AIRWAY STRETCH-ACTIVATED CONTRACTIONS

ASPECTS OF AIRWAY STRETCH-ACTIVATED CONTRACTIONS

ASSESSED IN PERFUSED INTACT BOVINE BRONCHIAL SEGMENTS

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A thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Doctor of Philosophy

McMaster University

DESCRIPTIVE NOTE

DOCTOR OF PHILOSOPHY (2011)　　　　　　　McMaster University

(Physiology and Pharmacology)　　　　　　　　　Hamilton, Ontario

TITLE:　　　　　　　　Aspects of Airway Stretch-Activated Contractions Assessed in Perfused Intact Bovine Bronchial Segments

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NUMBER OF PAGES:　　*ix*, 196

ABSTRACT

Asthma is a disease characterized by transient airway smooth muscle contraction leading to episodes of reversible airway narrowing. It affects over 300 million people worldwide and is implicated in over 250 000 deaths annually. The primary clinical features of asthma include airway inflammation, hyperresponsiveness, and remodeling. Generally, asthmatic patients experience exacerbations between periods of diminished symptoms. Interestingly, in addition to these above mentioned hallmarks, asthmatics have also been shown to react differently to ventilatory mechanical strain. This is most evident when assessing the effect of a deep inspiration (DI), clinically measured as a breath taken from functional residual capacity to total lung capacity, in healthy individuals *versus* asthmatics. These deep inspiratory efforts have been shown to produce a bronchodilatory response in healthy individuals, whereas in asthmatics, DIs are less effective in producing bronchodilation, can cause more rapid airway re-narrowing, and even bronchoconstriction in moderate to severe asthmatics. The mechanism by which a DI is able to cause bronchoconstriction remains ambiguous. Previous theories suggest that this phenomenon is intrinsic to airway smooth muscle (ASM) itself. However, the airway inflammation present in asthmatic airways may also add to the increased ASM contractility following stretch, by the release of mediators that can prime the contractile apparatus to react excessively in the presence of stretch.

Thus, collectively, the studies contained in this thesis are linked to the general theme of greater characterization of the signalling mechanisms that regulate airway stretch-activated contractions using a pharmacological approach in intact bovine bronchial segments, with the hope of providing novel insights into the mechanisms that regulate the DI-induced bronchoconstriction seen in asthmatics.

ACKNOWLEDGEMENTS

None of this work would have been possible without the help and guidance of Dr. Luke Janssen. As a supervisor, Luke is always approachable, open-minded, willing to help, and is an utmost pleasure to work for/with. As a mentor, he has a knack for listening to a complex problem, understanding/simplifying the issues at hand, and subsequently offering insightful solutions/suggestions. Most importantly, as a person, he is always good humored, intellectual, and demonstrates that a balance between a career in science and family is indeed possible.

Next, I would like to thank my supervisory committee, consisting of Dr. Mark Inman and Dr. Gerry Cox. These are two individuals for whom I hold the highest respect. Time and again, they have looked at my research with a critical eye while offering different perspectives, and always made an effort to probe me further with their astute questions and comments. They have also been instrumental in bestowing their knowledge and offering both scientific and career-based advice/direction.

I would also like to thank Tracy Tazzeo for her technical expertise. Ever since my first day in the Janssen Lab, she has been instrumental in helping me through the many technical issues I have encountered.

Although they were not directly involved in my research, I would sincerely like to thank my parents for always being my true support structure and for always giving me more, even at times when they had less. The time and effort

they have spent helping me achieve my full potential is greatly appreciated and will never be forgotten.

      Lastly, I would like to thank my wonderful fiancée Jennifer, for her unflagging patience, for always believing in/encouraging me, for listening to and critically commenting on my endless presentations, and (most importantly) for inspiring me to always be a better person.

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CHAPTER 1

Introduction

General Introduction

Mechanotransduction is defined as the sensing of mechanical stress and its conversion into a biochemical process. This phenomenon plays an essential role in the functioning of various cell types (bacterial, plant, and animal) (71; 113; 155), and physiological processes (blood pressure regulation, touch and pain sensation, vestibular function, and respiration) (22; 29; 30; 59; 60; 155). In the vasculature, blood pressure is strictly regulated by signaling pathways that respond to mechanical stress in order to ensure the precise control of blood flow under physiological conditions ranging from vigorous exercise to complete rest (45; 46; 93). At the turn of the last century (early 1900s), experiments performed using a dog hind-limb model of vascular perfusion, showed blood vessels responding to increased transmural pressure by constricting (12). This phenomenon was found to be independent of endothelial, neuronal, or hormonal input (54; 73; 146). Hence, this response was termed 'myogenic', emphasizing the fact that it is generated intrinsically by the vascular smooth muscle (VSM), without external influence. Similar to VSM, airway smooth muscle (ASM) are also subjected to constant mechanical stress due to each inhalation/exhalation cycle we perform.

The inappropriate contraction of ASM is a key feature of numerous obstructive airway diseases, including asthma, a chronic disease characterized by airway inflammation, obstruction, remodeling, and hyperresponsiveness (3; 6; 9; 10; 16; 90). However, aside from these hallmarks, asthmatics also differ from

healthy individuals in the way they respond to ventilatory mechanical stress. This stress typically produces beneficial (bronchodilatory) responses in healthy individuals, whereas in asthmatics, they have been shown to elicit harmful (bronchoconstrictory) responses (60; 104; 109; 138). More specifically, a deep inspiration (DI), which is clinically measured as a breath taken from functional residual capacity to total lung capacity, produces a bronchodilatory response in the airways of healthy individuals. Conversely, asthmatics are less effective in producing a bronchodilatory response to a DI, which in their case can even *cause* a bronchoconstriction (60; 81; 135; 138).

The central focus of this thesis is on the investigation of the underlying mechanisms behind isolated airway stretch-activated contractions ($R_{stretch}$) as a model for the DI-induced bronchoconstriction phenomenon seen in moderate to severe asthmatics. Studies were performed to characterize the effects of stretch - transmitted *via* acute transmural pressure loads - on ASM contraction, assessed *ex vivo* using perfused isolated intact bovine bronchial segments. Moreover, the potential for regulation of these $R_{stretch}$ responses by endogenous excitatory prostanoids, as well as the mechanistic similarities between the ASM $R_{stretch}$ and the vascular myogenic response were investigated using a pharmacological approach. This thesis is being presented in a "sandwich" format where the submitted/published papers act as the "body" of the manuscript, and are flanked by the *Introduction & Discussion,* which collectively serve to set the context, and explain the relevance and implications of this research project.

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Structure of the Airways

In order to stay viable, human tissues require a continuous supply of oxygen (O_2) and removal of carbon dioxide (CO_2). As a result, the main functions of the respiratory system are to obtain O_2 and allow it to diffuse into the circulatory system, while removing CO_2.

The respiratory system is composed of the lungs, the airways (this includes ASM), the central nervous system, the respiratory muscles (diaphragm, intercostals muscles, abdominal muscles) and the chest wall. However, due to the wide breadth of this topic area, the section below will elaborate only on the structure of the airways, with a special emphasis on the ASM.

Airways are classified as being conducting or respiratory. The conducting airways consist of the trachea, bronchi, and bronchioles. Their roles are to transport O_2 and CO_2, while inhibiting contaminants from reaching the distal lung. The respiratory airways mediate gas exchange through the alveoli and are located at the level of the respiratory bronchioles and alveolar ducts. The airways themselves are composed of the mucosa, submucosa, and fibrocartilagenous layers. The ASM is located in the submucosal layer, along with mucous glands, connective tissue and neurons (67). ASM is present from the trachea down to the alveolar ducts and take on different configurations depending on where it is located along the bronchial tree. The musculature of the first and second order human bronchi closely resembles that of the trachea. Conversely, that of the fourth to seventh order airways is substantially different in terms of the size and

arrangement of the muscle bundles, and appearance of the contractile myofilaments. Interestingly, mast cells also appear to be more intimately associated with the smooth muscle of these smaller airways. Furthermore, innervation of the smaller airways is much denser than that of the trachea and large bronchi (43).

At the level of the trachea and large bronchi, C-shaped cartilage pieces that support the airway are dorsally bound by ASM. On the other hand, ASM completely surrounds the small bronchi and bronchioles. In fact, as the airway size decreases distally, ASM occupies a larger proportion of the airway wall. Moreover, the cartilaginous portion of the airways can act as an afterload to the ASM by impeding its ability to contract and narrow the airway excessively. In fact, ASM contraction can only cause complete airway closure at the level of the smaller, less cartilagenous airways.

Abnormalities in the contractility of ASM are of great importance in obstructive airway diseases such as asthma. Excess ASM contraction can trigger symptoms of chest tightness, shortness of breath, and general difficulty of breathing by causing excessive airway narrowing and subsequent increase in airflow resistance (158).

Asthma

Asthma is a disease characterized by transient ASM contraction leading to episodes of reversible airway narrowing. It occurs in people of all ages but is particularly prevalent in children and young adults (98; 153). The primary clinical features of asthma include *airway inflammation*, *airway hyperresponsiveness* (AHR) - defined as ASM hypersensitivity and hyperreactivity to contractile stimuli (leftward-shift and increased maximal contractile force on a methacholine challenge test curve) (124) - and *airway remodeling* which includes epithelial damage & structural change, airway fibrosis and smooth muscle hypertrophy & hyperplasia (116). Asthmatic patients commonly experience exacerbations between periods of diminished symptoms.

Asthma affects 5 - 10% of the population, however many aspects of its etiology and pathogenesis remains uncertain (17; 58). Predisposing factors to asthma include both genetic and environmental elements. Many patients with atopic asthma often have a strong family history of the disease, suggesting genetic factors may play a role (20; 36). Candidate genes supposedly involved in the pathogenesis and predisposition to asthma include the beta-subunit gene for the high affinity IgE receptor (37), the beta-2 adrenergic receptor gene (42), as well as the disintegrin and matrixmetalloproteinase gene, ADAM 33 (80; 103; 157). Interestingly, there is no *Mendelian* inheritance pattern to asthma, suggesting a complex genetic etiology (36; 79).

Environmental predisposing factors have also been postulated. For example, early childhood microbial infections have been associated with later development of asthma (114; 139; 147). Conversely, other epidemiological studies suggest that there is an inverse relationship between allergic diseases and infections in early childhood (31; 127). The maturation of the immature immune system after birth is largely driven by exposure to microbes. In fact, animals protected from microbes produce excessive immune responses when immunized, and they do not develop normal immune regulation (125; 152). Thus, proponents of the "hygiene hypothesis" suggest that during childhood, microbial infections protect against the development of asthma later in life (150), by shifting the immunologic profile of the child towards a TH_1 pathway and away from a TH_2 pathway, which mediates allergic inflammation (161).

The major chief complaints during an asthma "attack" include shortness of breath, wheeze, and cough (136); features that accompany clinical/laboratory findings such as airway inflammation, airway hyperresponsiveness, increased airflow resistance, and decreased forced expiratory flow rates (158). Inflammatory cells play an important role in the pathology of asthma, observed histologically by the increases in leukocytes and lymphocytes in the asthmatic airway, coupled with the increased levels of TH_2 cytokines and inflammatory mediators found in sputum samples and bronchoalveolar lavage fluid of asthma patients (10; 161; 163). Interestingly, these pathways ultimately lead to ASM contraction through biological signaling. In fact, it is the excessive ASM

contraction that ultimately increases airflow resistance, impairing alveolar ventilation (3; 40; 62; 90; 161), thus leading to the chief complaints mentioned above.

Contractile Apparatus

ASM has historically been considered as being a multi-unit system, where each smooth muscle cell acts relatively independently of other smooth muscle cells, and cell-to-cell communication is poor compared to single-unit systems (i.e. gastrointestinal (GI) smooth muscle) (112; 148). However, in contrast to the paucity of gap junctions normally found coupling cells in a multi-unit system, electron micrographs have revealed the ASM cell to be well-connected physically to neighboring cells via gap-junctions, which provide pathways of low resistance for electrical signals to be transmitted. The number and size of gap junctions have in fact been shown to increase in ASM located towards the distal end of the airway tree (43; 100). More interestingly, ASM has been shown to exhibit spontaneous electrical slow waves (similar to that of GI smooth muscle) either continually (at rest and during excitation) or solely during excitation (75), which points to the fact that this smooth muscle subtype may actually exhibit features of both multi-unit and single unit systems. For now, however, the role of these slow waves in ASM physiology remains unclear.

Tone generation, which is another intrinsic property of smooth muscle, can be modified by changes in the external microenvironment. In ASM, this can occur *via* the release of pharmacological contractile stimuli, which includes numerous extracellular factors such as neuronal input from nerve endings that terminate within the airway wall, epithelial-derived factors, and inflammatory products released by circulating and tissue-bound inflammatory cells. These

mediators typically bind to their respective receptors located on the ASM plasma membrane, which leads to the activation of signaling pathways (described below) within the ASM cell, ultimately culminating in an increase in the net amount of phosphorylated myosin light chain.

The organization of the contractile apparatus in ASM is consistent with the sliding actin and myosin filament model. Actin filaments are composed of globular subunits arranged in a right-handed helix, while myosin thick filaments consist of six polypeptide chains; two heavy chains (~200 kDa), two regulatory light chains (~20 kDa), and two essential light chains (~17 kDa). The carboxy terminals of the heavy chains form a supercoiled α-helix, while the amino terminals form globular heads with a catalytic site for the hydrolysis of adenosine triphosphate (ATP). Actomyosin activation is initiated by the phosphorylation of the 20 kDa regulatory myosin light chain. Tension generated by the contractile filaments is transmitted throughout the cell *via* a network of actin filaments anchored to dense plaques at the cell membrane, where force is transmitted to the extracellular matrix *via* transmembrane integrins (137).

The initiating event in ASM contraction is usually a rise in intracellular Ca^{2+} concentration either from intracellular stores and/or from the extracellular space. This rise in cytosolic Ca^{2+} ultimately leads to the binding of Ca^{2+} to the protein calmodulin, which induces a conformational change in its structure. This Ca^{2+}-calmodulin complex then binds to myosin-light chain kinase (MLCK), resulting in the phosphorylation of serine residue-19 on the myosin light chain

associated with each myosin head. This then enhances the ATPase activity of myosin, resulting in the hydrolysis of ATP to adenosine diphosphate (ADP) and inorganic phosphate (Pi). As the ADP and Pi dissociate from the myosin head, a conformational change occurs that enables the myosin head to pull the bound actin filament, resulting in contraction (88). Conversely, smooth muscle also contains myosin light chain phosphatase (MLCP) which works against MLCK by continually removing the phosphates put on myosin light chains. Thus, it is essentially these two enzymes that exert the greatest influence on the contractility of ASM.

In addition to a rise in intracellular Ca^{2+} concentration, ASM contraction can also be regulated by calcium sensitization, achieved through the inhibition of MLCP. This phenomenon results in a net increase in the amount of phosphorylated myosin for a given level of Ca^{2+}, thus essentially lowering the threshold for contraction. The downregulation of MLCP activity can be achieved *via* two pathways. The first involves Diacylglycerol (DAG), a cleavage product of Phospholipase (PL) C that participates in Ca^{2+} sensitization by directly activating CPI-17, which subsequently inactivates MLCP (49). The second pathway involves the activation of the monomeric G-protein RhoA, by the binding of ligands to G-protein coupled receptors (GPCRs) coupled to $G\alpha_q$ or $G\alpha_{12,13}$. Once activated, RhoA translocates from the cytoplasm to the plasmalemma where it activates its downstream effector molecule Rho-kinase (ROCK). The latter

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directly phosphorylates MLCP thereby suppressing its phosphatase activity (143-145).

ASM Excitation-Contraction Coupling

Voltage-gated Ca^{2+} influx

In general, the excitation of muscle cells commences by a change in membrane potential which elicits the opening of voltage-gated Ca^{2+} channels (VGCCs). It is for this reason that many pharmacological treatments aimed at relaxing muscle tissue directly target these channels. Interestingly, contrary to cardiac and skeletal muscle, as well as vascular and GI smooth muscle subtypes, extracellular Ca^{2+} influx through VGCCs has been shown to play a minimal role in ASM contraction (74; 88), hence, the mixed opinions regarding the value of VGCC blockers in asthma (11; 41; 52).

VGCCs consist of an α_1 subunit that forms the core of the channel, and auxiliary subunits such as α_2, β, δ, and γ, that act to regulate the functional properties of the α_1 subunit (4; 149). In general, these channels are responsible for mediating the voltage-dependent influx of Ca^{2+} but may also be activated in a DAG-dependent fashion by agonists binding to GPCRs (156). Although both T-type and L-type VGCCs have been described in ASM (87; 156), it is suggested that the expression of T-type channels on ASM may be species dependent (64; 159). L-type Ca^{2+} channels possess a relatively large unitary conductance of ~25 pS. The resultant currents exhibit threshold and peak activation at ~-35 and ~+10 mV respectively and inactivate with a time-constant of ~24 ms. Conversely, T-type Ca^{2+} channels possess a conductance of ~10 pS, display threshold and peak activations at ~-60 and ~-20 mV respectively, and inactivate with both fast (1 ms)

and slow (34 ms) time constants (137). L-type channels are sensitive to inhibitors of the dihydropyridine class (i.e. nifedipine), while T-type channels are relatively resistant to these (87; 137).

Interestingly, another characteristic feature of the ASM cell is the membrane's propensity for electrical rectification subsequent to a depolarizing stimulus. This rectification behavior is thought to be due to the opening of voltage-dependent, large conductance, Ca^{2+}-activated K^+ channels (BK_{Ca}) which allow for a time-delayed, K^+ efflux, causing repolarization of the cell (128). In fact, K^+ channel antagonists can trigger considerable depolarization and contraction in ASM (39).

Agonist-induced contraction

In addition to a change in membrane potential, ASM contraction can also be elicited by pharmacomechanical coupling through the activation of GPCRs by neurotransmitters, biological mediators, and drugs. These receptors directly affect the contractile apparatus through second messengers without a change in membrane potential. The activation of the contractile apparatus follows an elevation of intracellular Ca^{2+} concentration involving the release of Ca^{2+} that is stored or sequestered in the sarcoplasmic reticulum (SR). Agonists, such as carbachol and serotonin contract ASM by activating their specific membrane receptors which triggers the generation of second messengers that release Ca^{2+} from the SR. Carbachol causes contraction primarily by binding to the M_3

muscarinic receptor (132; 134). Activation of the M_3 muscarinic receptor, a GPCR, activates its $G_{q/11}$-α subunit, which liberates inositol-1,4,5-trisphosphate (IP_3) following the phospholipase C (PLC) mediated hydrolysis of membrane-bound phosphatidylinositol-4,5-bisphosphate (PIP_2). IP_3 then triggers the release of intracellular Ca^{2+} from the SR by binding to its receptor, the IP_3-receptor (IP_3-R) (137). In addition to the M_3 receptor, the M_2 muscarinic may potentiate the carbachol-induced contraction by inhibiting cAMP-dependent relaxation. Activation of the M_2 receptor inhibits adenylyl cyclase through the activation of the $G_{-i/o}$-α subunit (131; 133; 151).

Serotonin has been shown to contract ASM in animals, however, its role in human airways remains debated (8). Similar to carbachol-induced contractions, in animals, serotonin-induced contractions are mediated by the activation of 5-HT_{2A} receptors, which leads to the activation of PLC, liberation of IP_3 and subsequent mobilization of Ca^{2+} from the intracellular stores (83).

Ca^{2+}-Handling

By and large, the many mechanisms that govern ASM contraction revolve around Ca^{2+}-handling. Intracellularly, Ca^{2+} concentration is very tightly regulated. As outlined above, the rise in intracellular Ca^{2+} concentration can be initiated either by Ca^{2+} influx from the extracellular space *via* plasmalemma-bound channels and/or by the release of internally sequestered Ca^{2+} by activation of the IP$_3$ receptor, involved in GPCR-mediated contractions. In addition to these processes, internally sequestered Ca^{2+} can also be released by the activation of ryanodine receptors, another class of Ca^{2+} channel located on the SR-membrane that are responsible for releasing Ca^{2+} from the SR in response to elevations in cytosolic Ca^{2+} (a phenomenon known as Ca^{2+}-induced Ca^{2+} release (CICR)). Ryanodine receptors are sensitive to the plant alkyloid, ryanodine. At low concentrations, ryanodine activates these receptors, subsequently inducing Ca^{2+} release; whereas at higher concentrations, Ca^{2+} conduction *via* these receptors is inhibited (141).

Following a rise in intracellular Ca^{2+}, either by influx or release from stores, cellular processes are initiated to reduce the intracellular Ca^{2+} concentration either by actively sequestering the cytosolic Ca^{2+} into the SR or by extrusion into the extracellular domain.

The sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is located on the SR and helps to regulate cytosolic Ca^{2+} levels by sequestering two Ca^{2+} ions into the SR for every ATP hydrolyzed. In fact, the pumping action of this ATPase is

responsible for the over 10 000-fold concentration gradient for Ca^{2+} that exists between the cytosol and the interior of the SR (115; 162). Interestingly, of the three major isoforms that have been identified to date (SERCA 1, 2 and 3), smooth muscle only expresses the SERCA 2 isoform (2).

Two major extrusion mechanisms exist that move free cytosolic Ca^{2+} into the extracellular domain. The first occurs actively through the plasma membrane Ca^{2+}-ATPase (PMCA). Similar to SERCA, PMCA uses energy from the hydrolysis of ATP to pump cytosolic Ca^{2+} across a substantial gradient into the extracellular space (26). In fact, it has been shown that inhibiting this ATPase results in elevated cytosolic Ca^{2+} levels as well as enhanced cholinergic responses (27; 91). The second mechanism for Ca^{2+} extrusion involves the sodium calcium exchanger (NCX), which is a non-ATP dependent plasmalemma-bound protein that facilitates bidirectional movement of Ca^{2+} and Na^+ ions across the plasmalemma (three Na^+ for every Ca^{2+}). In the forward mode, this exchanger uses the Na^+ gradient to facilitate the extrusion of Ca^{2+}, thus maintaining low cytosolic levels (91). However, since this process is passive, it is influenced by changes to both the Ca^{2+} and Na^+ gradients. Therefore, when cytosolic levels of Na^+ increase relative to declining Ca^{2+} levels, the exchanger can flip into reverse mode, and contribute to the influx of extracellular Ca^{2+} into the cytosol. NCX in its reverse mode provides a source of extracellular Ca^{2+} for refilling depleted intracellular stores (76; 77).

TxA$_2$ and ASM Contraction in Asthma

Abnormally functioning ASM can lead to significant morbidity. Examples include disease states characterized by bronchoconstriction – defined as the constriction of the airways due to the stiffening and excessive contraction of surrounding ASM. This subsequently leads to wheezing, chest tightness, and dyspnea (i.e. asthma exacerbations). ASM acts as the effector cell in the bronchoconstrictory pathway. Therefore, although precise mechanisms for the excessive bronchoconstriction present in asthma are still not completely understood, it is believed that changes in the asthmatic airway *milieu* may play an important role by pathologically affecting ASM excitation-contraction coupling and Ca^{2+}-handling. Interestingly, inflammatory mediators by themselves may directly affect ASM contraction (10). *Ex vivo* studies have shown that incubation of normal human ASM tissues with IgE or allergic serum elicited an ASM $R_{stretch}$ response (117; 118), a possible mechanism for the DI-induced bronchoconstriction seen in asthmatics.

In addition, among the numerous mediators released in asthmatic airways, prostanoids are both synthesized and released by bouts of airway inflammation as well as by mechanical stress (1; 129). Immunologic challenge of sensitized isolated perfused guinea pig lung *ex vivo*, and mechanical stretch of rat lung epithelial cells *in vitro*, both stimulated prostanoid synthesis and release (38; 130). Among the prostanoids that stimulate ASM, TxA$_2$ has attracted attention as a potential important mediator in the pathophysiology of airway

hyperresponsiveness due to the potency of its bronchoconstrictory ability (approx. two orders of magnitude more potent than other prostanoids) (14; 33; 48).

TxA_2 was originally identified in extracts of human platelets. Its synthesis occurs through a pathway similar to that of the other members of the prostanoid family. Upon cellular stimulation, arachidonic acid is liberated from phospholipids of cell membranes and converted into prostaglandin (PG) H_2 *via* the cyclooxygenase enzymes (COX-1 and/or -2). PGH_2 is then further converted into biologically active prostanoids (TxA_2, PGI_2, $PGF_{2\alpha}$, PGE_2 and PGD_2) by specific enzymes: TxA_2-synthase, PGI_2-synthase, $PGF_{2\alpha}$-reductase, PGE_2- and PGD_2-isomerases, respectively (48).

In the lung, TxA_2 is produced by a number of cells, including the epithelia, smooth muscle, and resident macrophages (48; 82; 122; 123). In aqueous solutions, TxA_2 is rapidly hydrolyzed to TxB_2, a stable and inactive metabolite. The short half-life of TxA_2 suggests that it functions in an autocrine/paracrine fashion and that its actions are limited to tissues in proximity to the source of its synthesis. Because of the instability of TxA_2, most experimental studies of TxA_2 biology have utilized the stable TxA_2 mimetic, U-46619 (68). Exposure of human tracheal rings to U-46619 results in concentration-dependent contractions (5). These *ex vivo* studies were consistent with studies in humans and other animals demonstrating that inhalation of U-46619 results in rapid bronchoconstriction (94; 105).

The actions of TxA_2, as well as those of other prostanoids, are mediated through binding to specific GPCRs. TxA_2 elicits its bronchoconstrictory effects by both directly binding to and activating thromboxane prostanoid (TP)-receptors on ASM (which signal through the $G_{q/11}$ family of G proteins), causing activation of PLC and increases in intracellular Ca^{2+} concentration (96), as well as by causing prejunctional release of ACh from cholinergic neurons (1; 89). Interestingly, in addition to exerting their effects by binding to their respective receptors, it has been shown that other bronchoconstrictory prostanoids (PGD_2 and $PGF_{2\alpha}$) can also bind to the TP-receptor (51).

Deep Inspiration

A deep inspiration (DI), clinically measured as a breath taken from functional residual capacity to total lung capacity, produces a bronchodilation in healthy individuals (104; 135). Asthmatics, however, exhibit airway hyperresponsiveness, characterized by exaggerated airway narrowing, or more seriously, complete airway closure when challenged with various non-specific stimuli (116; 124; 140). Airway responses to DIs differ in asthmatics in that they are less effective in producing bronchodilation, and can even *cause* bronchoconstriction (21; 60; 81; 112; 140). The likelihood of loss of bronchodilation and generation of bronchoconstriction following a DI correlates positively with the clinical severity of asthma (more common in severe asthmatics) and airway inflammation measured by bronchial biopsy (142). Interestingly, the manner by which a DI is able to cause bronchoconstriction remains ambiguous. Several theories suggest that this phenomenon is intrinsic to ASM itself. One suggestion is that in asthmatics, smooth muscle activation and tension generation cause an increase in ASM stiffness to the point where it stretches little during a DI (3). Another theory suggests that a DI-induced bronchoconstriction is a peripheral parenchymal hysteresis-associated event, related to the lung pressure-volume hysteresis curve. After lung inflation to TLC during a DI, the lower recoil pressures during deflation at any given volume can lead to smaller airways than before the DI was performed because of unloading of the ASM, which narrows the airways more than it would have otherwise (104).

Additionally, the airway inflammation and remodeling present in asthmatic airways may also add to the increased ASM contractility after stretch, by the release of stimuli that prime the contractile apparatus to react excessively in the presence of stretch (117).

Stretch-Activated Contractions

In the vasculature

In the vasculature, blood pressure is kept within a physiological range by signaling pathways that respond to mechanical stress (45; 46; 93). In 1902, Bayliss performed experiments using dog hind-limb which showed blood vessels responding to increased transmural pressure by constricting (12). This phenomenon was later termed a *myogenic response*, as it was an intrinsic property of the vascular smooth muscle (VSM), independent of neural, metabolic, or hormonal input (46; 54; 73; 146). According to Hill *et al*., stretch elicits VSM membrane depolarization (70; 73), activating L-type Ca^{2+} channels, thus causing Ca^{2+} influx (46). This subsequently activates the CICR mechanism *via* SR-bound RyR activation (50; 86), and causes a myogenic contraction (73), the magnitude of which has been shown to be limited by the activation of plasmalemma-bound large conductance Ca^{2+}-dependent K^{+} (BK_{Ca}) channels (18; 72; 86).

