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AIRWAY SMOOTH MUSCLE EXCITATION-CONTRACTION COUPLING
INvolvEMEnt oF SRC tyrosine kinase and calcium-handling in airway smooth muscle excitation-contraction coupling

by

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a thesis submitted to the school of graduate studies in partial fulfillment of the requirements for the degree master of science

mcMaster university

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DESCRIPTIVE NOTE

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TITLE: INVOLVEMENT OF SRC TYROSINE KINASE AND CALCIUM-HANDLING IN AIRWAY SMOOTH MUSCLE EXCITATION-CONTRACTION COUPLING

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ABSTRACT

Introduction
Asthma is a chronic respiratory disease that is becoming more prevalent. Airway hyperresponsiveness, a key feature of asthma, involves increased narrowing of the airways in response to bronchoconstricting agents. Airway smooth muscle (ASM) functioning is largely responsible for hyperresponsiveness yet the mechanisms behind excitation-contraction coupling are not fully understood. Src tyrosine kinase contributes to contraction in other smooth muscle types. Furthermore, STIM1, Orai1, IPLA2β and RyRs play a role in ASM excitation-contraction coupling.

Aim
We sought to determine whether Src activity is involved in serotonin (5-HT)- and acetylcholine (ACh)-induced ASM contraction. We also examined whether the gene expression of molecules involved in sarcoplasmic reticulum emptying and refilling is altered during airway hyperresponsiveness.

Methods
Bovine tracheal ASM strips were pre-treated with the non-specific tyrosine kinase inhibitor genistein (10^-4 M), src kinase family inhibitors PP1 (10^-5 M) and PP2 (10^-5 M) or vehicle and challenged with either 5-HT or ACh to determine the involvement of Src in contraction. Western blotting was used to examine Src activity following 5-HT or ACh treatment. Female BALB/c mice were exposed to an intranasal injection of [1.7μg/μl]
HDM extract or saline. Real time, reverse-transcriptase polymerase chain reaction was used to examine gene expression.

**Results**

Genistein, PP1 and PP2 significantly reduced 5-HT-induced ASM contractions and Src activity was significantly increased in response to 5-HT. ACh-induced contractions were significantly reduced by genistein, but not PP1 and PP2. However, Src activity was significantly increased by ACh. RyR3 mRNA expression was significantly increased, Orai1 was significantly decreased, and STIM1, IPLA2β, RyR1 and RyR2 were unchanged by HDM treatment.

**Conclusion**

These data suggest 5-HT-induced ASM contraction involves Src activity. However, ACh-induced ASM contractions might not require Src. The changes in RyR3 and Orai1 expression might alter Ca$^{2+}$-handling in such a way as to potentiate airway hyperresponsiveness but further investigation is required.
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<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness</td>
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<tr>
<td>ASM</td>
<td>Airway smooth muscle</td>
</tr>
<tr>
<td>BPT</td>
<td>Bronchial provocation test</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
</tr>
<tr>
<td>[Ca^{2+}]_i</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>cADPR</td>
<td>Cyclic ADP-ribose</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CIF</td>
<td>Calcium influx factor</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyclopiazonic acid</td>
</tr>
<tr>
<td>CRAC</td>
<td>Ca^{2+} release-activated current</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>FEV_1</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>GINA</td>
<td>Global Initiative for Asthma</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>HDM</td>
<td>House dust mite</td>
</tr>
<tr>
<td>I_{CRAC}</td>
<td>Calcium-Release Activated Calcium Current</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IN</td>
<td>Intranasal</td>
</tr>
<tr>
<td>IP_3</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IP_3R</td>
<td>Inositol 1,4,5-trisphosphate receptor</td>
</tr>
<tr>
<td>IPLA_2β</td>
<td>Ca^{2+}-independent phospholipase A_2 beta</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium potassium exchanger</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PIP_2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PP1</td>
<td>4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo-d-3,4-pyrimidine</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>PP2</td>
<td>4-Amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine</td>
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<tr>
<td>RBL</td>
<td>Rat basophil leukaemia</td>
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<td>RYR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SAM</td>
<td>Sterile alpha motif</td>
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<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
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<tr>
<td>SOC</td>
<td>Store-operated calcium</td>
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<tr>
<td>SOCE</td>
<td>Store-operated calcium entry</td>
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<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
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<td>Sarcoma</td>
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<tr>
<td>STIM1</td>
<td>Stromal interaction molecule 1</td>
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<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER 1: INTRODUCTION

1.1 Asthma

The rising number of asthma cases around the world is of great concern for public health. Asthma is a chronic respiratory disease which has become more prevalent over the last 25 years (Bousquet et al. 2005). The World Health Organization (WHO) estimates over 235 million people currently suffer from asthma. Furthermore, this disease is the most prevalent chronic disease among children (Beasley et al. 2000). Approximately half of the people who have asthma develop the disease before age 10, and most develop it before age 30 (Bijanzadeh et al. 2010). The magnitude of this disease places a huge stress on healthcare resources in many countries. In fact, asthma is a major cause of hospitalizations worldwide.

Although asthma is most widespread in developed western countries such as Canada and the U.S.A., the prevalence is also on the rise in developing countries. At the 61st World Health Assembly in 2008, the WHO Director-General stated, “asthma is on the rise everywhere” in her opening speech. This highlights the fact that asthma is not only a major public health problem in high-income countries; it is also a serious health problem in low-income countries. In all cases, asthma has the potential to make breathing so difficult that quality of life is dramatically reduced.

According to the Global Initiative for Asthma (GINA), asthma is characterized by hyperresponsiveness of the tracheobronchial tree that leads to a number of recurrent symptoms including breathlessness, wheezing, chest tightness and coughing. These symptoms differ in severity and frequency among asthmatic individuals and often become
worse at night or early morning, during physical activity and in cold temperatures. Although the causes of asthma are not entirely known, it is well recognized that this is a complex disease with both genetic and environmental risk factors. The highest risk of developing asthma is the combination of genetic predisposition and exposure to inhaled substances and particles that irritate the airways or induce allergic reactions (Bijanzadeh et al. 2010). Some of these environmental substances, which have been documented to trigger asthmatic responses, include cigarette smoke, air pollution, indoor irritants (e.g., house dust mite, pet hair) and outdoor irritants (e.g., pollen, moulds).

The smaller airways (e.g. bronchi) in the lungs, as opposed to the trachea, are most affected during an asthmatic attack. In particular, these airways become inflamed, clogged as a result of mucus build-up, and constricted as smooth muscle thickens around the airway. As a result, the airways narrow which reduces airflow into and out of the lungs and breathing becomes increasingly difficult. Since the prevalence of this multifactorial disease continues to increase globally, it has been estimated by the WHO that an additional 100 million people will suffer from asthma by 2025 (Masoli et al. 2004). Therefore, increasing our understanding of the physiological and molecular mechanisms of asthma is becoming increasingly important in order to develop novel therapeutic targets for this disease.

1.2 Airway Hyperresponsiveness

Airway hyperresponsiveness (AHR) is a universal and defining feature of asthma. It is characterized as an increased narrowing of the airways following exposure to a
constrictor agent (O’Byrne and Inman, 2003). Nearly all patients suffering from symptomatic asthma demonstrate AHR. AHR occurs as a result of both hypersensitivity and hyperreactivity in response to an airway constrictor or irritant. Hypersensitivity is the requirement of a smaller concentration of irritant to produce bronchoconstriction (O’Byrne and Inman, 2003). Hyperreactivity, on the other hand, is a greater degree of constriction in response to an irritant (O’Byrne and Inman, 2003). If the ASM of normal airways and asthmatic airways was compared using a dose response curve, a leftward shift in the curve would represent ASM hypersensitivity and an upward shift in the dose response curve would represent ASM hyperreactivity.

Measuring airway responsiveness is easily done in research clinics and laboratories using inhalation challenges with bronchoconstrictor agents such as methacholine or histamine. These so called bronchial provocation tests (BPT) are therefore capable of determining whether an individual has asthma. The amount of airflow obstruction, as indicated by the reduction in forced expiratory volume in 1 second (FEV$_1$), correlates with the increase in airway hyperresponsiveness (Ramsdale et al. 1984). Furthermore, the amount of AHR a patient has directly relates to their disease severity as well as the amount of treatment required to control their symptoms (Cockcroft et al. 1997).

Direct-acting stimuli are bronchoconstricting agonists that activate receptors present on the airway smooth muscle. Aside from histamine and cholinergic agonists, other examples of direct acting stimuli are chemical mediators cysteinyl leukotrienes C$_4$ and D$_4$ as well as prostaglandin D$_2$ and F$_2$. (Adelroth et al. 1986; Hardy et al. 1984;
Thomson et al. 1981). Indirect-acting stimuli can also initiate AHR. This type of stimulus can cause bronchoconstriction in asthmatics by acting on cells in the airway to release constrictor mediators such as acetylcholine from neurons or serotonin from mast cells (Ameisen et al. 1989; Cyphert et al. 2009; Weigand et al. 2009). Examples include exercise, hyperventilation and cold air as well as chemical mediators like mannitol and adenosine monophosphate (AMP) (Brannan et al. 1998; Cockcroft et al. 1997). These indirect stimuli have also been used in bronchial provocation tests in order to determine whether an individual has asthma or to determine the effectiveness of a particular treatment.

It is believed that the chronic inflammation associated with asthma accounts for the release of many mediators which drive a process called airway remodeling. Airway remodeling encompasses all of the structural alterations that occur in the airways of the asthmatic lung. Such alterations include epithelial changes, increased number of activated fibroblast/myofibroblasts, goblet cell hyperplasia, sub-epithelial fibrosis, vascular changes as well as increased airway smooth muscle mass (Davies et al. 2003; Al-Muhsen et al. 2011). The latter appears to be the primary cause of airway obstruction and AHR (Chiba et al. 2010; Lambert et al. 1993; Oliver & Black, 2006). This is because a larger ASM layer allows for an increased contractile ability of the airways and therefore greater degree of airway narrowing. James et al. (2009) showed that the thickness of the ASM layer represents the severity of the disease. They observed that the thickest layer of ASM is associated with fatal asthmatic cases in comparison to non-fatal and control patients.
There is heterogeneity in the evidence examining whether the ASM thickening is due to ASM cell hypertrophy or hyperplasia. A study examining this in fatal asthmatic cases observed an increase in both ASM cell number and size (Ebina et al. 1993). Others have observed only hypertrophy (in mild-to-moderate and severe cases) (Benayoun et al. 2003) or only hyperplasia (in mild-to-moderate cases) (Woodruff et al. 2004). It is likely that both of these processes contribute to the increased ASM mass. Furthermore, during airway remodeling, epithelial damage can allow greater amounts of bronchoconstricting agents to reach the ASM (O’Byrne and Inman, 2003). As a result, the larger ASM mass can contract more strongly which further narrows the airway and ultimately increases AHR.

1.2.1 Animal Models of Asthma

Animal models of allergic asthma have been instrumental in helping researchers develop an understanding of the basic cellular and molecular mechanisms of the disease (Epstein, 2004). Animal models of AHR and airway inflammation have been documented for a variety of species including horses, cats, sheep, guinea pigs, rats and mice (Kumar and Foster, 2002). Asthmatic conditions must be induced in animals which means sensitization to antigen is required for the development of asthma-like symptoms (Taube et al. 2004). It is likely impossible to generate an animal model which reproduces all of the features of the human disease, however following sensitization and respiratory tract challenges with antigen certain animals (eg. mice) develop signs that closely
resemble asthma. Examples of these signs include eosinophilic lung inflammation, increased IgE, mucous hypersecretion, airway remodeling and AHR (Epstein, 2004).

A variety of models have been generated using different animals and antigens in order to mimic asthma (Epstein, 2004). The most commonly used experimental animal is the mouse mainly because of the availability of genetically characterized in-bred strains, a well-described genome, and a well-characterized immune system (Taube et al. 2004). One commonly used antigen is ovalbumin (OVA). If OVA is given systematically in a series of sensitizing doses, conjugated with an aluminum based adjuvant prior to intrapulmonary challenge it is capable of inducing airway inflammation, airway remodeling and AHR (Inman et al. 1999, Leigh et al. 2002). Another commonly used antigen is house dust mite (HDM). HDM extracts are significant sources of indoor allergens (Neeno et al. 1996). In fact, the dust mite *Dermatophagoides pteronyssinus* (*Der. P*) is recognized as the most frequently implicated source of mite-related allergens in subjects with respiratory allergy (O’Brien et al, 1992). A benefit to using the HDM antigen is that allergic lung inflammation readily develops when administered through the airway without the need for an adjuvant or systemic sensitization (Cates et al. 2004; Johnson et al. 2004; Fattouh et al. 2005; Southam et al. 2007). In a mouse model, intranasal exposure to HDM can produce both acute and chronic airway inflammation with a characteristic Th2 type inflammatory response, airway remodeling as well as AHR. The ability to produce acute and chronic animal models is beneficial if a researcher is focusing on acute asthma rather than chronic asthma.
Mouse models of asthma, including the HDM model, are particularly useful tools because the researcher has the ability to perform invasive experimental procedures, which are less feasible in human patients. Using the proper laboratory tools, AHR can easily be evaluated in mouse models by measuring features including tracheal pressure changes during mechanical ventilation, pulmonary resistance, dynamic lung compliance and whole-body plethysmography (Epstein, 2004). Since AHR, among many other features of human asthma, develops in HDM-treated mice this is a useful model to examine molecular mechanisms involved in ASM excitation-contraction coupling. In particular, no studies have examined the expression levels of genes involved in Ca\(^{2+}\)-store emptying and refilling. Altered expression in these genes could account for increased contractility of the ASM and therefore the AHR as seen in asthmatic airways.

