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Heterogeneity of Airway Smooth Muscle Cells and Its Implications in Pathogenesis of Asthma

by

XUEFEI MA

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

Department of Physiology, Faculty of Medicine

University of Manitoba

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HETEROGENEITY OF AIRWAY SMOOTH MUSCLE CELLS AND ITS IMPLICATIONS IN PATHOGENESIS OF ASTHMA

BY

XUEFEI MA

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

of Manitoba in partial fulfillment of the requirements of the degree

of

DOCTOR OF PHILOSOPHY

Xuefei Ma ©1998

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ABSTRACT

The greatest importance of airway smooth muscle research is perhaps its application to the elucidation of the pathogenesis of allergic bronchospasm. Excessive shortening of airway smooth muscle plays an important role in the asthmatic attack. Almost all asthmatic attack occurs in central and/or peripheral bronchi, however, most studies of airway smooth muscle focus on the trachea because of difficulty in obtaining suitable bronchial samples. It has been generally believed that airway smooth muscles from trachea and bronchus are similar in their mechanical and biochemical properties and therefore one can be used as a model for the other. Whether this is true or not has never been proved. Rapidly accumulating data indicating the presence of heterogeneity of smooth muscle imply that that may not be true. Marked diversity in biophysical and biochemical properties of vascular smooth muscle has been demonstrated recently both at tissue and cellular levels. No corresponding studies have yet been reported in airway smooth muscle. The present project commenced with mechanical studies of airway smooth muscles from different generations of the canine airways. Two biophysically distinct groups of airway smooth muscle were detected along the canine ariway tree: an extrapulmonary group, including airway smooth muscles from trachea and bronchial generations 1 and 2, displayed relatively high contractility, and an intrapulmonary group, consisting of muscles from bronchial generation 3 to 6, showed a relatively low contractility. Enhanced expression of smooth muscle myosin light chain kinase and N-terminal isoform of smooth muscle myosin heavy chain in extrapulmonary airway smooth

muscle could probably contribute to the enhanced contactiltity of extrapulmonary muscle. At the single cell level, three morphologically, biophysically, and biochemically different types of contractile smooth muscle cells were identified in airway smooth muscle. Significant differences in proportions of these cells were detected between extra- and intrapulmonary airway smooth muscles. Increased numbers of cells with decreased contractility in intrapulmonary muscles might contribute to their reduced contractility compared with extrapulmonary muscles. Heterogeneous alterations in shortening properties were also observed in sensitized airway smooth muscle at tissue and single cell levels, these in turn may contribute to the non-homogeneous bronchospasm observed in asthmatic airways. Heterogeneity was also displayed in cultured tracheal smooth muscle cells subjected to prolonged serum deprivation. Typical patterns of hill-and-valley disappeared in serum-deprived smooth muscle cell in culture, instead, two phenotypically distinct populations of cells developed: 1. Elongated spindle-shaped cells, which expressed large amounts of smooth muscle contractile and regulatory proteins, usually formed bundles, and showed a surprising shortening capacity and velocity which was even greater than that of the freshly isolated cells; 2. Flat and stellate cells, which expressed very few smooth muscle contractile proteins and showed no contraction. A considerable increase of smooth muscle light chain kinase was detected in the newly developed super-contractile cells, but the myosin heavy chain N-terminal isoform which is believed to be responsible for the higher shortening velocity was not detected. Finally the shortening properties of human asthmatic airway smooth muscle were evaluated in single cells isolated from endobronchoscopic biopsies. Significantly increased shortening capacity and velocity were

found in asthmatic cells, which unraveled the long-lasting puzzle of whether the asthmatic airway smooth muscle possesses altered contractility. In general these systematic studies demonstrated a clear heterogeneity of airway smooth muscle (cells) with respect to structural, biophysical and biochemical properties; this was of pathophysiological importance. This is the first demonstration of heterogeneity of contractile airway smooth muscle cells. Establishment of *in vitro* smooth muscle cell culture which enables development of contractile smooth muscle cells may provide an important model for studying smooth muscle development and contraction.

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List of Abbreviations

ΔLmax	maximal capacity of shortening
Ach	acetylcholine
ASMC	airway smooth muscle cells
ASM	airway smooth muscle
BSM	bronchial smooth muscle
CaD	caldesmon
CaP	calponin
EDTA	ethylenediaminetetraacetic acid
kDa	kilodalton
Lo	optimal length of muscle for contraction
Lcell	relaxed cell length
MLC_{20}	regulatory, 20 kDa, smooth muscle myosin light chain
MLCK	myosin light chain kinase
MHC	myosin heavy chain
NM	non-muscle
PAGE	polyacrylamide gel electrophoresis
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
Po	isometric tension

Pre	resting tension
SDS	sodium dodecyl sulphate
SM1	204 kDa smooth muscle myosin heavy chain
SM2	200 kDa smooth muscle myosin heavy chain
SM	smooth muscle
SM-B	smooth muscle myosin heavy chain isoform with a 7-amino acid insert in it N-terminal head region
SMC	smooth muscle cells
TSM	tracheal smooth muscle
Vo	maximal velocity of shortening
V2	maximal velocity of shortening at 2 second
V8	maximal velocity of shortening at 8 second

Chapter 1. Introduction and Literature Review

1.1 Introduction

Because of the difficulty in obtaining suitable bronchial samples, most studies of airway smooth muscle focus on the trachea. It has been generally believed that airway smooth muscles from trachea and bronchus are similar in their mechanical and biochemical properties and therefore one can be used as a model for the other. Whether this is true or not has never been proven. Rapidly accumulating data indicating smooth muscle heterogeneity imples that this may not be true. Significant differences in contractility (defined as the maximal contractile properties in this thesis) has been identified in vascular smooth muscles from different segments of vessels (DiSanto et al., 1997). Also, recent reports have demonstrated that a single 1 mm wide smooth muscle strip consists of different types of cells, each type contributing to a distinct function, physiologically and/or pathologically (Archer, 1996; Chacko, 1996; Meer and Eddinger, 1996; Frid et al., 1994; Wohrley et al., 1995). Furthermore, smooth muscle isolated from different vessel segments consists of different types of cells resulting in their different responses to identical stimuli (Archer et al., 1996). In airway smooth muscle, no corresponding studies have been reported.

Although heterogeneity in the biophysical and biochemical properties of airway smooth muscle has never been demonstrated, differences in innervation and receptor distribution along the airway tree have been well established (Barnes et al., 1983; Grandordy et al., 1986; Nadel et al., 1971). In addition, non-homogeneous distributions

of bronchoconstriction during an acute asthma attack (McFadden et al., 1977; Takishima et al., 1991) and development of irregular smooth muscle hypertrophy and hyperplasia along chronic asthmatic airways have been well documented (Dunnill et al., 1969; Ebina et al., 1993). Structural heterogeneity of the airway tree is an outstanding feature. In the trachea, two-thirds of its perimeter consists of intact stiff cartilage rings, and smooth muscle directly attached to the cartilage, spans the remaining one-third. Therefore smooth muscle contraction is limited by the high resistance of the stiff cartilaginous rings. In the bronchus, the cartilage does not form intact rings; instead, it exists as separate soft cartilage plaques distributed around the lumen. An internal ring of smooth muscle surrounds the entire lumen and connects with superjacent cartilage plaques through soft connective tissue. Its contraction therefore is only limited slightly by these plaques. Lung parenchyma and cartilage may not provide significant resistance to muscle shortening. In fact, considerable narrowing of the the bronchial lumen may be achieved during smooth muscle contraction without any obvious cartilage movement (Iizuka et al., 1992; Mitchell and Sparrow, 1994). The structural heterogeneity of the airway tree implies that effective regulation of the airway lumen may require the presence of biophysically distinct types of smooth muscles along the tree, without which, improper regulation of airway lumen would occur *in vivo*. Therefore it is highly likely that heterogeneous airway smooth muscle exist along the airway tree, and that this heterogeneity is also important to normal airway functioning.

Perhaps the greatest importance of airway smooth muscle research is its application

to elucidate the pathogenesis of the asthmatic bronchospasm. Although the question of whether the intrinsic contractility of airway smooth muscle change in asthmatic patients has not been answered, altered contractility has been identified in ragweed pollen-sensitized canine airway smooth muscle (Jiang et al., 1992). Furthermore, bronchospasm resulting from increased airway smooth muscle shortening is a hallmark of asthma. Heterogeneous bronchospasm locations in asthmatic attacks have also been observed. For example, the asthmatic attack predominates in central (early asthmatic response) or peripheral bronchi (late asthmatic response), but rarely in the trachea (Metzger et al., 1985). Unfortunately, whether tracheal smooth muscle behaviors the same as bronchous muscle has never been proved, current information about airway smooth muscle obtained from studies of tracheal smooth muscle can not be applied to understanding disorders of bronchial muscle function. What is urgently needed, therefore, is to conduct studies to determine whether tracheal and bronchial smooth muscle cells are heterogeneous. This is an important objective since it is common practice to use the former as a model for the latter. Examining heterogeneity of airway smooth muscle at the tissue and cellular levels will facilitate, at the subcellular level, elucidation of its function, and, of its pathogenic role in asthma.

Precent studies of smooth muscle cell heterogeneity have mainly focused on their morphological, biochemical and proliferative characteristics. However problems arising from their heterogeneity have not been addressed in the previuos studies. Different smooth muscle phenotypes may exist *in vivo*. Smooth muscle cells are particularly prone to

heterogeneity because unlike skeletal muscle cells they are not terminally differentiated and can be affected by environmental stimuli. Broadly, smooth muscle cells may be divided into two types: contractile and non-contractile (the latter have been also termed secretory or synthetic). In primary culture, smooth muscle cells manifest a transition from a spindle-shaped contractile phenotype to a circular, poorly contractile, synthetic phenotype (Schwartz et al., 1986; Campbell and Campbell, 1993). Contractile cells are poorly proliferative, while synthetic cells are highly proliferative. Both contractile and synthetic types of smooth muscle cells exist *in vivo*. However, the primary function of mature smooth muscle cells *in vivo* is believed to be contraction. Studies from smooth muscle strips demonstrate that smooth muscles from different organs or even from different locations in the same organ, show significantly different contractile behaviors (Horiuti et al., 1989). Whether the intrinsic contractility of individual smooth muscle cells vary intercellularly has never been demonstrated. Investigation of this phenomenon is one of the major objectives of this project.

Smooth muscle contraction is regulated by the expression of precise amounts of smooth muscle contractile and regulatory proteins. Smooth muscle contraction is primarily initiated and regulated by phosphorylation of the regulatory, 20 kDa molecular weight, smooth muscle myosin light chain (MLC_{20}) by the key regulatory enzyme, smooth muscle myosin light chain kinase (smMLCK) (Kamm and Stull, 1985; Somlyo and Somlyo, 1994). Unlike skeletal muscle, the myosin ATPase activity and contractile properties of smooth muscle are positively correlated to the level of MLC_{20} phosphorylation. Currently,

four isoenzymes of smooth muscle myosin heavy chain (smMHC) have been detected. The most recently identified (SM-B), expresses a 7 amino acid insert in its N-terminal head region and has been shown an increased ATPase activity and velocity of actin filament movement *in vitro* when compared to those without the insert (Kelley et al., 1993; Babij, 1993; White, 1993). Expression of this isoenzyme is believed to be important in determining the contractility of smooth muscle. Other proteins such as caldesmon and calponin are also believed to regulate the contractile properties of smooth muscle. Therefore, heterogeneous smooth muscle expression of the contractile and regulatory proteins may contribute to the mechanical heterogeneity of airway smooth muscles.

The present project was designed to delineate the biophysical and biochemical heterogeneity of airway smooth muscle both at tissue and cellular levels and to determine the underlying mechanism of that heterogeneity. As the primary function of airway smooth muscle is contraction, our study began by investigating contractility of smooth muscle strips obtained from different generations of canine airways. This was followed by protein analysis of muscle strip homogenates to examine the biochemical heterogeneity of airway smooth muscles. This cellular heterogeneity was analyzed in freshly-isolated airway smooth muscle cells with respect to both biophysical and biochemical properties. To determine the significance of airway smooth muscle heterogeneity in the pathogenesis of asthma, the heterogeneity of pathogenic responses of airway smooth muscle was evaluated in ragweed pollen-sensitized canine airways both at the tissue and cellular levels.