In the airway

Similar to the vasculature, the airways are also constantly subjected to mechanical stress due to the inflation and deflation of the lungs. In the airways, force-induced changes in the physical properties of cell membranes have been shown to regulate ion channel conductance (69; 84; 85). Thus, the increase in intracellular Ca^{2+} concentration leading to smooth muscle contraction can be triggered by mechanotransductory events. Channel sensitivity to stretch occurs *via*

the fluctuation between an open and a closed position, in which stretching of the cell membrane causes a conformational change that thermodynamically favours the open position of the channel (69). Stretch-activated cation channels expressed in the airway belong to the transient receptor potential (TRP) family, which consists of members belonging mainly to the TRPC (canonical) and TRPV (vanilloid) subtype. Moreover, the L-type Ca^{2+} channel has also been cited as an important player in airway stretch-activated contractile responses ($R_{stretch}$) (61; 112; 148). The importance of Ca^{2+} influx following stretch was demonstrated through experiments performed on airway tissues exposed to mechanical stress (84; 85). Interestingly, this stress produces both relaxant and constrictor responses in ASM (110). Thus, airway stretch is suggested to be either beneficial (bronchodilatory) in healthy individuals or harmful (bronchoconstrictory) in asthmatics, as seen by the impaired bronchodilatory response and bronchoconstriction induced by a DI in asthmatics. Unfortunately, the underlying signaling mechanisms behind airway contractions in response to stretch remain ambiguous. Marthan and Woolcock suggested in 1989, that DI-induced bronchoconstrictions may be caused by an ASM myogenic response (similar to that seen in VSM), produced by the conversion of airway smooth muscle from a 'multiunit' to 'single unit' entity (112). Other suggestions involved possible roles for increased ASM stiffness in asthmatics (3), airway recoil pressures and the pressure-volume hysteresis curve (104), as well as airway inflammation and the release of inflammatory mediators (117).

Mechanotransduction

Mechanotransduction plays an important role in many physiological processes that require a cellular response to shear stress mediated through distortions of the cell membrane. In fact, the airway possesses a particularly complex relationship with the mechanical stimulus of ventilation. ASM contraction can be mediated by various stimuli: pharmacological, electrical, as well as mechanical. Mechanotransduction is not a finite single-step process but is rather a series of interrelated processes that involve the recruitment of cytoskeletal elements and signaling pathways.

ASM is continually subjected to changes in length and mechanical stress due to the oscillatory waveform of tidal ventilation. Lung volume changes and ASM tone are able to produce a variety of physiological effects pertaining to the resulting contractility of ASM. As stated above, in healthy individuals, a DI has been shown to decrease airway resistance (28; 29). Moreover, experiments in dogs have demonstrated that, changes in mechanical stress experienced during normal tidal breathing are able to modulate airway tone by lowering the airway resistance normally seen during bronchial challenge under static conditions. In other words, the increase in airway resistance in response to bronchial challenge is significantly lower during tidal breathing than under static conditions (65; 66). This data suggests that the effects of stretch and mechanical oscillation on airway smooth muscle may be important in maintaining the normal low levels of airway reactivity.

Inhibition of deep inspiration in healthy subjects has been shown to result in AHR similar to that observed in asthmatic subjects (19; 29; 140). Cyclic mechanical stretching of tracheal and bronchial strips showed a decrease in contractile force compared to strips maintained under static conditions (102; 120; 121). This suggests that the effects of mechanical oscillation on airway responsiveness result directly from the effects of oscillation on the airway. The degree of depression of force during length oscillation is directly correlated with both the frequency and magnitude of the oscillation. Thus, generally, when either the frequency or amplitude of the length oscillation is increased, force production decreases.

ASM as a Soft Glassy Material

Glass can possess characteristics of a highly malleable material (when heated) or one that is rigid (when cooled). It has been suggested that ASM adjusts its mechanical properties in a similar fashion, largely by remodeling its internal cytoskeletal structures to behave as a solid in absence of an external perturbation, and become more fluid-like in the presence of mechanical stress, as it takes on a state of perturbed equilibrium (99). In other words, in the absence of stretch, the ASM cell behaves more like a solid but when stretched, acts more like a fluid.

The theory of perturbed equilibrium explains that with each breath, lung inflation strains the airway smooth muscle. These periodic mechanical strain fluctuations are transmitted to the myosin head and cause it to detach from the actin filament much sooner than it otherwise would have in isometric circumstances. This premature detachment profoundly reduces the tension generation capability of myosin, typically by as much as 50% to 80% of its isometric (i.e., unperturbed) value and depresses total number of bridges attached by a similar extent (56; 57). During the state of perturbed equilibrium, ASM is expected to exhibit lower contractile forces, limited airway narrowing, an increased ability of DIs to stretch the muscle, and a corresponding transient dilatory response to a DI.

While this theory helps to explain why tidal breathing and deep inspirations are potent bronchodilators, it fails to explain why individuals with asthma are refractory to the beneficial effects of a DI. It is thought that due to

increased ASM muscle mass and the release of excitatory mediators in asthma, ASM stiffens and therefore stretches less, exhibiting a latched 'frozen' state where DIs can no longer perturb myosin binding, and the frozen, stiff phase fails to fluidize (55).

Regulation of ASM Contraction

By the epithelium

The airway epithelium is composed of a heterogeneous population of cells, forms the interface between the external environment and the airways, and acts as the first structural line of defense in the lung (78; 154). In fact, one of the hallmarks of the airway remodeling present in asthma is the disruption of the integrity of the epithelial barrier (13; 92; 101; 119). This damage to the airway epithelium can affect airway responsiveness in a number of ways. Firstly, the loss of epithelial integrity enables the invasion of unwanted foreign antigens deeper into the lung tissue, thereby eliciting an inflammatory response that may exacerbate asthma symptoms (16; 92). Secondly, the epithelial layer protects intra-epithelial nerves from being stimulated by the above-mentioned inhaled products. If this layer is damaged, the sensory nerves involved in neuropeptide release become exposed and bronchoconstriction can more easily be induced (101; 160). Thirdly, the epithelial layer has a metabolic function. Acetylcholine can be metabolized within the epithelial layer by cholinesterase (97). Thus, dysfunction of the epithelial layer could result in an increase in the concentration of this contractile agent. Lastly, the epithelial layer has a secretory function. It synthesizes mucous (32), cytokines, chemokines (78), and excitatory prostanoids such as PGD_2, $PGF_2\alpha$ and TxA_2 (48; 82), all of which can cause ASM contraction through activation of the ASM membrane-bound TP-receptor (33; 51).

By peripheral neurons

In general, the airways receive inputs from the autonomic nervous system. ASM is innervated by both sympathetic and parasympathetic nerves (67). In fact, *in vivo* and *in vitro* studies have shown that, when activated, airway nerves can markedly constrict bronchi (23-25), therefore playing a primary role in regulating airway caliber, whereby its dysfunction is likely to contribute to the pathogenesis of airways diseases. The predominant contractile innervation of ASM is parasympathetic and cholinergic in nature, shown by the maximal contractions of isolated ASM *in vitro*, caused by the activation of plasmalemma-bound M_3 receptors. In fact, knocking out the M_3 receptor abolishes bronchoconstriction induced by cholinergic stimulation (53). This highlights the role of the M_3 receptor in ASM contraction. Sympathetic-adrenergic nerve agonists can also evoke ASM contractions by the activation of α_1 and/or α_2 adrenoceptors. However, they have been shown to play little role in regulating human ASM tone (7; 63; 126). Lastly, the airways also possess non-cholinergic non-adrenergic innervation, such as rapidly adapting receptors and C-fibers (15; 24). Pulmonary C-fiber nerve endings are located in the lung parenchyma as well as within the airway mucosa and contain sensory neuropeptides, in particular the tachykinins substance P (SP) and neurokinin A (NKA) (34; 35; 160). These nerve endings are sensitive to increases in airway stretch and lung volume. The stimulation of pulmonary C-fiber receptors may result in a local axon reflex and cause the release of the sensory neuropeptides contained within it (35; 44; 160). SP and

NKA are potent inducers of airway smooth-muscle contraction (by binding to and activating the NK_1 and NK_2 receptors (respectively), located on the ASM plasmalemma) (47; 111), vasodilatation, bronchial edema and mucus hypersecretion, which are all symptoms of inflammatory airway diseases (15; 47; 95; 160). Electrically evoked bronchoconstriction *in vitro* and *in vivo* have been shown to be blocked by both NK_1 and NK_2 receptor antagonists (106-108). Therefore, neuropeptide release from these nerve endings, has been proposed to be important in the pathology of airway diseases such as asthma.

Aims of Thesis Research

A DI produces bronchodilation in non-asthmatic individuals, whereas in asthmatics they do not convey this protective effect, and can even cause bronchoconstriction. Furthermore, the mechanisms by which a DI is able to cause bronchoconstriction *via* an airway stretch-activated contractile response remains ambiguous. Previous theories suggest that this phenomenon is intrinsic to the ASM itself. However, the inflammation and remodelling present in asthmatic airways may also add to the increased ASM contractility following stretch, by the release of stimuli that can prime the contractile apparatus to react excessively to this mechanical stimulus. Thus, the specific aims of this research project are as follows:

AIM #1: Describe the stretch-activated contractile phenomenon in intact bovine bronchial segments that occurs upon transmural pressure loading

AIM #2: Investigate the role of TxA_2 in airway stretch-activated contractions

AIM #3: Investigate the role of Ca^{2+} influx *via* L-type Ca^{2+} channels in airway stretch-activated contractions

Overall Hypothesis

Perfused intact bovine bronchial segments produce a stretch-activated contraction upon transmural pressure loading, a phenomenon regulated by: contractile machinery priming (by excitatory mediators), the magnitude of the transmural pressure load, and Ca^{2+} influx through L-type Ca^{2+} channels.

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CHAPTER 2

Involvement of the neurokinin-2 receptor in airway stretch-activated contractions assessed in perfused intact bovine bronchial segments

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Abbreviations:

5-HT – 5-hydroxytryptamine (3-(2-aminoethyl)-1H-indol-5-ol)

ASM – Airway smooth muscle

CCh – Carbachol (2-carbamoyloxyethyl-trimethyl-azanium)

DI – Deep inspiration

ECM – Extracellular matrix

FRC – Functional residual capacity

L-732,138 – N-Acetyl-L-tryptophan-3,5-bis(trifluoromethyl) benzylester

MEN 10376 – [Tyr^1,D-Trp^{6,8,9},Lys^{10}]-NKA(4-10)

NK – Neurokinin

PAR-2 – Protease-activated receptor-2

RAR – Rapidly adapting pulmonary stretch receptors

$R_{stretch,x}$ – contraction evoked by an instantaneous stretch to x cmH$_2$0

SAR – Slowly adapting pulmonary stretch receptors

SP – Substance P

SR48968 – N - [(2S) - 4 - (4-acetamido-4-phenylpiperidin-1-yl) - 2 - (3,4-dichlorophenyl)butyl] - N - methylbenzamide

TLC – Total lung capacity

TRPV1 – Transient receptor potential vanilloid 1

TTX – Tetrodotoxin

VSM – Vascular smooth muscle

Abstract

The airway response to deep inspirations (DIs) in asthmatics has been shown to be ineffective in producing bronchodilation, and can even cause bronchoconstriction. However, the manner by which a DI is able to cause bronchoconstriction remains ambiguous. We sought to investigate the pathway involved in this stretch-activated contraction, as well as whether this contraction is intrinsic to airway smooth muscle (ASM). Briefly, intact bovine bronchial segments were dissected and side branches ligated, then mounted horizontally in an organ bath. Intraluminal pressure was measured under isovolumic conditions. Instantaneously opening and then closing the tap on a column of fluid 5-30 cm high evoked a sudden increase in intraluminal pressure (equivalent to the height of the column of fluid) followed by a stress relaxation response of the ASM. When tissues were stimulated with carbachol (10^{-8}M) or serotonin (10^{-7}M) for 10 min and the consequent agonist-evoked pressure response was dissipated manually, the response to the same transmural stretch was accompanied by a slowly-developing and prolonged increase in intraluminal pressure. This stretch-activated response was significantly diminished by the stretch-activated cation channel blocker gadolinium (10^{-3}M), the L-type Ca^{2+} channel blockers nifedipine (2×10^{-6}M), diltiazem (10^{-5}M), and verapamil (10^{-5}M), the sensory neurotoxin capsaicin (10^{-5}M), and the NK_2-receptor antagonists MEN 10376 (10^{-5}M) and SR48968 (3×10^{-6}M). These results show the ability of isolated airways to exhibit stretch-activated contractions, and suggest a role for stretch-activated cation

channels, sensory afferent neurons, the neurotransmitter NKA, as well as L-type Ca^{2+} channels in these isolated airway responses.

Introduction

Mechanotransduction, defined as the conversion of mechanical stress into biochemical information, is essential to the proper functioning of cells and organ systems (16). In the vasculature, blood pressure is strictly regulated by signaling pathways that respond to mechanical stress to ensure the precise control of blood flow under physiological conditions ranging from vigorous exercise to complete rest (9). In 1902, Bayliss performed experiments using dog hind-limb which showed blood vessels responding to increased transmural pressure by constricting. This phenomenon was later termed a *myogenic response*, as it was an intrinsic property of the vascular smooth muscle (VSM) independent of neural, metabolic, or hormonal input (6). Similar to the vasculature, the airways are also constantly subjected to mechanical stress due to the inflation and deflation of the lungs. This stress produces both relaxant and constrictor responses in airway smooth muscle (ASM) (25). Thus, airway stretch is suggested to be either beneficial (bronchodilatory) in healthy individuals or harmful (leading to airway hyperresponsiveness) in asthmatics.

A deep inspiration (DI) is clinically measured as a breath taken from functional residual capacity (FRC) to total lung capacity (TLC). DIs produce bronchodilation in non-asthmatic individuals, whereas in asthmatics they do not convey this protective effect, and can even cause bronchoconstriction (10; 24; 35). The mechanisms by which a DI is able to cause bronchoconstriction remain ambiguous. One suggestion is that smooth muscle activation and tension

generation cause an increase in ASM stiffness to the point where it stretches little during a DI. This can subsequently cause the ASM to enter a frozen state, where it stays in a high-stiffness, low-hysteresis latch state (2). Another theory suggests that a DI-induced bronchoconstriction is a peripheral parenchymal hysteresis-associated event, related to the lung pressure–volume hysteresis curve. Following lung inflation to TLC during a DI, the lower recoil pressures during deflation at any given volume can lead to smaller airways than before the DI was performed, due to unloading of the ASM, which narrows the airways more than it would have otherwise (24). Since the failure of bronchodilation following a DI depends on the degree of airway obstruction in asthma, severe asthmatics lose more than they gain when performing a DI. However, the airway inflammation and remodelling present in asthmatic airways may also add to the increased ASM contractility following stretch, by the release of stimuli that can prime the contractile apparatus to react excessively in the presence of stretch. Passive sensitization to IgE has been shown to unmask stretch-activated contractions in human airways *in vitro* (31), suggesting a role for inflammatory mediators.

In VSM, stretch-activated contractions are mediated in part through the release of substance P (SP) from sensory neurons (37). ASM tone is regulated in part by a subset of myelinated and unmyelinated sensory nerves, such as slowly-adapting (SAR) and rapidly-adapting (RAR) pulmonary stretch receptors, as well as C-fiber receptors. C-fibers terminate within the airway epithelium and in proximity to the ASM deep within the submucosa. These receptors are

nociceptive and respond to many of the mediators released by tissue damage. They are also polymodal and respond to both mechanical and chemical stimuli such as the sensory neurotoxin capsaicin (41). Capsaicin mediates its excitatory effects by binding to the vanilloid receptor, TRPV1 (14; 15; 26). When activated, C-fiber receptors release sensory neuropeptides including SP and neurokinin-A (NKA), both of which can exert a bronchoconstrictory response (22).

Recent single cell and tissue bath studies have shown that ASM *per se* can contract in response to stretch (25; 32). These responses are mediated by the opening of mechanically-gated stretch-activated cation channels (17). Pretreatment of guinea pig tracheal ASM strips with the stretch-activated cation channel blocker gadolinium (Gd^{3+}) significantly decreased isometric force generation after stretch (19).

In this study, we set out to investigate the effect of acute airway stretch on agonist-induced contraction in bovine bronchial segments, as well as the possibility that SP and NKA release can mediate stretch-activated contractions in these tissues. Moreover, we assessed the potential involvement of stretch-activated cation channels using the isolated bronchial segment technique previously described in (30).

Methods

Animals. All experimental procedures were approved by the McMaster University Animal Care Committee (McMaster University, Hamilton, ON, Canada) and conform to the guidelines set by the Canadian Council on Animal Care (Ottawa, ON, Canada). Lower lobes of lung were obtained from cows (200–500 kg) euthanized at a local abattoir and transported to the laboratory in ice-cold modified Krebs buffer solution (116 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.3 mM NaH_2PO_4, 23 mM $NaHCO_3$, 11mM D-glucose, 0.01 mM indomethacin), saturated with 95% oxygen - 5% carbon dioxide to maintain pH at 7.4. Upon receipt of the lobes of lung, intact bovine bronchial segments (2 mm diameter, 20 mm length) were carefully dissected free from surrounding parenchyma, excised, and immediately used or stored in modified Krebs solution at 4°C for up to 24 h.

Bronchial segment preparation. Following the dissection and excision of the bronchial segment, side branches were tightly ligated with surgical silk (4-O) as previously mentioned (23; 28). The ligated bronchial segment was then mounted horizontally in a 30 ml Mayflower organ bath (Hugo Sachs Elektronik, March-Hugstetten, Germany) containing warmed modified Krebs buffer solution (37°C) gassed with carbogen (95% O_2 – 5% CO_2) as previously mentioned by (29) with modifications. Briefly, both luminal ends of the airway were mounted on adjustable cannulae that allowed airways of different lengths to be mounted. The

airway lumen was filled with warmed modified Krebs solution gassed with carbogen *via* a jacketed-reservoir, the height of which set the baseline transmural pressure (~5 cmH$_2$O). This baseline pressure was selected to simulate the transmural pressure found in relaxed airways (33). The connectors at each end of the airway possessed 3-way taps, which could be opened to flush the airway with modified Krebs solution or closed to make the airway lumen isovolumic. The intraluminal pressure was recorded with a pressure transducer (Hewlett-Packard Medical Products, MA., USA) attached proximally to the airway. The pressure transducer output was fed through a pressure amplifier (Hewlett-Packard Medical Products, MA., USA) and data was digitally recorded using WinDaq DI-720 recording software (DataQ Instruments, OH., USA). Manual transmural pressure variation was induced by varying the height of perfusate in a column manometer attached distally to the cannulated airway.

The airway segment was mounted at 115% of its resting length (the latter being the length of the segment when dissected free from parenchyma at zero transmural pressure). This co-axial stretch has previously been shown to produce increased contractile responses compared to an airway segment mounted at resting length (23). Subsequently, a pressure test was performed to ensure that there were no leaks in the airway. The segment was then left to equilibrate for ~2 hours, during which the lumen and adventitia were regularly washed with fresh modified Krebs solution. Following tissue equilibration, transmural pressure was set to 5 cmH$_2$O by manually opening the 3-way tap which communicated with the

reservoir of Krebs buffer 5 cm higher than the bronchial segment; equilibration of pressures between the two compartments was essentially instantaneous. With the 3-way tap now closed (isovolumic condition), tissues were treated with 60 mM KCl and the contractile response (isovolumic increase in intraluminal pressure) was recorded in order to test viability. After washing four times, baseline pressure was then reset to ~ 5 cmH$_2$O by opening/closing the tap.

Tissue Baths. Following the tissue viability test, the airway was allowed 20 min of recovery time under isovolumic conditions. Subsequently, electric-field stimulation (EFS) responses were evoked at 5 min intervals until a uniform response was established (after approx. 3-4 repetitions) under isovolumic conditions. EFS was delivered by a train of pulses (60 volts, 2 ms pulse duration, and frequency of 20 pulses per second), evoked *via* circular electrodes placed above and below the airway in the organ bath, which were connected to a Grass S48 stimulator (Grass Technologies, RI., USA). The airway was then stretched by opening the 3-way tap which now communicated with a column of fluid 10-30 cm in height, allowing the pressure between the two to equilibrate instantaneously, and then closing the tap (*ii* and *iii* in Fig. 1A and 1B); this increased intraluminal pressure was maintained isovolumically for 3 minutes. Intraluminal pressure was then restored to baseline by opening/closing the tap and allowing equilibration with the reservoir 5 cm higher than the tissue (*iv* in Fig. 1A and 1B). The tissue was allowed 5 min recovery time. To mimic the increased airway tone seen in

asthmatic airways, this process was repeated following pretreatment with 10^{-8}M

carbachol (CCh) or 10^{-7}M serotonin (5-HT) added to the bath solution to induce

submaximal ASM tone under isovolumic conditions (*v* in Fig. 1A and 1B). Ten

minutes later, at which point agonist-induced tone had reached a plateau,

transmural pressure was reset to ~5 cmH$_2$O (by opening and closing the 3-way

tap; *vi* in Fig. 1A and 1B) before re-assessing airway contractile responses to

stretch (R$_{stretch}$) (*vii, viii* and *ix* in Fig. 1A and 1B). This protocol enabled the

investigation of the effects of mechanical stretch on intraluminal pressure

generation in the perfused isolated bronchial segment.

Pharmacological interventions. To investigate the pathway involved in airway

stretch-activated contractile responses, tissues were treated with a range of

different antagonists following assessment of stretch-activated contractions under

control conditions (where tissues were pretreated with CCh (10^{-8}M)). The

possible role for stretch-activated cation channels was tested by pretreating for 30

min with gadolinium (Gd^{3+}; 10^{-3}M), while a role for L-type Ca^{2+} channels was

assessed by pretreatment for 30 min with nifedipine (2×10^{-6}M), verapamil (10^{-5}M)

or diltiazem (10^{-5}M). To assess any potential neurogenic component of airway

constriction (3; 22; 41), we tested the effect of pretreating with: the Na$^+$-channel

blocker tetrodotoxin (TTX) (10^{-6}M; 10 min); the sensory excitatory neurotoxin

capsaicin (10^{-5}M; 20 min); the neurokinin-1 (NK$_1$) receptor antagonist L-732,138

(10^{-5}M (data not shown), 10^{-4}M; 30 min); or the NK$_2$ receptor antagonists MEN

10376 (10^{-7}M (data not shown), 10^{-6}M (data not shown), 10^{-5}M; 30 min) or SR48968 (3×10^{-6}M; 30 min).

Chemicals and solvents. L-732,138 was obtained from Biomol International L.P. (PA, USA). MEN 10376 was obtained from LKT Laboratories Inc. (MN, USA). SR48968 was kindly donated by Sanofi-Synthelabo Recherche (Montpellier, France). All other pharmacological agents were obtained from Sigma–Aldrich (ON, Canada). The 10 mM stock solutions were prepared in distilled water (CCh, 5-HT, diltiazem, Gd^{3+}), dilute acetic acid (TTX), absolute EtOH (nifedipine, verapamil, L-732,138) or DMSO (MEN 10376, SR48968). Dilutions of these were made in physiological medium; the maximal bath concentration of solvents did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity.

Statistical Analysis. Stretch-activated contractions ($R_{stretch}$) were quantified as the difference between the minima and the maxima observed in the transmural pressure recordings following a sudden isovolumic stretch (Fig. 1A). All responses were reported as means ± SEM; *n* refers to the number of animals. Statistical analyses comparing multiple groups were done using one-way ANOVA followed by the Bonferroni's multiple comparison *post-hoc* test; Statistical comparisons between paired groups were made using the Paired t-test; $P < 0.05$ was considered statistically significant.

Results

Airway stretch-activated contractions.　In resting tissues at a baseline transmural pressure of 5 cmH$_2$O, instantaneously opening and closing the tap communicating with a column of Krebs 10-30 cm high led to a sudden increase in transmural pressure (presumably accompanied by a small increase in luminal volume, though this was not measured) followed by a prolonged isovolumic stress relaxation response (*i-iii* in Fig. 1A and 1B). After restoring transmural pressure to baseline (by opening/closing the tap communicating with a 5 cm column of fluid and allowing some fluid to escape) (*iv* in Fig. 1A and 1B), the tissue was challenged with CCh (10^{-8}M) under isovolumic conditions (*v* in Fig. 1A and 1B): there was an increase in airway tone shown by a rise in active transmural pressure. When this cholinergic tone had stabilized, we reset transmural pressure to 5 cmH$_2$O (by opening/closing the tap and allowing fluid to exit the airway; *vi* in Fig. 1A and 1B) and allowed 5 minutes for the tissue to re-equilibrate under those new isovolumic conditions before re-assessing the response to a sudden pressure pulse (10-30 cmH$_2$O, as described above; *vii-ix* in Fig. 1A and 1B).　In contrast to what was seen in the absence of any underlying cholinergic stimulation (above), the instantaneous spike and transient decrease in transmural pressure (stress relaxation) were now followed by a slowly-developing and prolonged contraction (R$_{stretch}$), the magnitude of which increased with increasing pressure pulse amplitude (Fig. 2B). To determine whether that third component of the mechanical response was a uniquely cholinergic phenomenon, we repeated this

experiment using 10^{-7}M 5-HT and obtained the same relationship between test pressure pulse amplitude and magnitude of $R_{stretch}$ (Fig. 2C).

To characterize the mechanisms underlying $R_{stretch}$, all subsequent experiments used a standard test pulse of 30 cmH$_2$O since the contractile response ($R_{stretch,30}$) was maximal at this point (Fig. 2B and 2C) and since this mirrors the transmural pressure seen during a deep inspiration to TLC in humans (1; 36).

Relationship between agonist concentration and $R_{stretch,30}$. Next, we investigated the dependence of $R_{stretch,30}$ upon the degree of excitation produced by agonist-stimulation. Tissues were stimulated with varying concentrations of CCh or 5-HT for 10 minutes, after which transmural pressure was returned to 5 cmH$_2$O by allowing fluid to exit the lumen of the airway and 5 minutes given before evaluating the response to a transmural pressure pulse of 30 cmH$_2$O. Even when tissues were stimulated with CCh or 5-HT at concentrations which evoked little or no contractile response of their own (Fig. 3B and 3D), there was a substantial $R_{stretch,30}$ (Fig. 3A and 3C). The latter increased in magnitude with increasing degrees of excitatory stimulation, reaching a peak at 10^{-8}M CCh and 10^{-7}M 5-HT: these agonist concentrations were sub-maximally effective with respect to evoking a direct bronchoconstrictor response.

Effect of stretch-activated cation channel blockade on $R_{stretch,30}$. To investigate whether stretch-activated cation channels are involved in $R_{stretch,30}$, we used a

mechanosensitive cation channel blocker Gd^{3+} (4). Control responses were established upon pretreatment with 10^{-8}M CCh at a transmural pressure load of 30 cmH_2O prior to antagonist treatment. Gd^{3+} (10^{-3}M) significantly reduced airway $R_{stretch,30}$ compared to control (Fig. 4).

Effect of L-Type Ca^{2+} channel blockade on $R_{stretch,30}$. To investigate whether L-type Ca^{2+} channel blockade would affect $R_{stretch,30}$, we established our control responses upon pretreatment with 10^{-8}M CCh at a transmural pressure load of 30 cmH_2O before treating the airway segments with a variety of L-type Ca^{2+} channel blockers for 20 minutes and then re-evaluating $R_{stretch,30}$. Blockers included the dihydropyridine nifedipine, the phenylalkylamine verapamil, and the benzothiazepine diltiazem (8; 27; 40). Nifedipine ($2x10^{-6}$M), verapamil (10^{-5}M), and diltiazem (10^{-5}M) all abolished $R_{stretch,30}$ (Fig. 5).