### 1.3 Excitation-Contraction Coupling

The signal transduction pathway involved in ASM stimulation and contraction has been widely investigated (shown in fig. 1). An interaction between a contractile agonist (eg., acetylcholine, serotonin, histamine) and its respective G protein-coupled receptor (GPCR), in particular G\(_{\text{q11}}\), leads to the activation of phospholipase C (PLC) (Pelaia et al. 2008). PLC then catalyzes the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) into two molecules, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\(_3\)). The latter activates its receptor, IP\(_3\)R, which is located on the membrane of the “internal calcium (Ca\(^{2+}\))-store,” or the sarcoplasmic reticulum (SR). Since the IP\(_3\)R is a Ca\(^{2+}\) release channel, its activation leads to an elevation in intracellular Ca\(^{2+}\) concentration.
 ([Ca\textsuperscript{2+}],) (Berridge, 1993). The ryanodine receptor (RyR) – another Ca\textsuperscript{2+} release channel located on the SR membrane – is stimulated by cyclic ADP-ribose (cADPR) during agonist-induced Ca\textsuperscript{2+} signaling. The activation of RyR also contributes to the release of Ca\textsuperscript{2+} into the cytosol (Bai et al. 2009). Increased [Ca\textsuperscript{2+}], activates Ca\textsuperscript{2+}-dependent calmodulin which in turn triggers myosin light chain kinase (MLCK) to phosphorylate myosin light chain (MLC) leading to contraction (Pelaia et al. 2008).

5-HT and ACh are two main bronchoconstrictive mediators involved in asthma. Therefore, during an asthmatic response the release of these mediators can stimulate the ASM to contract. For example, an early study looking at a mouse model of allergic asthma suggested that upon antigen-challenge, mast-cell degranulation results in serotonin (5-HT) release (Ameisen et al. 1989). Acetylcholine (ACh) on the other hand, is released by cholinergic parasympathetic nerve fibres near the airway wall and binds to muscarinic ACh receptors directly on the ASM cells leading to contraction (Cyphert et al. 2009). In this study we examined whether ACh- and 5-HT-induced airway smooth muscle contraction involves Src tyrosine kinase activation. There have been no studies that examine this particular question within the excitation-contraction coupling phenomenon using whole airway smooth muscle tissue.

1.3.1 ACh and 5-HT Receptors

There are five subtypes of muscarinic acetylcholine receptors (mAChRs), referred to as M\textsubscript{1}-M\textsubscript{5}. They belong to the superfamily of G protein-coupled receptors that have seven transmembrane-spanning domains. M2 and M3 are the most abundantly expressed...
mACHRs in ASM and both influence ASM contraction through different pathways (Belmonte, 2005). In most species, including humans, ACh-induced ASM contraction is regulated by stimulation of M₃ mAChRs (Caulfield et al. 1998). M₃ mAChRs couple to the heterotrimeric G protein Gq₁₁, resulting in activation of PLC, production of IP₃ and increased [Ca²⁺], promoting activation of the contractile apparatus. However, M₂ mAChRs are generally expressed four-times more than M₃ mAChRs in ASM and are therefore also important for ASM contraction (Roffel et al. 1988).

M₂ mAChRs couple to G protein Gi and function to counteract the β₂ receptor-mediated ASM relaxant pathway by inhibiting cyclic adenosine monophosphate (cAMP) liberation and accumulation. This cAMP inhibition likely occurs due to M₂ mAChR-induced adenylate cyclase enzyme inhibition (Jones et al. 1987). M₂ mAChR activation might also induce ASM contraction by inhibiting calcium-activated potassium channels (also known as maxi-K or BK channels) that are coupled to β₂ receptors via the Gs signaling protein (Belmonte, 2005). That is, M₂ mAChR activation can promote a reversal of hyperpolarization mediated by the β₂ receptor and maxi-K channels which would lead to a degree of depolarization and contraction. Whether Src kinase can become activated by M₂ mAChR or M₃ mAChR stimulation to contribute to ASM contraction is unclear. However, studies in vascular and colonic smooth muscle suggest that M₂ activates Src kinase (Callaghan et al. 2004; Singer et al. 2002). Therefore it is plausible that M₂ mAChR or M₃ mAChR stimulation could activate Src to promote ASM contraction.
In general, seven 5-HT receptor families exist (5-HT₁R-5-HT₇R) and some families have many subtypes (ie., 5-HT₁₅, 5-HT₁₆). Of the seven receptor families, all are preferentially coupled to G proteins except those of the 5-HT₃R family which are ligand-gated Na⁺ and K⁺ channels. The first four 5-HT receptor families are the most well known with respect to the pathways in which they are involved. The 5-HT₁Rs are coupled to G protein Gᵢ/Gₒ and are negatively coupled to adenylyl cyclase (Humphrey et al. 1993). The 5-HT₂Rs, similar to M₃ mAChRs, are coupled to G₉ which stimulates PLC activity allowing for increased [Ca²⁺]. Finally, 5-HT₄Rs are coupled to Gₛ which activate adenylate cyclase.

The role of 5-HT receptors has been heavily investigated in brain tissue, however there is much less information pertaining to the role of 5-HT receptors in airway smooth muscle contraction. It appears as though the primary target of 5-HT is the 5-HT₂₅R in vascular (Lu et al. 2008) and airway smooth muscle (Tolloczko et al. 1995). In airway smooth muscle the 5-HT₂₅R likely causes Ca²⁺ release from the SR via the PLC/IP₃ pathway leading to ASM contraction (Tolloczko et al. 1995). While evidence indicates Src kinase plays a role in 5-HT₂₅-induced vascular smooth muscle contraction (Lu et al. 2008), it is not known whether Src kinase participates in any of these 5-HTR pathways to promote ASM contraction.

1.3.2 Src Tyrosine Kinase

Src is a member of the Src family of tyrosine kinases which are ubiquitously expressed in many cell types, including smooth muscle. This protein has long been
known for its importance in transducing signals within the cell which control a variety of processes such as proliferation, differentiation, motility, and adhesion (Bjorge et al. 2000). In general, Src family members are membrane-associated non-receptor protein kinases that can be activated by a number of different stimuli including neurotransmitters, autacoids, growth factors and cytokines (Bjorge et al. 2000). Specifically, the activity of the Src tyrosine kinase is regulated by tyrosine phosphorylation at two sites with opposing effects. Phosphorylation of tyrosine-527 in the carboxy-terminal tail (typically by protein Csk) renders the enzyme inactive (Hunter, 1987). Phosphorylation of the tyrosine-416 residue in the activation loop of the kinase domain upregulates its activity (Hunter, 1987). The activity of Src kinase can be studied using pharmacological blockers.

The most common method of inhibiting Src tyrosine kinase activity is through the use of the pharmacological blockers PP1 and PP2. These inhibitors function through binding to the Src catalytic domain in the ATP-binding site and adjacent hydrophobic pocket (Bishop et al. 2001). Many studies have used these related pyrazolopyrimidines to investigate the physiological roles of Src kinase. However, even though such studies are important for improving our knowledge of particular cellular processes, there are significant non-specific actions of these drugs. Thus the data from these studies should be interpreted carefully. This is because PP1 and PP2 were developed to inhibit the entire family of Src tyrosine kinases and not just Src kinase (Hanke et al. 1996). As a result, these inhibitors do not discriminate between the different kinases within the Src family which can mislead interpretation of the data (Hanke et al. 1996; Liu et al. 1999).
It has been documented that airway smooth muscle contraction depends, at least in part, on tyrosine kinase activity through GPCRs (Bois et al. 1997; Chopra et al. 1997). However, it is not yet clear which tyrosine kinase(s) is involved in this process. Studies examining vascular smooth muscle and cultured airway smooth muscle cells have pointed to Src tyrosine kinase as a potential player in contraction (Lu et al. 2008; Tolloczko et al. 2002). Furthermore, the signaling pathways in which Src kinase is involved also remain unclear.

Two studies in particular provide support for Src kinase as a key player in 5-HT-evoked smooth muscle contraction. Lu et al (2008) provide evidence for a central role of Src kinase in 5-HT-induced vascular smooth muscle contraction. They showed the Src tyrosine kinase inhibitor, PP2, prevented contraction triggered by 5-HT in an organ bath system. Using the HEK 293 T cell line, this same group suggests 5-HT2aR (the major serotonin receptor in vascular smooth muscle) and Src kinase may physically associate with one another to form a signaling complex. Another study using cultured rat tracheal smooth muscle cells showed that Src kinase can regulate 5-HT-induced Ca^{2+} release from the SR and therefore potentially contribute to airway contraction. Inhibition of Src kinase significantly reduced 5-HT-induced contraction by decreasing PIP2 levels which ultimately reduced inositol phosphate production (a key player in Ca^{2+} release from the SR through the IP3 receptor) (Tolloczko et al. 2002). These two studies indicate that 5-HT might activate Src tyrosine kinase to promote contraction in vascular smooth muscle as well as in cultured rat airway smooth muscle cells.
Limited data exist on the effects of muscarinic stimulation on tyrosine kinase phosphorylation in smooth muscle. Also, there have been no studies investigating ACh-induced Src kinase activity in airway smooth muscle contraction. In a study using cultured canine colonic myocytes, ACh-stimulated cells showed activation of Src kinase. In another set of experiments using the muscarinic antagonist, AF-DX 116, this same group demonstrated that Src activation is mediated through M2 receptors but not M3 receptors in colonic smooth muscle (Singer et al. 2002). This study provides evidence for ACh-evoked Src kinase activation in colon smooth muscle. However, these experiments did not examine whether Src kinase mediates the contraction of these colonic myocytes.

Moreover, using rabbit portal vein myocytes, Callaghan et al. (2004) showed M2 receptor stimulation of Cav1.2b channels (L-type Ca\(^{2+}\) channels) is coupled to a G\(\beta\gamma/PI3K/PKC\) pathway and that Src kinase is an important downstream mediator of this pathway. It is unclear whether Src kinase directly stimulates the Cav1.2b channels or activates an additional second messenger to enhance Cav1.2b activity and thus allow Ca\(^{2+}\) into the cell. Nevertheless, these studies reveal the ability of ACh to activate Src kinase in non-airway smooth muscle cell types.

### 1.4 Sarcoplasmic Reticulum (SR) Emptying/Refilling

During ASM muscle excitation the Ca\(^{2+}\)-store becomes depleted. It must then be refilled in order to relax the ASM and prepare for any subsequent excitatory stimulation. This Ca\(^{2+}\)-store depletion triggers an influx of external Ca\(^{2+}\) across the plasmalemma which is in turn used to refill the depleted store by the sarco/endoplasmic reticulum Ca\(^{2+}\)-
ATPase, or SERCA (Putney, 1986). This phenomenon is commonly referred to as store-operated calcium entry (SOCE) or capacitative Ca\(^{2+}\) entry (CCE), but the underlying mechanisms behind this pathway in ASM have remained elusive. Studies completed in non-muscle fields have been instrumental in identifying some key molecular players that contribute to SOCE by first releasing Ca\(^{2+}\) out of the store, sensing this depletion, and then refilling the store. These molecules include ryanodine receptor (RyR), STIM1, Orai1 and IPLA\(_2\).

1.4.1 SR Depletion Occurs Through Ryanodine Receptor (RYR1, RYR2, RYR3)

During ASM excitation-contraction Ca\(^{2+}\) mobilization out of, and back into the store is a critical process. Ca\(^{2+}\) release, or emptying, from the SR into the cytosol occurs through the activation of both inositol 1,4,5-trisphosphate receptors (IP\(_3\)R) and RyR. The former have long been recognized as the main method of SR Ca\(^{2+}\) release into the cytosol. However, there is a growing body of evidence that suggests RyRs might play a larger role in Ca\(^{2+}\)-store release than initially thought.