Apart from contraction, smooth muscle cells are also capable of proliferation. A number of studies have been carried out investigating the proliferative properties of smooth muscle cells. Enhanced proliferation of smooth muscle cells has been observed in hypertensive vascular walls and also in asthmatic airways, implying its importance in the pathogenesis of these diseases. Because of the unavailability of *in vitro* models demonstrating smooth muscle cell differentiation, very little is known about the development and differentiation of smooth muscle cells. To investigate the characteristics of smooth muscle development and differentiation, an *in vitro* smooth muscle cell culture system was developed, in which cultured dedifferentiated smooth muscle cells were induced to differentiate. This model allowed heterogeneity in biophysical and biochemical properties of these cells to be analyzed.

To further confirm the clinical importance of airway smooth muscle study, the contractility of asthmatic human bronchial smooth muscle cells was evaluated in single cells isolated from endobronchoscopic biopsies of airway smooth muscle obtained from living subjects. As stated above, the contractility of asthmatic airway smooth muscle has never been demonstrated. This is mainly because of the limitations of obtaining suitable airway smooth muscle samples from living asthmatic patients for conventional mechanical measurement at the tissue level. The current understanding of the pathogenesis of asthma is that it predominately consists of an allergic reaction, chronic airway inflammation and increases in cell growth. Mediators, such as histamine from allergic reactions, and leukotrienes arising from inflammatory reaction, are released and stimulate airway smooth

muscle contraction resulting in a reduction in airway lumen diameter. The importance of inflammation in the pathogenesis of asthma is further supported by successful clinical application of corticosteroids in controlling asthma attacks. In spite of increased efforts to elucidate the pathogenesis of asthma, and to develop new treatments, the occurrence and mortality of asthma are progressively increasing. Due to the fact that various stimuli induce an allergic asthmatic attack, it seems likely that the common effector lies in the peripheral effector organ, viz, the smooth muscle. Therefore delineation of airway smooth muscle contractility could elucidate the pathogenesis of asthma, and development of more effective therapeutic measures.

1.2 Heterogeneity of Smooth Muscle

Smooth muscle comprises the major component of the media layer of hollow organs. The primary function of mature smooth muscle is believed to be contraction, i.e. generation of force and shortening, and the consequent regulation of wall tension and lumen area of hollow organs. In addition, smooth muscle cells are also capable of synthesizing and releasing extracellular matrix proteins and some growth factors, and of proliferating in response to mitogens. Furthermore, the major pathologic changes in the arterial wall, atherosclerotic lesions, and asthmatic airways, are characterized by great structural (Haust 1971; Carroll et al., 1993) and biochemical (Katz et al. 1976) diversities possibly reflecting different mechanisms of their formation.

Great contributions to the understanding of the regulation of smooth muscle contraction was obtained from studies of vascular smooth muscle strips, while the biochemical properties of smooth muscle were studied mainly in chicken gizzard. These tissues were chosen purely as a matter of convenience. Therefore, many assumptions have to be made when applying these findings to airways. Some of these are as follows: smooth muscle consists of a homogeneous population of smooth muscle cells, no significant differences exist within smooth muscles obtained from different origins. However, recently accumulating evidence shows that phenotypically distinct populations of smooth muscle cells exist in smooth muscle, each possessing unique physiological and pathological roles. In addition, smooth muscles from different organs, different species,

or even from different sites of the same organs show significant differences in their mechanical and biochemical properties. The following review discusses the recent advances in this new and exciting area of smooth muscle research, to wit, heterogeneity of smooth muscle.

1.2.1 Cell-to-cell heterogeneity

Smooth muscle consists of heterogeneous populations of smooth muscle cells. Morphological and functionally distinct populations of smooth muscle cells were identified in various types of smooth muscle. Unlike skeletal muscle, smooth muscle cells are highly plastic. They are not known to terminally differentiate and are thus capable of phenotypic modulation. Phenotypes of smooth muscle cells, can therefore be influenced by environmental factors. In primary culture, smooth muscle cells undergo phenotypic modulation from contractile type to non-contractile type. Therefore, with regard to degree of differentiation, smooth muscle cells can be broadly divided into two populations: contractile and non-contractile (also called secretory or synthetic) (Campbell and Campbell, 1981), although some intermediate phenotypes may also exist. The vast majority of cells in mature smooth muscle are of the contractile type. The primary function of these cells is contraction. Their cytoplasm is filled with thick and thin myofilaments (Campbell et al., 1988; Devine and Somlyo, 1971; Chamley-Campbell et al., 1979). Non-contractile cells are actively engaged in synthesis of extracellular matrix

proteins and/or cell division; these cells contain few myofilaments but large amounts of free ribosomes, rough endoplasmic reticulum and mitochondria (Chamley-Campbell et al., 1979; Imai et al. 1970). Using the 200 kDa myosin heavy chain (SM2) as an index of smooth muscle maturation, Sartore et al. (1997) reported that both "immature" and fully differentiated smooth muscle cells were identified in adult arterial vessels. These immature smooth muscle cells show great potential for proliferation and migration in experimental models and in human vascular disease. The pattern of cell types obtained from developing vessels or those undergoing regenerative response to injury show, however, more pronounced phenotypic diversity. Smooth muscle cells isolated from fetal or neointimal blood vessels (Gordon et al., 1986; Schwartz et al., 1990; Van Neck et al., 1993; Cook et al., 1994; Lemire et al., 1994) as well as cultures of neointimal cells after balloon-catheter injury contain cells of epithelioid shape that contrast with spindle-shaped adult smooth muscle cells (Majesky et al., 1992; Orlandi et al., 1994). According to the developmental stage of the tissue used for culture, the ratio between cells of the epithelioid and spindle-shaped phenotypes is variable. In particular, epithelioid cells display characteristic features associated with immaturity (Ehler et al., 1995). Villaschi et al. (1994) reported that epithelioid and spindle-shaped cells exist in anatomically distinct regions of normal rat aorta. Medial smooth muscle cells were spindle-shaped and grew in hills-and-valleys in culture, showing features typical of most cultured smooth muscle cells. Cells from the intimal side of the media displayed a polygonal and epithelioid shape, grew mainly as a monolayer, and had a higher proliferative rate. In addition, medial smooth muscle cells also showed a marked heterogeneity. At least four

smooth muscle cell populations with unique lineages exist simultaneously within the mature pulmonary arterial and systemic aortic media, and these cells display considerable differences in their phenotype, growth, and matrix-producing capabilities (Frid et al. 1997). Heterogeneity in smooth muscle cells has been well-established in embryonic vessels (Mironov et al., 1995). The majority of embryonic aortic cells are smooth muscle cells, however, there is a regional difference in shape and synthetic states of these cells. The inner sublayer adjacent to the endothelium contains round and ovoid cells with a synthetic phenotype. In the intermediate sublayer, spindle-like cells ultrastructurally similar to smooth muscle cells are found. Cells of the outer sublayer resemble fibroblasts or poorly differentiated mesenchymal cells.

Heterogeneity in smooth muscle cells is also revealed in different patterns of protein expression. With regard to desmin (a cytoskeletal protein) expression, two types of smooth muscle cells have been identified in human fetal vessels: desmin-positive and desmin-negative. Smooth muscle cells in elastic and muscular arteries are desmin-negative, while those in femoral and bronchial veins are desmin-positive. In addition, differences in local distribution of these two types of cells have also been found. In umbilical cord arteries and veins, desmin-positive cells are largely localized to the outer layer of media, but not to the inner layer. In placenta, both desmin-positive and negative cells have been found (Nanaev et al. 1991; Osborn et al. 1987). With respect to distribution of smooth muscle and non-muscle myosin heavy chains, two other types of smooth muscle cells exist in the medial layer of normal adult aorta, namely cells that

contain smooth muscle (SM) myosin exclusively and cells that show the coexistence of SM and non-muscle type (NM) myosin isoforms. During experimental atherogenesis, the number of cells in the population with two myosin isoforms increases markedly (Zanellato et al. 1990). More recently, Eddinger and Meer (1997) reported that most single smooth muscle cells isolated from adult rabbit carotid express both SM1 and SM2, however, expression of these SM-MHC isoforms at the cellular level is nonuniform and highly variable. In addition, differences in expression of the smooth muscle myosin "inserted" isoform, SM-B, which contains an extra 7 amino acid insert in its N-terminal region, has also been observed; both SM-B positive and negative cells have also been identified in smooth muscle cells freshly isolated from canine airways (Ma et al., 1998).

Smooth muscle cells also differ in their membrane properties. Heterogeneity in distribution of K^+ channels has been observed in pulmonary smooth muscle cells. Four types of K^+ channels have been characterized in smooth muscle cells (Nelson and Quayle 1995): (1) the delayed rectifier channel (K_{DR}), (2) the Ca2+-dependent K+ channel (K_{Ca}), (3) the inward rectifier channel (K_{IR}), and (4) the ATP-dependent channel (K_{ATP}). In pulmonary arterial smooth muscle cells, some are enriched in K_{DR} channels whereas others have more K_{Ca} channels (Michelakis et al. 1997). With regard to the expression of voltage-gated Ca2+ currents identified as either low- or high voltage-activated (LVA and HVA), four distinct populations of smooth muscle cells have been identified in cultured aortic myocytes: LVA, HVA, LVA/HVA mixture, and none. HVA currents are expressed in the first five days in primary culture and after cells reach confluence (>15 days); LVA

are expressed transiently between 5 to 15 days in culture during which time cells are proliferative and show loss of contractility (Richard et al. 1992). Therefore, HVA and LVA may serve distinct cellular functions. HVA may be important in smooth muscle contraction, whereas LVA may contribute to cell proliferation.

Heterogeneity also exists in cultured smooth muscle cells. Two populations of smooth muscle cells have been identified in cultured human arterial smooth muscle cells: cells in the monolayered region are large and well spread, whereas the multilayers are composed of smaller cells with poor spreading on tissue culture plastic; cells in monolayers are highly adhesive to the plastic culture plate and have abundant filamentous actin; in contrast, cells from multilayers show low adhesion, and have very little filamentous actin (Bjorkerud, 1985). A spontaneous oscillation of cytoplasmic free calcium has been observed in cultured smooth muscle cells from guinea pig ileum. Each individual cell displays a regular pattern of oscillation. However, marked heterogeneity in frequency and amplitude of Ca^{2+} oscillation exists between neighboring cells. Furthermore, oscillation is not observed in all cells, but only in 25% to 80% of the cells, which differs from the batches of cells after 5 to 8 days of culture (Ohata et al., 1993). Suzuki (1994) reported that cultured tracheal smooth muscle cells showed heterogeneity in the threshold concentration of histamine required to increase $[Ca2+]_i$. Most recently, an *in vitro* smooth muscle cell culture system has been developed in which differentiated smooth muscle cells can be induced from dedifferentiated cultured cells through prolonged serum deprivation in the presence of insulin, transferrin, and selenium. Two

morphological, biophysical, and biochemical distinct populations of cells have been observed in serum-deprived tracheal smooth muscle cell culture (Ma et al., 1998b). One population is of a non-contractile phenotype. Its cells appear flat and circular, contain small amounts of smooth muscle contractile and regulatory proteins, and show no contractile responses to various contractile stimuli. The other population is of contractile type. Its cells are spindle-shaped, express large amounts of smooth muscle contractile and regulatory proteins, and demonstrate reversal contractile responses to various contractile stimuli.