Effect of capsaicin and TTX on $R_{stretch,30}$. To determine whether neurogenic mechanisms contributed to this $R_{stretch,30}$, we treated airway segments with the sensory excitatory neurotoxin capsaicin (10^{-5}M; 20 min) to induce desensitization of sensory afferents by causing depletion of neurotransmitters contained within their nerve terminals. Control responses were established upon pretreatment with 10^{-8}M CCh at a transmural pressure load of 30 cmH_2O, before treating the airway segments with neurotoxin. Treatment with 10^{-5}M capsaicin abolished $R_{stretch,30}$ (Fig. 6), thus, showing a vital role for sensory afferent neurons in this

phenomenon. Interestingly, TTX (10^{-6}M) did not significantly affect $R_{stretch,30}$ (although we did find it to be sufficient to abolish EFS-evoked responses; data not shown), suggesting that a TTX-resistant neural component might be involved in mediating these contractions (Fig. 6).

Effect of NK$_1$- and NK$_2$-receptor antagonists on $R_{stretch,30}$. Our results above suggest the involvement of a TTX-resistant sensory neural mechanism in $R_{stretch,30}$. Thus, we sought to determine the neurotransmitter implicated in these contractions by blocking either NK$_1$- or NK$_2$-receptors to assess the role of SP or NKA respectively. The NK$_1$ receptor antagonist L-732,138 (10^{-4}M) showed no significant effect on $R_{stretch,30}$, suggesting that airway stretch-activated contractions are not mediated by SP release from sensory neurons. A 10-fold lower concentration of L-732,138 (10^{-5}M) elicited no significant effect on $R_{stretch,30}$ (data not shown). Interestingly, blockade of NK$_2$ receptors by either MEN 10376 (10^{-5}M) or SR48968 (3×10^{-6}M) caused a significant reduction in $R_{stretch,30}$ suggesting an essential role for NKA (Fig. 7). Concentrations of 10^{-7}M and 10^{-6}M of MEN 10376 caused a dose-dependent but non-significant decrease in $R_{stretch,30}$ (data not shown).
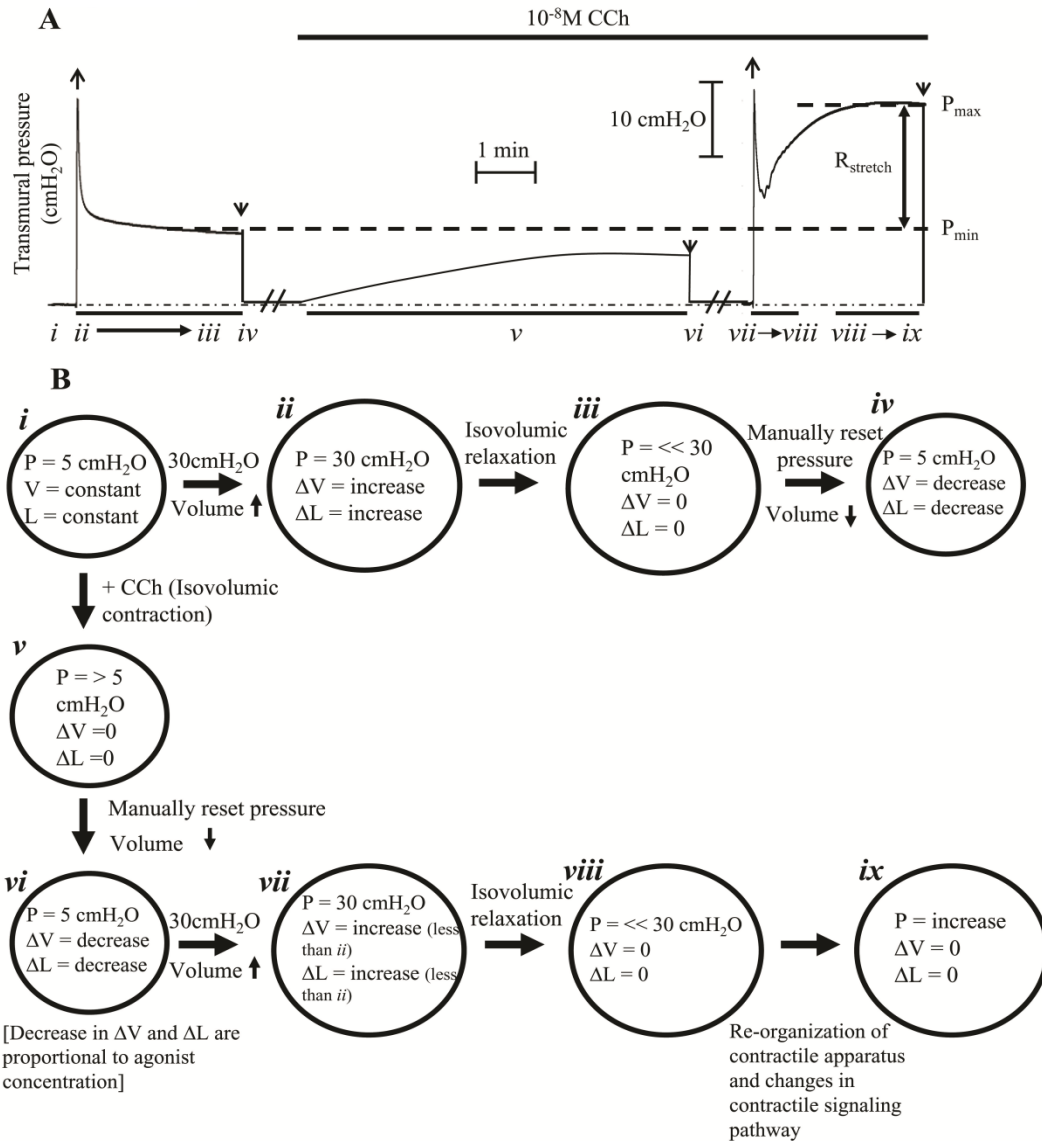
Fig 1. Experimental protocols for this study. (A) Pressure recording during the various manipulations used in our experimental protocol; details are given in the Methods, Results, and Discussion. Response to a pressure pulse stretch (30 cm H_2O) is indicated by "↑". Restoration of transmural pressure to 5 cmH$_2$O is indicated by "↓". R$_{stretch}$ was quantified as illustrated. Italicized labels refer to the cartoon drawings of airway cross-sections given in (B), summarizing (in a non-quantitative fashion) the changes in pressure (P), volume (V) and airway diameter (L) during the various steps in our experimental protocol.
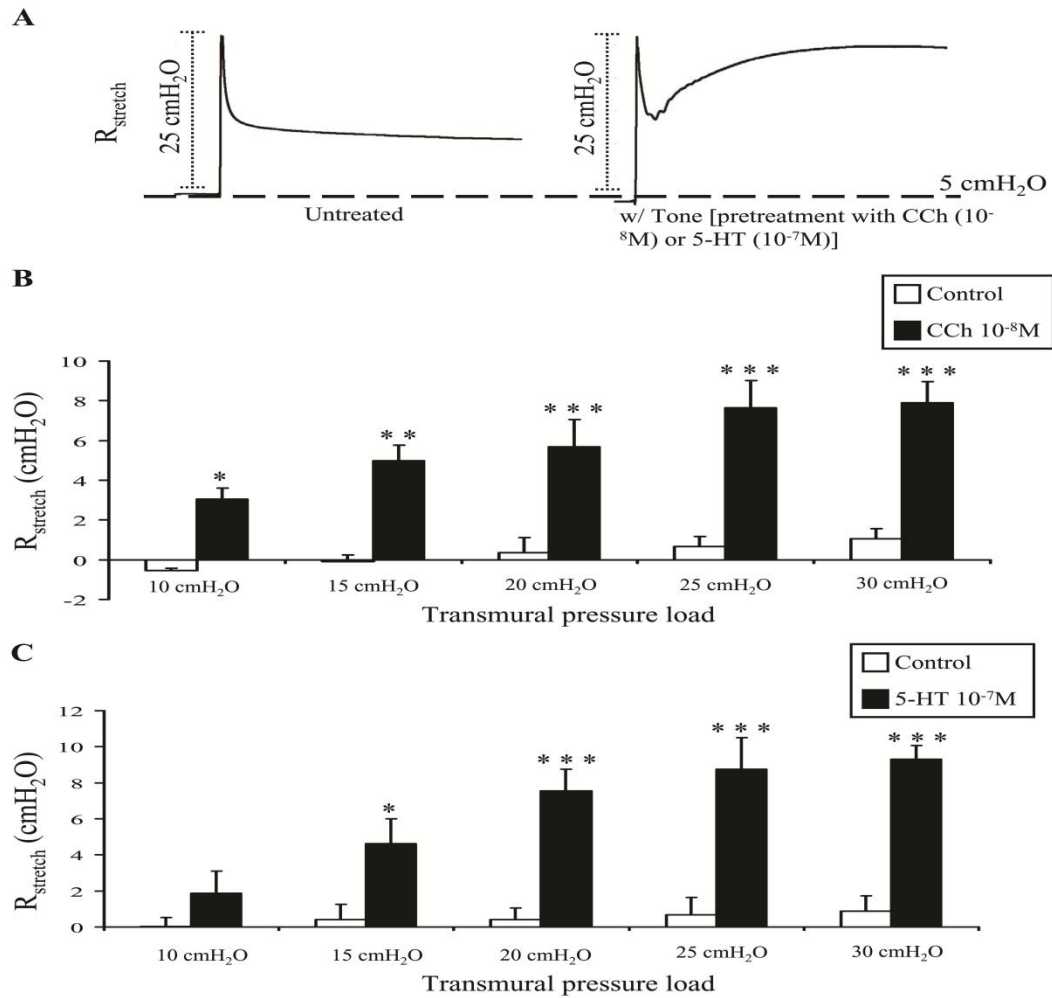
Fig 2. Effects of CCh and 5-HT pretreatment on bronchial responsiveness to stretch. Experiments were performed under isovolumic conditions. Agonists were added to the bath 10 min prior to the experimental protocol. (A) An instantaneous transmural stretch to 30 cmH_2O (from a baseline of 5 cm H_2O) elicited a contraction in airways pretreated with a contractile agonist (10^{-8}M CCh or 10^{-7}M 5-HT) but not in unpretreated tissues. Mean magnitudes of $R_{stretch}$ evoked by transmural pressures of 10-30 cmH_2O in the absence or presence of 10^{-8}M CCh (B) or 10^{-7}M 5-HT (C). $n = 6$ for both. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$
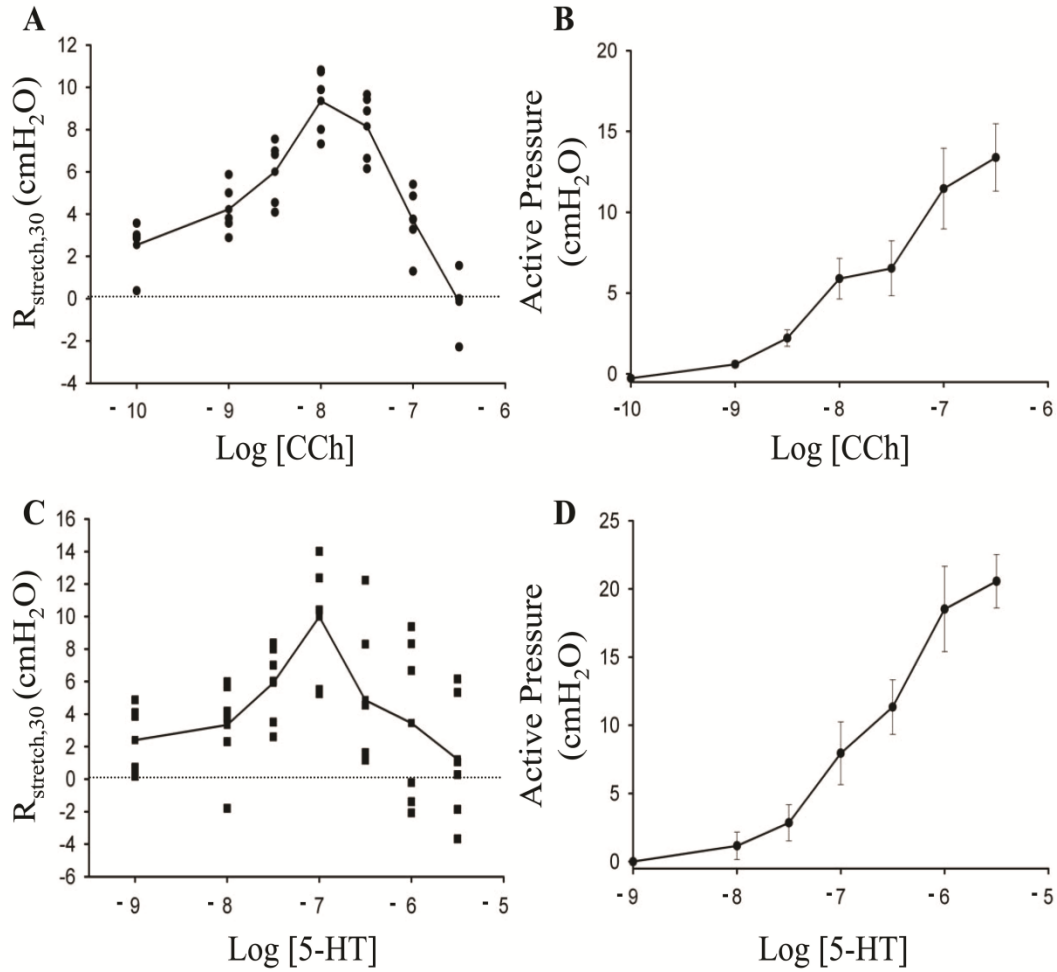
Fig 3. Relationship between contractile agonist concentration and $R_{stretch}$. Experiments were performed using the protocol illustrated in Figure 1B; agonists were added to the bath 10 min prior to the experimental protocol. Individual $R_{stretch,30}$ were measured at various concentrations of CCh (A) ($n = 5$) or 5-HT (C) ($n = 6$). Agonist-induced tone was measured under isovolumic conditions by pretreatment with increasing concentrations of (B) CCh or (D) 5-HT. $n = 6$ for both.
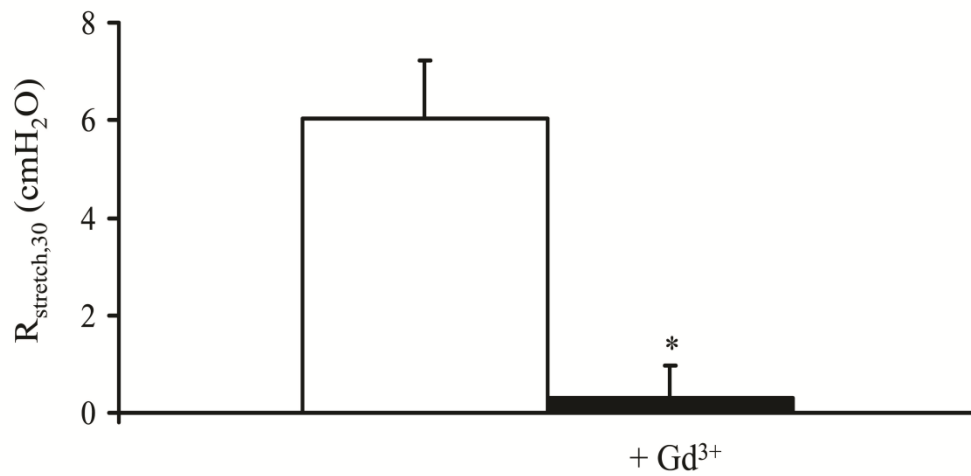
Fig 4. Effects of mechanically-gated cation channel blockade on $R_{stretch,30}$. Mean values of $R_{stretch,30}$ measured before (open bars) and during (closed bars) treatment with Gd^{3+} ($10^{-3}M$). $n = 6$. *, $p < 0.05$
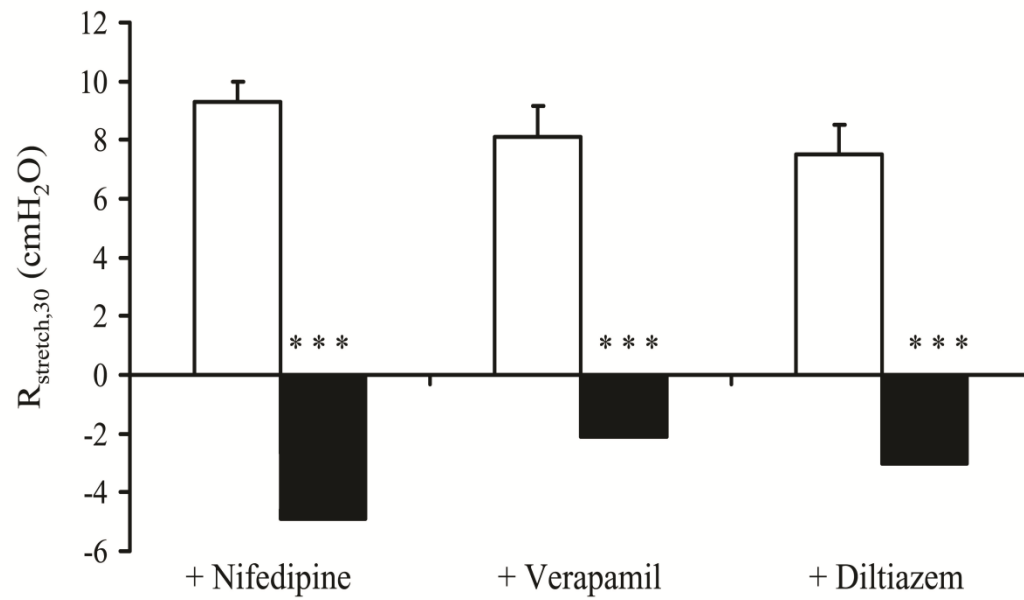
Fig 5. Effect of L-type Ca^{2+} channel blockers on R$_{stretch,30}$. Mean values of R$_{stretch,30}$ measured before (open bars) and during (closed bars) treatment with nifedipine (2×10^{-6}M; $n = 6$), verapamil (10^{-5}M; $n = 6$) or diltiazem (10^{-5}M; $n = 6$). ***, p < 0.001
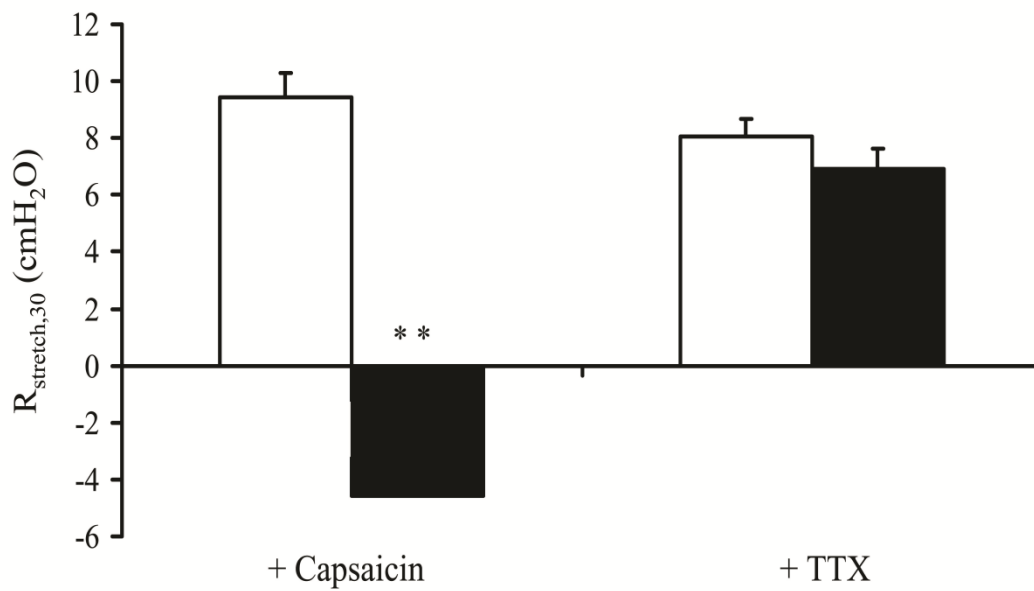
Fig 6. Effect of neurotoxin treatment (capsaicin and TTX) on R$_{stretch,30}$. Mean values of R$_{stretch,30}$ measured before (open bars) and during (closed bars) treatment with capsaicin (10^{-5}M; $n = 6$) or TTX (10^{-6}M; $n = 6$). **, p < 0.01
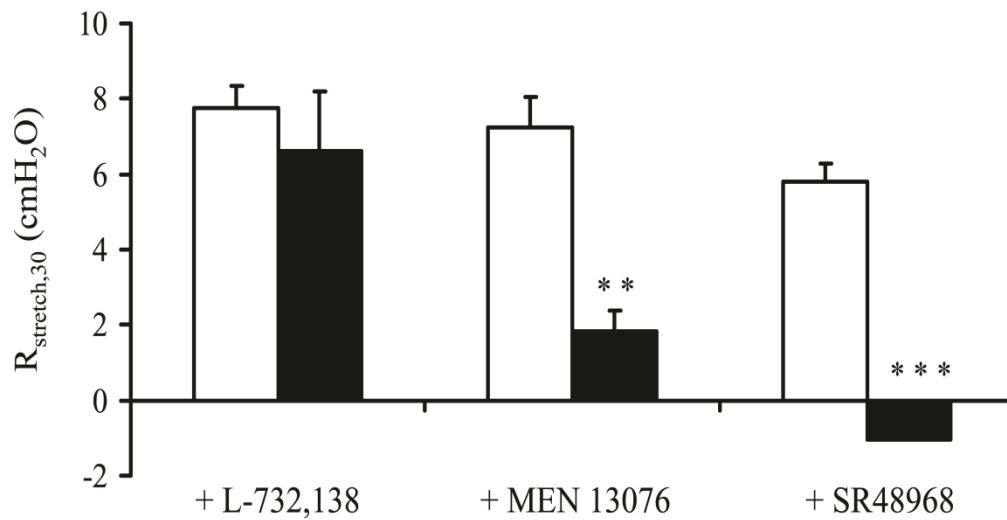
Fig 7. Effects of NK$_1$- and NK$_2$- receptor blockade on R$_{stretch,30}$. Mean values of R$_{stretch,30}$ measured before (open bars) and during (closed bars) treatment with the NK$_1$ receptor antagonist L-732,138 (10^{-5}M; $n = 6$) or the NK$_2$ receptor antagonists MEN 10376 (10^{-5}M; $n = 6$) or SR48968 (10^{-6}M; $n = 6$). **, $p < 0.01$; ***, $p < 0.001$

Discussion

There have been numerous reports of stretch eliciting a contractile response in ASM; however, most of these studies have used ASM cells or strips (13; 25; 31). Previous studies have also deemed ASM $R_{stretch}$ as a myogenic event (38; 39), suggesting an intrinsic property of ASM itself. However, an examination of the pathway involved in airway $R_{stretch}$ has previously not been addressed using perfused intact bronchial segments.

Here we describe the ability of perfused bovine bronchial segments to constrict in response to stretch, but only when pretreated with submaximally-effective, or even sub-threshold, concentrations of a contractile agonist (CCh and 5-HT). In Figure 1B we summarize the changes in pressure, volume and muscle length at different points in our experimental protocol. Due to the incompressibility of the liquid within the airway lumen, airway volume and muscle length remain constant during isovolumic conditions. The increase in pressure and muscle length seen in Figure 1B*ii* is attributed to a change in volume of fluid within the airway. The subsequent isovolumic loss of pressure immediately following a test pulse (Fig. 1B*iii*), on the other hand, is attributed to "stress relaxation" in the passive tissues: this may include processes such as fluidization of the cytoskeleton during the stretch and equilibration of the series and parallel elastic elements, although our experimental approach is not able to resolve these. Moreover, we hypothesize that following agonist pretreatment (*v* in Fig. 1A and 1B), the loss of pressure and decrease in muscle length seen in Figure

1B*vi* are related to the volume of fluid expelled from the airway lumen upon opening/closing of the 3-way tap: clearly, the change in muscle length would be proportional to the concentration of agonist used. As such, the changes in airway volume (and muscle length) upon eliciting an $R_{stretch,30}$ in Figure 1B*vii* would have been less than that seen in Figure 1B*ii*. At this smaller muscle length, the isovolumic stress relaxation is now followed by substantial force generation (Fig. 1B*ix*), which we attribute in part to re-organization of the contractile apparatus, as well as changes occurring in the contractile signaling pathway due to the presence of stretch and agonist activation. We do not, however, view this $R_{stretch}$ as being solely related to a change in the muscle's position on the length-tension curve, since it was seen even at concentrations of agonists which did not generate any tone on their own (and therefore there would be no change in airway diameter / muscle length during the transition from *v* to *vi* in Fig. 1A and 1B.

Discrepancies between stretch applied to intact airways *versus* isolated ASM bundles have been noted, where in intact airways, stretch promoted increased muscle contractility and the opposite effect is seen in ASM bundles (23; 32). These discrepancies may be species-related and/or attributed to different properties of different regions in the airway tree, where $R_{stretch}$ may be a more significant phenomenon in small resistance airways compared to larger airways. A possible explanation for the stretch-induced contraction we observe is that following priming of the contractile apparatus with an agonist, stretch caused fluidization of the cytoskeleton, and during redevelopment of force, the

contractile apparatus was able to regenerate force above and beyond pre-stretch levels. However, further experiments suggest the possibility that the $R_{stretch}$ phenomenon we observe in bovine bronchial segments may possess a neurogenic component.

DI-induced bronchoconstriction is abnormal in humans, given that it is only seen in moderate to severe asthmatics. Our bovine bronchial segments were not inflamed nor exhibited spontaneous tone, and did not manifest a stretch-induced contraction until they were pretreated with a contractile agonist (CCh or 5-HT), which we used to mimic the increased ASM tone seen in asthmatic airways. For our experimental setup, we chose to use a baseline transmural pressure of 5 cmH_2O, and maximal pressure pulse of 30 cmH_2O, to mimic pressures in the human lung at FRC and TLC, respectively (33). In fact, we have demonstrated that pretreatment with CCh or 5-HT, at concentrations which produce relatively little change in basal tension, can produce a fundamental change in ASM biophysical properties and elicit a prolonged $R_{stretch}$. Given that $R_{stretch}$ is only seen in the presence of an agonist, it would appear to be non-myogenic in nature. Others have also demonstrated an $R_{stretch}$ in ASM that required pretreatment with a pharmacological agent to prime the contractile apparatus, such as tetraethylammonium chloride, or a cholinergic agonist (38; 39). The authors of these studies interpreted this phenomenon as a functional transformation of multiunit-smooth muscle into a single-unit, mediated by a contractile agonist. Although others (Fredberg et al. 1997) have observed a

stretch-induced relaxation in bovine tracheal strips in the presence of an agonist

(Ach), this was done using oscillatory stretches. The authors concluded that these

tidal changes in length can cause an excess rate of detachment which is faster than

the rate of attachment, thus causing a net decrease in ASM force production. In

our setup, the airway stretch is static: as such the myosin and actin interactions

should have been able to return to a latch state. Thus, the discrepancies between

our data and those presented by Fredberg et al. appear to be due to differences in

experimental protocols.

Mechanotransduction is sometimes mediated in part through activation of

sensory neurons (37). Also, neurogenic mechanisms can contribute to airway

responsiveness (3; 22; 41). Therefore, to investigate whether airway $R_{stretch,30}$ is

also mediated by neuronal input, we treated the isolated airway segments with the

sensory neurotoxin capsaicin (11). In this study, capsaicin-induced depletion of

sensory nerve endings abolished the $R_{stretch,30}$ which was unmasked by CCh,

suggesting the involvement of sensory neurons. Surprisingly, we found this

sensory neuronal component to be unaffected by TTX. TTX-resistant channels

have previously been characterized on neurons controlling many different organ

systems, including $Na_v1.8$ and Na_x which are expressed on sensory C-fibers and

neurons in the peripheral nervous system with nerve endings in proximity of

smooth muscle, respectively (34).