The three mammalian isoforms of the RyR can be characterized as large homotetrameric ion channels that, as mentioned above, make up one of the two known groups of channels which release Ca\(^{2+}\) from the SR into the cytosol (Meissner, 1994). The physiological role and regulatory mechanisms of RyRs are most well known in skeletal and cardiac muscles whereby RyRs are important for muscle contraction (Franzini-Armstrong & Protasi, 1997). Of the three RyR isoforms, RyR1 and RyR2 are mainly expressed in skeletal and cardiac muscles, respectively. In these two types of
striated muscle the RyRs are located on the SR membrane and contribute to Ca\(^{2+}\) mobilization through distinct mechanisms.

First, in skeletal muscle, membrane depolarization activates L-type Ca\(^{2+}\) channels causing them to physically interact with RyRs thereby triggering a conformation change into an open position. This open orientation allows Ca\(^{2+}\) release into the cytosol (Fill & Copello, 2002). Secondly, in cardiac muscle, it is thought that instead of a physical interaction between plasma membrane bound L-type channels and RyRs, membrane depolarization induces a Ca\(^{2+}\) influx through the L-type channels. This Ca\(^{2+}\) influx activates RyRs which in turn allows Ca\(^{2+}\) to be released from the SR creating an even greater [Ca\(^{2+}\)]\(_c\) (Coronado et al. 1994). In both cases (skeletal and cardiac muscle), the increased levels of cytosolic Ca\(^{2+}\) leads to muscle contraction.

ASM cells appear to express all three types of RyRs: the two previously mentioned (RyR1 and RyR2) as well as RyR3 which has been identified as the brain isoform as it was first found to be highly expressed in the brain. However, there is still little known regarding the expression levels and physiological function of the RyRs in mammalian ASM cells. Du et al. (2005) examined the RyR expression in both mouse bronchi (whole tissue) as well as in cultured human ASM cells using RT-PCR and immunoblot techniques. Their observations revealed that all three isoforms were comparably expressed in both tissue types. Kannan et al (1997) support these findings as they also documented expression of all three RyRs in porcine tracheal smooth muscle cells using the same technique.
Some studies have begun to highlight the role of RyRs in ASM excitation contraction coupling. This was done by determining RyR localization within ASM cells as well as examining the effect of RyR antagonists following stimulation of ASM cells. Du et al. (2005) conducted immunofluorescence studies of RyRs in primary mouse ASM cells and discovered for the first time that RyR1 and RyR3 are differentially localized. RyR1 appears to be predominantly located at the periphery of the cell near the plasma membrane while RyR3 seems to be located close to the nucleus.

Aside from examining the localization of RyR1 and RyR3 in ASM cells, Du et al. (2005) also investigated the effect of ryanodine, a RyR antagonist at high concentrations, on carbachol-induced contractile responses of mouse bronchial rings as well as the carbachol-induced Ca\(^{2+}\) responses in cultured mouse ASM cells. In the case of carbachol-induced contractile responses of mouse bronchial rings, ryanodine suppressed contractions by approximately 40-60%. Similarly, by inhibiting RyRs in the cultured mouse ASM cells and measuring calcium responses via the Ca\(^{2+}\)-sensitive dye Fura-2, a significant suppression in Ca\(^{2+}\) response was shown. This provides evidence that RyRs may play a vital role in ASM excitation-contraction coupling by releasing Ca\(^{2+}\) from the store since RyR inhibition substantially reduces ASM contractibility.

In contrast, Tazzeo et al. (2008) suggest RyRs have no role in ASM contraction. Using a similar organ bath technique as Du et al. (2005) and exposing bovine tracheal ASM strips to ryanodine, Tazzeo et al. (2008) showed the contractile responses induced by various contractile agonists (ie., KCl, carbachol, serotonin) were unaltered. Interestingly, in some cases, these authors document increases in the contractile response
which opposes the findings of other studies examining agonist-induced ASM contractility in the presence of RyR antagonists (Bergner & Sanderson, 2002; Du et al. 2005; Du et al. 2006; Gerthoffer et al. 1988). The experimental evidence regarding the involvement of RyRs in ASM contraction is heterogeneous and further investigation is required to uncover the role of each sub-type in this process. Moreover, the expression levels of these receptors have not yet been examined in an animal model of asthma.

1.4.2 Stromal Interaction Molecule 1 (STIM1) Senses SR Depletion

The mammalian homologue of the stromal interaction molecule (STIM1) is located on the membrane of the endoplasmic reticulum and sarcoplasmic reticulum in non-muscle cells and muscle cells, respectively. Structurally, this single transmembrane, non-channel protein consists of a C-terminus extending into the cytosol and an N-terminus extending into the endo/sarcoplasmic reticulum lumen (Frischauf et al. 2008). Both regions, luminal and cytosolic, contain particular amino acid sequences important to the protein’s overall function. These include an EF-hand motif in the SR lumen as well as a polybasic tail and sterile alpha motif (SAM) within the cytosolic region (Frischauf et al. 2008). STIM1 is thought to ultimately contribute to SOCE through four main events: sensing Ca$^{2+}$-store depletion, oligomerization, translocation to the plasma membrane and activation of Ca$^{2+}$ release-activated current (CRAC) channels located on the plasma membrane (Baur et al. 2008).

Several studies using non-contractile cell types point to the involvement of STIM1 in SOCE by detecting Ca$^{2+}$-store depletion as well as conveying this information to
plasmalemmal ion channels. Experimental evidence suggests Ca\(^{2+}\) binds to the luminal EF-hand motif of STIM1 thus allowing it to monitor when Ca\(^{2+}\) levels change within the store. When EF-hand motifs were mutated in HeLa cells, therefore inhibiting the binding of Ca\(^{2+}\) to this region, SOCE occurred even when the Ca\(^{2+}\)-store was full (Liou et al. 2005). Furthermore, when the store was depleted, the EF-hand mutant was unable to induce SOCE (Liou et al. 2005; Zhang et al. 2005). This provides evidence for STIM1 as a Ca\(^{2+}\)-store sensor.

STIM1 is monomeric when Ca\(^{2+}\) levels are high within the Ca\(^{2+}\)-store because the EF-hand sites are occupied by Ca\(^{2+}\) (Liou et al. 2005). However, after the EF-hand motif senses Ca\(^{2+}\)-store depletion (EF-hand motif unbound to Ca\(^{2+}\)) oligomerization of STIM1 within the sarcoplasmic reticulum membrane is thought to occur (Zhang et al. 2005). In particular, this oligomerization is believed to take place between the SAM regions of individual STIM1 proteins. Using total internal reflection fluorescence microscopy STIM1 was shown to oligomerize in both HEK293 cells and human Jurkat leukaemic T cells after internal store depletion (Mercer et al. 2006). Following STIM1 oligomerization, these multimers translocate to regions extremely close (10-25nm) to the plasmalemma where they form defined punctae (Stathopulos et al. 2008).

A recent study has suggested STIM1 translocation involves the polybasic tail in the C-terminus of the protein. HeLa and RBL cells with mutant STIM1 proteins lacking the polybasic tail were shown to oligomerize but translocation failed to occur after store depletion (Liou et al. 2007). Nevertheless, once localized at these internal store-plasmalemma junctions, STIM1 interacts with plasmalemmal Ca\(^{2+}\) channels. Altogether,
these studies suggest STIM1 is an essential component of SOCE by translocating to the plasmalemma and activating Ca\(^{2+}\) channels following Ca\(^{2+}\)-store depletion. Although only one study has looked at the role of STIM1 in ASM SOCE, it appears as though STIM1 regulates SOCE in this tissue type as well. Selectively suppressing STIM1 in cultured human bronchial smooth muscle cells significantly reduced store-depletion evoked membrane currents (Peel et al. 2006). Moreover, silencing STIM1 abolished both cyclopiazonic acid (CPA)- and histamine-induced SOCE.

### 1.4.3 Orai1 Allows Extracellular Ca\(^{2+}\) Re-entry

The exact nature of the plasmalemmal ion channel(s) which are activated following store depletion and presumably contribute to store refilling have been of interest for two decades but still remain poorly understood. However, recent studies have indicated the possibility that following translocation of STIM1 to the plasmalemma it interacts with a cation channel referred to as Orai1.

Structurally, Orai1 consists of four transmembrane regions with both N- and C-termini extending into the cytosol (Vig et al. 2006). Furthermore, it is believed that Orai1 is a Ca\(^{2+}\)-release activated Ca\(^{2+}\) (CRAC) channel which is a subtype of SOCE channel. The Ca\(^{2+}\) current that flows through these CRAC channels is referred to as I\(_{\text{CRAC}}\). The discovery of Orai1’s involvement in SOCE can be attributed to the investigation of a number of patients suffering from severe combined immunodeficiency (SCID). These patients had T-cells lacking SOCE and I\(_{\text{CRAC}}\) (Feske et al. 2006). Further investigation using siRNA techniques determined that the mutated protein within the T-cells of the
patients was Orai1 (Feske et al. 2006). This was the first evidence that Orai1 was involved in SOCE.

Upon Ca\(^{2+}\)-store depletion, it appears as though STIM1 and Orai1 somehow interact to form the CRAC channel and thus allow an influx of Ca\(^{2+}\) across the plasma membrane. No difference in SOCE is observed when STIM1 or Orai1 are overexpressed alone in HEK293 cells, however overexpressing STIM1 and Orai1 together results in significantly larger I\(_{\text{CRAC}}\) currents (Mercer et al. 2006; Peinelt et al. 2006). This data suggests STIM1 and Orai1 interact synergistically to produce SOCE. The question then becomes, does STIM1 interact with Orai1 directly or indirectly to induce Orai1 channel opening? Initially, as mentioned above, it was believed that STIM1 translocated to the plasmalemma and an insertion of the C-terminus into the membrane activated Ca\(^{2+}\) entry (Huang et al. 2006). However, other studies support an interaction between particular amino acid residues on the C-terminal of STIM1 and the C-terminal of Orai1 (Li et al. 2007). This interaction causes four Orai1 molecules, which would normally be distributed throughout the plasmalemma, to come together and form the channel (Fahrner et al. 2009).

Previous studies from our laboratory have identified non-selective cation channels which become activated in ASM upon store depletion (Helli and Janssen, 2008; Helli et al. 2005). However, only one study has examined the Ca\(^{2+}\)-selective Orai1 channel in ASM. Using RNA interference techniques, Peel et al (2008) show that Orai1 is indeed expressed in cultured human bronchial smooth muscle cells. When these cells are transfected with Orai1 siRNA, a reduced thapsigargin- or CPA-induced Ca\(^{2+}\) influx
occurs. These data, similar to those of STIM1, suggest Orai1 plays a role in SOCE in ASM cells. It is not yet clear whether other molecules are involved in mediating the interaction between STIM1 and Orai1. Some believe Ca\(^{2+}\)-independent phospholipase A\(_2\) beta (IPLA\(_2\)) mediates this interaction.

### 1.4.4 Ca\(^{2+}\)-independent phospholipase A\(_2\) beta (IPLA\(_2\beta\)) mediates STIM1 and Orai1 Interaction

Ca\(^{2+}\)-independent phospholipase A\(_2\) (or IPLA\(_2\)) is a family of intracellular phospholipases that do not require Ca\(^{2+}\) for their activation and function (Bolotina, 2008). This family of regulatory enzymes, of which 5 isoforms have been identified, can be found in most mammalian cells (Balsinde & Balboa, 2005). They are generally known to function by catalyzing the breakdown of membrane phospholipids to free fatty acids and lysophospholipids. The IPLA\(_2\beta\) isoform appears to have a unique feature whereby it can bind the Ca\(^{2+}\)-CaM (calmodulin) complex because it has a CaM-binding domain in the C-terminus. This binding domain is a highly conserved region of the protein sequence when compared among various different species (Bolotina, 2008). Removal of CaM from the CaM-binding domain allows for an interaction between this binding domain and the protein’s active site, which ultimately results in IPLA\(_2\beta\) activity (Jenkins et al. 2001). On the other hand, when CaM binds to IPLA\(_2\beta\) the interaction between the binding domain and the active site is disturbed which promotes inhibition of the enzyme’s activity.

Smani et al. (2003 & 2004) illustrated the fact that IPLA\(_2\beta\) is a key molecular player in SOCE in primary mouse vascular SMCs and RBL cells. Since that time,
IPLA2βs role in SOCE has been confirmed in many other non-smooth muscle cell types including astrocytes (Singaravelu et al. 2006), skeletal muscle (Boittin et al. 2006), fibroblasts (Martinez & Moreno, 2005) and keratinocytes (Ross et al. 2007) and others. All of these studies observed full impairment of SOCE following either knock-down or inhibition of IPLA2β. The mechanism in which IPLA2β participates in order to activate SOCE is still not entirely understood. However, studies in non-muscle cells also have provided some insight into which molecules IPLA2β might interact with to induce SOCE including STIM1 and Orai1.