Single smooth muscle cells also show marked heterogeneity in contractility. Such cells isolated from guinea pig ileum display significant differences in their velocities (Vo) and capacities (ΔLmax) of unloaded shortening (Fang et al. 1993). Wide distributions in unloaded shortening velocity ranging from 0.3 to 2.06 μm/s have also been observed in freshly isolated vascular smooth muscle cells (Meer and Eddinger, 1997). Recent studies (Ma et al., 1997; Ma et al., 1998) from our laboratory demonstrate that there are at least three types of arbitrarily classified, contractile smooth muscle cells in canine airway smooth muscle based on their morphological and contractile parameters: type I cells with a mean length of 110 μm, show ΔLmax and Vo of 28 ± 3 % of their resting length (Lcell) and 8 ± 0.6 %Lcell/s, respectively; type IIA and IIB cells which have the same mean length of 200 μm, shorten with ΔLmax and Vo of 17 ± 2 %Lcell and 4 ± 0.3 %Lcell/s, and 58 ± 6 %Lcell and 10 ± 0.9 %Lcell/s, respectively.

The observation of smooth muscle cell heterogeneity raises the question as to how this phenotypic diversity is achieved. That is, are there specific subpopulations of phenotypically distinct smooth muscle cells that persist throughout all stages of development, or is the observed smooth muscle cell heterogeneity the result of a temporal and reversible "modulation" of a single phenotype. It is probable that multiple stable subpopulations of smooth muscle cells with distinct lineages exist within mature smooth muscle *in vivo* (Frid et al., 1994, Kim et al. 1997). Detection of smooth muscle cell heterogeneity in embryonic tissues (Mironov et al., 1995) further supports that notion. It is also possible that local unique growth factors and transcription factors generated during tissue development result in formation of tissue and/or regional specific phenotypes of smooth muscle cells (Cardoso, 1995). In addition, local interactions between mesenchyme (source of smooth muscle progenitor cells) and epithelial or endothelial cells (Minoo and King, 1994; Hungerford et al., 1996) during development may contribute to the development of diversity between smooth muscle in different tissues and at different sites in the same tissue. Finally, phenotypic plasticity of smooth muscle cells may also play an important role in *in vivo* smooth muscle cell heterogeneity. Phenotypic modulation from contractile to non-contractile type has been observed in freshly-isolated smooth muscle cells under primary culture (Chamley-Campbell et al., 1979). Recently, development of contractile smooth muscle cells from non-contractile smooth muscle cell culture has also been demonstrated (Ma et al., 1998b).

1.2.2 Segment-to-segment heterogeneity

Considerable heterogeneity in contractility has been demonstrated in airway smooth muscle along the airway tree. Using isometric tension as an index of smooth muscle contraction, small bronchi were shown to possess an increased contractile response to methacholine and histamine when they were compared with large bronchi (Shioya et al., 1989, Chitano et al., 1993). Heterogeneity in isotonic contractile properties has also been observed in our recent studies (Ma et al., 1996). Two groups of airway smooth muscles have been identified along the canine airway tree: an extrapulmonary group, which consists of smooth muscle from the trachea and 1st and 2nd generations of bronchi, and possesses enhanced maximal capacity (ΔLmax) and velocity (Vo) of shortening in response to electrical stimulation; and an intrapulmonary group, comprised of smooth muscles from 3rd to 6th generations of bronchi, and which shows lower shortening properties. Furthermore, marked heterogeneity of airway narrowing in response to bronchoconstricting stimuli has been observed. Histamine predominately contracts the small airways, while acetylcholine results in greater contraction of large airways compared with small airways (Brown et al. 1993; Jackson et al., 1981; Sekizawa et al., 1988). Leukotrienes predominantly result in contraction of smooth muscle in peripheral airways (Drazen and Austen, 1987). A very high degree of heterogeneity in responsiveness to contractile agonists such as methacholine, has further been identified across the airways of human lung using cultured lung explants (Minshall et al., 1997). Furthermore, membrane receptors have been found not to be evenly distributed in airway smooth

muscles along the airway tree. Muscarinic M_2 receptor density is greatest in large airways and least in peripheral airways (Barnes, 1987). On the other hand, histamine H_1 receptors distribute more densely in the smooth muscle of small airways than in those of large airways (Bradley and Russell, 1982; Barnes, 1987). Marked differences in adrenergic receptor distribution also exist. Beta-adrenoreceptors are present in high density throughout the airways, with the highest density being present in bronchioles; α-adrenoreceptors are sparse in large airways, but numerous in small bronchioles (Barnes et al., 1983). The heterogenous distribution of various receptors may contribute to the heterogeneous contractile responses of airway smooth muscles discussed above. Airway smooth muscles also differ in their innervation. The primary innervation of airway smooth muscle is the parasympathetic nervous system. Parasympathetic innervation is dense in bronchial smooth muscle but sparser in bronchiolar smooth muscle and virtually absent from terminal bronchioles (Richardson, 1979).

Marked pharmacological and mechanical heterogeneity has also been observed in vascular smooth muscle. Within isolated ophthalmociliary arteries, despite similar diameters the most distal segment, nearest the eye, developed the smallest Po compared to the middle segment and the most proximal segment (Yu, 1992). Contractions to phenylephrine, norepinephrine, and histamine were significantly potentiated in small rings, with or without endothelium, compared to large pulmonary artery rings with or without endothelium (Zellers and Vanhoutte, 1989). Angiotensin II-induced contractions are greater in the distal portions than in the proximal portions of mesenteric and coronary

arteries. In addition, mesenteric arteries responded to the peptide with a greater contraction than coronary arteries (Minami and Toda, 1988). A regional heterogeneity in the contractile response has also been demonstrated in the pulmonary artery to alpha and beta-adrenergic stimulation. The extrapulmonary arterial segments are found to be more sensitive to beta-stimulation than are the smaller, intrapulmonary segments. The intrapulmonary arterial segments, on the other hand, are more sensitive to alpha-stimulation than the extrapulmonary segments (Kolbeck and Speir, 1987). A recent report by DiSanto et al. (1997) demonstrates that smooth muscle from small muscular arteries shortens at Vo twice the value of that of the elastic large arterial smooth muscle. In addition to heterogeneity in contractile responses, heterogeneous relaxant responses have also been identified. Endothelium-dependent relaxation predominates in small pulmonary arteries compared with large pulmonary arteries (Kolbeck and Speir, 1987). Heterogeneous relaxation responses to nicorandil have been reported in epicardial coronary arteries. Nicoradil behaves predominately as a nitrate in large epicardial arteries rather than in small arteries (Matsumoto et al., 1997).

Segmental heterogeneity of smooth muscle is also revealed in their membrane properties. Harder (1983) reported that the middle cerebral artery of the cat possessed a higher resting membrane potential and depolarized to excess K^+ with a steeper slope than other peripheral arteries of the same animal. Furthermore, cerebral arteries from different areas of the brain exhibited distinctly different resting membrane properties as well as different responses to norepinephrine. In addition, cerebral arteries exhibited a high degree

of electromechanical coupling not always observed in other peripheral arteries. Topographical differences in membrane properties have also been observed in airway smooth muscle, the resting membrane potential of bronchial smooth muscle being more negative than that of tracheal (Souhrada et al., 1983; Inoue and Ito, 1986). Considerable segmental heterogeneity exists between pre- and post-glomerular vascular smooth muscle cells in ionic signaling events; preglomerular branch points show vascular smooth muscle enriched with calcium channels (Casellas and Carmines, 1996). Recently, regional differences in K^+ channel distribution have also been reported. Smooth muscle from conduit pulmonary arteries is rich in Ca^{2+}-dependent potassium channels (Kca), while smooth muscle from resistance pulmonary arteries is rich in delay rectifier potassium channels (K_{DR}) (Archer, 1996).

With regard to the expression of intermediate filament proteins, vascular smooth muscle cells are rich in vimentin. However desmin positive cells also exist. Capacitance and resistance arterial smooth muscle cells are predominately desmin-negative, while femoral and bronchial venous cells are mainly desmin-positive (Osborn et al, 1981; Nanaev et al, 1991). Differences in expression of smooth muscle contractile and regulatory proteins have also been demonstrated in smooth muscles from different segments of vascular tissue. Smooth muscle from small muscular arteries possesses a large amount of SM-B compared with that from large arteries (DiSanto et al., 1997). Furthermore, aortic smooth muscle displays near equal quantities of the a and b isoforms of the 17 kDa essential myosin light chain (MLC_{17}), whereas MLC_{17a} predominates in

femoral/saphenous arterial smooth muscles. In airway smooth muscle, increased expressions of smooth muscle α-actin, SM-B, and smooth muscle myosin type light chain kinase have been demonstrated in bronchial smooth muscle (Ma and Stephens, 1997).

Differences in characteristics of cell proliferation have been observed in smooth muscle cells from different segments of tissues. For example, the proliferative activity of venous smooth muscle cells is significantly lower than that of aortic (Denes et al., 1983). Smooth muscle cells derived from mesenteric resistance arteries proliferate at a rate that is 4 times slower than that of large artery cells (De Mey et al, 1991). Human vascular smooth muscle cells from different vessels also show heterogeneous proliferative responses to alpha-thrombin (Kanthou et al., 1995).

1.2.3 Tissue-to-tissue heterogeneity

Smooth muscles from various tissues can be broadly divided into tonic and phasic types on the basis of intrinsic mechanical properties determined by the coordinate expression of various ion channels and contractile protein isoforms (Somlyo and Somlyo, 1990). Phasic muscles as expected show phasic contractions, while tonic muscles are continually, partially contracted. Phasic smooth muscles generate action potentials and have faster shortening velocities, determined by the expression of fast myosin isoforms, a preponderance of the MLC_{17} isoform of the alkali myosin light chain, and a N-terminal

"inserted" myosin heavy chain isoform (Kelley et al., 1993; Somlyo, 1993). In contrast, tonic smooth muscles do not normally generate action potentials, have a relatively high content of acidic light chain isoform and have heavy chains which lack the N-terminal insert. In addition, phasic smooth muscles primarily contain smooth muscle γ-actin, while tonic muscles mainly express smooth muscle α-actin (Fatigati and Murphy, 1984). The typical phasic smooth muscles include those of the intestines, uterus, bladder, and the arteriole. Smooth muscles in large elastic arteries and trachea belong to tonic type.

Phasic smooth muscle is of a so-called single unit type. The single unit smooth muscle is sparsely innervated, but is rich in gap junctions between smooth muscle cells which enable the fast propagation of depolarization from cell to cell (Bozler, 1948; Kroeger and Stephens, 1975). This enables single unit smooth muscle to work as a functional unit. Most tonic smooth muscles are multi-unit. Multi-unit smooth muscle, on the other hand, has a rich innervation, but displays reduced numbers of gap junctions between smooth muscle cells. There are thus little direct electrical communications between smooth muscle cells in this type of smooth muscle. Airway smooth muscle does not fit neatly into either single unit nor multiple unit types, and has been allocated to an intermediate category (Burnstock, 1970).

1.2.4 Species to species heterogeneity

Smooth muscles from the same organs but from different species of animals may present remarkable differences in contractile responses. For example, esophageal smooth muscles from guinea pig and rabbit contract in response to serotonin via activation of 5-HT2 receptors, however, serotonin neither contracted nor relaxed canine esophagus, but relaxed the rat esophagus via 5-HT4 receptors (Cohen et al., 1994). Substantial heterogeneity in contractile response to agonists has also been identified in mesenteric resistance arteries of different species (Nielsen et al., 1991).

1.2.5 Pathophysiological significance of smooth muscle heterogeneity

As is clear from the foregoing, smooth muscle displays tremendous heterogeneity at various levels. The physiological significance of this heterogeneity is currently unclear. Because of the extremely extensive distribution and complicated functions of smooth muscles in various types of organs, and considerable heterogeneity in their physiological functions, and even within different sections of the same organ, the existence of smooth muscle heterogeneity is physiologically important. Phasic smooth muscles in arterioles may be important in the fine control of regional blood distribution, while tonic smooth muscles in large elastic arteries are required to maintain normal vascular tension necessary for maintaining average, steady levels of blood pressure and blood flow.