Upon demonstrating a TTX-resistant sensory neuronal pathway

involvement in $R_{stretch,30}$, we sought to characterize the neuronal pathway that

mediates this response. Of the numerous neurotransmitters found within airway sensory nerve terminals, SP and NKA have been shown to contribute to bronchoconstriction in asthmatics. The receptors for these neurotransmitters, NK_1 and NK_2, respectively, have been well-characterized in ASM (22). Using the NK_1-receptor antagonist L-732,138, as well as the peptide NK_2-receptor antagonist, MEN 10376 and the non-peptide NK_2-receptor antagonist SR48968, we found no significant difference upon blockade of NK_1-receptors, whereas NK_2-receptor blockade significantly decreased contractile responses, thus affirming a central role for those receptors (and for NKA) in airway $R_{stretch,30}$. These results are supported by a recent study that showed an NK_2-selectivity pertaining to bronchial hyperreactivity and suggested an importance for capsaicin-sensitive nerves in bronchoconstriction in mice (7). Another study found that NK_2 receptors played a predominant role in a guinea-pig model of mechanically-induced bronchoconstriction (5). Conversely, protease-activated receptor-2 (PAR-2) mediated, TTX- and capsaicin-sensitive neurons in murine small intestine did not reveal differences in NK_1 vs. NK_2 selectivity (42), as we observed, which could possibly be explained by species differences between bovine and murine or tissue differences between bronchi and small intestine.

Given that mechanotransduction often involves stretch-sensitive ion channels, we also probed the effect of various cation channel blockers on $R_{stretch,30}$. On the one hand, a significant inhibitory effect of Gd^{3+} implicated a central role for non-selective cation channels in these contractions; it is as yet

unclear if these are the same set of Gd^{3+}-sensitive channels which we have previously shown are activated by intracellular Ca^{2+} store depletion in bovine ASM cells (18). Nifedipine -- a dihydropyridine class of L-type Ca^{2+}-channel blocker -- was also tested, albeit originally as a negative control for Gd^{3+}. Surprisingly, nifedipine also abolished $R_{stretch,30}$. To determine whether this was a non-specific effect of nifedipine, we then employed two other structural classes of L-type Ca^{2+} channel blocker -- verapamil (a phenylalkylamine) and diltiazem (a benzothiazepine) -- and found these too abolished $R_{stretch,30}$. L-type Ca^{2+} channels have been well-characterized in ASM (12; 20); however, the electrophysiological and pharmacological properties of those channels are not consistent with an involvement in agonist-evoked responses (21). Nonetheless, our data clearly suggest that airway stretch-activated contractions may signal through a different pathway than agonist-evoked contractions due to their dependence on L-type Ca^{2+} channels.

In conclusion, our data suggest that airway $R_{stretch}$ may occur through a non-myogenic pathway (since pretreatment with a contractile agonist is required) and airway sensory C-fibers are involved in mediating $R_{stretch}$ in bronchial segments. Moreover, it appears that this mechanosensitivity is sensed by stretch-activated cation channels. Following an elevation in transmural pressure, we propose that stretch-activated cation channels located on C-fibers penetrating the airway wall are activated, resulting in the release of NKA from these nerve-endings. This NKA, in turn, binds to postjunctional NK_2 receptors located on the

smooth muscle to mediate an airway stretch-activated contraction. These results highlight an alternative pathway for potential therapeutic targeting in asthmatic patients where a bronchoconstrictory response to a DI may play a role in airway hyperresponsiveness.

Acknowledgements

We would like to thank Tracy Tazzeo for all of her technical assistance and for obtaining the bovine lungs used for this study. We would also like to thank Sanofi-Synthelabo Recherche for their generous sample of the NK_2-receptor antagonist SR48968.

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CHAPTER 3

TP-receptor activation amplifies airway stretch-activated contractions assessed in perfused intact bovine bronchial segments

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Author contributions:

Jeremy M Hernandez – Responsible for experimental design and data analysis; conducted all experiments; wrote the manuscript

Luke J Janssen – Supervision; guidance with study design; manuscript editing

Abbreviations:

5-HT – 5-hydroxytryptamine (3-(2-aminoethyl)-1*H*-indol-5-ol)

AH 6809 – 6-isopropoxy-9-oxoxanthene-2-carboxylic acid

AL 8810 – 9α,15R-dihydroxy-11.beta-fluoro-15-(2,3-dihydro-1H-inden-2-yl)-16, 17,18,19,20-pentanor-prosta-5Z,13E-dien-1-oic acid

ASM – Airway smooth muscle

CCh – Carbachol (2-carbamoyloxyethyl-trimethyl-azanium)

DI – Deep inspiration

FRC – Functional residual capacity

ICI 192605 – 4-(*Z*)-6-(2-*o*-Chlorophenyl-4-*o*-hydroxyphenyl-1,3-dioxan-*cis*-5-yl)hexenoic acid

Indo - Indomethacin (2-{1-[(4-chlorophenyl)carbonyl]-5-methoxy-2-methyl-1*H*-indol-3-yl}cetic acid)

MAPK – Mitogen-activated protein kinase

MEN 10376 – [Tyr^1,D-Trp^{6,8,9,}Lys^{10}]-NKA(4-10)

NK – Neurokinin

PD 95089 – 2-(2-Amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one

R_{CCh} – CCh-induced bronchial tone

$R_{stretch,x}$ – contraction evoked by an instantaneous stretch to x cmH$_2$0

R_{U46619} – U46619-induced bronchial tone

TLC – Total lung capacity

Tx – Thromboxane

U46619 – 9,11-dideoxy-9α,11α-methanoepoxy-prosta-5Z,13E-dien-1-oic acid

VSM – Vascular smooth muscle

Abstract

A deep inspiration (DI) produces bronchodilation in healthy individuals. Conversely, in asthmatics, DIs are less effective in producing bronchodilation, can cause more rapid airway re-narrowing and even bronchoconstriction in moderate to severe asthmatics. Interestingly, the manner by which a DI is able to cause bronchoconstriction *via* a stretch-activated contraction ($R_{stretch}$) is thought to correlate positively with airway inflammation. Asthmatic airway inflammation is associated with increased production of thromboxane A_2 (TxA_2) and subsequent TP-receptor activation, causing the heightened contractility of airway smooth muscle. In this study, we sought to investigate the effect of TxA_2 on airway $R_{stretch}$ using bovine bronchial segments. In brief, these intact bronchial segments (2mm dia.) were dissected, side branches ligated, and the tissues were mounted horizontally in an organ bath. $R_{stretch}$ was elicited by varying the transmural pressure under isovolumic conditions. Using a pharmacological approach, we showed a reduced $R_{stretch}$ response in tissues pretreated with indomethacin (Indo), a COX inhibitor; a result mimicked by pretreatment with the TP-selective receptor antagonist ICI 192605, the selective p42/p44 MAPK inhibitor PD 95089, and by airway epithelial denudation. U46619, a TP-receptor agonist elicited enhanced $R_{stretch}$ responses in a dose-dependent manner. Pretreatment with AH 6809, an EP_1/DP-selective receptor antagonist and AL 8810, an FP-selective receptor antagonist had no effect, suggesting EP, DP, and FP-receptor activation are not involved in amplifying ASM $R_{stretch}$. These data suggest a role for TP-

receptor activation and epithelial release of TxA_2 in amplifying airway $R_{stretch}$, thus providing novel insights into mechanisms regulating the DI-induced bronchoconstriction seen in asthmatics.

Introduction

Airways are constantly subjected to mechanical stress due to the inflation and deflation of the lungs. This stress can either produce beneficial (bronchodilatory) responses in healthy individuals or harmful responses (leading to airway hyperresponsiveness) in asthmatics (25). More specifically, a deep inspiration (DI), clinically measured as a breath taken from functional residual capacity to total lung capacity, produces a bronchodilatory response in ASM of healthy individuals. Conversely, in asthmatics DIs are less effective in producing bronchodilation, can cause more rapid airway re-narrowing, and even bronchoconstriction in moderate to severe asthmatics (12; 18; 24; 34). The mechanisms by which a DI is able to cause bronchoconstriction remain unclear; however, several theories have been postulated explaining how this might occur. Firstly, smooth muscle activation and tension generation may cause an increase in ASM stiffness to the point where it enters a frozen state, in other words, a pro-contractile, high-stiffness, low-hysteresis latch state (2). Others have reported DI-induced bronchoconstrictions to be a peripheral parenchymal hysteresis-associated event (24). Interestingly, our laboratory has shown, using perfused intact bovine bronchial segments, that airway stretch-activated contractions ($R_{stretch}$) are dependent upon baseline airway tone and the magnitude of airway stretch. Moreover, we have shown that in intact bovine bronchi, these responses possess non-myogenic characteristics due to the requirement of sensory neuronal input mediated by neurokinin (NK)-A acting through the NK_2-receptor (15). The

inflammation present in asthmatic airways may also amplify airway $R_{stretch}$ responses. Thus, in this study, we are investigating the role of selected inflammatory mediators in regulating airway $R_{stretch}$ responses.

Experiments performed *in vitro* demonstrated that passive sensitization caused $R_{stretch}$ responses in human airways (27), suggesting a role for inflammatory mediators in priming the contractile apparatus to react excessively in the presence of mechanical stress. Among the numerous mediators released in asthmatic airways, prostanoids are both synthesized and released by bouts of airway inflammation as well as by mechanical stress (1; 31). Immunologic challenge of sensitized isolated perfused guinea pig lung, and mechanical stretch of rat lung epithelial cells *in vitro*, both stimulate prostanoid synthesis and release (8; 32).

In the airway, the major sources of prostanoid synthesis and release include the epithelium, platelets, and alveolar macrophages (5; 16). Upon cellular stimulation, prostanoids are synthesized from arachidonic acid liberated from membrane phospholipids by the enzyme phospholipase (PL)-A_2 *via* a p42/44 MAPK-dependent mechanism (8). Arachidonic acid is then converted into PGH_2 *via* cyclooxygenase (COX)-1 and -2. This metabolite is then further converted, by enzyme-dependent reactions, into biologically active prostanoids, namely, prostaglandin (PG)I_2 and E_2, which produce bronchodilatory (airway protective) features, as well as PGD_2, $PGF_{2\alpha}$, and thromboxane (Tx)-A_2, which elicit bronchoconstriction (16). Among the prostanoids that stimulate ASM, TxA_2 has

attracted attention as a potential important mediator in the pathophysiology of airway hyperresponsiveness due to the potency of its bronchoconstrictory ability (approx. two orders of magnitude more potent than other prostanoids) (9). Furthermore, clinical studies have demonstrated increased TxA_2 concentration in the BAL fluid of asthmatic patients (4; 23; 31). TxA_2 elicits its bronchoconstrictory effects by both directly binding to and activating TP-receptors on ASM (which signal through the $G_{q/11}$ family of G proteins) (21), as well as by causing prejunctional release of ACh from cholinergic neurons (1; 19).

Using a pharmacological approach in intact bovine bronchial segments, as previously described in (26), our objective in this study was to determine the effects of the endogenous bronchoconstrictory prostanoids PGD_2, $PGF_{2\alpha}$, and TxA_2 on $R_{stretch}$ responses. In addition, we investigated the possible involvement of the airway epithelium, p42/44 MAPK, and the TxA_2-induced prejunctional ACh-release in amplifying these stretch-activated contractions.

Methods

Animals. All experimental procedures were approved by the McMaster University Animal Care Committee (McMaster University, Hamilton, ON, Canada) and conform to the guidelines set by the Canadian Council on Animal Care (Ottawa, ON, Canada). Lower lobes of lung were obtained from cows (200–500 kg) euthanized at a local abattoir and transported to the laboratory in ice-cold modified Krebs buffer solution (116 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.3 mM NaH_2PO_4, 23 mM $NaHCO_3$, 11mM D-glucose), saturated with 95% oxygen - 5% carbon dioxide to maintain pH at 7.4. Unless indicated otherwise, Krebs buffer did not contain the non-specific cyclooxygenase blocker indomethacin. Upon receipt of the lobes of lung, intact bovine bronchial segments (2 mm diameter, 20 mm length) were carefully dissected free from surrounding parenchyma, excised, and immediately used or stored in modified Krebs solution at 4^oC for up to 24 h.

Bronchial segment preparation. For a detailed description of our bronchial segment preparation protocol, please refer to our previous study (15). Briefly, following the dissection and excision of the bronchial segment, side branches were tightly ligated. The ligated bronchial segment was then mounted horizontally in a 30 ml Mayflower organ bath (Hugo Sachs Elektronik, March-Hugstetten, Germany) containing warmed modified Krebs buffer solution (37^oC) gassed with carbogen (95% O_2 – 5% CO_2). The airway lumen was also filled with

warmed modified Krebs solution gassed with carbogen *via* a jacketed-reservoir, the height of which set the baseline transmural pressure (~5 cmH$_2$O). This baseline pressure was selected to simulate the transmural pressure found in relaxed airways (29). The connectors at each end of the airway possessed 3-way taps, which could be opened to flush the airway with modified Krebs solution or closed to make the airway lumen isovolumic. Manual transmural pressure variation was induced under isovolumic conditions by varying the height of perfusate in a column manometer attached distally to the cannulated airway.

Subsequently, we briefly subjected the tissue to an increased transmural pressure load under isovolumic conditions to ensure there were no leaks in the airway. The segment was then left to equilibrate for ~2 hours. During this time, the lumen and adventitia were regularly washed with fresh modified Krebs solution. Following tissue equilibration, transmural pressure was set to ~ 5 cmH$_2$O. Under isovolumic conditions, tissues were treated with 60 mM KCl (administered extraluminally) and the contractile response (isovolumic increase in transmural pressure) was recorded to test for viability. After washing four times, baseline pressure was reset to ~ 5 cmH$_2$O.

Tissue Baths. To evaluate the effects of mechanical stretch on ASM contraction (measured by transmural pressure generation) in the isolated bronchial segment, we followed the protocol outlined in our previous publication (15). In brief, following the tissue viability test, the airway was allowed 20 min of recovery time

under isovolumic conditions. Subsequently, electric-field stimulation (EFS) responses were evoked at 5 min intervals until a uniform response was established (after approx. 3-4 repetitions) under isovolumic conditions. EFS was delivered by a train of pulses (60 volts, 2 ms pulse duration, frequency of 20 pulses per second, and 1.5 sec train duration). The airway was then subjected to a transmural pressure pulse of 30 cmH_2O, which was maintained for 3 minutes under isovolumic conditions. Transmural pressure was subsequently restored to baseline (~ 5 cmH_2O) and the tissue was allowed 5 min recovery time. To mimic the increased airway tone seen in asthmatic airways, this process was repeated following pretreatment with 10 nM carbachol (CCh) added to the bath solution to induce submaximal ASM tone under isovolumic conditions. When the agonist-induced tone (R_{CCh}) had reached a plateau (in approx. 10 min), transmural pressure was reset to ~5 cmH_2O before re-assessing airway contractile responses to stretch ($R_{stretch,30}$) (Fig. 1). The effects of selected contractile agonists on ASM tone was assessed by measuring the rise in transmural pressure in response to increasing concentrations of agonist under isovolumic conditions.

Pharmacological interventions. To investigate the pathway involved in amplifying airway stretch-activated contractions, tissues were pretreated extraluminally with a range of different antagonists, whereas the assessment of stretch-activated contractions under control conditions was performed on tissues treated with CCh in a concentration-dependent manner. The possible role for

COX was tested by pretreating for 20 min with indomethacin (Indo; 10 µM) (30), while the roles for EP_1/DP, FP, and TP-receptors were assessed by pretreatment for 20 min with AH 6809 (10 µM) (7), AL 8810 (10 µM) (35), and ICI 192605 (10 µM) (20), respectively (prior to treatment with incremental concentrations of CCh). To further confirm the role for TP-receptors in the amplification of $R_{stretch}$, tissues were pretreated with the TP-receptor agonist, U46619, in a concentration-dependent manner. To assess any potential cholinergic effect elicited by TP-receptor activation, as previously reported in (19), tissues were pretreated with the muscarinic receptor antagonist, atropine (1 µM; 20 min.) (33), prior to treatment with incremental concentrations of U46619. Lastly, to investigate the role of p42/p44 MAPK in amplifying ASM $R_{stretch}$, we pretreated tissues with the p42/p44 MAPK inhibitor PD 95089 (10 µM; 20 min.) (17), prior to treatment with incremental concentrations of either CCh or U46619.

Enzyme Immunoassay. TxA_2 levels were determined in the luminal fluid by measuring its immediate and stable metabolite thromboxane B_2 (TxB_2). A competitive enzyme immunoassay (EIA) for TxB_2 (Cayman Chemical Company, Ann Arbor, MI, USA) was used according to the manufacturer's instructions (detection limit: 11pg/ml). Briefly, following the CCh concentration-response protocol outlined in Figure 1, samples were obtained by collecting Krebs buffer solution from the luminal chamber of the tissue bath, which were immediately frozen at $-80^{o}C$. Control tissues were subjected to increasing concentrations of

CCh without transmural pressure pulses used to elicit airway stretch. Prior to beginning the EIA protocol, frozen samples were thawed at room temperature, lyophilized, and solubilized in EIA buffer. The samples were then applied to a 96-well plate pre-coated with mouse anti-rabbit IgG and incubated with TxB_2 antiserum and recovery tracer for 18h. Following incubation, the plates were washed 5x with wash buffer and developed in the dark for 1 hour using Ellman's reagent. TxB_2 concentrations were determined spectrophotometrically and calculated from the standard curve.

Epithelial denudation. To investigate the effect of airway epithelial denudation on $R_{stretch}$ responses, the luminal surface of the excised bronchial segment was subjected to mechanical denudation by carefully inserting and retracting a manual probe (3-4 times). Side branches were then ligated with surgical silk and airway segments were mounted onto the Mayflower organ bath as mentioned above.

Histology and staining. Histology procedures followed by staining with Hematoxylin & Eosin (H&E) were used to detect whether the manual probing method was successful in denuding the airway epithelium. Briefly, following excision, a sample of intact and epithelial-denuded airways were submerged in 10% buffered neutral formalin and stored for 48 hours. The tissues were subsequently fixed, embedded in paraffin wax, sliced to a thickness of 6μm with a

microtome (Leica, Richmond Hill, ON), placed on a glass slide, and stained with
H&E.

Chemicals and solvents. AH 6809, AL 8810, ICI 192605, U46619, and PD
95089 were obtained from Cayman Chemical Company (Ann Arbor, MI, USA).
All other pharmacological agents were obtained from Sigma–Aldrich (ON,
Canada). The 10 mM stock solutions were prepared in distilled water (atropine,
CCh), absolute EtOH (indomethacin), or DMSO (AH 6809, AL 8810, ICI
192605, PD 95089, U46619). Dilutions of these were made in physiological
medium; the maximal bath concentration of solvents did not exceed 0.1%, which
we have found elsewhere to have little or no effect on mechanical activity.

Statistical Analysis. Stretch-activated contractions ($R_{stretch}$) were quantified as the
difference between the minima and the maxima observed in the transmural
pressure recordings following a sudden isovolumic stretch (Fig. 1). All responses
were reported as means ± SEM; *n* refers to the number of animals. TxB_2 EIA
samples were run in duplicates and TxB_2 release was calculated in pg/ml (mean ±
SD). Data were fitted to a bell-shaped concentration-response curve which
allowed for the measurement of both log EC_{50} and E_{max}. Statistical comparisons
between groups were made using the paired or unpaired Student's *t-test*; $P < 0.05$
was considered statistically significant.

Results

Airway stretch-activated contractions. In resting tissues at a baseline transmural pressure of 5 cmH_2O, instantaneously subjecting the tissue to a transmural pressure load of 30 cmH_2O led to an instantaneous increase in transmural pressure followed by a more gradual and prolonged isovolumic stress relaxation response (Fig. 1, left). After restoring transmural pressure to baseline, the tissue was challenged with CCh (10 nM) under isovolumic conditions. When this cholinergic tone (R_{CCh}) had stabilized, we reset transmural pressure to 5 cmH_2O and allowed 5 minutes for the tissue to re-equilibrate under those new isovolumic conditions before re-assessing the response to a sudden pressure load (30 cmH_2O). In contrast to what was seen in the absence of any underlying cholinergic stimulation, the instantaneous spike and transient decrease in transmural pressure (stress relaxation) were now followed by a slowly-developing and prolonged stretch-activated contraction ($R_{stretch}$) (Fig. 1, right), the magnitude of which increased with increasing pressure pulse amplitude (Fig. 2A). A more detailed description of this protocol is outlined in our previous study (15).

To characterize the mechanisms underlying $R_{stretch}$ amplification, all subsequent experiments used a standard test pulse of 30 cmH_2O (in response to increasing concentrations of either the cholinergic agonist CCh, or the TP-receptor agonist U46619), since the contractile response ($R_{stretch,30}$) was maximal at this transmural pressure load (Fig. 2A), and since this mirrors the transmural pressure seen during a deep inspiration to TLC in humans (36).

Relationship between agonist concentration and $R_{stretch,30}$. We investigated the dependence of $R_{stretch,30}$ upon the degree of excitation produced by agonist-stimulation. There was a substantial $R_{stretch,30}$ even when tissues were stimulated with CCh at concentrations which evoked little or no direct tone of their own. $R_{stretch,30}$ increased in magnitude with increasing agonist concentrations, reaching a peak at 10 nM CCh, which was sub-maximally effective with respect to evoking direct bronchoconstrictor tone (Fig. 2B). As we have shown previously, higher levels of cholinergic stimulation led to progressively smaller $R_{stretch,30}$ responses.

Effect of COX inhibition on $R_{stretch,30}$. To investigate whether arachidonic acid metabolism is involved in $R_{stretch,30}$, we used indomethacin (Indo), a non-selective inhibitor of cyclooxygenase (COX) 1 and 2. All handling of tissues in the control group was done in Indo-free Krebs, while tissues in the treatment group were handled in Krebs containing Indo (10 μM). $R_{stretch,30}$ responses were established following each concentration of a CCh concentration-response protocol. Indo (10 μM) markedly and significantly reduced the E_{max} of airway $R_{stretch,30}$ responses compared to control ($p < 0.05$) (Fig. 3A), but no significant shift in the EC_{50} was observed (Fig. 3A), and there was no effect on R_{CCh} (Fig. 3B). These data suggest the importance of arachidonic acid metabolites generated by COX in amplifying the magnitude of airway $R_{stretch,30}$ responses without altering R_{CCh}.

Effect of EP$_1$, DP, FP, and TP-receptor antagonism on R$_{stretch,30}$ and R$_{CCh}$. To
investigate whether EP$_1$, DP, FP, and TP-receptor antagonism would affect
R$_{stretch,30}$, we pretreated the tissues with the selective EP$_1$/DP-receptor antagonist
AH 6809 (10 µM), the selective FP-receptor antagonist AL 8810 (10 µM), and
the selective TP-receptor antagonist ICI 192605 (10 µM) for 20 minutes, then
performed a CCh concentration-response protocol, where R$_{stretch,30}$ responses were
established following each concentration of CCh. Pretreatment with AH 6809 (10
µM), and AL 8810 (10 µM) had no effect, whereas ICI 192605 (10 µM)
significantly reduced the E$_{max}$ of R$_{stretch,30}$ responses compared to control (Fig. 3C).
No significant shift in the EC$_{50}$ was observed (Fig. 3C), and R$_{CCh}$ was not affected
(Fig. 3D). These data suggest that TP-receptor activation is involved in
amplifying the magnitude of airway R$_{stretch,30}$ responses without altering R$_{CCh}$.

**Effect of a TP-receptor agonist (U46619) on R$_{stretch,30}$ and agonist-induced
tone (R$_{U46619}$).** All tissues used in these experiments were completely handled in
Krebs with Indo (10 µM). To investigate the effect of a TP-receptor agonist on
R$_{stretch,30}$ responses, a concentration-response protocol was performed using the
selective TP-receptor agonist U46619 (0.1 nM – 1 µM), where R$_{stretch,30}$ responses
were established following each concentration of agonist added. Treatment with
U46619 elicited a concentration-dependent increase in R$_{stretch,30}$ responses with a
peak response of 10.90±0.92 cmH$_2$O occurring at a concentration of 0.1 µM (Fig.
4A). This R$_{stretch,30}$ response occurred with minimal R$_{U46619}$ (1.12±0.45 cmH$_2$O)

(Fig. 4B). These data further strengthen our hypothesis regarding TP-receptor involvement in airway $R_{stretch,30}$ responses by showing the ability of a selective TP-receptor agonist to elicit $R_{stretch,30}$ responses in a concentration-dependent manner.

To test for the effect of p42/44 MAPK inhibition on U46619-induced $R_{stretch,30}$ responses and R_{U46619}, tissues were pretreated with the p42/p44 MAPK inhibitor PD 95089 (10 µM; 20 min.), prior to treatment with incremental concentrations of U46619. Pretreatment with PD 95089 (10 µM) had no effect on the E_{max} or EC_{50} of the U46619-induced $R_{stretch,30}$ responses (Fig. 4A) or R_{U46619} (Fig. 4B).

Effect of airway stretch on TxA$_2$ release. To investigate the effect of airway stretch on the release of TxA_2, levels of this arachidonic acid metabolite were determined in the luminal media (Krebs buffer solution) by measuring its immediate and stable metabolite TxB_2 using a competitive EIA, as described above. Stretched tissues elicited a significant increase in TxB_2 concentration compared to controls ($p < 0.05$) (Fig. 5), suggesting the ability of mechanical stretch to cause the release of TxA_2 from the intact bovine bronchial segment.

Effect of epithelial denudation on $R_{stretch,30}$ and R_{CCh}. To determine whether the airway epithelium is a source of the stretch-induced TxA_2-release implicated in amplifying the $R_{stretch,30}$ response, we manually denuded the airway epithelium as

described above. A CCh concentration-response experiment was then performed, where $R_{stretch,30}$ responses were established following each concentration of CCh. H&E staining of airway tissues confirmed the efficacy of the manual denudation process (described above) in fully removing the airway epithelium, while leaving the lamina propria intact (Fig. 6A *i), ii)*). Epithelial denudation caused a significant reduction in the E_{max} of $R_{stretch,30}$ responses compared to control ($p<0.05$), but no difference in EC_{50} was observed (Fig. 6C). R_{CCh} (Fig. 6D) and maximal KCl-induced contraction (R_{KCl}) (Fig. 6B) were not affected.

Role of prejunctional ACh release in TP-receptor activation-induced $R_{stretch,30}$ and R_{U46619}. All tissues used in these experiments were completely handled in Krebs with Indo (10 µM). TP-receptor activation has been shown to contribute to ASM contraction by prejunctionally promoting ACh release from cholinergic neurons (1; 19). Thus, to determine whether this phenomenon is implicated in the amplification of $R_{stretch}$ responses, U46619-induced $R_{stretch,30}$ responses were generated in the presence of the muscarinic-receptor antagonist atropine (1 µM). $R_{stretch,30}$ responses were assessed at 0.10 µM U46619, a concentration shown to produce maximal $R_{stretch,30}$ responses, as described above. Pretreatment with atropine (1 µM) caused no significant changes in maximal U46619 $R_{stretch,30}$ or R_{U46619}, suggesting that the TP-receptor-induced $R_{stretch,30}$ and R_{U46619} responses are independent of prejunctional ACh release from cholinergic neurons (data not shown).

