be effected by modulation of capping, splicing or poly(A) tail addition processes. Therefore, it will be interesting to investigate the exact mechanism leading to decrease in KCNQ mRNA levels in allergen-sensitized guinea pig airways. Additionally, if the functional Kv7 channel protein level is found to be unaffected in allergen-sensitized guinea pig ASMCs, we will continue to perform experiments to investigate the mechanism of

the suppressive effect of bronchoconstrictor agonists on Kv7 channel activity as seen in the electrophysiological and PCLS studies (discussed in next section).

***Kv7 channel activity in ASMCs and its significance in AHR***

AHR, in simple terms, can be defined as airways that constrict "too easily" and "too much" in response to particular stimuli. A number of pharmacological (eg. bronchoconstrictor agonists), chemical (eg. allergens) and physical (eg. exercise) stimuli can lead to such an exaggerated response. The diagnosis of asthma is generally made when bronchoconstrictor agonists that act directly on ASMCs induce constriction of airways at lower than normal concentrations, or when airways constrict with a lower threshold of indirect bronchial provocation (e.g., exercise or inhalation of cold air). In agreement with these well documented facts, our bronchoconstrictor agonist dose-response studies using PCLS preparations showed increased sensitivity to histamine and methacholine in allergen-sensitized guinea pigs compared to the saline control. There was an approximate 4.5-fold decrease in the $EC_{50}$ value for histamine and ~2.5-fold decrease in the $EC_{50}$ value for methacholine in allergen-sensitized guinea pig airways compared to saline control. Importantly, in addition to the increased airway sensitivity, there was a notable increase in the $E_{max}$ values in allergen-sensitized airways indicating a concomitant increase in the magnitude of constriction. Thus the allergen-sensitized guinea pig airways constricted "too easily" and "too much" in response to bronchoconstrictor agonist, suggesting airway hyper-sensitization. The hyper-sensitization of airways was also confirmed by application of ovalbumin (chemical stimulus) to the allergen-

sensitized PCLS preparation. Passive challenge with 1 μg/ml of ovalbumin completely constricted airways that were actively sensitized.

The underlying mechanism for increased sensitivity of airways to multiple direct and indirect stimuli in asthma patients is one of the key questions that remain unanswered. In line with our working hypothesis, a possible mechanism for sensitization of ASMCs to multiple bronchoconstrictor stimuli could be a reduction in $K^+$ channel activity associated with an increase in electrical resistance of ASMC plasma membrane. According to Ohm's law, voltage is proportional to the product of current and resistance. Therefore, an increase in resistance would enable a small current to produce a larger change in voltage. In other words, a reduction in outward $K^+$ currents would increase membrane resistance and thereby serve to sensitize ASMCs to depolarizing stimuli (depolarizing stimuli include small increases in inward current, e.g., due to activation of $Cl^-$ or cation channels in response to bronchial provocation). Thus bronchial provocation would tend to induce greater membrane depolarization and activation of voltage-sensitive calcium channels (VSCC) when $K^+$ channel activity is reduced. Such a mechanism is consistent with the observation that the sensitivity of porcine trachealis muscle to histamine-induced contraction was enhanced in the presence of a nonspecific $K^+$ channel blocker (tetraethylammonium, 10 mM), and that this effect was abolished by the calcium channel blocker verapamil (Mitchell et al. 1987). If Kv7 channel activity in ASMCs were reduced in asthma, bronchial provocation by pharmacological or mechanical stimulation would more effectively depolarize the

cells to activate L-type VSCC and enhance airway constriction. Our preliminary results from Kv7 channel expression studies in allergen-sensitized airways indeed suggest a decrease in Kv7 channel expression level that may lead to reduced Kv7 channel activity. Conversely, increasing Kv7 channel activity would reduce ASMC electrical excitability and decrease the sensitivity to bronchial provocation. In agreement with this hypothesis, our PCLS studies in normal guinea pigs indicate a dose-dependent constriction of airways with histamine and attenuation of this response in the presence of Kv7 channel activator, retigabine. Similarly, despite their reduced expression in allergen-sensitized airways, we believe that an increase in the activity of the remaining Kv7 channels is likely to decrease the sensitivity of allergen-sensitized airways to bronchial provocation. In our investigation of this effect we observed a dose-dependent constriction of allergen-sensitized airways with histamine and significant attenuation of this response in the presence of Kv7 channel activator, retigabine. Therefore, these results provide us an insight in to how Kv7 channel expression and function in airways play a role in regulation of airway diameter. Importantly, the attenuation of bronchoconstrictor agoinst-mediated airway constriction by Kv7 channel activators in allergen-sensitized airways indicates promising application of Kv7 channel activators in treatment of airway hyperresponsiveness.