Following Ca^{2+}-store depletion the calcium influx factor (CIF) is produced in the store and eventually targets IPLA2β (Csutora et al. 2006, 2008). CIF production appears to directly depend on STIM1 activity as STIM1 knock-down or overexpression experiments showed significant decreases and increases of CIF, respectively (Csutora et al. 2008). Furthermore, this study indicated STIM1 deficiency and CIF deficiency in the NG115 cell line results in a reduction of SOCE whereas normal STIM1 expression in these cells results in typical CIF production and SOCE. It appears as though CIF production is associated with the glycosylation sites of the SAM region of the STIM1 molecule since mutations in this region prevents CIF production as well as SOCE (Csutora et al. 2008). It is likely that CIF production begins as a result of a Ca^{2+}-store depletion, or loss of Ca^{2+} binding to the EF-hand motif of STIM1 and that a conformational change in STIM1 allows glycosylation sites in the SAM domain to interact with CIF-generating machinery in the SR lumen (Bolotina, 2008).
It has been suggested that translocation of STIM1 from the SR membrane to the plasma membrane following store depletion and CIF production could promote efficient delivery of CIF to its plasma membrane target, IPLA$_{2}\beta$ (Boilotina et al. 2008). CIF has been shown to displace inhibitory CaM from IPLA$_{2}\beta$ and therefore activate the enzyme (Smani et al 2004). Once IPLA$_{2}\beta$ is activated it likely opens the SOCE channel, Orai1, on the plasma membrane and ultimately promote SOCE. Clearly there is mounting evidence that suggests the interaction of STIM1 and Orai1 is mediated by the CIF-induced activation of IPLA$_{2}\beta$ in the complex SOCE mechanism.

1.4.5 Sarco/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) pump

Although we did not examine the SERCA pump in the present study it is another important molecule in EC-coupling and should be briefly introduced. There are three members of the mammalian SERCA family, SERCA1, SERCA2 and SERCA3, but SERCA2 is the most highly expressed in smooth muscle (Grover and Khan, 1992; Hovnanian, 2007). SERCA is localized to the SR membrane in ASM cells and is critical for terminating increases in [Ca$^{2+}$]$_i$ which arise following agonist-induced ASM excitation. One of the main methods for reducing cytosolic Ca$^{2+}$ levels is pumping the Ca$^{2+}$ back into the SR by the SERCA pump. No other way of mobilizing Ca$^{2+}$ back into the SR has been identified. Interestingly, SERCA2 expression is significantly decreased in cultured ASM cells from subjects with moderately severe asthma as well as in ASM in vivo from patients with moderate and severe asthma (Mahan et al. 2009). In addition to SERCA, another main method for reducing cytosolic Ca$^{2+}$ levels involves the extrusion of
Ca\(^{2+}\) across the plasma membrane by sodium/potassium pump (NCX). The NCX is a plasma membrane pump which functions by extruding one Ca\(^{2+}\) ion in exchange for the import of three Na\(^{+}\) ions. However, the reverse-mode of the NCX has become well known as a source of Ca\(^{2+}\) influx for store-refilling (Algara-Suarez et al. 2007; Hirota and Janssen, 2007; Hirota et al. 2007; Sathish et al. 2011). Unpublished data from our laboratory suggests a significant increase in NCX gene expression in the same HDM mouse model of asthma as used in the present study. The changes in SERCA and NCX gene expression in the ASM of asthmatic airways prompted us to examine the expression of other genes which might influence Ca\(^{2+}\)-handling and ultimately contribute to AHR.
CHAPTER 2: ORGANIZATION OF THESIS

2.1 Purpose of Thesis

The purpose of the present thesis was to examine airway smooth muscle excitation-contraction coupling. In particular, we focused on two distinct but complementary signaling pathways: 1) exploring the involvement of a tyrosine kinase in bovine ASM contraction and 2) investigating the SOCE pathway in a mouse model of asthma.

We first investigated the role of Src tyrosine kinase in agonist-induced bovine ASM contraction. In particular, we were interested in whether ACh- and 5-HT-evoked ASM contraction requires Src kinase activation. Secondly, in order to investigate the SOCE pathway in a model of asthma, we examined whether genes in the SOCE pathway, which have been previously shown to participate in Ca\(^{2+}\)-store emptying and refilling, are differentially expressed in a murine model of chronic hyperresponsiveness compared to controls. These genes include RYR1, RYR2, RYR3, IPTA₂β, STIM1 and Orai1.

2.2 General Hypotheses

1) 5-HT- and ACh-induced ASM contraction will require Src tyrosine kinase activation.

2) There will be an increased expression of genes involved in the SOCE mechanism (RYR1, RYR2, RYR3, IPTA₂β, STIM1, Orai1) in the HDM group when the mRNA levels of HDM-treated (hyperresponsive) and control mice are compared.
2.3 Specific Aims & Outline of Experiments

**Specific Aim ➔ To demonstrate that Src kinase is involved in agonist-induced ASM contraction.**

**Study Design**

Muscle bath experiments were used to examine the effect of a general tyrosine kinase inhibitor (genistein) and Src kinase family inhibitors (PP1 and PP2) on ACh- and 5-HT-evoked contractions. Bovine ASM strips were utilized in these experiments. Furthermore, Western blot analysis was used to determine Src kinase activation in response to ACh and 5-HT. Non-airway smooth muscle studies have suggested that Src kinase is involved in smooth muscle contraction, but no studies have yet examined the role of Src kinase during agonist-induced contraction using whole ASM tissue. This provides novelty to the present study.

**Specific Aim ➔ To demonstrate a difference in RYR1, RYR2, RYR3, I\(\text{PLA}_2\)\(\beta\), STIM1 and Orai1 mRNA expression in the tracheal ASM of control mice compared to mice made hyperresponsive to house dust mite.**

**Study Design**

Female Balb/c mice were made hyperresponsive to HDM via 2 weeks of HDM antigen sensitization and 6 weeks of HDM challenge. Tracheal ASM was extracted and used for real-time RT-PCR analysis for various genes involved in the SOCE pathway. These genes include RYR1, RYR2, RYR3, I\(\text{PLA}_2\)\(\beta\), STIM1 and Orai1. There have been no studies that have examined the mRNA expression or protein levels of these particular genes that regulate the SOCE pathway in a model of asthma. Changes in the expression of one or more of these genes could lead to important pathological changes such as those seen in asthmatic patients. For example, increased expression of STIM1 and/or Orai1
could result in overfilling of the SR, leading to increased mechanical responsiveness of the airway smooth muscle upon excitatory stimulation (a key feature of asthma).
CHAPTER 3: METHODS

3.1 Animals

3.1.1 Mice

All measurements for RT-PCR experiments were conducted on female Balb/c mice, aged 12-16 weeks, purchased from Charles River Laboratories (Saint-Constant, Quebec, Canada). The mice were housed in environmentally-controlled specific pathogen free conditions using bioBubble technology for 1 week prior to any study, and throughout the duration of all experimental exposures. All procedures were reviewed and approved by the Animal Research Ethics Board at McMaster University (Hamilton, ON, Canada). After the mice were sacrificed, the tracheal ASM was dissected and the epithelium was removed. The ASM tissue was placed in centrifuge tubes containing RNAlater (Applied Biosystems, USA) and stored at -70°C.

3.1.2 Bovine

All measurements for muscle bath and Western blot experiments were done using airway smooth muscle from bovine trachea. Bovine trachea were collected from cows weighing 200–500 kg at a local abattoir and immediately placed in ice cold Krebs buffer (116 mM NaCl; 4.6 mM KCl; 1.2 mM MgSO₄; 2.5 mM CaCl₂; 1.3 mM NaH₂PO₄; 23 mM NaHCO₃; 11 mM D-glucose; 0.01 mM indomethacin; pH 7.4). Upon receiving trachea in the lab, the epithelium was removed and tracheal ASM strips (~2–3 mm wide, ~10 mm long) were excised and used immediately or stored at 4°C for use up to 48 h later.
3.2 House Dust Mite (HDM) Preparation and Delivery

HDM *Dermatophagoides pteronyssinus* extract (Greer Laboratories, Lenoir, NC) was re-suspended with sterile 1x phosphate buffered saline (PBS) (Invitrogen) to reach the specific concentration of 1.7µg/µl and frozen at -20ºC. The HDM was thawed at 4ºC overnight to be administered the next day. Mice were anesthetized in a gaseous anesthesia chamber using isoflurane (Abbot Laboratories, Montreal, Canada) and exposed to HDM through a 25µl intranasal (IN) installation (fig. 15). To control for the effects of IN HDM exposure, control mice were anesthetized in the same manner and challenged with saline (SAL) (25µl IN) following the same protocol. It should be noted that I did not complete the preparation and delivery of HDM.

3.3 Muscle Bath Technique

Epithelium-denuded bovine tracheal ASM strips were mounted vertically in organ baths using silk suture (Ethicon 4-O; Ethicon Inc., Somerville, NJ, USA) tied to a Grass FT.03 force transducer (Astro-Med Industrial Park, West Warwick, RI, USA) on one end and to a glass rod, which served as an anchor, on the other end. These were bathed in Krebs–Ringer’s buffer containing indomethacin (0.01 mM) and Nω-nitro-L-arginine (L-NNA; 10–4 M), bubbled with 95% O₂ and 5% CO₂, and maintained at 37ºC. Tissues were passively stretched to impose a preload tension of 1 gram. Isometric changes in tension were digitised at a rate of 0.5 seconds and recorded on the computer. Tissues were equilibrated for 1 hour before commencing the experiments, during which time they were challenged with 60 mM KCl three times, to assess the functional state of each tissue.
To assess the effects of ACh and 5-HT on Src phosphorylation during contraction, muscles strips were first treated with $10^{-5}$ M acetylcholine or $10^{-5}$ M serotonin. For example, muscle strips were treated with ACh for 0, 0.5, 1, 2, 3, 5, 10 and 15 minutes. Similarly, muscle strips were treated with 5-HT for 0, 0.5, 1, 2, 3, 5, 15 and 20 minutes. Upon completion of the contractile agonist challenge, muscle strips were quickly disassembled from the organ bath, flash frozen by immersion in liquid nitrogen and stored at -70ºC for protein analysis.

Other muscle bath experiments were conducted to determine the effect of certain inhibitors on ASM contraction. In one set of experiments, tissues were pre-treated with genistein ($10^{-4}$ M) or vehicle (dimethyl sulphoxide, DMSO) for 30 minutes before being challenged with increasing concentrations of ACh or 5-HT ($10^{-10}$ M – $10^{-5}$ M) which were added in a cumulative fashion. In a second set of experiments, tissues were pre-treated with PP1 ($10^{-5}$ M), PP2 ($10^{-5}$ M) or DMSO vehicle for 1 hour before being challenged with increasing concentrations of ACh or 5-HT ($10^{-9}$ M – $10^{-5}$ M) which were added in a cumulative fashion. In a third set of experiments, tissues were pre-treated with Purvalanol A ($10^{-7}$ M), Kenpaullone ($10^{-6}$ M), SP600125 ($10^{-6}$ M) or the DMSO vehicle for 1 hour after which the 5-HT concentration-response relationship was examined, also in a cumulative manner.

3.4 RNA Isolation

Mouse tracheal ASM samples were thawed on ice and then centrifuged for 10 minutes at 4º C and 12,000 rpm. The RNA later solution was decanted and replaced with
200 µl ice cold nuclease free water. The samples were kept cold with dry ice and then sonicated before the addition of 500 µl ice cold trizol. After a brief mix, the samples were allowed to sit for 30 minutes at room temperature. 300 µl of chloroform was added and the samples were shaken for 30 minutes at room temperature before undergoing centrifugation for 25 minutes at 4ºC and 12,000 rpm. The clear supernatant was added to new centrifuge tubes containing 500 µl ice cold isopropanol and incubated at -80ºC for 2 hours. The samples were placed on ice and then centrifuged for 20 minutes at 4ºC and 12,000 rpm. The supernatant was decanted and 15 µl ice cold nuclease-free water was added to the sample. The pellet was then mixed into the nuclease-free water and placed on ice for 2 hours. The total mRNA concentration of these isolated samples was measured using a NanoDrop Spectrophotometer ND-1000 (Thermal Scientific, USA).

3.5 Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

1 µg of total RNA was reverse-transcribed with 10x reaction buffer (Life Technologies), amplification grade DNase I (Life Technologies) and nuclease-free water. This mixture was incubated at room temperature for 15 min before the addition of 25mM EDTA (Life Technologies) and incubated again for 15 min at 65ºC. The samples were cooled on ice for 1 min, after which, random primers (Life Technologies) and 10 mM dNTP mix (Life Technologies) were added to the samples and incubated at 65ºC for 5 min and then cooled on ice. 5x 1st stand (Life Technologies), 0.1M DTT (Life Technologies), RnaseOUT Recombinant (Life Technologies) and SUPERSCRIPT II RT (Life Technologies) were added to each sample. Samples were incubated at 25ºC for 10
min, 42ºC for 50 min, 70ºC for 15 min and then maintained at 4ºC. RNase H (Life Technologies) was added before the samples were incubated at 37ºC for 20 min.