Role of p42/44 MAPK in airway $R_{stretch,30}$ and R_{CCh}. Stretching airway epithelial cells *in vitro* has been shown to increase prostanoid synthesis and release through a MAPK-dependent mechanism (8). We investigated the possible role of p42/p44 MAPK in the amplification of $R_{stretch,30}$ by pretreating tissues with the selective p42/p44 MAPK inhibitor PD 95089 (10 µM) for 20 minutes. A CCh concentration-response protocol was then performed, where $R_{stretch,30}$ responses were established following each concentration of CCh. PD 95089 (10 µM) significantly reduced $R_{stretch,30}$ E_{max} responses compared to control (p<0.05), but no difference in EC_{50} was observed (Fig. 7A). R_{CCh} was not affected (Fig. 7B), suggesting that the amplification of airway $R_{stretch,30}$ responses is dependent on p42/p44 MAPK activation, while R_{CCh} is not.
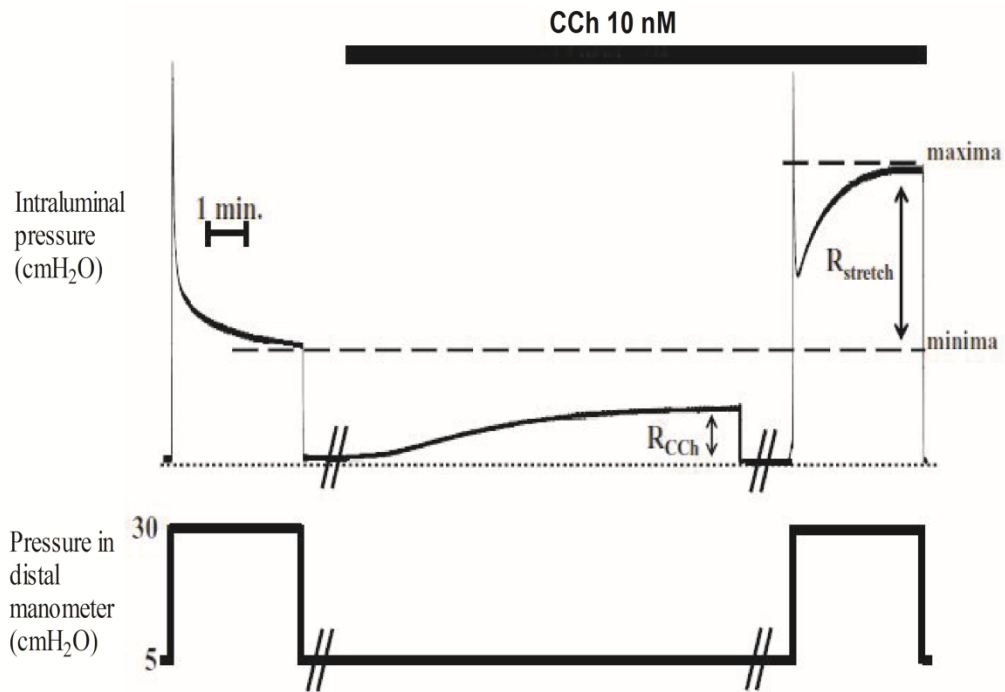
Fig 1. Representative experimental trace. Pressure recording during the various manipulations used in our experimental protocol; details are given in the Methods, Results, and Discussion. All experiments were performed under isovolumic conditions. The responses to cholinergic stimulation (R_{CCh}) and to pressure pulse stretch ($R_{stretch}$) were quantified as illustrated.
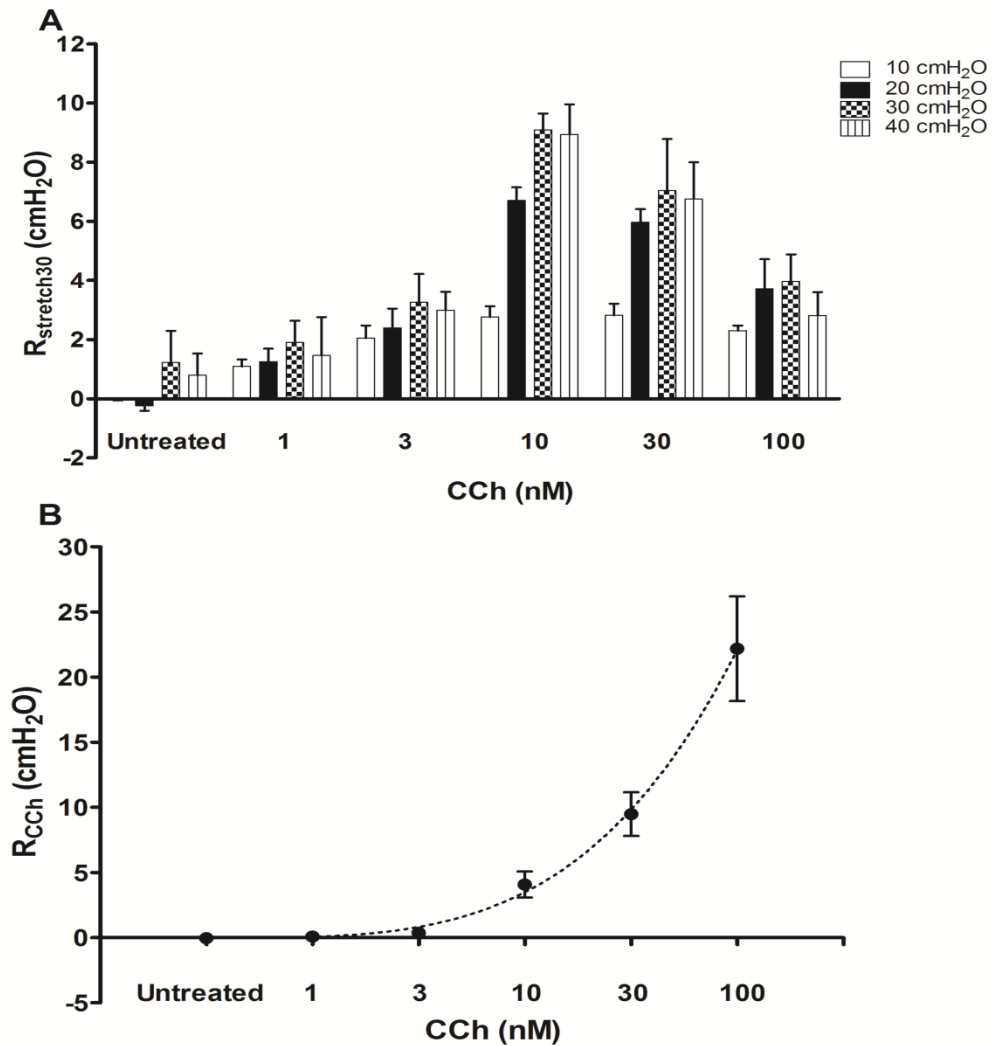
Fig 2. Relationship between CCh concentration, pressure pulse magnitude, and bronchial responsiveness to stretch. Agonist was added to the bath 10 min prior to the experimental protocol. (A) Mean magnitudes of $R_{stretch}$ evoked by transmural pressures of 10-40 cmH_2O and CCh concentrations of 1 nM – 0.1 μM ($n = 6$). (B) Bronchial tone in response to increasing concentrations of CCh (R_{CCh}) (1 nM – 0.1 μM) ($n = 6$).
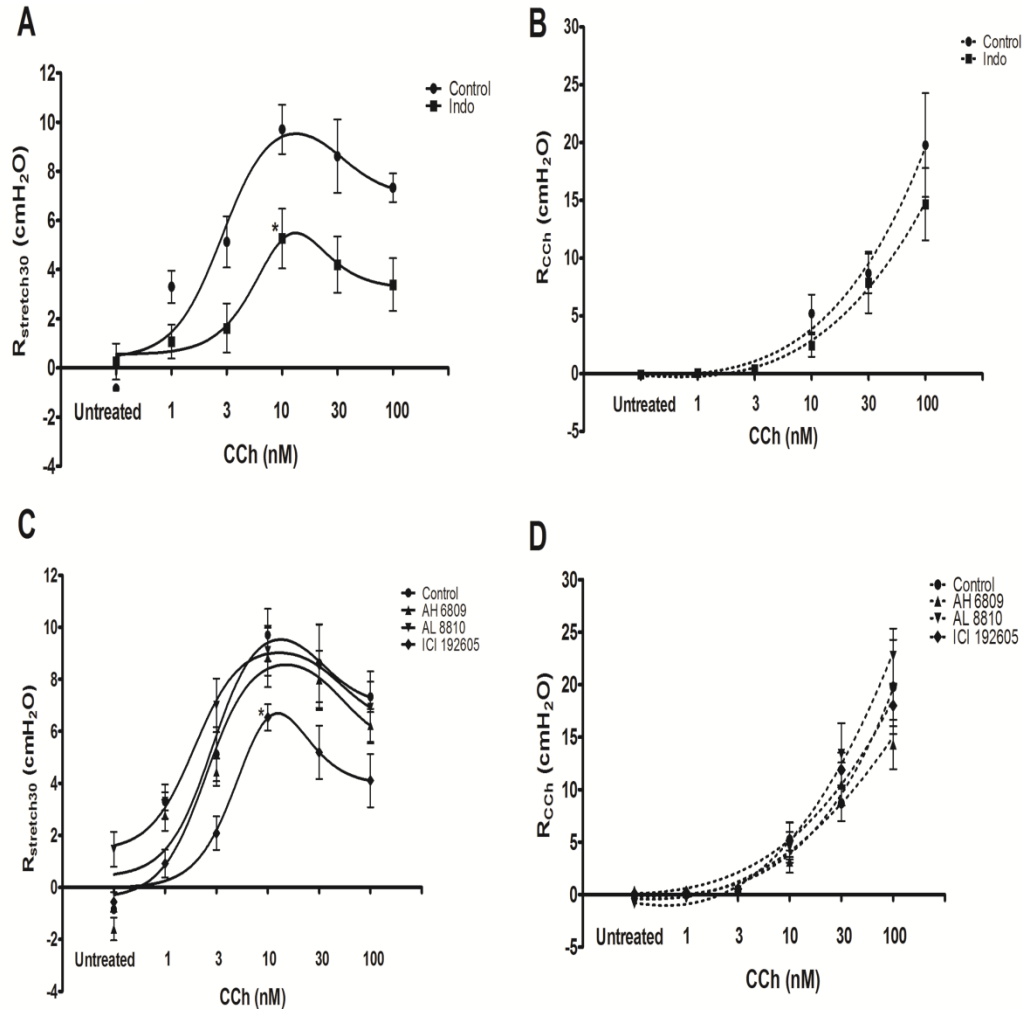
Fig 3. Effect of excitatory prostanoids on $R_{stretch,30}$ and R_{CCh}. $R_{stretch,30}$ response is represented by a solid line, while R_{CCh} is represented by a broken line. Effect of COX inhibition on $R_{stretch,30}$ (A) and R_{CCh} (B). All handling of tissues in the control group was done in Indo-free Krebs, while tissues in the treatment group were handled in Krebs pretreated with Indo (10 µM). Effect of selective prostanoid receptor antagonism on $R_{stretch,30}$ (C) and R_{CCh} (D). The EP$_1$/DP-selective receptor antagonist AH 6809 (10 µM), FP-selective receptor antagonist AL 8810 (10 µM), or TP-selective receptor antagonist ICI 192605 (10 µM) was added to the bath 20 min prior to the experimental protocol. $R_{stretch,30}$ response and R_{CCh} were measured at each CCh concentration (1 nM – 0.1 µM) under isovolumic conditions. ($n = 6$). *, $p < 0.05$
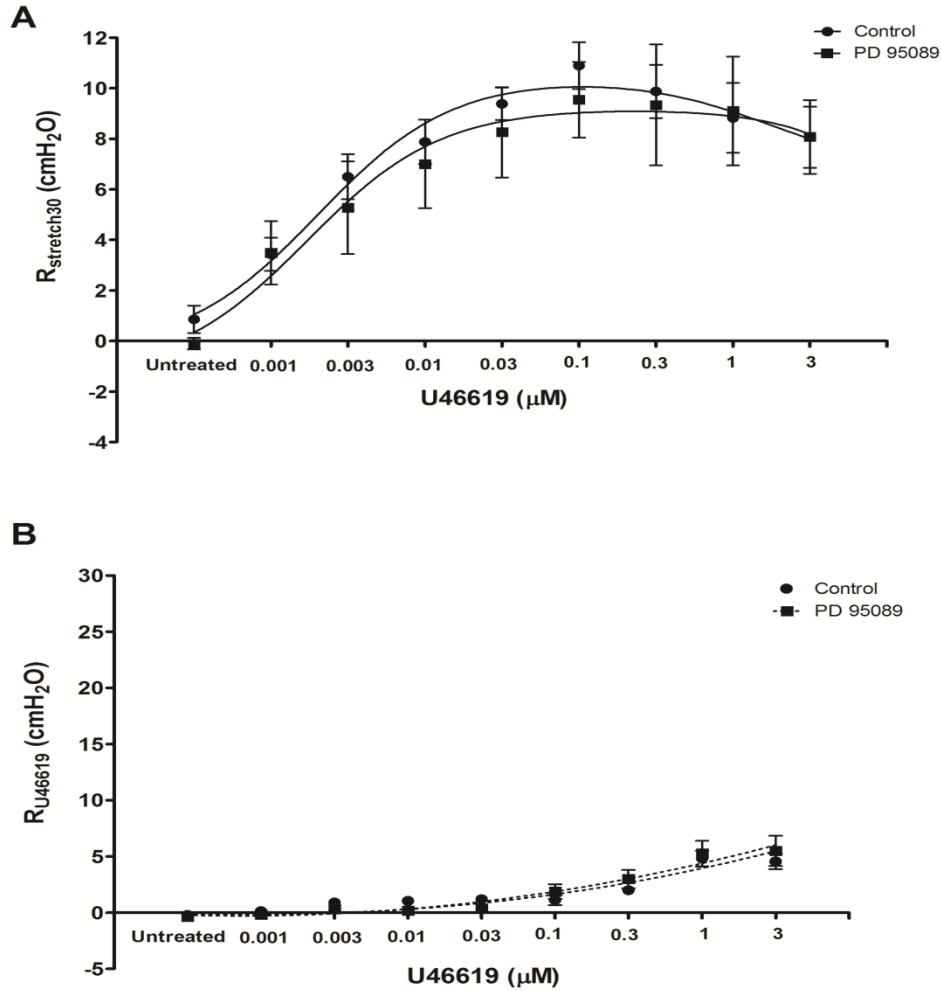
Fig 4. Effect of the TP-receptor agonist U46619 on $R_{stretch,30}$ and U46619-induced tone (R_{U46619}). $R_{stretch,30}$ response is represented by a solid line, while R_{U46619} is represented by a broken line. (A) $R_{stretch,30}$ response and (B) R_{U46619} were measured at each U46619 concentration (1 nM – 3 µM) under isovolumic conditions. The p42/44 MAPK inhibitor PD 95089 (10 µM) was added to the bath 20 min prior to the experimental protocol. ($n = 6$).
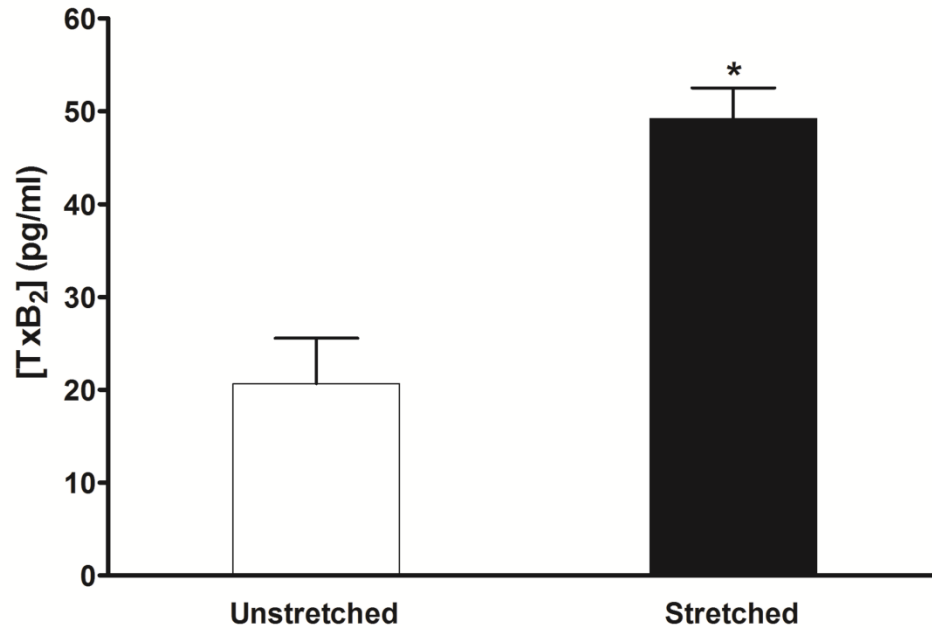
Fig 5. Effect of bronchial stretch on TxB$_2$ release. Experimental details are outlined in the Methods section. Mean values of TxB$_2$ concentrations in the luminal perfusate measured by competitive EIA in unstretched (Control) tissues (open bar), and in stretched tissues (solid bar). (n = 4). *, $p < 0.05$. Detection limit: 11pg/ml.
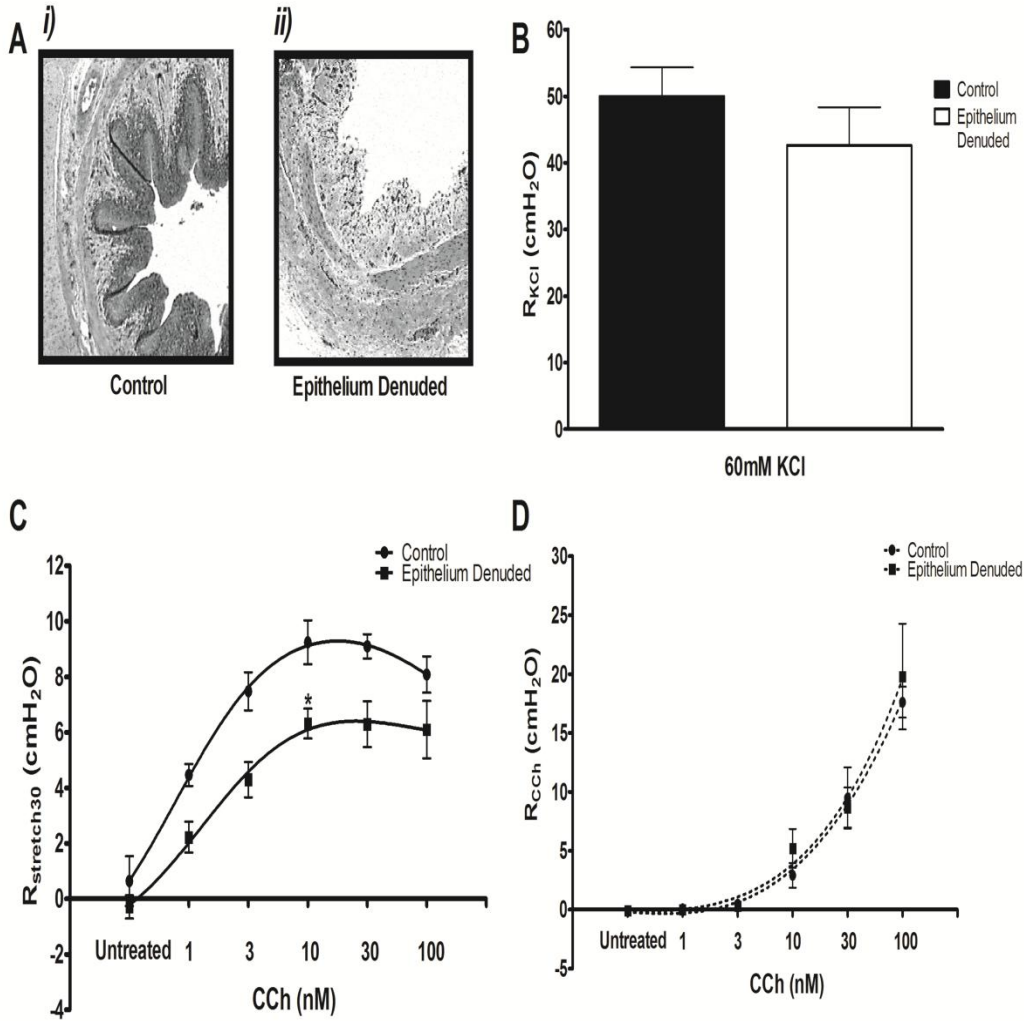
Fig 6. Effect of epithelial denudation on $R_{stretch,30}$, R_{CCh}, and maximal response to KCl (R_{KCl}). (Ai) & (Aii) H&E-stained bronchial cross-sections demonstrate the efficacy of our epithelial denudation technique (outlined in the Methods section) (100x magnification) ($n = 5$). (C) $R_{stretch,30}$ response and (D) R_{CCh} were measured at each CCh concentration (1 nM – 0.1 μM) under isovolumic conditions ($n = 6$). $R_{stretch,30}$ response is represented by a solid line, while R_{CCh} is represented by a broken line. (B) R_{KCl} (responses to 60 mM KCl) was measured 20 min prior to subjecting the tissues to $R_{stretch,30}$ responses in control (solid bar) vs. denuded (open bar) tissues ($n = 6$). *, $p < 0.05$
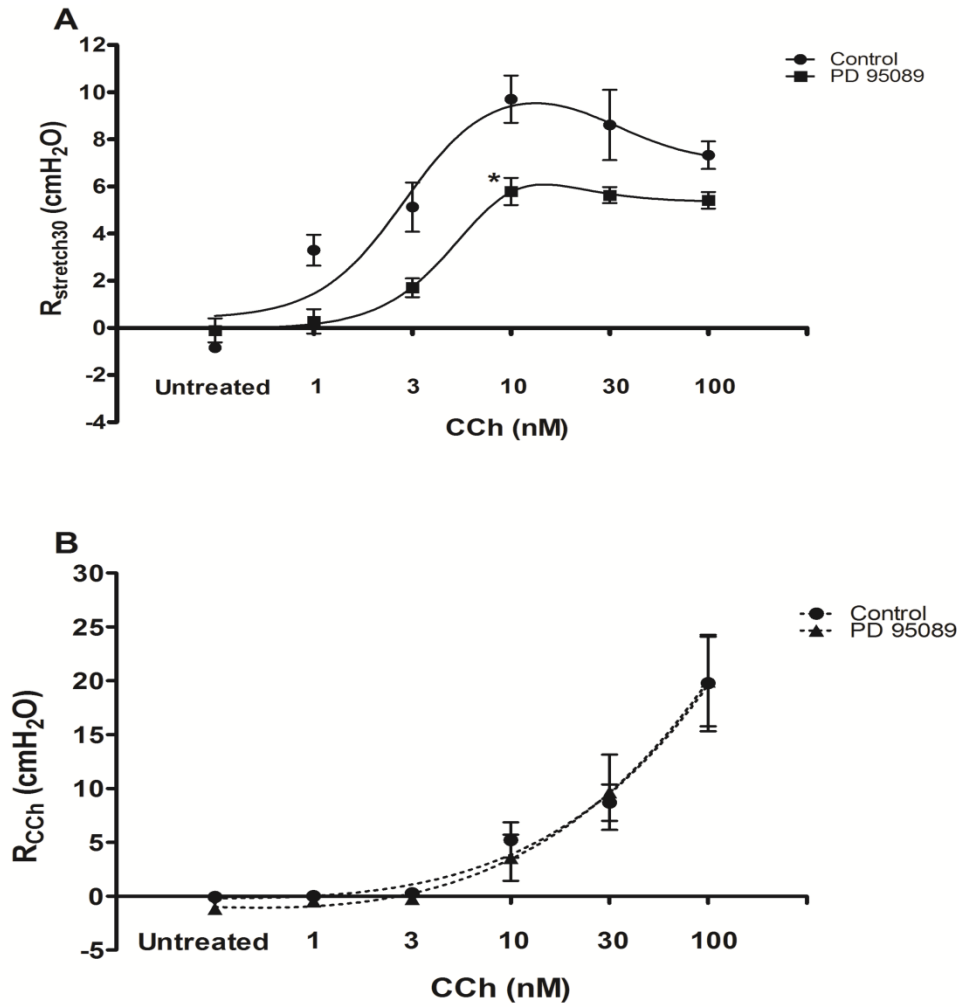
Fig 7. Effect of p42/44 MAPK inhibition on $R_{stretch,30}$ and R_{CCh}. $R_{stretch,30}$ response is represented by a solid line, while R_{CCh} is represented by a broken line. The p42/p44 MAPK-inhibitor PD 95089 (10 µM) was added to the bath 20 min prior to the experimental protocol. (A) $R_{stretch,30}$ response and (B) R_{CCh} were measured at each CCh concentration (1 nM – 0.1 µM) under isovolumic conditions ($n = 6$). *, $p < 0.05$

Discussion

In this study, we investigated the effects of the endogenous bronchoconstrictory prostanoids PGD_2, $PGF_{2\alpha}$, and TxA_2 on $R_{stretch}$ responses using a pharmacological approach in intact bovine bronchial segments. In addition, we provide evidence to suggest the involvement of airway epithelium-derived TxA_2 and p42/44 MAPK in the amplification of these $R_{stretch}$ responses.

The concept of stretch inducing a contractile response in ASM is not a novel finding, as it has been previously reported by research groups using both *in vitro* and *ex vivo* preparations (13; 25; 27). The novelty of our studies lies in the fact that while previous studies have deemed ASM $R_{stretch}$ to be a myogenic event (37; 39), intrinsic to ASM itself, we have demonstrated that this may not be entirely accurate. Using intact bovine bronchial segments, we have shown that airway $R_{stretch}$ are dependent upon contractile machinery priming and the magnitude of airway stretch. Moreover, in intact bovine bronchi, these responses possess non-myogenic characteristics due to the requirement of sensory neuronal input mediated by neurokinin (NK)-A acting through the NK_2-receptor (15).

In Figures 1&2 of this study, we show that contractile machinery priming is required for airway $R_{stretch}$, since these responses occur only when pretreated with submaximally-effective, or even sub-threshold concentrations of CCh. At higher agonist concentrations, the airway segment experiences a lower pre-load volume at the baseline transmural pressure of 5 cmH_2O due to its higher contractile state, and the stimulation may render the airway too stiff and non-

compliant to be able to produce adequate strain following the transmural pressure pulse to generate an $R_{stretch}$ response. Thus, airway smooth muscle contraction *per se* may not be the main driver for the $R_{stretch}$ response, because as shown in Figure 2, higher concentrations of CCh, which produced greater bronchial tone, generated smaller $R_{stretch}$ responses. In order to elicit an $R_{stretch}$ response, our data suggest that the airway merely needs to first be 'primed' with a submaximal concentration of CCh, and that too much agonist will impede the $R_{stretch}$ response even though bronchial tone is highly elevated. Because of this, our data are best-fit by a bell-shaped curve, showing stimulation at low-concentrations and inhibition at high-concentrations, rather than a sigmoidal curve.

Interestingly, airway inflammation present in asthmatic airways, as shown by *ex vivo* experiments using passively sensitized human airways (27), may add to $R_{stretch}$ responses by the release of stimuli (such as excitatory prostanoids) that prime the contractile apparatus to react excessively in the presence of mechanical stress. Animal studies have demonstrated that these excitatory arachidonic acid metabolites can in fact be synthesized and released by bouts of airway inflammation as well as by mechanical stress (1; 8; 31; 32).

In this study, we sought to investigate the possibility that airway $R_{stretch}$ responses may be amplified by the stretch-induced release of excitatory prostanoids. As prostanoids are not typically stored intracellularly after being synthesized, we investigated their role in airway $R_{stretch}$ by inhibiting COX, a key enzyme in the prostanoid synthesis pathway (16), present in the airways (38), and

susceptible to inhibition by Indo (6). Figures 3A&B show the ability of Indo to significantly reduce the magnitude of $R_{stretch,30}$ without altering R_{CCh}, suggesting a role for excitatory prostanoids in $R_{stretch,30}$ independent of agonist-induced tone generation. In fact, in comparing Fig. 3 to Fig. 4, we see that $R_{stretch,30}$ is of similar magnitude in the presence of CCh *versus* U46619, even though the former generates a much larger bronchial tone than the latter. Thus, it is possible that airway $R_{stretch,30}$ responses possess both tone-dependent and –independent characteristics, where upon reaching a threshold baseline tone, $R_{stretch,30}$ responses can be significantly augmented with minute increases in concentration of contractile stimuli which are insufficient to alter the airway tone directly.

Upon demonstrating the efficacy of COX-inhibition in significantly reducing airway $R_{stretch,30}$, we sought to investigate the roles of selected prostanoid receptors (DP, FP, and TP) in amplifying airway $R_{stretch,30}$ responses. Figures 3C&D show the inability of DP- or FP-receptor antagonism to alter the magnitude of $R_{stretch,30}$ responses, while TP-receptor antagonism significantly reduced these responses, suggesting the involvement of TP-receptor activation in amplifying $R_{stretch,30}$. No alteration in R_{CCh} was present following treatment with the TP-receptor antagonist (ICI 192605 10 μM), strengthening our hypothesis that $R_{stretch,30}$ responses may indeed possess both tone-dependent and –independent characteristics. Furthermore, the TP-receptor agonist U46619 generated $R_{stretch,30}$ responses in a concentration-dependent manner, largely independent of R_{U46619} and of prejunctional release of ACh from cholinergic neurons, as shown in past

studies (1; 19). Interestingly, PGD_2 and $PGF_{2\alpha}$ have also been shown to exert their effects by binding to the TP-receptor (10; 23), which signal through the $G_{q/11}$ family of G proteins in ASM (21), reinforcing the importance of TP-receptor activation in these $R_{stretch,30}$ responses. In our previous study (15), experiments were performed on tissues bathed in Krebs solution containing 10 μM Indo, which would have completely inhibited COX and blocked prostanoid synthesis. Interestingly, $R_{stretch}$ responses were still elicited, suggesting that these $R_{stretch,30}$ responses were comprised of the component that is TP receptor-independent. Conversely, in our present study, we performed our control experiments using Indo-free Krebs solution and observed a significant increase in the magnitude of $R_{stretch,30}$ compared to tissues treated with 10 μM Indo (Fig. 3A), which we attributed to TP-receptor activation, suggesting that TP-receptor activation leads to an amplification of $R_{stretch}$ responses but is not actually required for $R_{stretch}$ to occur.