The structural heterogeneity of the airway tree is an outstanding feature. The

smooth muscle in the trachea spans one-third of its circumference, and directly attaches to stiff cartilage rings. The shortening of the muscle is therefore limited by the strong resistance generated by the cartilage rings. However, cartilages in bronchus do not form intact rings, but instead exist as separate soft plaques. The smooth muscles in the bronchus surround the entire circumference with attachment to cartilage plaques through soft connective tissue, and their shortening is not loaded heavily as occurs in tracheal smooth muscle. Therefore, the reduced contractility found in bronchial smooth muscle may provide a self-limiting mechanism preventing these airways from excessive narrowing during smooth muscle activation in physiological situation (Ma et al. 1996). The co-existence of phenotypically distinct smooth muscle cells may be important in maintaining homeostasis of tissue function. This is particularly important in the case of the mammalian artery wall where the smooth muscle cell is the only cell type present within the media, and must therefore be responsible for both contraction and maintaining the integrity of wall (Chamley-Campbell and Campbell, 1981). Contractile differentiated smooth muscle cells are primarily responsible for contraction, while proliferative non-contractile smooth muscle cells are actively engaged in synthesis of extracellular matrix (Chamley-Campbell and Campbell, 1981) and/or replication and development of new contractile cells (Majesky et al., 1992; Ma et al. 1998b). Differentiation in expression of smooth muscle contractile protein isoforms in different smooth muscles may be important in determining their contractility (Garland et al., 1982; Garland et al., 1983). Increased expression of the fast myosin heavy chain isoform SM-B in intestinal and small muscular arterial smooth muscles contributes to rapid shortening of these muscles (Kelley et al., 1993; DiSanto et

al., 1997). Smooth muscles consist of remarkably heterogeneous populations of smooth muscle cells, their mechanical behaviors therefore differ in tandem with to the proportions of different populations of smooth muscle cells within them. Predomination of K_{Ca} enriched smooth muscle cells in conduit pulmonary smooth muscle results in a bi-phasic response, predominated by relaxation, to hypoxia, and considerable relaxation to NO stimulation, due to increased activity of K_{Ca} channels. On the other hand, hypoxia inhibits K_{DR} current, and consequently causes constriction of resistance pulmonary arteries in which the majority of smooth muscle cells are enriched in K_{DR} channels, and NO-induced relaxation is found to be much less compared with conduit pulmonary arteries (Archer et al., 1996). In airway smooth muscle, an increased number of smooth muscle cells with low contractility confers reduced contractility on bronchial smooth muscle compared to tracheal smooth muscle (Ma et al. 1997).

Heterogeneity of smooth muscle also has significant implication in the pathogenesis of diseases. It has been demonstrated that specific types of smooth muscle cells may contribute to unique roles in the development of pathology. Pathological lesions in atherosclerosis, restenosis, and hypertension, as well as neointimal thickening in injured vessels are facilitated by the presence of immature types of smooth muscle cells (Sartore, et al. 1997). In addition, regional differences in hypertensive vascular walls have been observed. Intimal thickening and smooth muscle hypertrophy are restricted to large arteries such as aorta, while the smooth muscle cell population in resistance small vessels is stable and does not change (Pauletto et al. 1995). Distinct subpopulations of smooth

muscle cells within the pulmonary arterial media display increased proliferative behavior and increased production and deposition of extracellular matrix in hypoxia-induced pulmonary hypertension (Stenmark and Mecham, 1997). Heterogeneity of airway smooth muscle cells also plays important roles in the pathogenesis of asthma. Non-homogeneous airway narrowing is an outstanding feature of asthma (Ebina et al., 1990). Heterogeneous development of smooth muscle hypertrophy and hyperplasia has also been observed in asthmatic airways (Ebina et al., 1993). Our recent study in ragweed pollen-sensitized canine airways demonstrates that sensitization alters contractility of airway smooth muscle and its constituent cells in a heterogeneous manner. Bronchial smooth muscles show greater increases in their shortening properties than tracheal muscles, and smooth muscle cell type IIA (described above) displays the greatest increase in $\Delta Lmax$ and Vo the among three contractile smooth muscle cells identified in airway smooth muscle (Stephens et al. 1998).

1.3 Regulation of Smooth Muscle Contraction

Contraction of smooth muscle is primary controlled by levels of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. Increase of $[Ca^{2+}]_i$ activates Ca^{2+}/calmodulin (CaM)-dependent myosin light chain kinase (MLCK) by forming a complex and consequently phosphorylates the 20 kDa myosin light chain (MLC_{20}), subsequently resulting in an increase in myosin ATPase activity and cross-bridge cycling. However, MLC_{20} phosphorylation and force development are not always linearly related. During smooth muscle contraction, elevation of MLC_{20} phosphorylation is usually seen in the phase while force is developing, while during the subsequent plateau phase force is still maintained but the levels of phosphorylation drop considerably. Therefore, other alternative and parallel pathways must exist for the regulation of force maintenance. The basic sliding filament mechanism of muscle contraction cannot account for this behaviour. Some of the factors that regulate smooth muscle contraction will now be discussed.

1.3.1 Smooth muscle myosin

The importance of myosin is indicated by the fact that smooth muscle is capable of generating a similar amount of active stress (force per cross-sectional area of muscle) as skeletal muscle in spite of the fact that it contains only one-fifth the amount of myosin of skeletal muscle (Murphy et al. 1974). In theory, this enhanced capacity in force

generation could be the result of a greater unitary force, and/or an increased duty-time of the single smooth muscle cross-bridge. Results obtained from an *in vitro* motility assay demonstrate that there is no difference in cross-bridge duty-time between smooth muscle and skeletal myosin (Harris and Warshaw, 1993). The standard *in vitro* motility assay however, is carried out under the condition of zero-load, and cross-bridge function is proved to be strain dependent (Ford et al. 1977). Therefore, these results may not be suitable in interpretation of properties of cross-bridge force generation. Utilizing a novel technique VanBuren et al. (1994) demonstrated that the smooth muscle myosin head produced force 3 to 4 times greater than that of skeletal myosin. Recent studies using a novel double laser trap showed that smooth muscle myosin displays similar values in unitary displacement and unitary forces as skeletal myosin, however, the duty-time of smooth muscle myosin was about 3 to 4 times longer than that of skeletal myosin (Guilford et al., 1997), implying that an increased duty-time is responsible for the enhanced force-generating capacity of smooth muscle myosin.

As discussed in the previous section, smooth muscles from different origins vary in their contractility. It is possible that alterations at the level of the contractile and regulatory smooth muscle proteins may explain these differences. One of the factors which determines the velocity of smooth muscle shortening is cross-bridge cycling rate, which is, determined in turn, mainly by the Mg^{2+} ATPase activity of the N-terminal myosin head. Smooth muscle myosin II is a hexamer, composed of two heavy chains and two pairs of light chains of 20 kDa (MLC_{20}) and 17 kDa (MLC_{17}). A variety of isoforms of

myosin heavy chain and light chains have been described. Differences in distributions of isoforms may contribute to contractile differentiation of various smooth muscles.

Four isoforms of smooth muscle myosin heavy chain have been identified to date. Two of them differ in having an extra sequence in the C-terminal tail in the SM-1 isoform which does not exist in the SM-2 isoform (Nagai et al., 1989). Increased expression of SM-1 has been reported in rat uterus from estrogen-treated animals which is associated with increased maximum velocity of shortening of the smooth muscle (Sparrow et al, 1988; Hewett et al. 1993). It is thought that the C-terminal end of the smooth muscle myosin heavy chain is primarily involved in thick filament assembly (Somlyo, 1993) and since it is distant from the myosin head, it is difficult to envision how alteration in the C-terminus would lead to changes in the ATPase activity of the myosin head. It is speculated that intermolecular contacts between the tail of one molecule and the S2-hinge region of an subjacent molecule in the thick filament may modulate the contractility of the myosin head (Cai et al., 1995). However, *in vitro* motility assay shows no differences in ATPase activity and velocity of translocation of actin filaments in vitro between SM-1 and SM-2 (Kelley et al., 1993). The two other isoforms display a difference in their N-terminal region, SM-B contains an extra 7 amino acid insert in its N-terminal head region close to the ATP-binding site near the junction of the 25- and 50-kDa domains (Babij, 1993; Kelley, et al., 1993; White et al., 1993). This isoform possesses an enhanced ATPase activity which is about 2 times higher and a velocity of actin filament translation *in vitro* which is 2.5 fold faster than those of SM-A which does not contain any insert

(Kelley et al, 1993). Increased expression of SM-B in intestinal (Kelley et al., 1993) and small arterial (DiSanto et al., 1997) smooth muscles contributes to the enhanced contractility of these small arteries compared to that of smooth muscle from large blood vessels. Recent studies on chimeric myosins, where the surface loops obtained from different myosin II isoforms were inserted into a common smooth muscle myosin backbone, demonstrated that the N-terminal insert near the catalytic region of myosin was important but not the primary determinant in determining smooth muscle myosin ATPase activity and *in vitro* movement of actin filaments (Sweeney et al., 1998; Rovner et al., 1997). However, dissociation of the content of SM-B expression from contractility of smooth muscle has also been noted. Haase and Morano (1996) reported a decrease of SM-B expression, while the maximal shortening velocity of smooth muscle increased in pregnant rat myometrium fibers. Siegman et al. (1997) found no correlation between the amount of SM-B and shortening velocity in mouse megacolon. Our recent studies of arrested contractile smooth muscle cells showed that these cells did not express SM-B, but possessed elevated contractility compared with freshly isolated cells in which SM-B was present (Ma, et al., 1998b).

In addition, two isoforms of MLC_{17} have also been identified in smooth muscle. An increased content of the more acidic isoform MLC_{17a} has been found to be associated with enhanced contractility of smooth muscle (Malmqvist and Arner, 1991; Morano et al. 1993). However, exchange of the MLC17a with to MLC17b did not alter in vitro motility of vascular myosin heavy chain (Kelley et al., 1993). Isoforms of myosin regulatory light

chain (MLC_{20}) were also detected in smooth muscle (Monical et al., 1993). Decreased expression of the smooth muscle isoform of MLC_{20} was identified in proliferating smooth muscle cells, while no detectable changes in expression of non-muscle isoform of MLC_{20} was found in smooth muscle cells in culture. However, there was no evidence for differential phosphorylation of the SM and NM MLC_{20} isoforms in response to activating or relaxing agents suggesting that there is no functional difference between them.

In summary, smooth muscle expresses various isoforms of smooth muscle myosin. The importance of myosin isoform distribution in determining smooth muscle contractility needs further investigation.

1.3.2 Myosin light chain kinase

Various types of myosin light chain kinase (MLCK) have been described, including a smooth muscle type (smMLCK) present in mature, differentiated muscle cells, non-muscle type (nmMLCK), embryonic type, and endothelial type (Gallagher et al., 1995; Fisher and Ikebe, 1995). While the function(s) of the other types of MLCK is/are unclear, smMLCK plays a critical role in regulation of smooth muscle contraction. smMLCK is a highly specific protein kinase, with the only known physiological substrate being myosin II. It specifically phosphorylates Ser^{19} of MLC_{20}. In the presence of high concentrations of MLCK or during maximal levels of muscle contraction, phosphorylation

of Thr^{18} also occurs (Ikebe et al., 1986), but dual phosphorylation appears unlikely to have broad physiological significance in intact smooth muscle tissues. The primary regulator of smMLCK is the Ca^{2+}-CaM complex. Increase of intracellular Ca^{2+} triggers a signaling pathway leading to smooth muscle contraction. One molecule of calmodulin (CaM) is capable of binding four Ca^{2+} ions. Formation of the Ca^{2+}-CaM complex increases CaM affinity for MLCK, which subsequently results in formation of a Ca^{2+}-CaM-MLCK complex and activation of smMLCK, which in turn, phosphorylates Ser^{19} of MLC_{20}, activates actin-activated myosin Mg^{2+} ATPase activity and leads to cross-bridge cycling (Sweeney et al., 1994). Relaxation is probably regulated by a reversal of the contraction process. Resequestration of Ca^{2+} by the sarcoplasmic reticulum by cessation of stimulation results in disassociation of the Ca^{2+}-CaM-MLCK complex and inactivates MLCK. At the same time activation of smooth muscle myosin light chain phosphatase dephosphorylates the phosphorylated MLC_{20} thus deactivating the actomyosin ATPase, which finally leads to smooth muscle relaxation (Somlyo and Somlyo, 1994).