RNA expression was quantified using a TaqMan Gene Expression Assay (Applied Biosystems). Samples were added to a 96-well plate which contained master mix (Life Technologies), nuclease-free water and primers for the gene of interest (Life Technologies). The relative expression of the target genes was calculated using the comparative method (\(2^{-\Delta\Delta CT}\)) as highlighted in Bookout and Mangelsdorf (2003).

3.6 Protein Extraction

Frozen tissues were taken from the -70ºC freezer and placed in liquid nitrogen. Tissues were pulverized into a fine powder using a porcelain mortar and pestle which were first cooled on dry ice. The powder was collected in a 20 mL glass tissue grinder (Kontes Glass Co., Vineland, New Jersey, USA) and homogenized in the presence of a RIPA lysis buffer (Cell Signaling Technology, Danvers, Massachusetts, USA) which contained 20 mM Tris-HCl; 150 mM NaCl; 1 mM Na₂EDTA; 1 mM EGTA; 1% NP-40; 1% sodium deoxycholate; 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate; 1 mM Na3VO4; 1 µg/ml leupeptin; 1mM phenylmethylsulfonyl fluoride; 10 mM sodium fluoride, 4 µg/mL aprotinin. Samples were transferred to 1.5 mL Eppendorf tubes, allowed to sit on ice for 30 minutes and then centrifuged at 4ºC and 12,000 rpm for 15 min. The supernatant was collected and frozen at -80ºC.

To determine the total protein concentration of each sample, the Bradford protein assay was conducted using Bio-Rad assay reagent (BioRad). The Ultrospec 2100 pro
UV/Visible spectrophotometer (GE Healthcare) was used to analyze both the bovine serum albumin (1 mg/mL) samples, which produced a standard curve, as well as the experimental ASM samples.

### 3.7 Western Blots

After diluting the samples in 5X loading sample buffer and boiling for 5 minutes, equal amounts of protein were subjected to sulphate polyacrylamide gel electrophoresis (SDS–PAGE) using 10% separating gels and Tris-glycine SDS running buffer (25 mM Tris, 129 Glycine, 3 mM SDS) at 100 volts for 90 minutes at room temperature. The resolved proteins were electrophoretically transferred to a nitrocellulose blotting membrane (Amersham Biosciences, Germany) in a transfer buffer (25 mM Tris, 129 Glycine, 20% methanol) at 260 mA for 90 minutes.

Membranes were blocked for 60 minutes with 5% nonfat milk in TBST (Tris Buffered Saline and 0.5% Tween 20) at room temperature. Blots were incubated overnight with the appropriate anti-Src rabbit monoclonal (1:1000; Cell Signalling, Danvers, MA, U.S.A.) or anti-phospho-Src rabbit monoclonal (1:500; Cell Signalling, Danvers, MA, U.S.A.) primary antibody at 4°C. β-actin was used as a loading control and membranes were incubated with actin primary antibody (1:4,000; Santa Cruz Biotechnology, Inc.) as described above. Membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000; Cell Signalling, Danvers, MA, U.S.A.) or donkey anti-goat (1:4,000; Santa Cruz Biotechnology, Inc.) secondary antibody for 2 hours at room temperature.
For chemiluminescent detection of the membrane, Amersham ECL Western Blotting Detection Reagents (GE healthcare, UK) were used to detect total Src and actin. Supersignal West Femto Detection Reagents (Thermo Scientific, USA) were used to detect phospho-Src. In both cases, reagents were used according to the manufacturer’s specifications. The membrane was incubated in the chemiluminescent reagent for 2 minutes at room temperature. The membrane was then drained of excess reagent and placed in the film cassette. Autoradiography film (GE Healthcare, UK) was placed on top of the membrane in the film cassette and the film was exposed for an amount of time which allowed for protein bands to become clearly visible.

Densitometric analysis of Western blots was performed by Scion Image software. The amount of signal in the blot was quantified by optical density of the bands of interest. The specific proteins of interest were determined by comparison to a Precision Plus Protein Standard (Bio-Rad Laboratories, CA).

Levels of phosphorylated Src are expressed as a percent of total Src levels and β-actin in each group.

3.8 Statistical Analysis

All values are expressed as mean and standard error of mean (SEM). Significance of differences between control and treatment groups was determined by analysis of variance. Upon finding significance with analysis of variance, an unpaired t-test was performed. For all analysis, p values <0.05 were considered statistically significant.
CHAPTER 4: RESULTS

4.1 Effect of a non-selective tyrosine kinase inhibitor (genistein) on 5-HT- and ACh-evoked contractions

We first examined the effect of genistein, a non-selective tyrosine kinase inhibitor, on 5-HT- and ACh-evoked contractions using bovine tracheal airway smooth muscle strips. These experiments were done in the muscle bath apparatus. Tissues were pretreated with genistein (10^-4 M) or control vehicle (DMSO) for 30 min before the concentration-response relationship for either 5-HT (10^{-10} M – 10^{-5} M) or ACh (10^{-10} M – 10^{-5} M) was examined. Responses were standardized as a percent of the third control response to 60 mM KCl obtained during the equilibration period (~30 min before treatment with genistein or with vehicle).

Genistein (10^-4 M) completely abolished the contractile responses of tracheal airway smooth muscle preparations to 5-HT (10^{-10} M – 10^{-5} M) (n=7; fig. 2). However, genistein had far less of an effect against Ach-evoked contractions, although their effect did reach statistical significance at 10^{-7} M and 10^{-5} M Ach (n=5; fig. 6).

4.2 Effect of specific Src tyrosine kinase inhibitors (PP1 and PP2) on 5-HT- and ACh-evoked contractions

The above data suggests the involvement of a tyrosine kinase in 5-HT-evoked airway smooth muscle contraction, but not in ACh-evoked contractions. Furthermore, there is increasing evidence for Src tyrosine kinase as a key molecule in the contraction of various types of smooth muscle. Thus, we examined the effect of specific Src tyrosine kinase inhibitors on 5-HT- and ACh-evoked contractions using bovine tracheal airway
smooth muscle strips. Tissues were pretreated with PP1 (10^{-5} \text{ M}), PP2 (10^{-5} \text{ M}) or vehicle (DMSO) for 1 hour before the concentration-response relationship for either 5-HT (10^{-9} \text{ M} – 10^{-5} \text{ M}) or ACh (10^{-9} \text{ M} – 10^{-5} \text{ M}) was examined.

Both PP1 (n=6; 10^{-5} \text{ M}) and PP2 (n=6; 10^{-5} \text{ M}) significantly suppressed contractile responses to 5-HT (10^{-9} \text{ M} – 10^{-5} \text{ M}) (fig. 3). In fact, PP1 and PP2 reduced 5-HT-evoked contractions by 85% and 82%, respectively at 10^{-5} \text{ M}. Furthermore, neither PP1 (n=6; 10^{-5} \text{ M}), nor PP2 (n=6; 10^{-5} \text{ M}) had an effect on the bovine tracheal airway smooth muscle contractions in response to ACh (10^{-9} \text{ M} – 10^{-5} \text{ M}) (fig. 7). These data suggest Src participates in 5-HT-induced contractions but not in ACh-induced contractions.

4.3 Effect of other protein kinase inhibitors on 5-HT-evoked contractions

PP1 and PP2 have been reported to inhibit a number of other protein kinases in addition to Src tyrosine kinase (Bain et al. 2003 & 2007). These additional kinases include Lck, CSK, and CK1δ. To account for the potential non-specific inhibition of PP1 and PP2 to Src kinase we chose to selectively block Lck, CSK, and CK1δ during 5-HT-induced bovine ASM contraction. Purvalanol A, Kenpaullone and SP600125 were used to specifically inhibit Lck, CSK, and CK1δ, respectively.

Bovine tracheal ASM strips were pretreated with Purvalanol A (10^{-7} \text{ M}), Kenpaullone (10^{-6} \text{ M}) and SP600125 (10^{-6} \text{ M}) or control vehicle (DMSO) for 1 hour before the concentration-response relationship for 5-HT (10^{-9} \text{ M} – 10^{-5} \text{ M}) was examined. Responses were standardized as a percent of the third control response to 60 mM KCl obtained during the equilibration period (~30 min before treatment with the kinase
inhibitors or vehicle). Purvalanol A (10^{-7} M; n=6), Kenpaullone (10^{-6} M; n=6) and SP600125 (10^{-6} M; n=6) had no significant effect on the contractions of tracheal airway smooth muscle preparations in response to 5-HT (10^{-9} M – 10^{-5} M) (fig. 4). This confirms that the reduction in 5-HT-induced contractions in the presence of PP1 and PP2 (fig. 3) are not a result of Lck, CSK, or CK1δ activity. We next investigated Src activation in 5-HT- and ACh-induced contractions using Western blotting.

4.4 Effect of contractile agonists on levels of Src tyrosine kinase activation

To examine whether Src tyrosine is activated (Tyr-416 phosphorylated) in response to 5-HT or ACh, Western blot analysis was performed on airway smooth muscle preparations which were treated with these agonists at 10^{-5} M in the muscle bath system.

Compared to the control treatment (zero seconds of agonist), levels of Tyr-416 phosphorylated Src kinase were significantly increased after 1, 2, 3 and 5 minutes of exposure to 10^{-5} M 5-HT (n=6; fig. 5). There is a steady increase in Src kinase phosphorylation which reaches its peak activity at 3 minutes, at which time dephosphorylation begins. At 3 minutes of 5-HT treatment there is a 2.3 fold increase in Src kinase phosphorylation. Levels of Src phosphorylation are expressed as a percent of total Src levels and β-actin in each group.

Surprisingly, after ACh treatment (10^{-5} M), Src kinase phosphorylation is also significantly increased at 1, 2, 3 and 5 minutes in comparison to the control group (n=2; fig. 8). The largest amount of Src phosphorylation occurs at 3 minutes at which time dephosphorylation begins. At 3 minutes of ACh treatment there is a 2.7 fold increase in Src
kinase phosphorylation. Levels of Src phosphorylation are expressed as a percent of total Src levels and β-actin in each group.

4.5 Effects of HDM treatment on gene expression

It is well recognized that alterations in the regulation of \([Ca^{2+}]_i\) is a key feature of the pathophysiology of airway diseases such as asthma (Tao et al. 1999). For this reason, we examined whether the expression of various genes, which are involved in Ca\(^{2+}\)-store release and Ca\(^{2+}\)-store re-uptake, are altered as a result of the murine house dust mite treatment model. A 25 µl intranasal injection of [1.7 µg/µl] HDM extract or saline (control) was used during the sensitization and challenge periods.

Ryanodine receptors, of which there are three isoforms, exist on the sarcoplasmic reticulum membrane. Following agonist-induced stimulation of a GPCR, RyRs become activated and mobilize Ca\(^{2+}\) into the cytosolic space. We examined the mRNA expression of each of the three RyR isoforms (RyR1, RyR2 and RyR3) in the HDM treated mice as well as in the control mice. There was no significant difference in the mRNA expression of RyR1 in the HDM-treated mice (n=18) compared to the controls (n=13) (p>0.05; fig. 9). Similarly, the HDM treatment (n=9) did not significantly effect the mRNA expression of RyR2 compared to the control group (n=7) (p>0.05; fig. 10). The mRNA expression of RyR3, on the other hand, was significantly increased in the HDM-treated mice (n=6) when compared to the control group (n=4) (p=0.005; fig. 11). We also examined the mRNA expression of genes involved in replenishing the SR with Ca\(^{2+}\) including STIM1, Orai1 and IPLA\(_2\)β.
Since STIM1 has been shown to be an important molecule for sensing Ca\textsuperscript{2+}-store depletion we examined its expression. The HDM treatment did not have a statistically significant effect on the mRNA expression of STIM1 (n=13) when compared to the control mice (n=10) (p=0.079; fig. 12). The store-operated Ca\textsuperscript{2+} channel, Orai1, becomes activated by STIM1 following Ca\textsuperscript{2+}-store depletion. Orai1 mRNA expression was significantly reduced in the HDM group (n=9) compared to the control group (n=12) (p=0.003; fig. 13). The interaction between STIM1 and Orai1 is mediated by IPLA\textsubscript{2}\beta and therefore the activation of IPLA\textsubscript{2}\beta is critical for store operated calcium entry. However, IPLA\textsubscript{2}\beta mRNA expression was not significantly affected by the HDM treatment (n=8) compared to the control group (n=9) (p>0.05; fig. 14).
CHAPTER 5: FIGURES