Because of its potency as a bronchoconstrictor (approx. 2 times more potent than other prostanoids) (9), and its increased concentration in the BAL fluid of asthmatic patients (5; 23; 31), TxA_2 has attracted attention as a potential important mediator in the pathophysiology of asthma. Here, we showed a significantly increased release of TxB_2, the immediate and stable metabolite of TxA_2, following transmural pressure loading using a competitive EIA (Fig. 5), demonstrating the ability of mechanical stretch to cause TxA_2 release from the airway, as previously shown in cultured rat lung epithelial cells (8).

Moreover, we show the ability of epithelial denudation to significantly reduce $R_{stretch,30}$ to similar levels as that done by COX-inhibition and TP-receptor antagonism (Fig. 6), strengthening previous reports of the epithelium being a major source of prostanoid synthesis and release in response to mechanical stress (5; 8; 16). Upon cellular stimulation, prostanoids are synthesized from arachidonic acid liberated from membrane phospholipids by the enzyme PLA_2 *via* a MAPK-dependent mechanism (8). Animal studies support that p42/p44 MAPK activation contributes to airway inflammation and hyperresponsiveness (11), and plays an essential role in stretch-induced prostanoid release from airway epithelium (8). In this study, we demonstrated the ability of a p42/p44 MAPK inhibitor to significantly reduce $R_{stretch,30}$ responses (Fig. 7), showing a role for p42/p44 MAPK in $R_{stretch,30}$ responses. Thus, using our preparation, we suggest that the p42/p44 MAPK activation occurs at the airway epithelial level prior to TxA_2 synthesis and release, as shown in (8).

DI-induced bronchoconstriction is an abnormal phenomenon in humans, since it is only seen in moderate to severe asthmatics. Our bovine bronchial segments were not inflamed nor exhibited spontaneous tone, and did not manifest a stretch-induced contraction until they were pretreated with a contractile agonist (CCh or U46619), used to mimic the increased ASM tone seen in asthmatic airways. Previous studies have also demonstrated an $R_{stretch}$ in ASM that required pretreatment with a pharmacological agent to prime the contractile apparatus, such as tetraethylammonium chloride, or a cholinergic agonist (37; 39). Although

others (3; 14; 29) have observed that stretch caused reductions in airway responses to cholinergic stimulation in canine and porcine bronchi, contrasting reports have shown both a lack of stretch-induced relaxation as well as constriction in intact bovine bronchi (15; 22). Although differences in experimental protocols exist between reports, questions have been raised as to whether these differences may be species-related, where bovine ASM is unique in its response to mechanical stretch by behaving more like the asthmatic phenotype (28). These discrepancies may also be attributed to properties of different regions in the airway tree, where $R_{stretch}$ may be more significant in small resistance airways compared to larger airways.

In conclusion, our data suggest that airway $R_{stretch}$ may be amplified by bronchoconstrictory prostanoids, namely TxA_2, synthesized in a p42/p44 MAPK-dependent manner and released by the airway epithelium in response to stretch. These results highlight an alternative pathway for potential therapeutic targeting in asthmatic patients where a bronchoconstrictory response to a DI may play a role in airway hyperresponsiveness.

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CHAPTER 4

The role of L-type calcium channel activation in airway stretch-activated contractions

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Jeremy M Hernandez – Responsible for experimental design and data analysis; conducted all experiments; wrote the manuscript

Luke J Janssen – Supervision; guidance with study design; manuscript editing

Abbreviations:

5-HT – 5-hydroxytryptamine (3-(2-aminoethyl)-1*H*-indol-5-ol)

ASM – Airway smooth muscle

BAY K8644 – Methyl 2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-1,4-dihydropyridine-3-carboxylate

BK_{Ca} channel – Large conductance Ca^{2+} channel

CICR - Calcium-induced calcium release

CCh – Carbachol (2-carbamoyloxyethyl-trimethyl-azanium)

DI – Deep inspiration

FRC – Functional residual capacity

IbTx – Iberiotoxin

ICI 192605 – 4-(*Z*)-6-(2-*o*-Chlorophenyl-4-*o*-hydroxyphenyl-1,3-dioxan-*cis*-5-yl)hexenoic acid

KCl – Potassium chloride

MAPK – Mitogen-activated protein kinase

MEN 10376 – [Tyr^{1},D-Trp^{6,8,9}Lys^{10}]-NKA(4-10)

NK – Neurokinin

RAR – Rapidly adapting pulmonary stretch receptors

$R_{stretch,x}$ – contraction evoked by an instantaneous stretch to x cmH_2O

SR – Sarcoplasmic reticulum

SR48968 – N-[(2S)-4-(4-acetamido-4-phenylpiperidin-1-yl)- 2-(3,4-dichlorophenyl)butyl]-N-methylbenzamide

TLC – Total lung capacity

TRPV1 – Transient receptor potential vanilloid 1

TTX – Tetrodotoxin

Tx – Thromboxane

U46619 – 9,11-dideoxy-9α,11α-methanoepoxy-prosta-5Z,13E-dien-1-oic acid

VSM – Vascular smooth muscle

Abstract

The vascular myogenic response, an intrinsic property of the vascular smooth muscle (VSM), is reliant on membrane depolarization, activation of L-type Ca^{2+} channels, Ca^{2+}-induced Ca^{2+}-release (CICR) (*via* ryanodine receptor activation), and BK_{Ca} channels. Similar to the vasculature, airways are also affected by constant mechanical stress. This is most evident when comparing the effects of a deep inspiration (DI) in healthy individuals *versus* asthmatics, where airway stretch has been shown to produce a bronchodilatory response in the former population, while in the latter, they are less effective in producing bronchodilation, can cause more rapid airway re-narrowing, and even bronchoconstriction (*via* an ASM stretch-activated contractile response ($R_{stretch}$)) in moderate to severe asthmatics. In our previous publications pertaining to the $R_{stretch}$ phenomenon, we focused mainly on determining the differences between the vascular myogenic response and the airway $R_{stretch}$. In this study, however, our objective was to further characterize the signaling mechanisms that regulate airway $R_{stretch}$ responses, while investigating the similarities between these and the vascular myogenic response. Using a pharmacological approach in intact bovine bronchial segments, we showed the ability of CCh, KCl, NKA, and U46619 to generate $R_{stretch}$ responses in a concentration-dependent manner that were sensitive to the L-type Ca^{2+} channel antagonist nifedipine (3 μM), the ryanodine receptor antagonist ryanodine (10 μM) and the BK_{Ca} channel antagonist iberiotoxin (30 nM), suggesting a role for the L-type Ca^{2+} channel, ryanodine

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receptor, and BK_{Ca} channel in these responses, thus providing novel insights into

mechanisms regulating the DI-induced bronchoconstriction seen in asthmatics.

Introduction

In the vasculature, blood pressure is strictly regulated by signaling pathways that respond to mechanical stress to ensure the precise control of blood flow under physiological conditions ranging from vigorous exercise to complete rest (2). This phenomenon, termed a *myogenic response*, has been shown to be an intrinsic property of the vascular smooth muscle (VSM) (1), reliant in part on the activation of L-type Ca^{2+} channels. According to Hill *et al*., stretch elicits VSM membrane depolarization, activating L-type Ca^{2+} channels, thus causing Ca^{2+} influx. This subsequently activates the Ca^{2+}-induced Ca^{2+}-release (CICR) mechanism *via* sarcoplasmic reticulum (SR)-bound ryanodine receptor (RyR) activation, and causes a myogenic contraction (9), the magnitude of which has been shown to be limited by the activation of plasmalemma-bound large conductance Ca^{2+}-dependent K^+ (BK_{Ca}) channels (8).

Similar to the vasculature, airways are also constantly subjected to mechanical stress with each inhalation-exhalation cycle, a phenomenon that may lead to effects that are either beneficial in healthy individuals or harmful in moderate to severe asthmatics (16). This is most evident when assessing the effect of a deep inspiration (DI), clinically measured as a breath taken from functional residual capacity to total lung capacity, in healthy individuals *versus* asthmatics. These deep inspiratory efforts have been shown to produce a bronchodilatory response healthy individuals, whereas in asthmatics, DIs are less effective in producing bronchodilation, can cause more rapid airway re-narrowing, and even

bronchoconstriction in moderate to severe asthmatics (3; 10; 15; 24). Interestingly, the mechanisms by which a DI is able to cause a stretch-induced bronchoconstriction remain ambiguous. Previous studies have suggested airway stretch-activated contractions ($R_{stretch}$) to be a myogenic event (27; 29), implying it to be an intrinsic property of the airway smooth muscle (ASM) cell itself. However, we have previously shown that this may not be the case, since in intact bovine bronchial segments, stretch-activated contractile responses are not completely intrinsic to the smooth muscle in that they require neuronal input mediated by NKA and can be amplified by excitatory prostanoids released from the epithelium acting through TP receptors (6; 7). In this study, we are looking to further characterize the signalling mechanisms involved in regulating these airway $R_{stretch}$ responses.

In our two above mentioned publications regarding the ASM $R_{stretch}$ response, which collectively act as the preamble to this study, we focused on determining the differences between the vascular myogenic response and the airway $R_{stretch}$. In this study, however, we set out to show some of the similarities between these two phenomena by further investigating the roles of L-type Ca^{2+} channels, membrane depolarization, CICR (SR-bound RyR involvement), and plasmalemma-bound BK_{Ca} channels in airway $R_{stretch}$. Furthermore, our laboratory has previously shown that agonist-induced excitation-contraction coupling in ASM is independent of Ca^{2+}-influx through L-type Ca^{2+} channels (shown by their insensitivity to the L-type Ca^{2+} channel blocker nifedipine (13)); however, we

have recently demonstrated that airway $R_{stretch}$ is completely abolished by treatment with nifedipine, which suggests a role for L-type Ca^{2+} channels is these responses (6).

Thus, using a pharmacological approach in intact bovine bronchial segments and the Mayflower tissue bath apparatus, as previously described in (18), our objective in this study was to further characterize the signaling mechanisms that regulate airway $R_{stretch}$ responses, while investigating the similarities between these and the well-know characteristics of the vascular myogenic response, with the hope of providing novel insights into mechanisms regulating the DI-induced bronchoconstriction seen in asthmatics.

Methods

Animals. All experimental procedures were approved by the McMaster University Animal Care Committee (McMaster University, Hamilton, ON, Canada) and conform to the guidelines set by the Canadian Council on Animal Care (Ottawa, ON, Canada). Lower lobes of lung were obtained from cows (200–500 kg) euthanized at a local abattoir and transported to the laboratory in ice-cold modified Krebs buffer solution (116 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.3 mM NaH_2PO_4, 23 mM $NaHCO_3$, 11mM D-glucose, 0.01 mM indomethacin), saturated with 95% oxygen - 5% carbon dioxide to maintain pH at 7.4. Upon receipt of the lobes of lung, intact bovine bronchial segments (2 mm diameter, 20 mm length) were carefully dissected free from surrounding parenchyma, excised, and immediately used or stored in modified Krebs solution at 4°C for up to 24 h.

Bronchial segment preparation. For a detailed description of our bronchial segment preparation protocol, please refer to our previous study (6). Briefly, following the dissection and excision of the bronchial segment, side branches were tightly ligated. The ligated bronchial segment was then mounted horizontally in a 30 ml Mayflower organ bath (Hugo Sachs Elektronik, March-Hugstetten, Germany) containing warmed modified Krebs buffer solution (37°C) gassed with carbogen (95% O_2 – 5% CO_2). The airway lumen was also filled with warmed modified Krebs solution gassed with carbogen *via* a jacketed-reservoir,

the height of which set the baseline transmural pressure (~5 cmH$_2$O). This baseline pressure was selected to simulate the transmural pressure found in relaxed airways (22). The Mayflower apparatus also possessed 3-way taps, which could be opened to flush the airway with modified Krebs solution or closed to subject the airway to isovolumic conditions. Manual transmural pressure variation was induced under isovolumic conditions by varying the height of perfusate in a column manometer attached distally to the cannulated airway.

Subsequently, we briefly subjected the tissue to an increased transmural pressure load under isovolumic conditions to ensure there were no leaks in the airway. The segment was then left to equilibrate for ~2 hours. During this time, the lumen and adventitia were regularly washed with fresh modified Krebs solution. Following tissue equilibration, transmural pressure was set to ~ 5 cmH$_2$O. Under isovolumic conditions, tissues were treated with 60 mM KCl (administered extraluminally) and the contractile response (isovolumic increase in transmural pressure) was recorded to test for viability. After washing four times, baseline pressure was reset to ~ 5 cmH$_2$O.

Tissue Baths. To evaluate the effects of mechanical stretch on ASM contraction (measured by transmural pressure increase) in the isolated bronchial segment, we followed the protocol outlined in our previous publication (6). In brief, following the tissue viability test, the airway was allowed 20 min of recovery time under isovolumic conditions. Subsequently, electric-field stimulation (EFS) responses

were evoked at 5 min intervals until a uniform response was established (after approx. 3-4 repetitions) under isovolumic conditions. EFS was delivered by a train of pulses (60 volts, 2 ms pulse duration, frequency of 20 pulses per second, and 1.5 sec train duration). The airway was then subjected to a transmural pressure pulse of 30 cmH_2O, which was maintained for 3 minutes under isovolumic conditions. Transmural pressure was subsequently restored to baseline (~ 5 cmH_2O) and the tissue was allowed 5 min recovery time. To mimic the increased airway tone seen in asthmatic airways, this process was repeated following pretreatment with 10 nM carbachol (CCh) added to the bath solution to induce submaximal ASM tone under isovolumic conditions. When the agonist-induced tone had reached a plateau (in approx. 10 min), transmural pressure was reset to ~5 cmH_2O before re-assessing airway contractile responses to stretch ($R_{stretch,30}$). The effects of a contractile agonist on ASM tone was assessed by measuring the rise in transmural pressure in response to increasing concentrations of agonist under isovolumic conditions.

Pharmacological interventions. To investigate the pathway involved in eliciting airway $R_{stretch,30}$ responses, tissues were treated extraluminally with a range of different agonists and antagonists. The role of ASM depolarization was tested by treating tissues with KCl in a concentration-dependent manner, while the role of L-type Ca^{2+} channels was assessed by pretreatment for 20 min with its respective channel blocker Nifedipine (3 μM) (6) and opener BAY K8644 (in a

concentration-dependent manner). To assess the role of the NK_2-receptor in $R_{stretch,30}$ responses, tissues were treated with its antagonist, SR48968 (3 µM; 30 min) (6), or agonist NKA in a concentration-dependent manner. Lastly, to investigate the potential role of plasmalemma-bound BK_{Ca} channels and sarcoplasmic reticulum (SR)-bound ryanodine receptors, tissues were pretreated with Iberiotoxin (IbTx) (30 nM; 30 min.) (21), and ryanodine (10 µM; 30 min.) (28), respectively.

Chemicals and solvents. SR48968 was kindly donated by Sanofi-Synthelabo Recherche (Montpellier, France). Ryanodine was obtained from Cedarlane (ON, Canada). All other pharmacological agents were obtained from Sigma–Aldrich (ON, Canada). The 10 mM stock solutions were prepared in distilled water (CCh, NKA), absolute EtOH (indomethacin, nifedipine, ryanodine), dilute acetic acid (iberiotoxin), or DMSO (SR48968, BAY K8644). Dilutions of these were made in physiological medium; the maximal bath concentration of solvents did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity.

Statistical Analysis. Stretch-activated contractions to 30 cmH_2O transmural pressure ($R_{stretch,30}$) were quantified as the difference between the minima and the maxima observed in the transmural pressure recordings following a sudden isovolumic stretch (as shown in Hernandez et al., 2008). All responses were

reported as means $\pm$ SEM; *n* refers to the number of animals. $R_{stretch,30}$ data were fitted to a Bell-Shaped concentration-response stimulation curve and agonist-induced tone data were fitted to a sigmoidal concentration-response stimulation curve, both of which allowed for the measurement of EC_{50} and E_{max} values. Statistical comparisons between groups were made using the paired or unpaired Student's *t-test*; $P < 0.05$ was considered statistically significant.

Results

Airway $R_{stretch}$ responses. To characterize the mechanisms underlying $R_{stretch}$ responses, all experiments used a standard test pulse of 30 cmH_2O, since this mirrors the transmural pressure seen during a deep inspiration to TLC in humans (26). Moreover, we have previously shown the contractile response ($R_{stretch,30}$) to be at its maximum at this transmural pressure load. A detailed description of the protocol used to elicit airway $R_{stretch}$ responses is outlined in our previous publication (6).

In this study, we investigated the role of L-type Ca^{2+} channels in airway $R_{stretch}$ responses, while reconciling our previous findings regarding the dependence of NK_2-receptor activation and the ability of TP-receptor activation to amplify these responses.

In Figure 1A, we demonstrated the ability of a cholinergic agonist (CCh) to elicit $R_{stretch,30}$ responses in a concentration-dependent manner, with a peak response occurring at 10 nM CCh. As we have shown previously (7), higher levels of cholinergic stimulation led to progressively smaller $R_{stretch,30}$ responses. In Figure 1B, we pretreated tissues with 10 nM CCh, then measured $R_{stretch,30}$ responses following consecutive 30 cmH_2O transmural pressure pulses elicited at 10 minute intervals in order to demonstrate that these responses did not wane with successive transmural pressure pulses.

Effect of L-type Ca^{2+} channel antagonism on $R_{stretch,30}$ responses. To investigate the dependence of $R_{stretch,30}$ responses upon L-type Ca^{2+} channel activation, a concentration-response protocol was performed using the L-type Ca^{2+} channel antagonist nifedipine (0.1 nM – 3 µM), where $R_{stretch,30}$ responses were elicited following each concentration of antagonist added. Control responses were elicited with 10 nM CCh prior to treatment with nifedipine. Tissues were incubated with nifedipine for 20 min prior to eliciting an $R_{stretch,30}$ response. Treatment with nifedipine elicited a concentration-dependent reduction in $R_{stretch,30}$ responses with total abolishment occurring at a concentration of 3 µM (Fig. 1C). Nifedipine had no effect on CCh-induced tone (R_{CCh}) (Fig. 1D). Tissues were bathed in Indo-free Krebs.

Effect of the L-type Ca^{2+} channel agonist BAY K8644 on $R_{stretch,30}$ responses. To further investigate the role of L-type Ca^{2+} channels in $R_{stretch,30}$ responses, we performed a concentration-response protocol using the selective L-type Ca^{2+} channel agonist BAY K8644 (0.1 nM – 3 µM), where $R_{stretch,30}$ responses were evoked 20 minutes following each concentration of BAY K8644 added. Treatment with BAY K8644 elicited a concentration-dependent increase in $R_{stretch,30}$ responses with a peak response occurring at a concentration of 30 nM, a response that was unaffected by pretreatment with the NK_2-receptor antagonist SR48968 (3 µM) (Fig. 2). These data further strengthen our hypothesis regarding L-type Ca^{2+} channel involvement in airway $R_{stretch,30}$ responses by showing the

ability of the selective L-type Ca^{2+} channel agonist BAY K8644 to elicit $R_{stretch,30}$ responses in a concentration-dependent manner. Moreover, the inability of the NK_2-receptor antagonist SR48968 to affect these responses suggests that L-type Ca^{2+} channel activation occurs downstream of NK_2-receptor activation during $R_{stretch,30}$ response generation.

Effect of NK_2-receptor activation on $R_{stretch,30}$ responses. To determine the effect of nifedipine on NK_2-augmented $R_{stretch,30}$ responses, we performed a concentration-response protocol using the selective NK_2-receptor agonist NKA (0.1 nM − 3 µM), where $R_{stretch,30}$ responses were established 20 minutes after each concentration of NKA was added. Treatment with NKA elicited a concentration-dependent increase in $R_{stretch,30}$ responses with a peak response occurring at a concentration of 30 nM. Interestingly, this response was totally abolished by pretreatment with the L-type Ca^{2+} channel antagonist nifedipine (3 µM) (Fig. 3), strengthening our theory regarding the importance of NK_2-receptor activation in airway $R_{stretch,30}$ responses and further suggesting that L-type Ca^{2+} channel activation occurs downstream of NK_2-receptor activation during $R_{stretch,30}$ response generation.

Effect of KCl-induced membrane depolarization on $R_{stretch,30}$ responses. To investigate the dependence of airway $R_{stretch,30}$ responses upon membrane depolarization, as seen in the VSM myogenic response, a concentration-response

protocol was performed using KCl (5 – 35 mM), where $R_{stretch,30}$ responses were established following each concentration of agonist added. Treatment with KCl elicited a concentration-dependent increase in $R_{stretch,30}$ responses with a peak response occurring at a concentration of 25 mM. Not surprisingly, this response was totally abolished by pretreatment with the L-type Ca^{2+} channel antagonist nifedipine (3 µM) (Fig. 4), once again showing the importance of L-type Ca^{2+} channel activation in $R_{stretch,30}$ responses.

Effect of nifedipine on TP-receptor agonist (U46619)-induced $R_{stretch,30}$ responses. In our previous publication, we showed the ability of TxA_2 released by the airway epithelium to amplify $R_{stretch,30}$ responses (7). Here, we are investigating the possible involvement of the L-type Ca^{2+} channel in U46619-induced responses by pretreating the tissues with nifedipine (3 µM). A concentration-response protocol was performed using U46619 (0.1 nM – 3 µM), where $R_{stretch,30}$ responses were measured 20 minutes after each concentration of U46619 was added. Treatment with U46619 elicited a concentration-dependent increase in $R_{stretch,30}$ responses with a peak response occurring at a concentration of 0.1 µM. This response was totally abolished by pretreatment with the L-type Ca^{2+} channel antagonist nifedipine (3 µM), suggesting that U46619-induced $R_{stretch,30}$ response generation is L-type Ca^{2+} channel dependent (Fig. 5).

Role of SR-bound RyRs in R$_{stretch,30}$ responses. RyRs have been shown to be involved in eliciting vascular myogenic responses through a CICR mechanism. Thus, we decided to explore the role of RyRs in airway R$_{stretch,30}$ responses by pretreating tissues with the RyR antagonist ryanodine (10 µM) for 30 min prior to subjecting the tissues to a CCh (10 nM) -induced R$_{stretch,30}$ response. Ryanodine treated tissues exhibited a significant reduction in R$_{stretch,30}$ responses compared to control (p<0.05), suggesting involvement of SR-bound RyRs in airway R$_{stretch,30}$ responses (Fig. 6), as seen in the VSM myogenic response.