The activity of smMLCK can be further regulated by various other factors. Phosphorylation of a specific serine residue in the region of the CaM-binding domain of MLCK by several kinases including PKA, CaM kinase II and PKC reduces its affinity for Ca^{2+}-CaM and consequently its phosphorylating activity (Hashimoto and Soderling, 1990; Nishikawa et al., 1984; Nishikawa et al., 1984). Phosphorylation of MLCK by CaM kinase II occurs *in vivo*, and exerts an inhibitory effect thus providing a physiological mechanism for Ca^{2+}-desensitization of smooth muscle contraction (Tansey et al., 1994).

Protein kinase G (PKG) has also been reported to phosphorylate another serine site, C-terminal to the CaM-binding domain, however no changes in the affinity for Ca^{2+}-CaM have been demonstrated (Stull et al., 1993). Ca^{2+}-sensitization can be achieved by inhibition of myosin light chain phosphatase (MLCP) activity that normally dephosphorylates Ser^{19} of MLC_{20}. Arachidonic acid, released by agonists is a Ca^{2+}-sensitizing agent. It increase MLC_{20} phosphorylation and causes contraction of smooth muscle by inhibiting myosin light chain dephosphorylation without changes in intracellular Ca^{2+} concentration (Gong et al., 1992). Phorbol esters on the other hand result in Ca^{2+}-sensitization via activation of PKC (Itoh et al. 1988; Andrea and Walsh, 1992).

It is clear that smooth muscle myosin ATPase is activated by phosphorylation of MLC_{20}. Unphosphorylated myosin does however show a very low actin-activated Mg^{2+}-ATPase activity (Sellers et al., 1982). The molecular mechanism by which phosphorylation of MLC_{20} leads to an increase in ATPase is currently under active investigation. Two intact myosin heads are proved to be required, because phosphorylation of single-headed myosin has no effect on its ATPase (Cremo et al., 1995). Changes in net charge resulting from phosphorylation of MLC_{20}, spatial constraints that are satisfied by a phosphate moiety, and the proper positioning of the C-terminal region of MLC_{20} may all contribute to phosphorylation-dependent regulation of smooth muscle myosin Mg^{2+} ATPase activity (Sweeney et al., 1994; Trybus, 1989; Trybus and Chatman, 1993).

During sustained contraction such as occurs on high K^{+} stimulation, smooth muscle

is known to enter a state where $[Ca^{2+}]i$ and MLC20 phosphorylation decline to low levels, despite the fact that the tone is maintained. The phenomenon of Ca^{2+}-dependent force maintenance without detectable elevations in phosphorylation is termed as latch (Dillon et al., 1981), which is a state in which cross-bridge cycling rate is considerably reduced. Hai and Murphy (1988) proposed that dephosphorylation of an attached cross-bridge resulted in the formation of a latch cross-bridge, where its detachment rate was greatly reduced. However, there are notable exceptions that cannot be explained by this model. For example, permeabilization of intact smooth muscles generally results in loss of the latch phenomenon (Moreland et al., 1992). Additionally, uncoupling between the force-velocity relationship and MLC_{20} phosphorylation has been observed in various situations (Gunst et al., 1992; Gunst et al., 1994; Katoch and Moreland, 1995). Finally, a major problem with the latch-bridge concept is that no direct demonstration of its existence has been made to date.

1.3.3 Thin Filament Associated Regulation of Contraction

Although it has been reported that smMLCK activity is "sufficient and necessary" for smooth muscle contraction (Itoh et al., 1989), alternative mechanisms for the regulation of smooth muscle contraction have been proposed such as thin filament associated regulatory proteins that modulate contractility by altering interaction between actin and myosin resembling what occurs in the regulation of skeletal muscle contraction.

Thin filament regulation is one of several mechanisms invoked to explain the latch phenomenon. Smooth muscle thin filaments contain tropomyosin, but unlike skeletal muscle, they lack troponin. Proteins analogous to troponin have been identified however, these include caldesmon (CaD) (Sobue et al., 1981) and calponin (CaP) (Lehman and Kaminer, 1984; Takahashi et al., 1986). Accumulating evidence supports the physiological roles of CaD and CaP in regulation of smooth muscle contraction.

1.3.3.1. Caldesmon

Smooth muscle CaD is an elongated protein consisting of a single polypeptide chain extending from 75 to 80 nm in length with a molecular weight of 90 kDa, although it migrates as a 130- to 140-kDa protein on SDS-PAGE. CaD binds to F-actin, tropomyosin, myosin, and Ca^{2+}/CaM, and is involved in modulation of actomyosin ATPase activity (Sobue and Sellers, 1991). Binding of CaD to actin inhibits actin-activated Mg2+-ATPase activity of smooth muscle myosin primarily by competitively reducing the affinity between actin and myosin.ADP.Pi (Hemric and Chalovich, 1988; Horiuchi et al., 1991; Marston and Redwood, 1992). It is also suggested that CaD may act by slowing a rate-limiting step of the ATPase cycle (Marston and Redwood, 1992). In addition to the thin filament proteins, CaD also binds myosin. Because of its binding properties, CaD can act as a cross-linker of actin and myosin (Katayama and Ikebe, 1995). It has been suggested that cross-linking of actin to myosin by CaD might result in a

"tethering" that may contribute to CaD's ability to slow thin filament velocity in vitro (Fraser and Marston, 1995).

Caldesmon induced ATPase inhibition is regulated through phosphorylation of the protein by several protein kinases such as CaMKII, casein kinase II, PKA, cdc2kinase, MAP kinase and PKC (Childs et al., 1992; Ikebe and Reardon, 1990; Ikebe and Hornick, 1991; Mak et al., 1991; Wawrzynow et al., 1991), which are shown to phosphorylate CaD either *in vitro* or *in vivo*. MAP kinase is the most likely one to be responsible for the in vivo phosphorylation (Adam and Hathaway, 1993). MAP kinase-catalyzed phosphorylation sites on purified chicken gizzard CaD are consistent with one of the sites that are phosphorylated *in situ* (Childs and Mak, 1993). Phosphorylation of smooth muscle CaD by PKC has been reported to block its inhibitory effect on the actomyosin ATPase or to inhibit actin binding to CaD (Ikebe and Hornick, 1991; Tanaka et al., 1990); others have reported no significant changes (Vorotnikov and Gusev, 1991). Ca2+/CaM binding to CaD provides another mechanism suggested for regulating CaD's inhibitory activity. CaM has no effects on ATPase inhibition by CaD in the absence of Ca^{2+}, a complete reversal of inhibition by CaD occurs in the presence of relatively high concentration of Ca^{2+}/CaM (Horiuchi et al., 1991; Smith et al., 1987). Other Ca2+ binding proteins have also been proposed as CaD regulators, such as caltropin and the 21-kDa S100 protein (Fujii et al., 1990; Mani et al., 1992).

The *in vitro* studies cited above demonstrate that CaD has the actin binding and

myosin ATPase inhibitory activities required for functioning as a thin filament regulatory protein. However, evidence from *in vivo* studies is still not complete, and the establishment of CaD-related regulation of smooth muscle contraction needs further investigation.

1.3.3.2 Calponin

Smooth muscle calponin (CaP) provides another candidate for a thin filament associated regulatory protein that inhibits smooth muscle contraction. CaP is also an elongated molecule consisting of a single polypeptide with a molecular mass of 32 kDa (Stafford et al., 1995). Binding of CaP to F-actin inhibits smooth muscle myosin ATPase activity (Winder and Walsh, 1990). Similar to its action on ATPase activities, CaP inhibits the *in vitro* motility of actin filaments, full inhibition of actin motility being achieved at a CaP concentration around 1 to 2 μM (Shirinsky et al., 1992). Calponin is also seen to increase filament binding in a motility assay, and at subinhibitory concentrations, the presence of CaP increases the capacity of smooth muscle to overcome the load exerted on the cycling cross-bridges (Haeberle, 1994). This effect is interpreted as slowing down of the cross-bridge dissociation rate from actin, leading to an increased number of attached, slowly cycling cross-bridges, and a consequent increase in force.

Calponin can be phosphorylated by PKC *in vitro*, with dramatic lowering of the

affinity of CaP for F-actin, which reduces its inhibition of actin-activated myosin Mg^{2+}-ATPase activity (Walsh et al. 1996). The principal site of phosphorylation by PKC has been identified as serine 175 (Winder et al., 1993). Calponin has also been reported to be phosphorylated by CaMKII (Winder and Walsh, 1990). A calponin phosphatase has also been isolated from chicken gizzard (Winder et al., 1992). Dephosphorylation of CaP restores actin binding and actomyosin ATPase inhibition.

The physiological role of CaP in regulation of smooth muscle contraction has by no means been substantiated yet. Exogenously applied CaP has been demonstrated to be capable of inhibiting both force and shortening velocity of a skinned smooth muscle strip (Horowitz et al., 1996). However, there is no agreement whether CaP phosphorylation does occur *in vivo*. Several studies found that CaP became phosphorylated upon stimulation (Pohl et al., 1997; Winder et al., 1993), while others could not confirm this finding, even when using the same tissue and stimulation method (Adam et al., 1995; Barany and Barany, 1993).

1.3.4. Cytoskeletal Proteins

Smooth muscles show a marked plasticity in their mechanical properties (Gunst et al., 1993). The traditional sliding filament mechanism can not explain this phenomenon. Reorganization of cytoskeletal structures during smooth muscle activation has been

suggested to regulate plasticity of smooth muscle contraction (Gunst et al., 1995; Pavalko et al. 1995). Phosphorylation of cytoskeletal proteins has been observed during smooth muscle contraction. The phosphorylation levels of the dense-plaque proteins talin and paxillin have been correlated with force development in tracheal smooth muscle activated by various stimuli (Pavalko et al. 1995; Wang et al., 1996). Talin and paxallin have been implicated in the linkage of actin filaments to the cell membrane (via integrin) of smooth muscle (Turner et al., 1991). Thus the function of proteins that attach actin to the cell membrane may be regulated by phosphorylation. It has been proposed that in resting muscle, interactions between actin filaments and the plasma membrane may be relatively week, allowing for greater plasticity and increased potential for remodeling of cytoskeletal-membrane attachments; an increase in phosphorylation of talin and paxillin intensifies the attachments between actin filaments and membrane at dense-plaques during contraction (Pavalko et al. 1995). It has been demonstrated that paxillin undergoes tyrosine phosphorylation during smooth muscle contraction (Wang et al. 1996), therefore, the evidence cited above also demonstrates the significance of tyrosine phosphorylation in the regulation of smooth muscle contraction. Phosphorylation of intermediate filament proteins has also been reported during smooth muscle contraction (Park and Rasmussen. 1986). The physiological significance of this phosphorylation in the regulation of smooth muscle contraction however, needs further investigation.

1.3.5 Protein Kinase C

Involvement of protein kinase C (PKC) in the regulation of smooth muscle contraction has been implicated in phorbol ester-induced slow, sustained contractions (Jiang and Morgan, 1987; Danthuluri and Deth, 1984), and related phorbol compounds that do not activate PKC failed to elicit a contractile response. This was confirmed by addition of PKM (the constitutively active catalytic fragment of PKC generated by trypsin digestion) in inducing a slow, sustained contraction of skinned single smooth muscle cell (Collins et al. 1992). Phorbol esters are thought to substitute for DAG and activate PKC. In some cases, a transient increase in intracellular Ca^{2+} has been found on the addition of the active phorbol ester (Rembold and Murphy, 1988). The mechanism whereby phorbol esters elicit this increase has not been elucidated. In other cases, however, phorbol esters do not cause an increase in $[Ca^{2+}]_i$ or MLC_{20} phosphorylation (Itoh and Lederis, 1987; Jiang and Morgan, 1987). This Ca^{2+}-independent PKC mediated-regulation of smooth muscle contraction may contribute to the development of the latch bridge discussed above. Various isoenzymes of PKC have been detected in smooth muscle, Ca^{2+}-independent PKCε has been suggested to induce slow, sustained smooth muscle contraction in response to phorbol ester stimulation (Horowitz et al., 1996). In addition, translocation of PKCε from the cytosol to the surface membrane has been observed during smooth muscle contraction (Khalil et al. 1992).