Figure 1: Signal transduction pathway involved in airway smooth muscle contraction. Stimulation of a GPCR via a contractile agonist activates phospholipase C-beta (PLCβ) which exerts its enzymatic activity on phosphatidylinositol 4,5-bisphosphate (PIP2) promoting the production of second messenger molecules inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 activates its receptor (IP3R) on the sarcoplasmic reticulum (SR) membrane resulting in Ca2+ release. Ryanodine receptors (RyR) are activated by cyclic adenosine diphosphate ribose (cADPR) to release Ca2+ from the SR. This Ca2+ release is necessary for the activation of Ca2+-calmodulin-dependent myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chain (MLC), leading to ASM contraction, which is terminated by MLC phosphatase (MLCP). Furthermore, Ca2+ can enter ASM cells via store-operated Ca2+ entry (SOCE) channels on the plasma membrane. Ca2+ depletion of the SR activates the stromal interaction molecule 1 (STIM1) leading to Ca2+-independent phospholipase A2 beta (IPLA2β) activity which can then open the SOCE channel Orai1. Figure modified from Bolotina (2008) and Pelaia et al. (2008).
Figure 2: Role for tyrosine kinases in airway smooth muscle contraction in general. Mean concentration–response relationship for serotonin (5-HT) in bovine tracheal smooth muscle in the absence (*) versus presence of genistein ($10^{-5}$ M; ---; n=7). *p<0.05.
Figure 3: Role for Src tyrosine kinase in airway smooth muscle contraction. Mean concentration–response relationship for serotonin (5-HT) in bovine tracheal smooth muscle in the absence (*) versus presence of PP1 (\(10^{-5}\) M; n=6) or PP2 (\(10^{-5}\) M; n=6). *p<0.05.
Figure 4: Role of protein kinases Lck, CSK and CK1δ in airway smooth muscle contraction. Mean concentration–response relationship for serotonin (5-HT) in bovine tracheal smooth muscle in the absence (•) versus presence of Purvalanol A, Kenpaullone, or SP600125 (n=6).
Figure 5: Western blot analysis examining the effect of serotonin (5-HT) on Src phosphorylation in bovine airway smooth muscle. (A) Time course of 5-HT-induced Src kinase phosphorylation. Bovine airways smooth muscle strips were stimulated with 5-HT (10^{-5}M) and tissue lysates were resolved by SDS-PAGE and immunoblotted with anti-Src antibodies to detect the phosphorylated and total Src proteins. Both total Src as well as beta-actin were used as a loading control. (B) Relative p-Src expression (n=6; *p<0.05). Data is presented as mean and SEM.
Figure 6: Role for tyrosine kinases in airway smooth muscle contraction in general. Mean concentration–response relationship for acetylcholine (ACh) in bovine tracheal smooth muscle in the absence (*) versus presence of genistein (10^{-5} M; - - ; n=5). *p<0.05.
Figure 7: Role for Src tyrosine kinase in airway smooth muscle contraction. Mean concentration–response relationship for acetylcholine (ACh) in bovine tracheal smooth muscle in the absence (•) versus presence of PP1 (–;10^-5 M; n=6) or PP2 (——;10^-5 M; n=6). *p<0.05.
Figure 8: Western blot analysis examining the effect of acetylcholine (ACh) on Src phosphorylation in bovine airway smooth muscle. (A) Time course of ACh-induced Src kinase phosphorylation. Bovine airways smooth muscle strips were stimulated with ACh (10^{-5}M) and tissue lysates were resolved by SDS-PAGE and immunoblotted with anti-Src antibodies to detect the phosphorylated and total Src proteins. Both total Src as well as beta-actin were used as a loading control. (B) Relative p-Src expression (n=4; *p<0.05). Data is presented as mean and SEM.
Figure 9: RT-PCR analysis of RyR1 mRNA expression in the tracheal airway smooth muscle of control (n=13) and house dust mite (n=18) treated mice. House dust mite treatment did not affect IPLA2 mRNA expression in comparison to the control group (p=0.910).
Figure 10: RT-PCR analysis of RyR2 mRNA expression in the tracheal airway smooth muscle of control (n=7) and house dust mite (n=9) treated mice. House dust mite treatment did not affect RyR2 mRNA expression in comparison to the control group (p=0.874).
Figure 11: RT-PCR analysis of RyR3 mRNA expression in the tracheal airway smooth muscle of control (n=4) and house dust mite (n=6) treated mice. House dust mite treatment did not affect RyR3 mRNA expression in comparison to the control group (p=0.005).
Figure 12: RT-PCR analysis of STIM1 mRNA expression in the tracheal airway smooth muscle of control (n=10) and house dust mite (n=13) treated mice. House dust mite treatment did not influence STIM1 mRNA expression in comparison to the control group (p=0.079).
Figure 13: RT-PCR analysis of Orai1 mRNA expression in the tracheal airway smooth muscle of control (n=9) and house dust mite (n=12) treated mice. In comparison to the control group, Orai1 mRNA expression was significantly reduced in the HDM treatment group (*p<0.00298).
Figure 14: RT-PCR analysis of IPLA\textsubscript{2} beta mRNA expression in the tracheal airway smooth muscle of control (n=8) and house dust mite (n=9) treated mice. House dust mite treatment did not affect IPLA\textsubscript{2} beta mRNA expression in comparison to the control group (p=0.865).
Figure 15: House dust mite (HDM) sensitization and challenge protocol. Female Balb/c mice received HDM (25 µl IN), 5 days per week for 2 weeks at a concentration of 1.7µg/µl. Control mice received 25µl IN saline (SAL). Mice were sacrificed 24 hours after final challenge period.
CHAPTER 6: DISCUSSION

6.1 Summary

In summary, this study showed that 5-HT-induced airway smooth muscle contraction might require activation of Src tyrosine kinase. ACh-induced airway smooth muscle contraction, on the other hand, does not appear to require the activation of Src tyrosine kinase. Furthermore, we show a decreased expression of the Orai1 gene and an increased expression of the RyR3 gene in the murine house dust mite model of allergic asthma.

6.2 5-HT-induced ASM contraction

The role of tyrosine kinases in smooth muscle contraction has only recently been a target of investigation and is thus still unclear. We examined the role of Src kinase in 5-HT-evoked airway smooth muscle contraction in order to increase our understanding of excitation-contraction coupling in this tissue type.

5-HT is a contractile agonist of airway smooth muscle cells and the levels of 5-HT surrounding the asthmatic airway has been reported to be increased (Ameisen et al. 1989). This increase in 5-HT levels could contribute to airway hyperresponsiveness and therefore makes it a physiologically relevant agonist to examine. We first treated bovine airway smooth muscle strips with the non-selective tyrosine kinase inhibitor, genistein, in order to examine whether tyrosine kinases contribute to 5-HT-induced contraction. Treatment with genistein resulted in complete abolition of the contractile response, suggesting a role for a tyrosine kinase in the ASM contractile process (fig. 2). These
results are supported by Tolloczko et al (2000) who treated cultured rat tracheal smooth muscle cells with genistein (40 µM) and subsequently stimulated the cells with 5-HT (1µM). They, too, show a significant decrease in ASM contraction (decrease in surface area of the cells).

Inhibition of Src kinase using PP1 and PP2 allowed us to determine whether this kinase played a role in ASM contraction in response to 5-HT. The central role of Src in bovine ASM is supported by the robust blockade of 5-HT-induced contraction by PP1 and PP2 (fig. 3). Moreover, these results are supported by Western blot analysis in the present study. We demonstrate significantly increased Src kinase activation (phosphorylation at the tyrosine-416 site) during 5-HT-induced ASM contraction (fig. 5). The findings of the present study are consistent with previous studies which also suggested a potential role for Src kinase in cultured airway smooth muscle cells (Tolloczko et al. 2002) as well as in vascular smooth muscle (Alioua et al. 2002; Banes et al. 1999; Lu et al. 2008).

It is generally accepted that GPCR stimulation via contractile agonist and subsequent activation of PLC leads to the breakdown of PIP₂ into two second messengers: IP₃ and DAG. IP₃ activates the IP₃ receptor on the SR membrane causing Ca²⁺ release from the store and subsequent Ca²⁺ influx from the extracellular space. This increase in [Ca²⁺]ᵢ is necessary for ASM contraction. It has been reported in human embryonic kidney (HEK)-293 cells that PIP₂ levels might be regulated by a tyrosine-kinase-dependent mechanism (Rümenapp et al. 1998). However, the mechanism(s) by which tyrosine kinase(s) activity regulates GPCR-induced Ca²⁺ entry are poorly defined.
especially in airway smooth muscle. Tolloczko et al. (2002) investigated this phenomenon in cultured rat tracheal airway smooth muscle cells. They reported that the Src family kinase inhibitor, PP1, significantly reduced 5-HT-induced contractions as well as $[\text{Ca}^{2+}]$$_i$. Furthermore, PP1 treatment in these cells resulted in significantly reduced inositol phosphate production and significantly reduced PIP$_2$ levels following 5-HT stimulation. These results indicate that Src tyrosine kinase might regulate ASM cell contraction by regulating PLC$\beta$. This is because PLC$\beta$’s substrate is PIP$_2$, and since PIP$_2$ levels are reduced, less IP$_3$ is liberated which ultimately reduces the amount of Ca$^{2+}$ release from the SR. Although airway smooth muscle and vascular smooth muscle cells do not behave exactly alike both fields can still learn from one another. For example, Src kinase has been shown to contribute to 5-HT-induced vascular smooth muscle contraction in a different manner as compared to that proposed in airway smooth muscles by Tolloczko et al. (2002). Therefore the functional implications of the results reported in this study might also be explained in work done in vascular smooth muscle.

One such explanation involves the activation of the ERK MAPK pathway. Banes et al (1999) reported that PP1 significantly reduced 5-HT-induced contractions of rat aortic smooth muscle strips in a muscle bath environment similar to that used in the present study. Furthermore, Src kinase was shown by these authors to activate the ERK MAPK pathway in cultured rat aortic smooth muscle cells. The ERK MAPK pathway has been reported to participate in contraction of vascular (Adam et al., 1989; Jin et al., 1996) and airway smooth muscle (Sakai et al. 2010).
Another possible explanation for how Src might be involved in 5-HT-induced ASM contraction is through the inhibition of MaxiK channels. Pretreating endothelium-denuded human coronary arterial rings with LavA (a Src kinase inhibitor) and subsequently stimulating the rings with 5-HT resulted in a 50 percent reduction in contraction (Alioua et al. 2002). This provides further support that Src kinase is a significant player in the contractile response to 5-HT. Interestingly, in HEK293 T cells, this group showed a close localization of Src to MaxiK channels which are large conductance voltage K$^+$ channels. In this set of experiments it was reported that Src phosphorylates the alpha subunit of the MaxiK channel, leading to its inhibition, following 5-HT stimulation. Phosphorylation of the MaxiK alpha subunit prevents channel activity by decreasing its effectiveness in responding to Ca$^{2+}$ and voltage. As a result, extrusion of K$^+$ by MaxiK is prevented and the cell becomes depolarized which leads to Ca$^{2+}$ channel activation and ultimately contraction. Although this contractile mechanism involving MaxiK inactivation by Src kinase has not been explored in ASM, this might be an avenue for future investigation. This is because potassium channels are also important players in regulating the voltage across the ASM cell membrane.

Finally, Src activation might be initiated by the 5-HT$_{2A}$ receptor. Recently, Src kinase was found to be a key component for 5-HT$_{2A}$ receptor-mediated contractions in rat aortic smooth muscle (Lu et al. 2008). They show that PP2 nearly abolished 5-HT-induced contractions in endothelium-denuded aortic rings. These findings are consistent with the results of the present study whereby PP2 drastically reduced bovine ASM contractions (fig. 3). Furthermore, similar to the present study, Src kinase activity was
significantly increased in the rat aortic smooth muscle in response to 5-HT stimulation as confirmed by a Src kinase assay. In a separate set of experiments using HEK 293 T cells it was discovered that Src kinase and 5-HT$_{2A}$ are localized close to one another at the cell membrane (Lu et al. 2008). In ASM, whether Src kinase is activated primarily by the 5-HT$_{2A}$ receptor, as opposed to other 5-HT receptors (ie., 5-HT$_3$R), and thereafter exerts its contractile effects is still unknown. However, like vascular smooth muscle, the 5-HT$_{2A}$ receptor is also expressed abundantly in ASM cells (Fernandez-Rodriguez et al. 2010) and could potentially be the primary source of Src kinase activation.