Role of plasmalemma-bound BK$_{Ca}$ channels in R$_{stretch,30}$ responses. We explored the role of BK$_{Ca}$ channels in R$_{stretch,30}$ responses by pretreating tissues with the BK$_{Ca}$ antagonist iberiotoxin (IbTx) (30 nM) for 30 min prior to subjecting the tissues to a CCh (10 nM)-induced R$_{stretch,30}$ response. IbTx treated tissues exhibited a significant augmentation in R$_{stretch,30}$ responses compared to control (p<0.05), suggesting the involvement of BK$_{Ca}$ channels in airway R$_{stretch,30}$ responses (Fig. 7), similar to that seen in the VSM myogenic response.
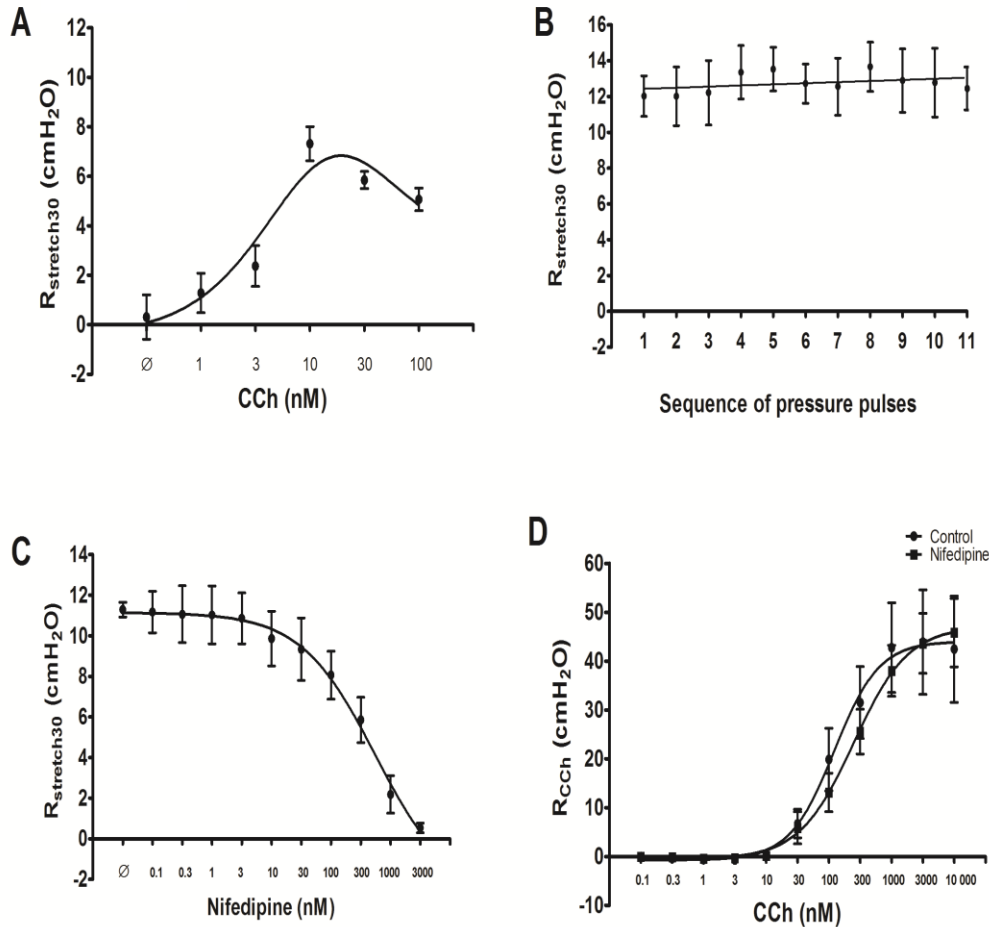
Fig 1. Effect of Nifedipine on CCh-induced $R_{stretch30}$ response and tone. (A) Mean magnitudes of $R_{stretch}$ evoked by a transmural pressure pulse of 30 cmH_2O ($R_{stretch,30}$) at CCh concentrations of 1 – 100 nM. CCh was added to the bath 10 min prior to each transmural pressure pulse. ($n = 6$). (B) Effect of consecutive transmural pressure pulses on $R_{stretch30}$ responses in tissues pretreated with 10 nM CCh. Tissues were subjected to consecutive 30 cmH_2O transmural pressure pulses at 10 minute intervals ($n = 6$). (C) Effect of increasing concentrations of nifedipine (0.1 – 3000 nM) on CCh-induced $R_{stretch,30}$ responses. Each concentration of nifedipine was added to the bath 20 min. prior to each transmural pressure pulse. ($n = 6$). (D) Effect of nifedipine on CCh-induced bronchial tone. Nifedipine was added to the bath 20 min. prior to the concentration-response protocol. ($n = 6$).
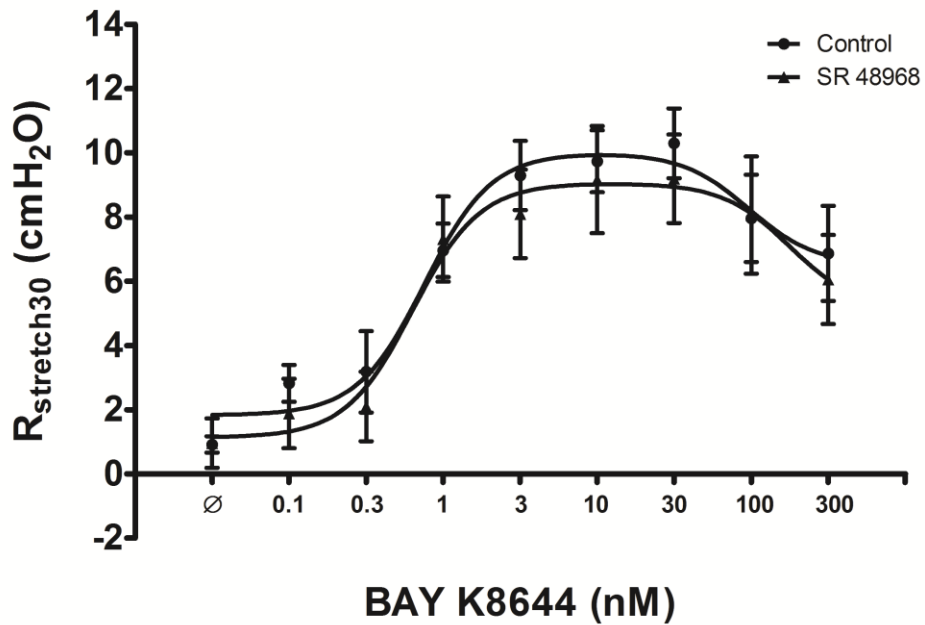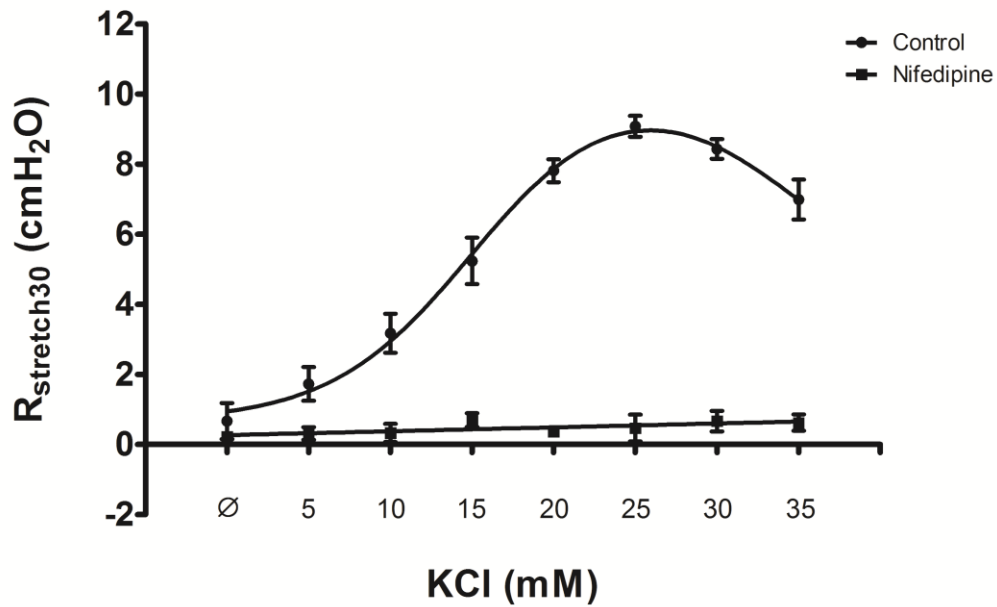
Fig 2. Effect of an L-type Ca^{2+} channel agonist (BAY K8644) on R$_{stretch30}$ responses. R$_{stretch,30}$ responses were measured at each BAY K8644 concentration (0.1 – 300 nM) under isovolumic conditions. The L-type Ca^{2+} channel agonist BAY K8644 was added to the bath 20 min prior to each transmural pressure pulse. ($n = 6$). The NK$_2$-receptor antagonist SR48968 was added to the bath 30 min prior to the concentration-response protocol. ($n = 6$).
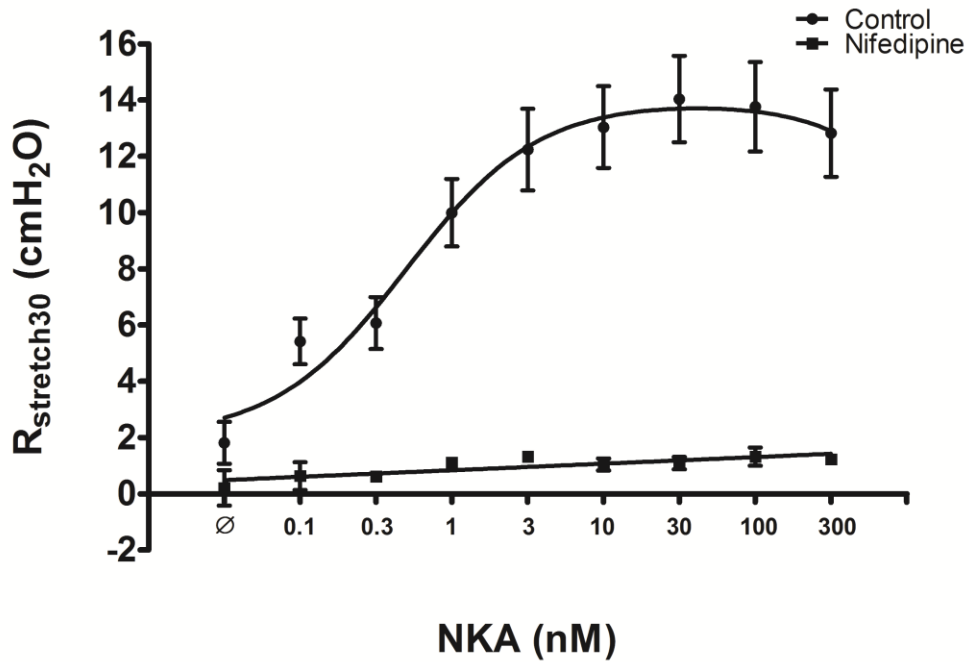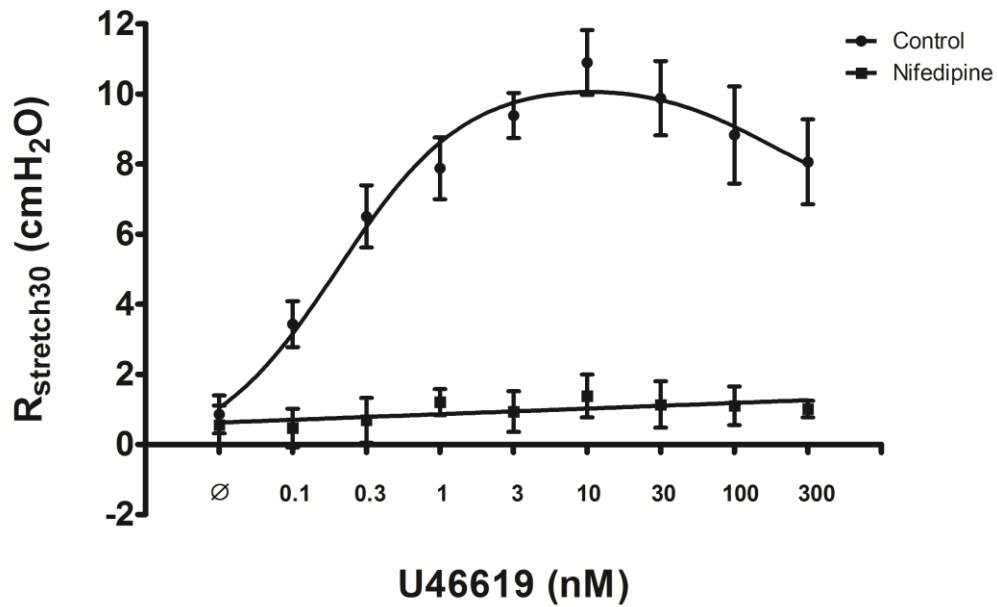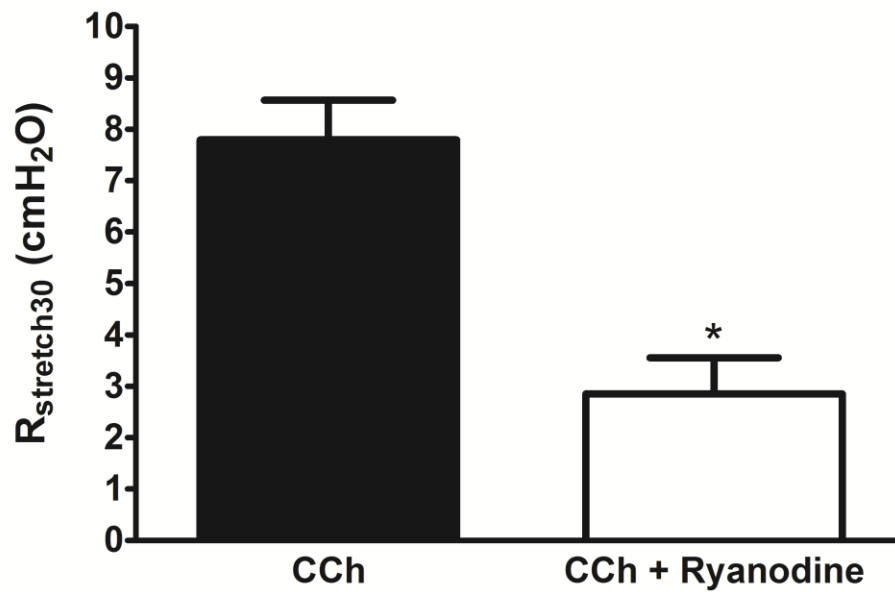
Fig 3. Effect of NKA on $R_{stretch30}$ responses. $R_{stretch,30}$ responses were measured at each NKA concentration (0.1 – 300 nM) under isovolumic conditions. The NK_2-receptor agonist NKA was added to the bath 20 min prior to each transmural pressure pulse. ($n = 6$). Nifedipine was added to the bath 20 min. prior to the concentration-response protocol. ($n = 6$).

Fig 4. Effect of KCl-induced membrane depolarization on $R_{stretch30}$ responses.
$R_{stretch,30}$ responses were measured at each KCl concentration (5 – 35 mM) under isovolumic conditions. The KCl was added to the bath 10 min prior to each transmural pressure pulse. ($n = 6$). Nifedipine was added to the bath 20 min. prior to the concentration-response protocol. ($n = 6$).

Fig 5. Effect of the TP-receptor agonist U46619 on $R_{stretch30}$ responses. $R_{stretch,30}$ responses were measured at each U46619 concentration (0.1 – 300 nM) under isovolumic conditions. The U46619 was added to the bath 30 min prior to each transmural pressure pulse. ($n = 6$). Nifedipine was added to the bath 20 min. prior to the concentration-response protocol. ($n = 6$).
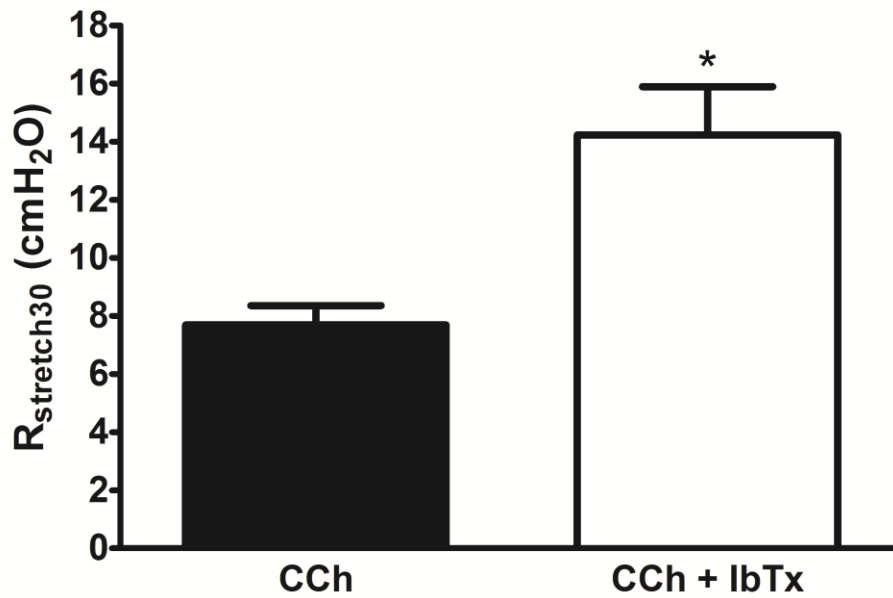
Fig 6. Effect of ryanodine receptor antagonism on $R_{stretch30}$ responses. Control tissues were pretreated with 10 nM CCh, while experimental tissues were treated with 10 nM CCh + 10 μM ryanodine. $R_{stretch,30}$ response was measured under isovolumic conditions. The CCh was added to the bath 10 min prior to the transmural pressure pulse. ($n = 6$). Ryanodine was added to the bath 30 min. prior to the transmural pressure pulse. ($n = 6$). *, P<0.05

Fig 7. Effect of the BK$_{Ca}$ channel opener iberiotoxin on R$_{stretch30}$ responses. Control tissues were pretreated with 10 nM CCh, while experimental tissues were treated with 10 nM CCh + 30 nM Iberiotoxin (IbTx). R$_{stretch,30}$ response was measured under isovolumic conditions. The CCh was added to the bath 10 min prior to the transmural pressure pulse. ($n = 6$). IbTx was added to the bath 30 min. prior to the transmural pressure pulse. ($n = 6$). *, P<0.05

Discussion

Numerous groups have published reports of stretch eliciting a contractile response in ASM (5; 17; 19; 29). However, to our knowledge, we are the first to have examined the signaling pathway involved in airway $R_{stretch}$ using perfused intact bronchial segments. Our previous publications have demonstrated that airway $R_{stretch}$ responses are dependent upon contractile machinery priming and the magnitude of airway stretch. Moreover, in intact bovine bronchi, these responses require input mediated by NKA acting through the NK_2-receptor (6), and can be amplified by excitatory prostanoids released from the epithelium acting though the TP-receptor (7). In this study, we further investigated the signaling mechanisms that regulate airway $R_{stretch}$ responses, while being the first to demonstrate similarities between these above mentioned responses and the well-know characteristics of the vascular myogenic response, by showing the involvement of membrane depolarization, L-type Ca^{2+} channels, CICR, and BK_{Ca} channel activation in airway $R_{stretch}$ responses, using a pharmacological approach.

In Figure 1A, we demonstrated the ability of a contractile agonist (CCh) to generate an $R_{stretch,30}$ response in a concentration-dependent manner, which peaked at 10 nM and decreased thereafter, suggesting that contractile machinery priming is in fact required for airway $R_{stretch}$ responses to occur. These responses occurred using submaximally-effective CCh concentrations, and decreased at higher concentrations due to the lower preload volume and higher contractile state of the airway. A more detailed description of this phenomenon can be found in

our previous publications (6; 7). In addition, we provided evidence for the robustness of our airway $R_{stretch,30}$ responses by showing that they do not wane with sequential transmural pressure pulses (Fig. 1B). In Figure 1D, we showed the inability of the L-type Ca^{2+} channel antagonist nifedipine to affect CCh-induced airway tone (R_{CCh}), consistent with previous findings from our laboratory outlining the independence of ASM agonist-induced excitation-contraction coupling on Ca^{2+}-influx through L-type Ca^{2+} channels (12; 13). Interestingly, in Figure 1C, we show the ability of nifedipine to significantly reduce airway $R_{stretch,30}$ responses in a concentration-dependent manner, demonstrating the requirement of L-type Ca^{2+} channel activation in airway $R_{stretch,30}$ responses but not in R_{CCh}.

In VSM, L-type Ca^{2+} channels play an active role in regulating vascular tone, keeping blood pressure within a physiological range, and protecting peripheral capillaries from pressure-induced damage by eliciting a myogenic response to vessel stretch. As such, L-type Ca^{2+} channel antagonists have been shown to be very useful in helping to control the unwanted tone present in pathological conditions such as hypertension (20). Interestingly, although ASM also ubiquitously expresses membrane-bound L-type Ca^{2+} channels, their role in agonist-induced excitation-contraction coupling remain ambiguous, due to its insensitivity to L-type Ca^{2+} channel blockers (4; 12; 13). Nonetheless, the prominence of L-type Ca^{2+} channels in ASM suggest they in fact play an

important role in this tissue, which according to our data may pertain to contractile responses to stretch.

Upon demonstrating the efficacy of nifedipine in significantly reducing airway $R_{stretch,30}$, we sought to further investigate the role of L-type Ca^{2+} channels in these responses by pretreating tissues with the selective L-type Ca^{2+} channel agonist BAY K8644. Figure 3 shows the ability of BAY K8644 to generate $R_{stretch,30}$ responses in a concentration-dependent manner, strengthening our hypothesis that $R_{stretch,30}$ responses indeed involves L-type Ca^{2+} channel activation. Thus, we looked to further demonstrate some of the similarities between the ASM $R_{stretch,30}$ and the vascular myogenic response.

Membrane depolarization, BK_{Ca} channel activation and CICR (through RyR activation) have all been implicated as important regulators of the vascular myogenic response (11). Therefore, in this study, we sought to investigate their relevance in the regulation of airway $R_{stretch,30}$ responses. In Figure 4, we showed the ability of KCl-induced membrane depolarization to generate $R_{stretch,30}$ responses in a concentration-dependent manner, a response that was abolished by nifedipine (3 μM) pretreatment, suggesting that the importance of membrane depolarization in the generation of airway $R_{stretch,30}$ responses lies in its role in activating L-type Ca^{2+} channels. In Figures 6 and 7, we demonstrated that the RyR antagonist ryanodine (10 μM) significantly reduced airway $R_{stretch,30}$ responses, whereas the BK_{Ca} channel antagonist IbTx (30 nM) significantly enhanced them. These data suggest that while RyR activation may help in the

generation of $R_{stretch,30}$ responses by increasing the cytosolic concentration of Ca^{2+} through its release from internal stores following Ca^{2+}-influx from L-type Ca^{2+} channels (CICR), BK_{Ca} channel activation may play a role in limiting the magnitude of these responses by hyperpolarizing the membrane.

In our two previous publications regarding the ASM $R_{stretch}$ response, which collectively act as the preamble to this study, we have shown that in intact bovine bronchial segments, $R_{stretch}$ responses are not completely intrinsic to the smooth muscle cell *per se* since they require input mediated by NKA binding to the plasmalemma-bound NK_2-receptor, and can be amplified by excitatory prostanoids released from the epithelium acting though membrane-bound TP-receptors (6; 7). We were surprised by the ability of three different classes of L-type Ca^{2+} channel antagonists (verapamil, diltiazem and nifedipine) to abolish $R_{stretch,30}$ (6). L-type Ca^{2+} channels have been well-characterized in ASM (4; 12); however, the electrophysiological and pharmacological properties of those channels are not consistent with an involvement in agonist-evoked responses (13). Nonetheless, our data clearly suggest that airway stretch-activated contractions may signal through a different pathway than agonist-evoked contractions due to their dependence on L-type Ca^{2+} channels. Thus, in this study, we also sought to further characterize the role of L-type Ca^{2+} channels in the signaling mechanism behind these responses.

In Figures 3 and 5, we showed the ability of NKA and U46619 to elicit $R_{stretch,30}$ responses in a concentration-dependent manner; responses which were

abolished by nifedipine (3 µM), suggesting that L-type Ca^{2+} channel activation occurs downstream of NK_2 and TP-receptors, both of which are classified as G-protein coupled receptors (GPCRs). Moreover, in Figure 2, we demonstrated the inability of the NK_2-receptor antagonist SR48968 to significantly affect BAY K8644-induced $R_{stretch,30}$ responses, further strengthening our hypothesis regarding the L-type Ca^{2+} channel activation occurring downstream of NK_2-receptor activation.

NKA-mediated excitatory postsynaptic potentials have been shown to be significantly reduced by voltage-gated Ca^{2+} channel antagonists in rat dorsal horn neurons (14). In addition, studies using single VSM cell electrophysiology protocols have shown potential interactions between voltage-dependent Ca^{2+} currents and GPCR activation (23; 25). Thus, together with the data obtained from our two previous publications pertaining to the ASM $R_{stretch,30}$ response, we propose that following an elevation in transmural pressure, the plasmalemma-bound postjunctional NK_2-receptor located on the smooth muscle mediates an airway stretch-activated contraction by interacting with and activating the L-type Ca^{2+} channel, subsequently causing CICR through activation of the RyR. These results highlight an alternative pathway for potential therapeutic targeting in asthmatic patients where a bronchoconstrictory response to a DI may play a role in airway hyperresponsiveness.

Acknowledgements

We would like to thank: Tracy Tazzeo, for obtaining the bovine lungs used in this study and Dr. Gerard Cox, for providing the Mayflower tissue bath.

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CHAPTER 5

Discussion

General Discussion

Asthma is an inflammatory airway disease characterized by exaggerated bronchoconstriction to neurotransmitters, inflammatory mediators, and other contractile stimuli (5; 6; 21; 58). In addition, asthmatic airways have also been shown to react differently to mechanical strain (39; 67; 91), most notably demonstrated by the difference between the effects of a DI in healthy individuals *versus* asthmatics (39; 50; 89; 91). As stated in **Chapter 1**, these deep inspiratory efforts have been shown to produce a bronchodilatory response in healthy individuals, whereas in asthmatics, they are less effective in producing bronchodilation, can cause more rapid airway re-narrowing, and can even cause bronchoconstriction in moderate to severe asthmatics (39; 54; 67; 89). Although this phenomenon has been observed and documented for over 30 years (39), the mechanisms behind it remain ambiguous, with numerous theories having been put forth. Marthan and Woolcock suggested in 1989, that DI-induced bronchoconstrictions may be caused by an ASM myogenic response (similar to that seen in VSM), produced by the conversion of airway smooth muscle from a 'multiunit' to 'single unit' entity (71). Other suggestions involved possible roles for increased ASM stiffness in asthmatics (3; 37), airway recoil pressures and the pressure-volume hysteresis curve (67), as well as airway inflammation and the release of inflammatory mediators (78) (as discussed in **Chapters 2 – 4**).

Thus, the objectives of this thesis were to investigate the signaling pathways involved in generating airway contractions in response to stretch

($R_{stretch}$), demonstrate the role of excitatory prostanoids in the regulation of these abovementioned responses, and compare the properties of $R_{stretch}$ in ASM with well-known properties of the vascular myogenic response. As such, we hypothesized that perfused intact bronchial segments produce a stretch-activated contraction upon transmural pressure loading which is regulated by: contractile machinery priming (by excitatory mediators), the magnitude of the transmural pressure load, and Ca^{2+} influx through L-type Ca^{2+}-channels.

Experimental Approach

We tested our hypothesis, by using a variety of pharmacological agents outlined in **Chapters 2 – 4** to selectively activate and/or block the signaling pathways in question. Moreover, the bulk of our experiments were conducted using the Mayflower apparatus (as shown in **Appendix 1**), which is a horizontal tissue bath that allowed for cannulation and pressurization of the intact bovine bronchial segment (as described in **Chapters 2 – 4**). The Mayflower apparatus was used (instead of isometric airway strips mounted vertically in a conventional tissue bath) because it allowed for the structural and geometrical integrity of the airway to be kept intact, in order to better simulate airway narrowing in the intact lung (77). In 1989 H.W. Mitchell *et al.* compared contractile data from isometric airway strips to that from perfused intact airway segments and demonstrated that as the airway narrows, the resulting change in circumference is not linearly related to the resistance within the airway. Moreover, they suggested that airway dimensions and geometry can make a particularly important contribution to responsiveness, since similar levels of muscle contraction could cause greater increases in resistance in bronchioles than in bronchi, because of their smaller initial diameter (77). They also showed that in small airways prepared cylindrically, contractile agonists were able to cause complete airway closure by a combination of smooth muscle shortening and by the consequent folding of the mucosal layer (74; 76). In contrast, isometric strips of airway tissue was shown to develop more than twice as much active force than is actually needed to cause

complete airway closure as seen by intact airway segments (75; 77). As a result, in smaller airways only the threshold or lower regions of the dose-response curve from experiments using isometric airway strips may be of physiological relevance. Therefore, it is important to take these airway properties into consideration when interpreting data regarding airway responsiveness using isometric strips and extrapolating them to the *in vivo* setting.

Investigation of Airway Stretch-Activated Contractions

Airways are subjected to mechanical stress with every inhalation-exhalation cycle we perform. As a result, numerous reports regarding the effects of stretch on ASM contraction have been published (42; 70; 78). Some groups have suggested that ASM $R_{stretch}$ is a myogenic event (71; 95; 101). In this study, we decided to test this theory by examining the pathway involved in generating airway $R_{stretch}$ responses using perfused intact bronchial segments.

In **Chapter 2**, we described the ability of perfused bovine bronchial segments to constrict in response to stretch, but only when pretreated with submaximally-effective, or even sub-threshold, concentrations of a contractile agonist (CCh or 5-HT). A stress relaxation response was observed in untreated tissues, possibly due to fluidization of the cytoskeleton during the stretch (62), and equilibration of the series and parallel elastic elements, although our experimental approach was not able to resolve this. In the presence of a contractile agonist, however, this stress relaxation response was followed by substantial force generation. We do not view this $R_{stretch}$ as being solely related to a change in the muscle's position on the length-tension curve, since it was seen even at concentrations of agonists which did not generate any tone on their own.

In fact, we showed that pretreatment with CCh or 5-HT, at concentrations which produce relatively little change in basal tension, can produce a fundamental change in ASM biophysical properties and elicit an $R_{stretch}$. Moreover, $R_{stretch}$ is only seen in the presence of an agonist. Others have also demonstrated an $R_{stretch}$

in ASM that required pretreatment with a pharmacological agent to prime the contractile apparatus, such as tetraethylammonium chloride, or a cholinergic agonist (95; 101). Interestingly, our experiments suggest the possibility that the $R_{stretch}$ phenomenon we observe in bovine bronchial segments may possess a neurogenic component.

Mechanotransduction is sometimes mediated by activation of sensory neurons (92). Also, neurogenic mechanisms can contribute to airway hyperresponsiveness (14; 61; 106). Airway sensory nerves (such as pulmonary C-fibers) relay sensory information to the central nervous system so that appropriate changes in the motor outputs may occur (8). The effects of stimulation of sensory nerves include various respiratory defense reflexes, such as cough and bronchoconstriction through the release of excitatory neuropeptides found within the nerve (18). Sensory nerve endings in the airways form specialized receptors shown originally by single-fiber recordings, performed *in vivo* in anaesthetized animals (17). A characteristic feature of C-fibers is their chemosensitivity and, in particular, their sensitivity to capsaicin, the principal pungent component of *Capsicum* peppers (34; 35; 105). Capsaicin acts by binding to and stimulating the specific membrane-bound receptor, the vanilloid VR1 receptor, now known to be a ligand-gated ion channel, and causing an excitatory depletion the neuropeptides contained within the C-fiber nerve ending (34; 36; 105). In addition to pulmonary C-fibers, neuroepithelial cell bodies, which are cells closely associated with non-myelinated nerves in the lungs are also a source of excitatory neuropeptides

within the lungs as they have been shown to contain SP, NKA, and CGRP (1; 104; 105). In this study, capsaicin-induced depletion of sensory nerve endings abolished the airway $R_{stretch}$ response suggesting the involvement of sensory neurons.

Of the numerous neurotransmitters found within airway sensory nerve terminals, SP and NKA have been shown to contribute to bronchoconstriction in asthmatics. In this study, we showed that NK_2-receptor (the receptor for NKA) blockade significantly decreased contractile responses, thus affirming a central role for these receptors (and for NKA) in airway $R_{stretch}$. These results are supported by a previous study that showed an NK_2-selectivity pertaining to bronchial hyperreactivity and suggested an importance for capsaicin-sensitive nerves in bronchoconstriction in mice (31). Another study found that NK_2 receptors played a predominant role in a guinea-pig model of mechanically-induced bronchoconstriction (20).