**Pharmacological targeting of Kv7 channels in ASMCs**

Bronchoconstrictor agonists depolarize ASMC membrane potentials positive to the L-type VSCC activation threshold which is likely to activate L-type VSCC

leading to influx of $Ca^{+2}$ and bronchoconstriction (Hirota et al. 2007). Therefore, an effective way of antagonizing agonist induced bronchoconstrictor effect is to block L-type VSCC. As discussed in chapter 1, a number of clinical trials were conducted in the early 1980's to evaluate the efficacy of L-type $Ca^{2+}$ channel blockers (CCBs) in the treatment of asthma. The results were inconsistent, although almost all the clinical trials revealed at least a subset of patients who benefited from treatment with either verapamil or nifedipine (Fish et al. 1984). Taken as a whole, the clinical trials revealed that asthma patients benefit from treatment with CCBs, but the efficacy of these agents is limited because of adverse side effects associated with systemic administration and limitations of formulation that prevent delivery of effective doses of verapamil or nifedipine by inhalation (Fish et al. 1984). Our ongoing studies have indicated that the bronchoconstrictor effect of histamine at lower concentration (≤ 1 μM) is effectively attenuated by CCBs like verapamil. However, verapamil is not effective in attenuating the effect of histamine used in higher concentrations (10 μM), thus indicating that at high agonist concentration, the bronchoconstrictor effect of histamine is independent of L-type VSCC. This is in agreement with the previously published study from our laboratory, showing concentration dependent signaling of $G_{q/11}$ receptor agonist vasopressin (AVP) in vascular smooth muscle cells (VSMCs) (Henderson et al. 2007). The local concentration of histamine in normal and allergen-sensitized airways is not known but given the fact that CCBs show significant protection against bronchoconstriction induced by exogenously administered histamine in human subjects (Cuss et al.

1985). It remains to be determined whether the local histamine concentration in asthmatic airways is in the range where CCBs are effective.

The Kv7 channel activators, by means of their membrane repolarizing property, can ultimately have the same effect of reducing $Ca^{2+}$ influx in ASMCs as with CCBs therapy. Although our studies suggest reduced expression of Kv7 channels in allergen-sensitized airways, it was also observed that Kv7 channel activator, retigabine, can attenuate the bronchoconstrictor agonist-mediated constriction, possibly by enhancing the activity of the remaining Kv7 channels. In general, Kv7 channel activators have well-established clinical safety profiles when administered systemically, and it may also be possible to develop inhalational formulations of these drugs, which would reduce off-target effects. Furthermore, if the Kv7 channel subunit stoichiometry in ASMCs differs from that of channels found in other tissues, it may be possible to develop drugs that have selectivity for ASMC Kv7 channels further improving their safety profile.

If we reproducibly measure decreased level of KCNQ mRNA in allergen-sensitized guinea pig airways, we will focus our efforts in identifying transcriptional or post-transcriptional events that lead to reduced KCNQ mRNA levels. Depending on the mechanism responsible for reduced expression, gene therapy would be a viable therapeutic approach. Gene therapy has shown tremendous potential in the treatment of disease like cystic fibrosis by replacement of mutated Cystic Fibrosis Transmembrane Regulator (CFTR) gene (Corbyn 2012). Gene thearpy is a promising approach as it targets the cause of a disease rather than just controlling the

symptoms. However, the success of this approach would depend on the mechanism that is responsible for reduced mRNA levels in allergen-sensitized airways.

**Summary**

In summary, our results reveal a novel and fundamental mechanism for regulating airway diameter. Our studies demonstrate that the activity of Kv7 potassium channels in ASMCs under resting conditions is essential to maintain the relaxed state of the airways. Preliminary findings suggest that bronchial provocation of airways with a known allergen reduces the expression of Kv7 channels. Ultimately, this may lead to potentiation of the bronchoconstrictor effects mediated by bronchoconstrictor agonists, and thereby account for airway hypersensitization. Our results also provide evidence that despite the reduced expression of Kv7 channel in diseased airways, Kv7 channel activators are still effective in attenuating the bronchoconstrictor effect of a bronchoconstrictor agoinst, indicating that these drugs may be useful in the treatment of airway hyperconstriction in asthma or other airway diseases. After confirming that there is a proportionate decrease in the functional Kv7 channel protein as a result of reduced KCNQ genes expression, it will be interesting to investigate the underlying mechanisms of the decreased Kv7 channel expression upon bronchial provocation.

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## VITA

Priyanka P. Kakad was born on June 25, 1985 in a small town in India called Jorve. Priyanka completed her bachelor's degree in Pharmacy in 2007. She then moved to the United States and received her research training in the laboratory of Dr. Tirumalai Rangasamy at the University of Rochester Medical Center, Rochester, New York.

In August 2009, Priyanka was accepted in to the Loyola University Chicago to pursue her graduate studies in the area of Molecular Pharmacology and Therapeutics. She joined the laboratory of Dr. Kenneth L. Byron in June 2010. Her master's thesis in the Byron laboratory focuses on studying the role of Kv7 potassium channels as a potential target in the treatment of asthma.

Upon completion of her master's degree, Priyanka will be conducting her PhD research under the guidance of Dr. Samuel Young at the Max Plank Florida Research Institute in Jupiter, Florida.