Thin filament-associated regulation may contribute to PKC mediated Ca^{2+}-independent smooth muscle contraction (Walsh et al., 1996). Smooth muscle caldesmon and calponin are substrates for PKC. Phosphorylation of CaD and CaP has been identified

in intact smooth muscle during contraction (Adam et al., 1989; Mino et al., 1995). Caldesmon and calponin have been suggested to inhibit smooth muscle actomyosin Mg^{2+}-ATPase activity, with phosphorylation diminishing the inhibitory properties of CaD and CaP. It is expected thus, smooth muscle contraction could be achieved without changes in $[Ca^{2+}]_i$ and MLC_{20} phosphorylation via activation of PKC.

1.4 Airway Smooth Muscle and Asthma

While the functions of airway smooth muscle are still not clear, alterations in airway smooth muscle mechanical and biochemical properties have been identified in asthmatic and/or sensitized airways. Asthmatic airways show a specific and non-specific hyperresponsiveness to various stimuli. Increased contractility has been reported in ragweed pollen sensitized airway smooth muscle (Jiang et al., 1992). Enhanced contractility has also been identified in passively-sensitized human airway smooth muscle (Black et al., 1989). Most recently, the contractility of human asthmatic airway smooth muscle has been measured at single cell level, and found to be significantly increased compared with those of non-asthmatic muscle (Stephens et al., 1998b). The increased contractility of asthmatic muscle may result in excessive narrowing of airways, and lead to airway hyperreactivity. The molecular mechanisms for the increased contractility of asthmatic muscle are still not known, however, increased content and enzymatic activity of smooth muscle type myosin light chain kinase may contribute to these changes (Jiang et al., 1992b). Smooth muscle overgrowth has also been demonstrated in asthmatic airways. Heterogeneous smooth muscle cell hypertrophy and hyperplasia have been detected in human asthmatic airways (Ebina et al., 1993). Furthermore, airway smooth muscle cells from the hyperreactive animal model show increased proliferation (Zacour; Martin, 1996). This increase in smooth muscle mass could cause an increase of bronchoconstriction in response to agonist simply by increase of airway wall thickness and encroachment on the lumen (Wiggs et al., 1992). The most obvious evidence of airway

smooth muscle involvement in the asthma attack is the universal clinical experience that β-agonists can almost always relieve an acute asthmatic attack. Therefore, studies of airway smooth muscle should facilitate elucidation of pathogenesis of asthma and development of new treatment of asthma.

1.5 References

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Chapter 2. Material and Methodology

2.1 Biophysical Studies.

2.1.1 Measurement of contractility of smooth muscle strip.

Smooth muscle strip preparation. Ragweed pollen sensitized mongrel dogs or their healthy litter-mates were anaesthetized by an intravenous injection of 30 mg/kg wt of pentobarbital sodium. This was followed by rapid removal of the cervical trachea and lung, after which the dogs were sacrificed by an intravenous injection of saturated KCl. The trachea and lung were placed immediately in separate beakers of ice-cold, aerated Krebs-Henseleit solution composed of (mM): NaCl, 115; $NaHCO_3$, 25; NaH_2PO_4, 1.38; KCl, 2.51; $MgSO_4$-$7H_2O$, 2.46; $CaCl_2$, 1.91; and dextrose, 5.56. The tracheobronchial tree was dissected away from the lung parenchyma and placed in cold, fresh Krebs-Henseleit solution. Bronchial generations were identified according to a method reported by Shioya et al. (1987), with the trachea as generation 0 (G0), main bronchi generation 1 (G1), and so on. Tracheal smooth muscle strips were obtained with the connective tissues carefully dissected away under a binocular microscope as described by Stephens et al. (1988). Bronchial smooth muscle strips were prepared essentially as reported by Jiang et al. (1990). Since the smooth muscle fibre orientation may be complex at bifurcations of the bronchial tree (Macklin, 1929), bronchial rings were cut out from nonbranching segments of the each generation of bronchus. After careful removal of cartilage and epithelium from a bronchial ring, a bronchial smooth muscle strip was cut out in the radial direction

(Ebina et al., 1990).

In vitro mechanical study. Measurements of mechanical muscle strip function were conducted essentially as described by Seow and Stephens (1986). A smooth muscle strip was mounted in an organ bath containing Krebs-Henseleit solution aerated with 95% O_2-5% CO_2 mixture at 37°C. The lower end of the strip was anchored to a clamp at the bottom of the bath, and the upper end was connected to an electromagnetic lever system (Brutsaert et al., 1971; Mitchell and Stephens, 1983), with braided 7-0 surgical thread. The strip was equilibrated in the bath for 1.5-2 hours, during which time it was stimulated electrically for 10 seconds at every 10 min by a 20 V, 60 Hz alternating voltage applied to platinum electrodes positioned on either side of the muscle. Steady state was achieved when the muscle developed a steady tetanic tension under stimulation. The optimal length (Lo) was obtained by stretching the muscle strip and measuring the isometric tetanic tension. The length that was associated with the development of maximum active isometric tension (Po) was identified as Lo, and the passive tension at this state was the resting tension (Pre). All experiments to be described were conducted at Lo. Conventional length-tension curves were determined as previously described by Stephens et al.(1988). Maximum shortening capacity and zero-load velocity of shortening were obtained by applying critically damped quick release techniques (Seow and Stephens, 1986). To determine the behaviour of Vo as a function of time, zero-load clamps were applied at times from 1 to 10 second at 1 second intervals in the course of a contraction of the muscle which has a contraction time of 11 seconds. The instantaneous force and

displacement produced by the muscle were recorded and analyzed with the help of a computer and analog-to-digital converter.

2.1.2 Measurement of contraction in single cells

Preparation of freshly isolated contractile airway smooth muscle cells. Preparation of contractile airway smooth muscle cells were carried out as described by Wade and Sims (1993). Briefly, tracheal and bronchial (generation 3 to 6) smooth muscles dissected out free of cartilage, epithelium, and vasculature were firstly cut into strips approximately 1-2 mm wide and 1 cm long, and then incubated at 37°C in Hank's solution with 10 mM taurine, 400 units/ml collagenase, 30 units/ml papain, 1 mg/ml BSA, and 1 mM DTT for 45 and 20 min respectively. After enzymatic digestion, the tissue was washed with Ca^{2+} free Krebs-Henseleit solution several times, and cells were finally dispersed by gentle trituration with a Pasteur pipette in Ca^{2+} free Krebs-Henseleit solution, and then stored on ice for study within 6 hours. Most freshly isolated cells appeared fully relaxed with smooth and shining sarcolemmal membranes as seen under an inverted microscope. Addition of relaxant agents such as norepinephrine, atropine, and isoproterenol did not cause further relaxation of cells. These cells showed reversible contractile responses to ACh, histamine, KCl, and single pulse electrical stimulation.

Preparation of contractile single cells from arrested smooth muscle cell culture. Arrested

smooth muscle cells were prepared as described in section 2.3. Arrested cultures were first washed three times with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS), which was pre-warmed to 37°C. Cells were lifted by addition of PBS containing trypsin (0.05%)/EDTA (0.53 mM) and occasional gentle shaking. Lifted cells were then stored at 4°C, without washing or any other disturbance, for further study. This was critical to keep serum deprivation-induced spindle-shaped cells in relaxed state. Measurement of cell shortening was conducted within an hour.

Measurement of single cell contraction. All studies on isolated smooth muscle cells were conducted at room temperature. Freshly, isolated cells were added to a custom designed chamber containing 1 ml aerated Krebs-Henseleit solution, and allowed to settle for 5 min, after which the chamber was slowly perfused with fresh Krebs-Henseleit solution. Cells did not adhere to the bottom of the chamber, because no detectable force (<1 pN) could be measured to move the cells settled down using a custom designed, extra-sensitive single cell lever system. Cell length was measured with an inverted microscope. For analysis of length distribution, lengths of all cells in a field were measured except for those with obvious contracture which could be clearly recognized by the presence of blebs emanating from the sarcolemma. Shortening capacity of cells was measured applying 10 Hz, 35 volts, 1 ms width bi-polar electric pulse stimulation, with one spot electrode placed approximately 10 μm away from the cell, and the other at a random position in the chamber. Throughout the experiment, images of the cells were monitored and recorded via a CCD videocamera mounted on the microscope. The images were then digitized, and

analyzed with a computer program ("peak 5") for maximum shortening capacity and maximum velocity of shortening.

2.2. Biochemical studies

2.2.1 Preparation of smooth muscle protein homogenate

Tracheal and bronchial smooth muscle. Tracheal and bronchial smooth muscle were dissected out free of cartilage, connective tissue, vasculature, and epithelium as described above. The tissues were minced into small pieces with scissors, quickly frozen in liquid nitrogen, and pulverized with a crescent dental amalgam mixer for 30 seconds. Protein homogenates were prepared by mixing the obtained powder in protein extraction buffer (10 μl/mg wet weight of tissue) which contained tris(hydroxymethyl) aminomethane (Tris).HCl (50 mM, PH 7.6), 0.3% sodium dodecyl sulfate (SDS), 0.6 mM β-mercaptoethanol, 20 μl/ml leupeptin, 250 μm phenylmethylsulfonylfluoride (PMSF), 50 mg/ml soybean trypsin inhibitor, and 10 μg/ml DNAse I, and then heated at 95°C for 10 min to fully solubilize and denature the proteins. The homogenates were then centrifuged at 10,000g for 10 min at 4°C and stored at -20°C for electrophoretic analysis within a month.

Cultured smooth muscle cells. Cultured smooth muscle cells were collected by trypsinization beginning from the day of confluence, and every three days thereafter during serum deprivation, until day 15. The collected cells were washed twice by resuspension in ice cold PBS following centrifugation. Crude protein homogenates were prepared in Tris lysis buffer containing 1.5% NP-40 (PH 7.6) to which protease inhibitors (leupeptin, PMSF, and soybean trypsin inhibitor) were added fresh. Cells were disrupted by pipetting several times. Samples were finally stored at -20°C until used for electrophoretic analysis. The storage period did not exceed one month.

2.2.2 Protein Assay

The protein content of all samples was measured using the Bio-Rad Protein Assay Kit with a Milton Roy Spectronic 1001 Plus spectrophotometer. Bovine serum albumin (BSA), Fraction V (Sigma Chemicals, St. Louis) was used as a relative protein standard for all assays.

2.2.3 Western blot analysis

Protein homogenates were fractionated by SDS-polyacrylamide electrophoresis (PAGE) on 8 × 10 cm minigels as first described by Laemmli (1970), and then transferred

to nitrocellulose, as described by Stephens et al. (1991). Blots were blocked overnight at 4°C in 10mM Tris buffered saline with 0.1% Tween (TTBS), containing 3% non-fat dried milk powder. Blots were then incubated in primary antibody (1:1000 monoclonal mouse-anti-sm-MLCK, Sigma; 1:10,000 monoclonal mouse-anti-sm α-actin, Boehringer; 1:10,000 polyclonal rabbit-anti-sm myosin heavy chain (both SM1 and SM2), Groeschel-Stewart; 1:1,000 polyclonal rabbit-anti-SM-B, gift from Dr. R. Low, Univ. Of Vermont; 1:7500 rabbit-anti-nm-MLCK polyclonal antibody, gift from Dr. Gallagher, Indiana U; and 1:10,000 rabbit anti-nm-MHC (NMA and NMB), gift from Dr. R. Adelstein, Natl. Institutes of Health, Bethesda, MD) diluted in TTBS containing 1% milk for 2 two 3 hours at room temperature. Blots were then washed with TTBS containing 1% milk three times, and incubated for 40 min at room temperature with biotinylated secondary antibodies (Amersham Life Science, Oakville, ON) diluted 1:1000 in TTBS containing 1% milk powder. Blots were rinsed again with TTBS and finally incubated for 40 min at room temperature with streptavidin-horse radish peroxidase conjugate (Amersham Life Science, Oakville, ON) diluted 1:5,000 in TTBS. Semi-quantitative staining of specific proteins was achieved using chemiluminescence detection; blots were washed in TTBS and subsequently dipped, for one min, into luminol substrate solution (Amersham). Chemilumigrams were developed on Hyperfilm-ECL (Amersham); the normal exposure times ranged from 30 sec to 5 min. An LKB Ultroscan XL laser densitometer was employed to scan the developed films for estimation of protein content. No detectable cross-reactivity of the muscle and non-muscle protein isoforms was found using antibodies for non-muscle MHC, non-muscle MLCK, smooth muscle MHC, and smooth muscle

MLCK. All values were normalized to total protein loaded onto the gels to allow for comparison between samples.