The present study is the first to show that 5-HT-induced ASM contraction requires Src kinase activation in whole airway smooth muscle tissue. It is clear that the downstream effector(s) of 5-HT-induced Src kinase activity are not yet well understood and that the effector(s) might be different depending on the smooth muscle type being examined (ie., airway or vascular). However, aside from the proposed role that Src kinase regulates PLC$\beta$ during 5-HT-induced ASM contraction (Tolloczko et al. 2002), future studies in the airway field could utilize what has previously been shown (and reported above) in vascular smooth muscle. For example, studies could examine which 5-HT receptor primarily activates Src kinase. In addition, once activated, examining whether Src kinase mediates ASM contraction by regulating the ERK MAPK pathway and/or $K^+$ channels (ie., MaxiK) would increase our knowledge of excitation-contraction coupling.
6.3 Acetylcholine-induced airway smooth muscle contraction

Acetylcholine, like 5-HT, is a neurotransmitter with potent contractile effects on ASM. In fact, ACh can contribute to airway hyperresponsiveness since its levels are increased in asthmatic airways. This occurs through its release from cholinergic parasympathetic nerve fibres that reside near the airway smooth muscle (Cyphert et al. 2009). The released ACh can directly bind to receptors on ASM cells leading to contraction. Since ACh levels are increased during the asthmatic response it is a physiologically relevant contractile agonist to use in the present study.

In ASM cells, ACh can mediate contraction through M2 and M3 muscarinic receptors with M2 representing the larger population (Mack and Barnes, 1990; Haddad et al. 1994). M2 receptors, which are coupled to heterotrimeric GTP-binding protein (G protein)G\textsubscript{i}/G\textsubscript{o}, inhibit adenylate cyclase and activate both voltage-gated Ca\textsuperscript{2+} channels (ie., L-type) and non-selective cation channels (Zhou et al, 2008). M3 receptors are coupled to G\textsubscript{q}/G\textsubscript{11} and stimulate PLC and phosphatidylinositol production which initiates Ca\textsuperscript{2+} release from the internal store (Zhou et al, 2008). These events mediated by M2 and M3 promote changes in intracellular Ca\textsuperscript{2+} levels required to cause contraction. However, the signaling pathways that mediate these events are not fully understood. The present study is the first to investigate whether Src kinase is required for ACh-induced ASM contractions.

Bovine ASM segments were pre-treated with the non-selective tyrosine kinase inhibitor, genistein, to examine the effect of tyrosine kinase activity during ACh-evoked ASM contraction. Although treatment with genistein reduced ACh-induced contractions
at concentrations of $10^{-7}$M and $10^{-5}$M (to the point where statistical significance was reached; fig. 6), there was far less of an effect against these contractions compared to those induced by 5-HT (fig. 2). Ultimately these results suggest that ACh-induced contractions do not largely depend on tyrosine kinase activity. It was not surprising that the Src family kinase inhibitors PP1 and PP2 had no effect on ASM contractions in response to ACh (fig. 7). This indicates that Src kinase is not necessary for ACh-induced ASM contraction.

Interestingly, Src kinase activity was still significantly increased in tissues treated with ACh (fig. 8). This is, in part, consistent with a previous study from canine colonic smooth muscle cells which also reports increased Src activity following M$_2$ muscarinic receptor stimulation (Singer et al 2002). However, their results contrast ours as they provide evidence, albeit indirect, that Src might play a role in contraction. That is, ERK MAPK becomes activated as a downstream event of Src kinase activation in the canine colonic smooth muscle cells. As previously mentioned, ERK MAPK has been identified as a player in the contraction of various smooth muscle types including colonic smooth muscle and ASM (Ihara et al. 2008; Sakai et al. 2010).

L-type Ca$^{2+}$ channels on ASM cell membranes contribute to the influx of Ca$^{2+}$ during the contractile response following ASM stimulation. Recently, ACh has been shown to stimulate M$_2$ receptors on rat portal vein myocytes and activate L-type Ca$^{2+}$ channels through a second messenger pathway (Callaghan et al. 2004; Callaghan et al. 2006). In particular, M$_2$ receptors coupled to the G-protein, G$_i$, activate a transduction cascade involving downstream mediators PI3K, PKC and Src kinase. Src kinase appears
to activate L-type Ca\(^{2+}\) channels which, at least in part, contribute to smooth muscle contraction. Therefore, this evidence for Src kinase regulating L-type Ca\(^{2+}\) channels contrasts our findings that suggest no role for Src kinase in ASM contractions. The question then becomes: why does Src kinase appear to regulate molecules that contribute to ACh-induced contraction (ie. ERK MAPK and L-type Ca\(^{2+}\) channels) in these non-airway smooth muscle types when our data suggest Src kinase is not necessary for ACh-induced ASM contraction.

One possible explanation is that there are functional differences between airway, vascular and colonic smooth muscle whereby muscarinic stimulation with ACh results in the activation of different pathways. For example, although the M\(_2\)/G\(_i\)/PI3K/PKC/Src kinase pathway seems to regulate L-type channels in vascular smooth muscle cells, this channel might not be regulated by the same pathway in ASM cells. Another simplistic explanation is that since Singer et al. (2002) and Callaghan et al. (2004) did not examine contractions in their studies, ERK MAPK and L-type Ca\(^{2+}\) channels do not play a large enough role in the contractile process to control its onset.

### 6.4 Involvement of other Src kinase family members in ASM contraction

PP1 and PP2 inhibit several members of the Src kinase family in addition to Src including Lck, Fyn and Hck. The activity of these kinases can not be discriminated from that of Src because Lck, Fyn and Hck become inhibited at relatively low concentrations (ie., IC\(_{50}\) ~5-20nM) of PP1 and PP2 whereas Src kinase is inhibited at relatively high concentrations (IC\(_{50}\) of ~0.3\(\mu\)M in vitro) (Hank et al. 1996; Waltenbeger et al. 1999).
Therefore, using a concentration of 10µM of PP1 and PP2 would indeed inhibit Src kinase as well as Lck, Fyn and Hck. Studies using PP1 and PP2 should be interpreted carefully.

Recently, RT-PCR analysis confirmed the expression of all nine Src family members in the lungs and main bronchi of rats (Sakai et al. 2010). These include Src, Fyn, Lck, Lyn, Hck, Yes, Blk, Fgr and Frk. It has become apparent that PP1 and PP2 also inhibit some non-Src family kinases including CSK, p38 MAPK, CK1δ, CSK, RIP2, and GAK (Bain et al. 2003; Bain et al. 2007). Therefore, we could not be certain that the decreased 5-HT-induced contraction occurred due to specific Src kinase inhibition. In addition, it is possible that other studies examining Src kinase activity, such as those referenced throughout this discussion are not specifically inhibiting Src kinase. To account for some of this non-specific inhibition in the present study, we blocked Lck, CSK and CK1δ by pre-treating the bovine smooth muscle with kenpaullone, purvalanol and SP600125, respectively. These blockers did not have any effect on the contractile response with 5-HT (fig. 4) which ruled out any contribution of Lck, CSK and CK1δ in 5-HT-induced contractions. Continuing experiments that block the Src kinase family members as well as the other non-Src family member kinases, will more precisely determine whether Src kinase is contributing to contraction.

6.5 Src kinase and Ca$^{2+}$-handling

Airway smooth muscle contraction critically depends on the increase in [Ca$^{2+}$], from the intracellular Ca$^{2+}$-store as well as from the extracellular space. However, little is
known about the modulation of $[\text{Ca}^{2+}]_i$ by tyrosine kinases. In rat tracheal smooth muscle cells, the inhibition of tyrosine kinases with genistein has been shown to significantly decrease peak calcium responses and contraction following stimulation with 5-HT (Tolloczko et al. 2000). These inhibitory effects of genistein on $\text{Ca}^{2+}$ responses were reported in experiments employing $\text{Ca}^{2+}$ and $\text{Ca}^{2+}$-free medium. This indicates the involvement of a tyrosine kinase in SR $\text{Ca}^{2+}$ release following excitation by 5-HT. Other studies using vascular smooth muscle have reported similar findings of the involvement of tyrosine kinases in $\text{Ca}^{2+}$ signaling following GPCR stimulation (Liu et al. 1996; Semenchuck and Di, 1995).

Tolloczko et al (2002) examined the possibility of Src kinase as the specific tyrosine kinase responsible for the increases in $[\text{Ca}^{2+}]_i$ observed in their previous study. PP1 and an anti-pp60 Src antibody were used to examine the specific effects of Src kinase on 5-HT-induced $\text{Ca}^{2+}$ signaling. Both PP1 and the anti-pp60 Src antibody significantly decreased intracellular $\text{Ca}^{2+}$ levels in response to 5-HT. This Src kinase dependent intracellular $\text{Ca}^{2+}$ increase is attributed to $\text{Ca}^{2+}$ release from the $\text{Ca}^{2+}$-stores since Src kinase appears to regulate PLCβ levels (Tolloczko et al. 2002). Therefore, during agonist-induced stimulation of ASM cells, Src kinase appears to be involved in the regulation of intracellular $\text{Ca}^{2+}$ levels which are required for contraction.

Moreover, Src kinase has also been proposed to contribute to $\text{Ca}^{2+}$ entry from the extracellular milieu by way of L-type $\text{Ca}^{2+}$ channels and store-operated channels. Over a decade ago, Wijetunge et al (2000) provided electrophysiological evidence for the modulation of L-type $\text{Ca}^{2+}$ channels by Src kinase in vascular smooth muscle cells. More
recently, Callaghan et al (2004 and 2006) have shown that L-type Ca\(^{2+}\) channels are regulated by Src kinase in cultured rat portal vein myocytes. L-type Ca\(^{2+}\) channels are important plasma membrane channels during agonist-induced contraction as they contribute to Ca\(^{2+}\) influx from extracellular space, allowing for the sustained increases in [Ca\(^{2+}\)]\(_i\). In addition, Babnigg et al (1997) provided evidence for the involvement of Src kinase in store-operated Ca\(^{2+}\) entry using fibroblasts. However, whether Src kinase regulates SOCE in airway smooth muscle remains to be investigated. Overall, these studies suggest Src kinases might play a large role in modulating airway smooth muscle contraction through regulating [Ca\(^{2+}\)]\(_i\) following agonist-induced excitation and potentially contributing to the altered smooth muscle functioning (AHR) seen in asthma.

### 6.6 Gene expression and HDM model

It is recognized that functional changes in ASM, for example changes in the regulation of [Ca\(^{2+}\)]\(_i\), might be a key feature of the pathophysiology of airway diseases such as the airway hyperresponsiveness seen in asthma (Amrani et al. 2004; Jude et al. 2008; Tao et al. 1999). However, the precise molecular changes that account for this airway hyperresponsiveness are still under investigation. Alternatively, some studies suggest that there might not be any functional difference in ASM between asthmatics and non-asthmatics (Armour et al. 1984; Bai, 1990; Bai, 1991). The present study examined the expression of various genes involved in Ca\(^{2+}\)-handling of ASM. In particular, we were interested in whether the genes involved in Ca\(^{2+}\) release and Ca\(^{2+}\) re-uptake from the SR were altered in a murine HDM model of airway hyperresponsiveness. This is the first
study to investigate the changes in gene expression of molecules related to the regulation of store-operated Ca\(^{2+}\) entry.

### 6.6.1 Decreased Orai1 Expression

Upon agonist-induced GPCR stimulation, the Ca\(^{2+}\) store becomes depleted which increases \([\text{Ca}^{2+}]_i\), allowing for ASM contraction. Following store depletion, STIM1 is activated on the SR membrane and promotes the opening of Orai1. Orai1 activation allows the entry of Ca\(^{2+}\) from the extracellular milieu which can then be pumped back into the store by SERCA. This leads to ASM relaxation and preparation for subsequent stimulation. The results of the present study do not support the hypothesis as Orai1 expression was significantly decreased in response to chronic HDM treatment (fig. 13) compared to the control treatment. The explanation for this decreased level of Orai1 in mice made hyperresponsive to HDM is not apparent. However, one potential explanation might involve the sodium-calcium exchanger (NCX).

The NCX is a plasma membrane associated channel used to rapidly extrude Ca\(^{2+}\) out of the cell by transporting one Ca\(^{2+}\) ion in exchange for the import of three Na\(^{+}\) ions. Recently, a growing body of evidence has suggested a role for the reverse-mode of the NCX in Ca\(^{2+}\) influx and SR refilling in ASM (Algara-Suarez et al. 2007; Hirota and Janssen, 2007; Hirota et al. 2007; Sathish et al. 2011). Ca\(^{2+}\) influx through the reverse-mode of the NCX is driven by depolarization as well as increased intracellular Na\(^{+}\) concentration regulated by non-selective cation channels (Hirota and Janssen, 2007). Unpublished data from our laboratory has suggested a significant increase in NCX
expression in the chronic HDM model. Other unpublished experiments from our laboratory examined the intracellular Ca\(^{2+}\) response (to contractile agonists) of ASM cells from HDM-treated mice and control mice. No significant difference in intracellular Ca\(^{2+}\) response was observed between treatment groups. Therefore, the decrease in Orai1 expression seen in the present study might be a compensatory effect of the increased NCX expression as an attempt to regulate Ca\(^{2+}\) entry. As such, the increased NCX expression and the decreased Orai1 expression could “balance” each other, thus promoting an unchanged net Ca\(^{2+}\) influx thereby protecting the cell from calcium overload or calcium toxicity. These findings might support the literature suggesting no functional difference in the ASM of asthmatics compared to non-asthmatics. However, these explanations are purely speculation and further investigation is required to determine more defined links between NCX and Orai1 expression as well as their relation to [Ca\(^{2+}\)].