Given that mechanotransduction often involves Ca^{2+} influx (40; 44; 52; 53), we also probed the effect of various Ca^{2+} channel blockers on $R_{stretch,30}$. L-type Ca^{2+} channels have been well-characterized in ASM (41; 56), and have been implicated in the regulation of the vascular myogenic response (as described in **Chapters 1 and 4**); however, the electrophysiological and pharmacological properties of these channels are not consistent with an involvement in agonist-evoked responses (49; 57). In this study, we showed Ca^{2+} influx through L-type Ca^{2+} channels to be essential in the generation of $R_{stretch}$ responses.

Thus, our findings clearly suggest that airway stretch-activated contractions may signal through a different pathway than agonist-evoked contractions due to their dependence on L-type Ca^{2+} channels. Moreover, we have also shown the involvement of airway sensory C-fibers as well as NK_2 receptors in mediating these $R_{stretch}$ responses.

Regulation of $R_{stretch}$ Responses by TP-Receptor Activation

Experiments performed *in vitro* demonstrated that passive sensitization caused $R_{stretch}$ responses in human airways, suggesting a role for inflammatory mediators in priming the contractile apparatus to react excessively in the presence of mechanical stress. Among the numerous mediators released in asthmatic airways, prostanoids are both synthesized and released by bouts of airway inflammation as well as by mechanical stress (2; 85). Immunologic challenge of sensitized isolated perfused guinea pig lung, and mechanical stretch of rat lung epithelial cells *in vitro*, both stimulate prostanoid synthesis and release (19; 86).

In this study, we investigated the effects of the endogenous bronchoconstrictory prostanoids PGD_2, $PGF_{2\alpha}$, and TxA_2 on $R_{stretch}$ responses using a pharmacological approach in intact bovine bronchial segments. In addition, we provided evidence to suggest the involvement of airway epithelium-derived TxA_2 and p42/44 MAPK in the amplification of these $R_{stretch}$ responses. As prostanoids are not typically stored intracellularly after being synthesized, we investigated their role in airway $R_{stretch}$ by inhibiting COX, a key enzyme in the prostanoid synthesis pathway (51), present in the airways (96), and susceptible to inhibition by Indo (9), which significantly reduced the magnitude of $R_{stretch,30}$ without altering R_{CCh}, suggesting a role for excitatory prostanoids in $R_{stretch,30}$ independent of agonist-induced tone generation.

Upon demonstrating the efficacy of COX-inhibition in significantly reducing airway $R_{stretch,30}$, we sought to investigate the roles of selected prostanoid

receptors (DP, FP, and TP) in amplifying airway $R_{stretch,30}$ responses and showed

the inability of DP- or FP-receptor antagonism to alter the magnitude of $R_{stretch,30}$

responses, while TP-receptor antagonism significantly reduced these responses,

suggesting the involvement of TP-receptor activation in amplifying $R_{stretch,30}$.

Furthermore, the TP-receptor agonist U46619 generated $R_{stretch,30}$ responses in a

concentration-dependent manner, largely independent of R_{U46619}. Interestingly,

PGD_2 and $PGF_{2\alpha}$ have also been shown to exert their effects by binding to the TP-

receptor (28; 33; 65), reinforcing the importance of TP-receptor activation in

these $R_{stretch,30}$ responses. In **Chapter 2**, experiments were performed using tissues

bathed in Krebs buffer solution containing 10 µM Indo, which would have

completely inhibited COX and blocked prostanoid synthesis. Interestingly, $R_{stretch}$

responses were still elicited, suggesting that these $R_{stretch,30}$ responses were

comprised of the component that is TP receptor-independent. Conversely, in

Chapter 3, we used Indo-free Krebs solution and observed a significant increase

in the magnitude of $R_{stretch,30}$ compared to tissues treated with 10 µM Indo, which

we attributed to TP-receptor activation, suggesting that TP-receptor activation

leads to an amplification of $R_{stretch}$ responses but is not actually required for $R_{stretch}$

to occur.

Because of its potency as a bronchoconstrictor (approx. 2 times more

potent than other prostanoids) (7; 16; 26), and its increased concentration in the

BAL fluid of asthmatic patients (30; 69; 84; 85; 94), TxA_2 has attracted attention

as a potential important mediator in the pathophysiology of asthma. Here, we

showed a significantly increased release of TxB_2, the immediate and stable metabolite of TxA_2, following transmural pressure loading using a competitive EIA, demonstrating the ability of mechanical stretch to cause TxA_2 release from the airway, as previously shown in cultured rat lung epithelial cells (19).

Moreover, we show the ability of epithelial denudation to significantly reduce $R_{stretch,30}$ to similar levels as that done by COX-inhibition and TP-receptor antagonism, strengthening previous reports of the epithelium being a major source of prostanoid synthesis and release in response to mechanical stress (19; 26; 51; 82; 83; 85). Upon cellular stimulation, prostanoids are synthesized from arachidonic acid liberated from membrane phospholipids by the enzyme PLA_2 *via* a MAPK-dependent mechanism (19). Animal studies support that p42/p44 MAPK activation contributes to airway inflammation and hyperresponsiveness (29), and plays an essential role in stretch-induced prostanoid release from airway epithelium (19). In this study, we demonstrated the ability of a p42/p44 MAPK inhibitor to significantly reduce $R_{stretch,30}$ responses, showing a role for p42/p44 MAPK in $R_{stretch,30}$ responses. Thus, using our preparation, we suggest that the p42/p44 MAPK activation occurs at the airway epithelial level prior to TxA_2 synthesis and release, as shown in (19).

We therefore propose that airway $R_{stretch}$ may be amplified by bronchoconstrictory prostanoids, namely TxA_2, synthesized in a p42/p44 MAPK-dependent manner and released by the airway epithelium in response to stretch.

L-type Ca^{2+} channels and R$_{stretch}$

Chapters 2 and 3, which collectively act as the preamble to **Chapter 4**, have demonstrated that airway R$_{stretch}$ responses are dependent upon contractile machinery priming and the magnitude of airway stretch. Moreover, in intact bovine bronchi, these responses require input mediated by NKA acting through the NK$_2$-receptor, and can be amplified by excitatory prostanoids released from the epithelium acting though the TP-receptor. In this study, we further investigated the signaling mechanisms that regulate airway R$_{stretch}$ responses, while being the first to demonstrate similarities between these abovementioned responses and the vascular myogenic response, by showing the involvement of membrane depolarization, CICR, and BK$_{Ca}$ channel activation in airway R$_{stretch}$ responses, using a pharmacological approach.

In VSM, L-type Ca^{2+} channels play an active role in regulating vascular tone, keeping blood pressure within a physiological range, and protecting peripheral capillaries from pressure-induced damage by eliciting a myogenic response to vessel stretch (22; 23; 60). As such, L-type Ca^{2+} channel antagonists have been shown to be very useful in helping to control the unwanted tone present in pathological conditions such as hypertension (79). Interestingly, although ASM also ubiquitously expresses membrane-bound L-type Ca^{2+} channels (56; 102), their role in agonist-induced excitation-contraction coupling remain ambiguous, due to the insensitivity of the latter to L-type Ca^{2+} channel blockers (49; 57). Nonetheless, the prominence of L-type Ca^{2+} channels in ASM suggest they in fact

play an important role in this tissue, which according to our data may be the case in $R_{stretch}$ responses.

Upon demonstrating the efficacy of nifedipine in significantly reducing airway $R_{stretch,30}$, we sought to further investigate the role of L-type Ca^{2+} channels in these responses by pretreating tissues with the selective L-type Ca^{2+} channel agonist BAY K8644. In this study, we showed the ability of BAY K8644 to unmask $R_{stretch,30}$ responses in a concentration-dependent manner, strengthening our hypothesis that $R_{stretch,30}$ responses indeed involves L-type Ca^{2+} channel activation. Interestingly, in the airways, L-type Ca^{2+} channels have been visualized on the ASM as well as on other structural elements of the airway wall buried within the epithelial layer (24). Therefore, to investigate the location of these channels in our bovine tissue, airways were manually denuded of their epithelial layer (using the protocol outlined in **Chapter 3**) and the tissues were subjected to a concentration-response protocol using increasing concentrations of nifedipine, where an $R_{stretch,30}$ was elicited at each concentration of nifedipine added. As seen in **Appendix 2**, nifedipine still caused a reduction in airway $R_{stretch30}$ responses in a concentration dependent manner albeit starting from a lower baseline magnitude, ruling out the involvement of L-type Ca^{2+} channels on cells buried within the epithelial layer. Next, we looked to further demonstrate some of the similarities between the airway $R_{stretch,30}$ and the vascular myogenic response.

Membrane depolarization (45; 48), CICR (through RyR activation) (32; 55), and BK_{Ca} channel activation (12; 47; 55) have all been implicated as important regulators of the vascular myogenic response. Therefore, in this study, we sought to investigate their relevance in the regulation of airway $R_{stretch,30}$ responses. Here, we showed the ability of KCl-induced membrane depolarization to unmask $R_{stretch,30}$ responses in a concentration-dependent manner, a response that was abolished by nifedipine pretreatment, suggesting that the importance of membrane depolarization in the generation of airway $R_{stretch,30}$ responses lies in its role in activating L-type Ca^{2+} channels. Interestingly, changes in membrane potential may play a role in modulating Ca^{2+} sensitization by directly activating RhoA leading to the downstream inhibition of MLCP (although this pathway does not seem to play a major role in the generation of airway $R_{stretch}$ since our responses were abolished by nifedipine pretreatment). Some groups have reported that voltage-gated Ca^{2+} influx may mediate this effect through the activation of calmodulin or protein kinase (PK) C, whereas others have suggested that depolarization alone may inhibit MLCP through a RhoA-dependent pathway (13; 59; 68; 73; 88; 103). This phenomenon has been shown to occur in ASM, however the exact mechanism by which this occurs remains unknown.

Additionally, in this study, we demonstrated that the RyR antagonist ryanodine significantly reduced airway $R_{stretch,30}$ responses, whereas the BK_{Ca} channel antagonist IbTx significantly enhanced them. These data suggest that while RyR activation may help in the generation of $R_{stretch,30}$ responses by

increasing the cytosolic concentration of Ca^{2+} through its release from internal stores following Ca^{2+}-influx from L-type Ca^{2+} channels (CICR), BK_{Ca} channel activation may play a role in limiting the magnitude of these responses by extruding K^+ into the extracellular space and thus lowering the membrane potential.

NKA and U46619 were also shown to elicit $R_{stretch,30}$ responses in a concentration-dependent manner; responses which were abolished by nifedipine, suggesting that L-type Ca^{2+} channel activation occurs downstream of NK_2 and TP-receptors, both of which are classified as GPCRs. Moreover, we demonstrated the inability of the NK_2-receptor antagonist SR48968 to significantly affect BAY K8644-induced $R_{stretch,30}$ responses, further strengthening our hypothesis regarding the L-type Ca^{2+} channel activation occurring downstream of NK_2-receptor activation.

NKA-mediated excitatory postsynaptic potentials have been shown to be significantly reduced by voltage-gated Ca^{2+} channel antagonists in rat dorsal horn neurons (66). In addition, numerous studies using single cell electrophysiology protocols in VSM and ASM have shown potential interactions between voltage-dependent Ca^{2+} currents and contractile agonist activation of GPCRs (87; 90; 102).

Thus, together with the data obtained from our two previous publications pertaining to the ASM $R_{stretch,30}$ response, we propose that following an elevation in transmural pressure in ASM tissue that possesses submaximal baseline tone,

the membrane-bound postjunctional NK_2-receptors located on the smooth muscle mediate an airway $R_{stretch}$ response by interacting with and activating L-type Ca^{2+} channels, allowing an increase in their open-state probability either directly or *via* a second messenger-coupling pathway, subsequently causing CICR through the activation of RyRs. Moreover, as shown in **Chapters 3** and **4**, the magnitude of the $R_{stretch}$ response can be amplified by TP-receptor activation and limited by BK_{Ca} activation, respectively (**Appendix 3**).

Study Limitations

The assessment of airway responsiveness *via* the intact perfused bronchial segment technique requires the intact airway to be harvested, manipulated, dissected, and mounted onto the tissue bath, while maintaining physiological normalcy. The actions of the investigator must be such that harm to the airway is minimized. A proper dissection of the bovine airway is possible, allowing for the measurement of airway contraction and relaxation using chemical stimuli, stretch or EFS. However, the dissection process for the isolated airway segment is not perfect, and tissue responses may not be suitable for analysis due to the destruction of the airway during dissection. Moreover, the dissection may lead to a leak in the tissue, making isovolumic analysis of the airway impossible. To assess these limitations, following tissue equilibration, each airway was stimulated by EFS and 60mM KCl to establish viability, and underwent a pressure test to ensure that there were no leaks present.

Pharmacological blockers and agonists play a vital role in the determination of the receptors and signal transduction pathways involved in stretch-induced ASM contractions. However, although pharmacological blockers are selective for certain receptors, many are not specific and may be able to interact with other receptors. Therefore, in order to decrease the occurrence of this phenomenon, we were careful to not use excessive concentrations of pharmacological blockers in order to ensure ourselves that the effect of the blocker is strictly due to binding to its selective receptor. Moreover,

concentration-response experiments were performed to ensure that the effect of the blocker is in fact concentration-dependent.

Although the Mayflower tissue bath proved to be useful for measuring ASM $R_{stretch}$ responses while keeping the structural and geometrical integrity of the airway segment intact, it did possess a significant limitation: the isovolumic setup used for this project did not allow for any measurements of ASM length changes during experimental protocols. Unlike striated muscle, maximal force generation in ASM is associated with a very broad length change due to its extensive length-adaptation capabilities. This length-adaptation may be triggered by mechanical strain (i.e. transmural pressure variations) (11), as performed throughout this project. Moreover, prolonged exposure to contractile agonists enables ASM (held at constant length) to exhibit force-adaptation properties, shown by its ability to generate a contractile force beyond the maximal level previously generated (at constant length). This has been suggested to occur by a cytoskeletal rearrangement process that allows the non-tension generating elements of the cytoskeleton to bear force, thus freeing up actin and myosin to generate more force (10). These mechanisms for ASM force generation in response to mechanical stress, and the resulting length-tension relationship have been extensively studied in the past (25; 38; 93; 97), and may provide additional insights into fully characterizing the ASM $R_{stretch}$ response. Unfortunately, the experimental setup for this project did not permit any measurement of ASM

length changes, and thus did not allow the author of this thesis to speculate where the tissue lies on the length-tension curve during the various protocols performed.

We have thus far shown that *ex vivo*, a quick stretch of the airway (due to an increase in transmural pressure), is able to evoke a stretch-induced contraction similar to that seen in the Bayliss myogenic response. However, it is important to acknowledge that our experiments were performed on an animal model (bovine airways). Although, the bovine airway is a robust model for studying the structure and function of human airways, we must remember that variations between the structure and function of human airways and that from other species still exists. Thus, caution was exercised when interpreting results from our experiments in order to not extrapolate their significance too far.

Lastly, DI-induced bronchoconstriction is an abnormal phenomenon in humans, since it is only seen in moderate to severe asthmatics. Our bovine bronchial segments were not inflamed nor exhibited spontaneous tone, and did not manifest a stretch-induced contraction until they were pretreated with a contractile agonist, used to mimic the increased ASM tone seen in asthmatic airways. Previous studies have also demonstrated an $R_{stretch}$ in ASM that required pretreatment with a pharmacological agent to prime the contractile apparatus, such as tetraethylammonium chloride, or a cholinergic agonist (95; 101). Although others (4; 43; 81) have observed that stretch caused reductions in airway responses to cholinergic stimulation in canine and porcine bronchi, contrasting reports have shown both a lack of stretch-induced relaxation as well as

constriction in intact bovine bronchi (46; 63). Although differences in experimental protocols exist between reports, questions have been raised as to whether these differences may be species-related, where bovine ASM is unique in its response to mechanical stretch by behaving more like the asthmatic phenotype (80). These discrepancies may also be attributed to properties of different regions in the airway tree, where $R_{stretch}$ may be more significant in small resistance airways compared to larger airways.

Conclusions and Future Directions

Using a pharmacological approach and the Mayflower tissue bath, the global objective of this thesis was to investigate the effect of an acute transmural pressure load on perfused intact bovine bronchi and to characterize the resulting response. Following the completion of this research project, we are able to conclude that an acute transmural pressure load is indeed able to alter the contractile state of bovine bronchial segments by eliciting an $R_{stretch}$ response that possesses neurogenic characteristics due to the involvement of C-fibers and the release of the neuropeptide NKA. Furthermore, our data provided evidence suggesting a role for excitatory prostanoids in amplifying these responses through the activation of the TP-receptor. Lastly, we demonstrated several similarities between the airway $R_{stretch}$ response and the vascular myogenic response by outlining the importance of Ca^{2+} influx through L-type Ca^{2+} channels as well as the involvement of CICR and BK_{Ca} channels in these responses.

However, a complete understanding of a pathophysiological mechanism can only be accomplished by comprehending the relationships between both the structural and functional aspects of the phenomenon in question. Through our use of pharmacological agents and the Mayflower apparatus, the studies outlined in this thesis were able to characterize the functional aspects of airway $R_{stretch}$ responses by using ASM contraction as the primary outcome measurement. However, in order to paint a clearer picture of the signaling pathways involved in regulating airway $R_{stretch}$ responses, our functional measurements may be

combined with molecular, imaging, and histological techniques that will allow for the visualization of the structural aspects of ASM $R_{stretch}$ responses, such as the location of the C-fibers in question, and their proximity to both the ASM and the epithelium (using immunohistochemistry), the identity of the mechanosensor involved in eliciting $R_{stretch}$ responses (by western blot), as well as further characterization of the stretch-induced Ca^{2+} mobilization in ASM (using Ca^{2+} fluorimetry).

Medical research is an ongoing process that exists to challenge the existing dogma in attempts to improve the quality of life of those afflicted by illness. However, to improve human health, scientific discoveries must be translated into practical applications.

Cigarette smoking is a major risk factor in the development of airway hyperresponsiveness and COPD (98). The chemicals and pollutants present in cigarettes directly affect airway smooth muscle contractility leading to an increase in the severity of asthma symptoms (100). Cigarette smoking can also cause acute bronchoconstriction and an increased frequency of exacerbations, as demonstrated by the higher number of emergency department visits as a result of exacerbations among cigarette smoking asthmatics (18; 20). Moreover, Tashkin *et al.* showed a dose-dependent effect of cigarette smoking on airway responsiveness (99), which has now been suggested to involve RhoA-mediated Ca^{2+} sensitization (15), increased release of thromboxane A_2 (TxA_2) (72), sensory afferent neural activation (64), reduced neutral endopeptidase activity (27), and subsequently,

elevated NKA concentration in BALF (107). Unfortunately, little is known about the effect of cigarette smoking on airway responses to stretch, which can cause a bronchoconstriction in certain individuals with airway hyperresponsiveness. Interestingly, many of the hallmarks associated with the pathologic asthmatic airway that predispose it to bronchoconstrict in response to a DI can also be artificially induced or worsened by cigarette smoke exposure. This may explain the abovementioned finding of worsening severity of symptoms in asthmatic smokers. Thus, an investigation into the yet unexplored effect of cigarette smoke exposure on ASM contractile responses to stretch will provide insight into the mechanisms involved in $R_{stretch}$ responses following cigarette smoke exposure and may highlight an alternative pathway for potential therapeutic targeting in asthmatic smokers who experience acute bronchoconstriction and an increase in the severity of asthma symptoms.

Lastly, translational research typically begins in the laboratory ("the bench") before progressing to the clinical level (the patient's "bedside"). However, scientists are increasingly aware that this bench-to-bedside approach to translational research is really a two-way street. Basic scientists provide clinicians with new tools for use to treat patients in the clinic, and clinical researchers make novel observations about the characteristics of a disease that often stimulate basic investigations.

In **Chapter 3**, we showed the ability of excitatory prostanoids to amplify airway $R_{stretch}$ responses and TP-receptor activation to be the major pathway

involved. Moreover, the TP-receptor antagonist BAY u3405 (Seratrodast) has been shown to be efficacious in treating airway hyperresponsiveness in some populations. Our experimental design demonstrates a potential role for TP-receptor activation in DI-induced bronchoconstriction, which has previously not been shown. Therefore, we propose that prior to the dismissal of the TP-receptor antagonist as an adequate treatment for asthmatics, perhaps studies focused on asthmatic patients with DI-induced bronchoconstriction in particular should be conducted, as this will plant the seed for future experiments aimed at developing individualized treatments for asthmatic patients.
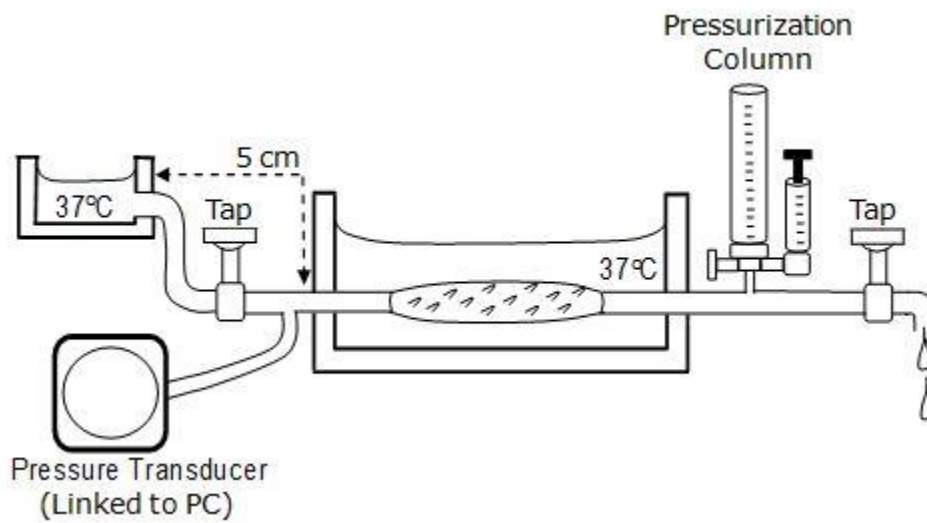
Appendix 1. Illustration of the Mayflower apparatus (simplified).
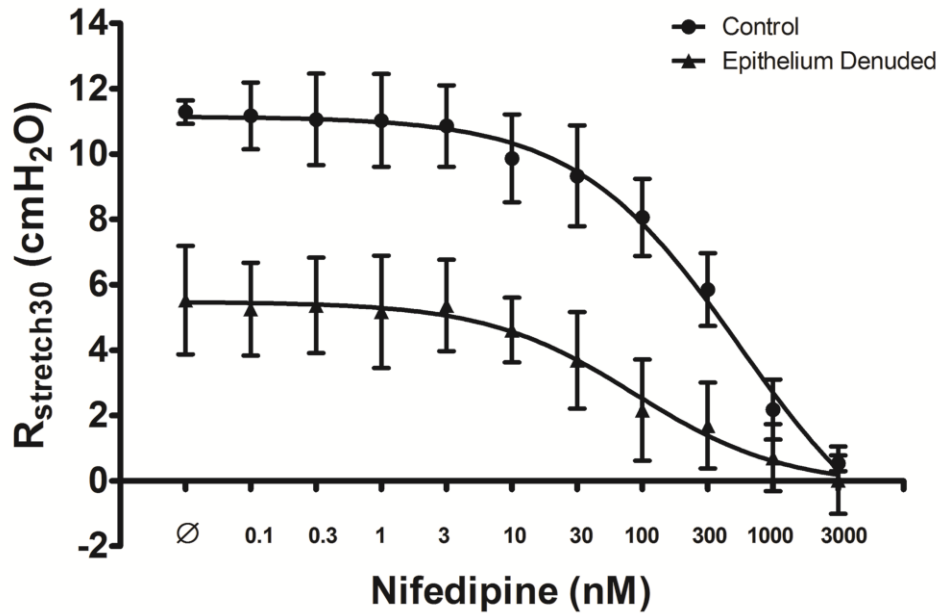
Appendix 2. Effect of Nifedipine and epithelial denudation on CCh-induced $R_{stretch30}$ responses. Effect of increasing concentrations of nifedipine (0.1 – 3000 nM) on CCh-induced $R_{stretch,30}$ responses. Each concentration of nifedipine was added to the bath 20 min. prior to each transmural pressure pulse. Baseline responses were established in the presence of 10 nM CCh. (Control: $n=6$; Denuded: $n=5$).
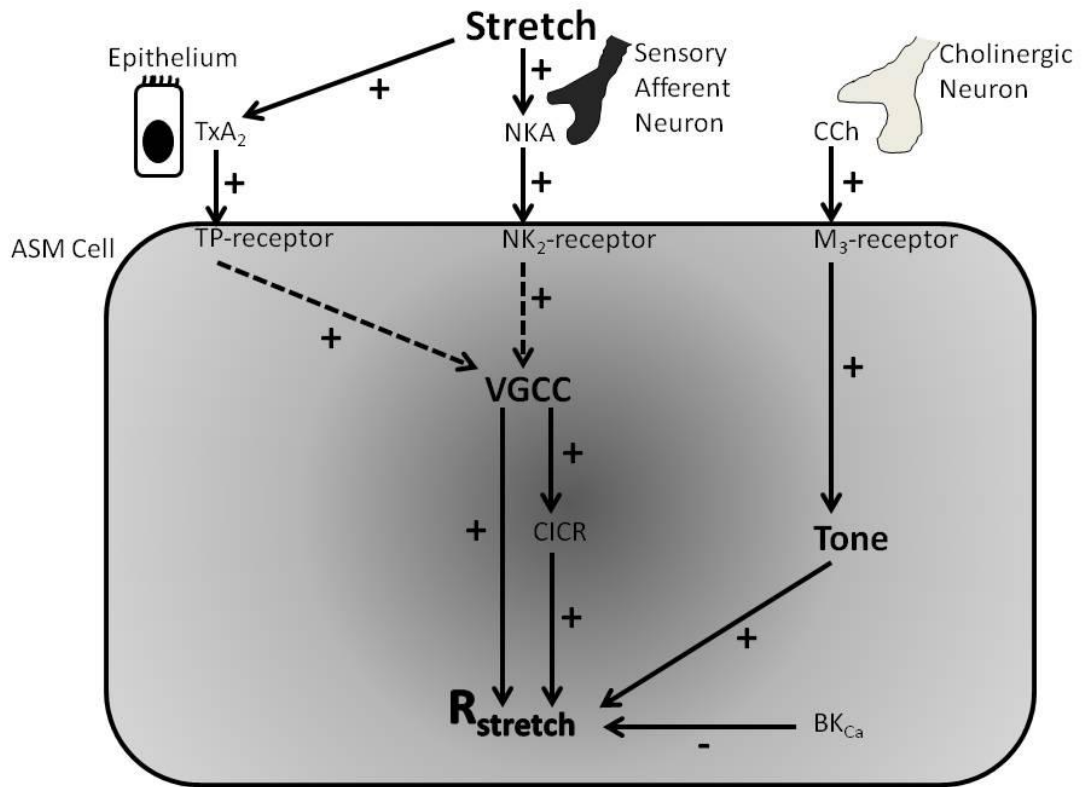
Appendix 3. Illustration depicting the ASM R$_{stretch}$ signaling pathway. (Dashed lines represent direct or indirect activation/augmentation of the voltage-gated Ca^{2+} channel (VGCC).)

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September 21, 2011

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Dear Mr. Hernandez:

This is to grant you permission to include the following articles in your thesis entitled, "Aspects of Airway Stretch-Activated Contractions Assessed in Bovine Bronchial Segments" for McMaster University:

Jeremy Mark Hernandez, Gerard Cox, and Luke Jeffrey Janssen, Involvement of the Neurokinin-2 Receptor in Airway Smooth Muscle Stretch-Activated Contractions Assessed in Perfused Intact Bovine Bronchial Segments, *J Pharmacol Exp Ther* November 2008 327:503-510

Jeremy Mark Hernandez and Luke Jeffrey Janssen, TP-receptor activation amplifies airway stretch-activated contractions assessed in perfused intact bovine bronchial segments, *J Pharmacol Exp Ther* July 18, 2011 jpet.111.182246; published ahead of print July 18, 2011, doi:10.1124/jpet.111.182246

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