2.2.4 RT-PCR

To study the level of the message of a newly identified smooth muscle myosin heavy chain isoform which possesses a 7 amino acid insert in its N-terminal head in cultured tracheal smooth muscle cells at different time points of serum deprivation, reverse transcription-polymerase chain reaction analysis was performed as described by Meer and Eddinger (1996). Total RNA was extracted from airway smooth muscle strip or cultured cells with the use of TRIZOL® Reagent (GIBCOBRL). RNA (1.0 μg) was then added to the RT reaction mixture to a final volume of 10 μl and incubated at 37°C for 2 h. PCR was performed using two oligonucleotide primers, corresponding to rabbit stomach smooth muscle myosin, flanking the 21-nucleotide exon in the N-terminal head that encodes the difference sequence between inserted and non-inserted smMHC isoforms (Babij, 1993). Thirty cycles of amplification were performed. The PCR products generated with the use of these primers were 162 and 141 bp, corresponding to smMHC mRNA with or without the 21-nucleotide insert. These products were separated on 4% agarose gel, stained with ethidium bromide and visualized under UV light.

2.3 Smooth muscle cell culture

Tracheas were excised from anesthetized, 6-12 month old mongrel dogs and placed into ice-cold calcium-free Krebs' solution. Trachealis muscle was dissected, cleaned of serosa, vasculature, and epithelia at room temperature, and washed four times in Hank's Balanced Salt Solution (HBSS) containing 100 mg streptomycin/ml, 100 U penicillin/ml under aseptic conditions. The muscle was then minced thoroughly with fine scissors, resuspended in digestion buffer (HBSS containing 600 U collagenase/ml (Gibcol/BRL), 8 U type IV elastase/ml (Sigma), and 1 U type XXVII Nagarse protease/ml (Sigma)). Cells were isolated by serial digestion (three stages, 45 min each) with vigorous shaking at 37°C. The fractions were pooled, filtered through 70 μm nylon mesh, and then washed and diluted in culture medium (DMEM) containing 10% fetal bovine serum. After their number was estimated by counting with a model Z_{BI} Coulter counter, the cells were plated into 100-mm plastic culture dishes at a density of 5000 cells/cm^2 and allowed to attach for 36 hrs. Cells were grown at 37°C in a humidified atmosphere consisting of 95% air/5% CO_2. The medium was then replaced with fresh medium containing 10%FBS and antibiotics every 72 hrs. For all the studies, only the primary cultures were used unless otherwise stated. Cultures reached confluence normally within 6 to 7 days. For preparation of arrested smooth muscle cells, cultures were switched to serum-free medium (F-12/Redu-serum) containing insulin, transferrin, and selenium upon confluence, and starved for up to 15 days. Spindle-shaped contractile phenotype of cells emerged beginning from the third day of deprivation and increased in number during continued deprivation. Cells

were counted in 5 field segments (upper-right and -left, lower-right and -left, and center) directly from each culture dish under a phase-contrast microscope; five dishes were counted for each time point, and the final data were expressed as the arithmetic average of the counts obtained from five dishes.

2.4 Fluorescent Immunocytochemistry

Freshly isolated cells were plated in 6-well dishes containing 22 × 22 mm Rat Tail Collagen, and Type I coated glass coverslips (Becton Dickinson). When the cells attained confluence they were arrested for 10 days, by withdrawing serum, the coverslips and attached cells were rinsed with PBS and fixed in 1% paraformaldehyde/PBS (pH 7.6) for 15 min at 4°C. They were subsequently permeabilized using 0.1% Triton X-100/PBS for 15 min at 4°C, and then rinsed with PBS and used immediately for immunostaining or stored in PBS containing 0.05% Sodium azide for a maximum of 10 to 14 days. For immunostaining, the coverslips were incubated in blocking solution (PBS containing 5% normal goat serum, and 0.1% Tween-20) for 2-4 hrs at 4°C in a humidified chamber. After rinsing with PBS containing 1% BSA and 0.1% Tween-20, the coverslips were incubated with primary antibodies diluted in PBS containing 1% BSA and 0.1% Tween-20 overnight in a cold room. The dilutions of antibodies were as follows: 1:200, rabbit-anti-nmMLCK; 1:25, mouse-anti-smMLCK; 1:25, mouse-anti-sm-α-actin; 1:25, rabbit-sm-MHC; 1:25, mouse-anti-β-tubulin. Negative controls were incubated in PBS containing 1% BSA and 0.1% Tween-20 without primary antibody. The coverslips were then

incubated for 1-2 hours at 4°C with secondary antibodies diluted at 1:100 to PBS containing 1% BSA and 0.1% Tween-20 according to manufacturer's instructions. The secondary antibodies used included: 1) FITC-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch, Code Number 515-095-003), 2) FITC-conjugated donkey anti-rabbit IgG (Amersham Life Science, Catalogue Number N1034), 3) Cy3-conjugated goat anti-mouse IgG and IgM, Jackson ImmunoResearch, Code Number115-165-044), and 4) Cy3-conjugated sheep anti-rabbit IgG (Jackson ImmunoResearch, Code Number111-165-003). Thereafter coverslips were washed three times with PBS, twice with double-distilled water, and finally subjected to nuclear staining using Hoechst 33342 dye (20μg/ml bisbenzamide). Stained coverslips were mounted on glass slides using anti-fade media (85% glycerol/1mM p-phenylenediamine/100mM Tris-HCL, pH 7.4). Prepared slides were finally stored in the dark at -20°C until they were viewed and photographed using a Nikon Diaphot microscope equipped with epifluorescence optics and a 35mm camera.

2.5 Mechanical studies of human asthmatic bronchial smooth muscle cells.

Selection and evaluation of human subjects. Five normal subjects and five subjects with asthma were recruited to this study. Normal subjects had no history of disease, no medication and no occupational exposure to sensitizing agents. In asthmatic subjects the diagnosis was made according to the definition of the American Thoracic Society (1987). Only subjects who were taking only inhaled $\beta 2$ agonist on demand were included in the

study. No subjects had upper and lower airway infections during the month preceding the bronchoscopy. All were current non-smokers. Three asthmatic subjects had smoked less than 7 pack-years and quit more than 4 years ago. The evaluation included a medical history, a physical examination, skin prick tests to common allergens (animal dander, dust mites, pollens, and molds obtained from Omega, Montreal, Canada), a spirometry and measurement of airway responsiveness to areosolized methacholine according to standardized procedures (Juniper et al., 1992). Concentrations of methacholine up to 256 mg/ml were used and the responses were expressed as the provocative concentration of methacholine inducing a 20% fall in FEV1 (PC20). The study was approved by the Laval Hospital Ethics Committee and all subjects provided written informed consent for their participation in the study.

Bronchoscopy and bronchial biopsies. Before bronchoscopy, a 200-μg dose of salbutamol was given to the asthmatic subjects using a metered-dose inhaler (Glaxo Wellcome, Mississauga, Ontario, Canada). Oxygen was administered, and vital signs, electrocardiogram and oximetry were monitored throughout the endoscopy. After local anesthesia of the throat, larynx and trachea with 4% lidocaine, a flexible bronchoscope (Olympus OES 10 fiberscope, Olympus, Marklan, Ontario, Canada) was introduced into the bronchial tree. Bronchial anesthesia was completed with 2% lidocaine up to a total 400 mg. Eight specimens from 3rd to 6th generation bronchi were taken from segmental or lobar carinae of the right lung. These specimens were suspended in modified Krebs-Henseleit solution and kept on ice until processing. After the bronchoscopy, inhaled

salbutamol was administered again if necessary and subjects were observed until it was felt they could resume their normal activity.

Surgical specimens. Third to 6th-generation bronchi were obtained from nonpathologic regions of lobectomy specimens within 30 min of surgery. Each surgical specimen was immediately placed in cooled Krebs-Henseleit solution. Airway smooth muscle was dissected out after careful removal of cartilage and epithelium.

Preparation of single cells and measurement of their contractility. Preparation of contractile cells was carried out using a method similar to that described above for canine airway smooth muscle. Four to 6 biopsies were pooled and incubated in enzymatic solution for 45 min. After enzymatic digestion, the tissue was washed with Ca^{2+}-free Krebs-Henseleit solution several times, cells were finally dispersed by gentle trituration with a Pasteur pipette in Ca^{2+} free Krebs-Henseleit solution, and then stored on ice for study within 6 hours. Contractility of these cells was measured as stated in the section entitled "measurement of single cells contraction".

2.6 Statistics

Data were expressed as mean ± standard error (SE). Po was normalized with respect to cross-sectional area of muscle as described by Jiang et al.(1991). General linear

model (GLM) analysis of variance (GLM ANOVA) was employed to analyze the differences in mean values of mechanical parameters among different airway generations. Duncan's new multiple range test was used as a complementary analysis to the ANOVA, so as to determine which generations were responsible for differences indicated by ANOVA, with P set at ≤ 0.05. Cluster analysis (Hartigan, 1975) was performed to identify functional groupings of airway generations. The unpaired two-tailed Student's t-test was used to compare mean values between two groups as needed. Cell length distribution was analyzed using the MIX program: an interactive program for fitting mixtures of distribution by finding a set of overlapping component distributions that gives the best fit to the histogram, which was developed by Dr. P. D. M. Macdonald of McMaster University and marketed by Ichthus Data Systems. The statistical method used to fit the mixture distribution to the data is maximum-likelihood estimation for grouped data. The goodness-of-fit chi-square statistic is used to test how well the mixture distribution fits the histogrom overall. This program enabled us to identify the different cell populations.

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Chapter 3. Biophysical and biochemical heterogeneity of airway smooth muscle obtained from different generations of canine airways

3.1 Background

The structure and physiological functions of airway segments are believed to be dependent on airway generation (Horsfield 1974). Central airways seem to mainly control overall airway resistance to airflow and gas distribution, while peripheral airways mainly control the regional ratios of perfusion to ventilation. Though the physiological function of airway smooth muscle is still unclear, airway size, and consequently airway resistance and regional ventilation/perfusion ratios must be regulated by airway smooth muscle contraction. Therefore, the heterogeneity of airway smooth muscle may be of great importance in determining topographical functional differences of airway generations. It may also be involved in the pathogenesis of acute asthma, potentially determining a nonhomogeneous distribution of bronchoconstriction (Ebina et al., 1990; McFadden, 1977; Takishima, 1991), and in chronic asthma, determining a nonhomogeneous development of airway smooth muscle hypertrophy and hyperplasia (Dunnill et al., 1969; Ebina et al., 1993). Therefore, studies of the heterogeneity of airway smooth muscle function along the airway tree may facilitate elucidation of airway smooth muscle physiological function and pathogenesis of asthma as well.

Heterogeneity in smooth muscle contractile responsiveness has been reported in several species (Brown et al., 1993; Chitano et al., 1993; Fleisch and Calkins 1976; Gauthier et al., 1992; Shioya et al., 1987), however, direct evidence of heterogeneity in airway smooth muscle mechanical properties is still lacking, since all these studies were

conducted in whole airway tissues, which included cartilage and epithelium. The existence of these structures will modulate airway smooth muscle mechanical behaviour (Aizawa et al., 1988; Jiang and Stephens, 1990; Stuart-Smith 1990). In these studies, furthermore, only isometric contraction was examined, instead of shortening capacity and velocity of shortening which are more important parameters in regulating airway resistance *in vivo* (Stephens et al., 1988). Hence, in the present experiments, we dissected out airway smooth muscle strips and carefully removed cartilage and epithelium from the trachea down to bronchial generation 6. The isometric and isotonic mechanical properties of these strips were compared. Results showed that canine airway smooth muscle could be divided into two groups: extrapulmonary vs intrapulmonary. The extrapulmonary group consisted of smooth muscle from trachea and bronchial generation 1 and 2, which manifested increased contractility. The intrapulmonary group included smooth muscle from bronchial generation 3 to 6, and showed reduced contractility compared with the extrapulmonary.