As eluded to above, the decreased Orai1 expression (our data) together with increased NCX expression (unpublished data) does not necessarily indicate altered Ca\(^{2+}\)-handling in the HDM model. However, Orai1 and NCX are membrane channels which regulate Ca\(^{2+}\) entry from the extracellular space. Once the Ca\(^{2+}\) has entered the cell it must be pumped back into the store through the SERCA pump (located on the SR membrane) in order to relax the smooth muscle cell and thus prevent further contraction. It has been recently reported that SERCA2 mRNA and protein expression is significantly decreased in cultured ASM cells from patients with moderate asthma as well as in vivo in tissue sections of endobronchial biopsies from moderate to severe asthmatics (Mahn et al.)
2009). Therefore, regardless of whether Ca$^{2+}$ entry is unaltered (by the balance between Orai1 and NCX expression), impaired replenishment of the SR via SERCA could alter Ca$^{2+}$-handling in such a way as to promote AHR. This is because Ca$^{2+}$ levels in the cytoplasm of asthmatic ASM cells might be higher - and therefore activate the contractile apparatus to a greater degree in comparison to those of non-asthmatic ASM cells. Mahn et al (2009) also reported an increased time interval taken for bradykinin-induced elevations in [Ca$^{2+}$]$_i$ to return to baseline in asthmatic ASM in comparison to healthy subjects. In other words, following agonist-induced SR depletion the [Ca$^{2+}$]$_i$ took a longer time to be pumped back into the SR by SERCA which might account for the increased ASM contraction seen in asthma. That is because more Ca$^{2+}$ will be available to the contractile apparatus for a longer period of time. Restoring the function of SERCA might be a therapeutic approach for treating asthma.

### 6.6.2 Increased RyR3 Expression

Our findings of a significantly increased expression of RyR3 in the HDM treatment (fig. 11) compared to the control treatment support the idea that there are functional differences in the ASM of asthmatic airways compared to those of non-asthmatics. The RyR is a Ca$^{2+}$-release channel localized to the SR membrane and the RyR3 isoform has been previously shown to be primarily localized to the portion of the SR facing the deep cytosol (Du et al. 2005). An increased expression of RyR3 might promote a larger than normal Ca$^{2+}$-release from the SR into the deep cytosol. Since the contractile apparatus is also located in this region of the ASM cell, an increased Ca$^{2+}$
release from the RyR3 could ultimately increase ASM contraction and promote AHR. Support for this possibility comes from studies examining cADPR-evoked Ca\textsuperscript{2+} release from the RyR.

Cyclic ADP-ribose (cADPR) is a second messenger and a RyR activator which has been implicated as an important signaling molecule regulating the effects of cytokines on ASM responsiveness (Amrani et al. 2004). The transmembrane glycoprotein, CD38, functions to generate cADPR from nicotinamide adenine dinucleotide phosphate (Amrani et al. 2004). Interestingly, CD38 expression is increased in ASM cells by various inflammatory cytokines including TNF\textsubscript{α}, interleukin-1\textsubscript{β} and interleukin-13 (Deshpande et al. 2003; Deshpande et al. 2004). These studies by Deshpande and his colleagues have shown that the activation of the CD38/cADPR pathway induces ASM hyperresponsiveness since cADPR inhibitors eliminate TNF\textsubscript{α} and IL-13 effects on agonist-induced Ca\textsuperscript{2+} responses. Furthermore, Franco et al (2001) show the addition of extracellular cADPR enhances ACh-evoked Ca\textsuperscript{2+} responses and contractile responses in bovine ASM cells. Since these cytokines are increased during the asthmatic response (Broide et al. 1992; Thomson et al. 2011) they could contribute, through cADPR production, to enhanced intracellular Ca\textsuperscript{2+} responses by releasing Ca\textsuperscript{2+} through the RyR3. This change in Ca\textsuperscript{2+} signaling could then lead to increased ASM contractility as seen in the AHR of asthma.

In addition to the functional ASM changes leading to contraction, increased RyR3 expression might also contribute to structural ASM changes seen during asthma. One of these structural changes is an increased smooth muscle mass which is a key feature of the
airway remodeling process in the asthmatic airway. Both ASM cell proliferation and migration are necessary cellular events leading to thickening of the smooth muscle layer. Intracellular Ca\(^{2+}\) plays a central role in ASM proliferation and migration (Gerthoffer et al. 2008; Lipskaia et al. 2009). The increased RyR3 expression shown in the present study might promote an increased Ca\(^{2+}\) release into the deeper cytosol which could contribute to signal transduction. For example, Ca\(^{2+}\) binds to and activates high affinity binding proteins such as phosphatase calcineurin which then activates two transcription factors: nuclear factor of activated T cells (NFAT) and nuclear factor-κB (NF-κB). Both NFAT and NF-κB have been reported to contribute to airway remodeling and therefore AHR in asthma (Crabtree and Olson, 2002; Poynter et al. 2004). Furthermore, alterations in [Ca\(^{2+}\)] have also been shown to activate gene transcription which is important for the proliferation and migration processes (Dolmetsch et al. 1998; Perez-Zoghbi et al. 2009).

It has been recently reported that healthy human ASM cells treated with the inflammatory cytokines TNFα and IL-13 have significantly reduced SERCA2 expression and function (Sathish et al. 2009). These findings reiterate the idea that, during allergic asthma, an increased [Ca\(^{2+}\)] as a result of heightened RyR3 expression and diminished SERCA2 expression might promote AHR. Aside from influencing the contractile apparatus (as previously mentioned), these changes in Ca\(^{2+}\)-handling might also promote AHR by increasing ASM mass through processes such as proliferation and migration.
6.6.3 Unchanged STIM1, IPLA2B, RyR1 and RyR2 expression

Our data showing no difference in STIM1, IPLA2β, RyR1 and RyR2 expression in the HDM model do not support our hypothesis because we expected the expression of these genes to be increased compared to the controls. While some studies demonstrate functional differences (i.e., Ca\textsuperscript{2+}-handling) in asthmatic ASM compared to healthy ASM (Mahn et al. 2009), others have proposed that there might not be substantial functional differences with respect to excitation-contraction coupling (Armour et al. 1984; Bai, 1990; Bai, 1991). The latter could help to explain why there were no differences in the expression of the majority of the genes examined in the present study.

It is well established that asthmatic airways demonstrate increased thickness of the ASM layers, especially the ASM that lines the smaller airways. This increase is likely attributed to both hyperplasia and hypertrophy (Ebina 1993). Greater muscle mass generates more force and shortening of the muscle ultimately resulting in greater decrease in diameter (narrowing) than a normal airway (James et al. 1989). Perhaps changes in ASM function (i.e., Ca\textsuperscript{2+}-signaling) are playing no substantial role in AHR and that changes in ASM structure (i.e., proliferation) are mainly contributing to the differences in ASM behaviour of asthmatic airways compared to normal ones. If that is the case, there might not be a need for an increased expression of genes regulating Ca\textsuperscript{2+}-handling.

6.7 Limitations

One limitation to the present thesis is the use of a mouse model to explore Ca\textsuperscript{2+}-handling in airway hyperresponsiveness, and bovine model to study Src signaling.
Species-related differences in signaling and coupling mechanisms is a common problem in physiological studies. Mouse models of asthma are not an exact imitation of the human disease (Shapiro, 2006; Wenzel and Holgate, 2006). One important difference between humans and mice is that mice do not spontaneously develop asthma as humans do. Mice require the artificial sensitization with an experimental allergen to develop airway disease. Therefore, an assumption of the current study is that the processes involved for the development of allergic asthma in the mouse model are somewhat similar to the process involved in sensitization in patients with allergic asthma.

Another limitation associated with the murine samples was the difficulty in dissecting the smooth muscle from the bronchi. The airways of mice are extremely small and great care was taken when dissecting the smooth muscle from the bronchi so that cartilage and other tissue types were not also present in the sample. However, it is possible for these samples, which were used for RT-PCR, to contain a small amount of these other tissue types. For example, it is virtually impossible to visually identify the epithelial layer directly above the smooth muscle. In order to denude the epithelium, forceps were lightly rubbed overtop of this area. It is possible that not all of the epithelial cells were removed and thus remained in the sample. Ultimately, this could influence the results from our gene expression experiments.

Bovine tissue was dissected upon arrival, placed in Kreb’s buffer and refrigerated for use the next day. Similar to that of the mouse ASM dissection, great care is taken to eliminate other tissue types from the smooth muscle strips but a small amount of connective tissue might remain on the samples. Another possible limitation is that
decreased viability of the tissue might occur by the time it was used for the experiments. It is also possible that the various kinases examined in the present study were in some way affected by the amount of time the tissue was away from the in vivo environment.

An important limitation is the non-specific effects of the PP1 and PP2. Both blockers inhibit all of the Src tyrosine kinase family members as well as various other protein kinases highlighted by Bain et al (2003 and 2007). As a result, we are unable to determine whether Src kinase was necessary for ASM contraction. Similarly, the primary antibodies used to detect both phosphorylated and total Src kinase in the Western blot experiments might cross-react with other Src family members. Therefore, we could not be certain that we were specifically targeting Src kinase in the muscle bath experiments or the Western blot experiments.

6.8 Future Directions

There is mounting evidence in various smooth muscle types, including ours in airway smooth muscle, that Src tyrosine kinase as a key player in smooth muscle contraction. However, since the majority of these studies have employed PP1 and PP2 (Src family member inhibitors), it is still not completely apparent whether Src kinase is contributing to contraction. It could be that another Src family member or even an unrelated protein kinase is playing a role in contraction because they too can be blocked by these drugs. Future studies should continue to use specific blockers of these other potential kinases to establish which is/are involved in ASM contraction. Assuming the particular kinase involved is Src, studies should investigate its downstream effector(s)
following agonist-induced ASM contraction in order to create an understanding to which pathway(s) Src belongs.

According to the results of the present study, it appears as though the participation of Src kinases in ASM contraction is GPCR receptor-dependent. That is, Src kinase contributes to 5-HT-induced contraction but not ACh-induced contraction. One particular avenue for future experiments might focus on which particular 5-HT receptor induces Src activity. Although 5-HT-induced Src activity occurs through the 5-HT$_{2A}$ receptor in vascular smooth muscle it is not yet clear whether this is true of ASM. As the 5-HT$_{2A}$ receptor is also expressed abundantly in ASM future experiments should examine this hypothesis. Furthermore, future investigations could examine the effect of other ASM agonists (i.e., histamine) on Src kinase activity and whether contractions in response to these agonists requires this kinase activity. This would increase our general understanding of ASM excitation-contraction coupling which is always an important step before designing therapeutic targets for asthma in the future.

Finally, the present study examined the mRNA expression of different genes involved in SOCE using RT-PCR analysis. However, the amount of mRNA expression in a cell does not always correlate with the amount of protein expression in that same cell. For example, the present study reported an increased RyR3 mRNA expression in HDM treated mice but we cannot be certain that RyR3 protein expression is also increased as no protein analysis was conducted. Post-translational modifications might take place potentially keeping the RyR3 protein expression the same as in control samples. The amount of ASM obtained from a mouse is so minute that Western blotting is not possible
for each mouse sample alone. It is important for future experiments to pool ASM samples from multiple mice of the same treatment group in order to conduct Western blotting analysis on the genes investigated in this study. This would complement the RT-PCR data and give a more indicative representation of the protein expression of molecules in the SOCE pathway.

6.9 Conclusions

The aim of the present study was to enhance our knowledge of excitation-contraction coupling by examining the potential role of Src kinase in ASM contraction as well as by investigating the expression levels of genes involved in Ca\(^{2+}\)-store emptying/refilling. Experiments done using other smooth muscle cell types have suggested a role for Src kinase in contraction but this is the first study to examine the role of Src kinase in whole ASM tissue. The results suggest 5-HT-induced ASM contraction involves Src tyrosine kinase activity. However, ACh-induced ASM contractions do not require this kinase. ACh-induced Src kinase activity might be required for other ASM cell processes not examined by this study. The present study also employed the commonly used HDM mouse model of allergic asthma in an effort to identify changes in the gene expression of molecules that regulate ASM Ca\(^{2+}\)-handling (store emptying/refilling). We show a significant increase in RyR3, a significant decrease in Orai1, and no change in STIM1, IPLA\(_{2}\)β, RyR1 and RyR2 gene expression. It is unclear whether the changes in RyR3 and Orai1 expression alter Ca\(^{2+}\)-handling in such a way as to potentiate airway hyperresponsiveness.
REFERENCES