Contractility of smooth muscle is regulated by its contractile and regulatory proteins. Differences in expression of these proteins between different types of smooth muscle could account for differences in their contractility. The regulation of smooth muscle contraction is regulated mainly by smooth muscle myosin light chain kinase (smMLCK). Increased expression and/or activity of smMLCK would result in increase of contractility of smooth muscle because of increased activation of myosin ATPase activity through an enhanced level of regulatory myosin light chain (MLC20) phosphorylation. Contractility of smooth muscle could also be determined by expression of smooth muscle

myosin heavy chain (smMHC) isoforms. Four smMHC isoforms have been identified thus far (Rovner et al., 1986; White et al., 1993). Two isoforms, SM1 and SM2, varied in their C-terminal regions but showed no differences in their ATPase activity and velocity of translocation of actin filaments *in vitro* (Kelley et al., 1993). Currently no correlation has been found between the distribution of SM1 and SM2 and contractility of smooth muscle. The two most recently identified N-terminal isoforms of smMHC, SM-A and SM-B, are believed to be important in determining the contractility of smooth muscle. SM-B with an extra 7 amino acid insert in its N-terminal region showed significantly elevated ATPase activity and actin filaments motility *in vitro* (Kelley et al., 1993). Increased velocity of shortening (Vo) was found in smooth muscle which expressed greater amounts of SM-B (DiSanto et al., 1997). Therefore, to further understand molecular mechanisms underlying contractile differences of airway smooth muscle, expression of smMLCK and SM-B were compared between extra- and intrapulmonary smooth muscles.

3.2 Objectives

Identification of biophysical and biochemical heterogeneity of airway smooth muscle tissue along the airway tree.

3.3 Hypothesis

1. Airway smooth muscles from different generations of airway possess different contractility.

2. In addition, biochemical properties also differ in smooth muscles obtained from different sites along the airway.

3. Differences in amount of smooth muscle contractile and regulatory protein expression contribute to mechanical differences in mechanical properties of airway smooth muscle.

3.3 Protocol

Airway smooth muscle strips were dissected out free of cartilage, epithelium, connective tissue, and vasculature from canine trachea down to bronchial generation 6.

The mechanical properties of these samples, such as maximal isometric force (Po), maximal isotonic zero-load shortening capacity (ΔLmax), and maximal velocity of shortening (Vo) at the 2 and 8 second points of a contraction (V2, which represents cycling rate of normal cross-bridges; and V8, which represents cycling rate of latch cross-bridge), were measured employing an electro-magnetic lever system and compared among different generations of airways. Smooth muscles were then grouped statistically based on their mechanical properties employing cluster analysis. Finally, the biochemical properties of these samples were analyzed using western blotting, and compared among groups to identify the biochemical basis of mechanical heterogeneity.

3.4 Results

Biophysical studies:

In vitro mechanical studies of airway smooth muscle strips free of both cartilage and epithelium, showed that there were significant differences in ΔLmax, V2 and V8, and in the ratio of V8 to V2, between different generations of airway from trachea to bronchial generation 6, without any obvious changes in normalized Po (Table 1). The mechanical properties of airway smooth muscle decreased progressively down the airway tree. The magnitudes of the alteration varied with different mechanical parameters: 45.68% decrease in ΔLmax, 50.19% decrease in V2, 69.52% in V8, and 39.13% decrease in V8/V2 from trachea to bronchial generation 6.

Cluster analysis after pooling all mechanical data without distinguishing airway generations indicated that airway smooth muscle from trachea down to bronchial generation 6 can be divided into two groups based on their mechanical behaviour, one with higher mechanical performance-higher ΔLmax, V2, V8, and V8/V2, the other with lower. Cluster analysis by pooling mean values from different generations of airway tree further indicated that the group with higher mechanical performances consisted of smooth muscle from extrapulmonary airways containing trachea and bronchial generation 1 and 2. Because of their location we called this group the extrapulmonary group; the second

group referred to as the intrapulmonary group and characterized by lower mechanical performances, consisted of bronchial generations 3 to 6. No statistical differences in Po were found among them (Fig. 1). There were statistically significant differences in ΔLmax, V2, V8, and V8/V2 between these two groups (Fig. 2). GLM ANOVA showed that there were no significant differences in mechanical properties between bronchial generations within intrapulmonary group, though the data did show a trend of decreases from bronchial generation 3 to 6 (Table 1). In the extrapulmonary group, except for V8 and V8/V2, the other mechanical parameters of smooth muscle did not show significant differences among bronchial generations within the group.

In consonance with no differences in Po, there were no significant differences in the characteristics of active length-tension curves between trachea and bronchial generation 5 (Fig. 3). Comparison of zero-load velocity-time relationships revealed a significant difference between trachea and bronchial generation 5 (Fig. 4). Smooth muscle from bronchial generation 5 developed maximum shortening velocity earlier than the tracheal smooth muscle though its magnitude was less, and also decreased more rapidly and to a greater extent. It was also found that smooth muscle from bronchial generation 5 relaxed faster than muscle from the trachea, both in isometric contraction and isotonic shortening with EFS stimulation.

Biochemical studies:

To investigate molecular mechanisms which may be responsible for the differences in contractility between extra- and intrapulmonary smooth muscles, tracheal (representing the extrapulmonary group) and bronchial (from generation 3 to 6) smooth muscles were dissected out free from cartilage and epithelium, and subjected to Western-blot analysis employing specific antibodies. No significant differences were identified in protein content of total smooth muscle myosin heavy chain between extra- and intrapulmonary smooth muscles (Fig. 5). Extrapulmonary smooth muscle showed significantly increased expression in sm-α-actin, and sm-MLCK when compared with intrapulmonary muscle (Fig. 6). A significantly increased expression of SM-B was also identified in extrapulmonary smooth muscle both at protein and messenger level (Fig. 7). Approximately a 9 times difference in SM-B protein content was identified between TSM and BSM, while only a twice difference was found for SM-B mRNA. No explanation for such a difference was available to date. Differences in mRNA and protein stability and/or protein translation efficiency may contribute to this difference.

Table 1. Mechanical parameters of airway smooth muscle from different generations of airway

Generation	Po(mN/mm2)	ΔLmax(Lo%)	V2(Lo%/sec)	V8(Lo%/sec)	V8/V2
0(8)	165.1±20.2	70.6±4.1[a,b,c,d]	32.3±3.8[a]	22.3±1.9[a,b,c,d,e,f]	0.69±0.04[a,b,c,d,e]
1st(6)	166.5±22.1	61.2±3.8[e]	24.2±4.4	14.2±2.2[a,g]	0.60±0.05[f,g,h]
2nd(8)	171.4±19.1	62.3±4.1[f,g]	31.7±3.8[b]	13.9±1.9[b,h]	0.45±0.04[a,f]
3rd(10)	147.9±17.1	48.3±3.7[a]	22.6±3.4	10.4±1.7[c]	0.49±0.04[b]
4th(7)	187.1±20.1	50.4±4.4[b,h]	20.8±4.1	10.2±2.1[d]	0.48±0.04[c]
5th(7)	166.5±20.5	47.1±3.5[c,f]	19.2±4.1	8.3±2.1[e]	0.42±0.04[d,g]
6th(6)	151.4±18.2	38.4±2.7[d,e,g,h]	16.1±2.3[ab]	6.8±1.5[f,g,h]	0.42±0.04[e,h]

Values are means ± SE; Po was normalized with respect to muscle cross-sectional area; numbers of samples are indicated in parentheses; for each column, groups denoted by the same letter are significantly different revealed by Duncan's new multiple test with P set at 0.05.

Figure 1. Isometric Tension of Extrapulmonary and Intrapulmonary Canine Airway Smooth Muscles.

No statistically significant difference was found in isometric tension (Po) between extra- and intrapulmonary canine airway smooth muscles. Extra-SM: extrapulmonary airway smooth muscles, including smooth muscles isolated from trachea, and bronchi generation 1 and 2; Intra-SM: intrapulmonary airway smooth muscle, including smooth muscles isolated from bronchi 3 to 6.

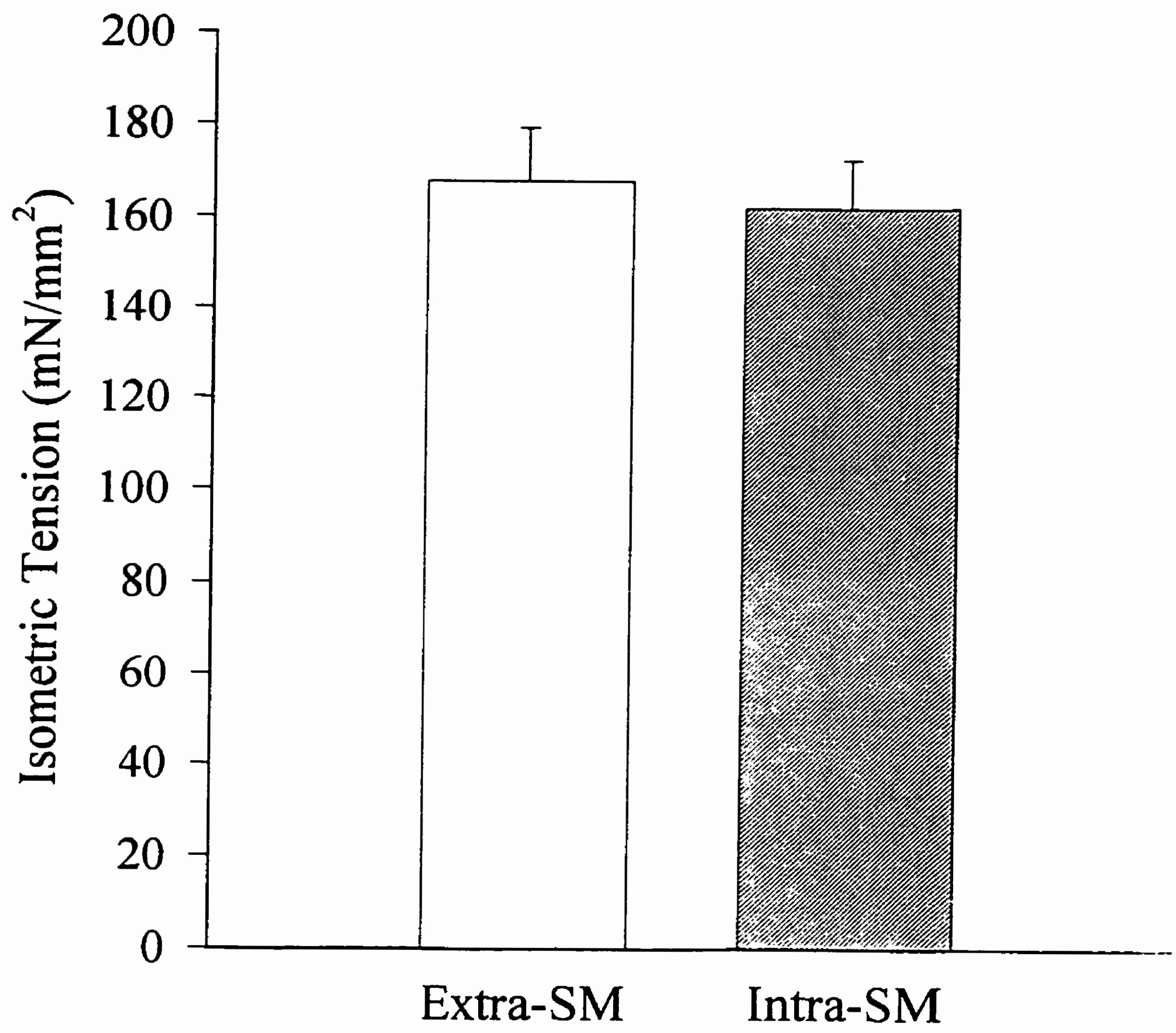
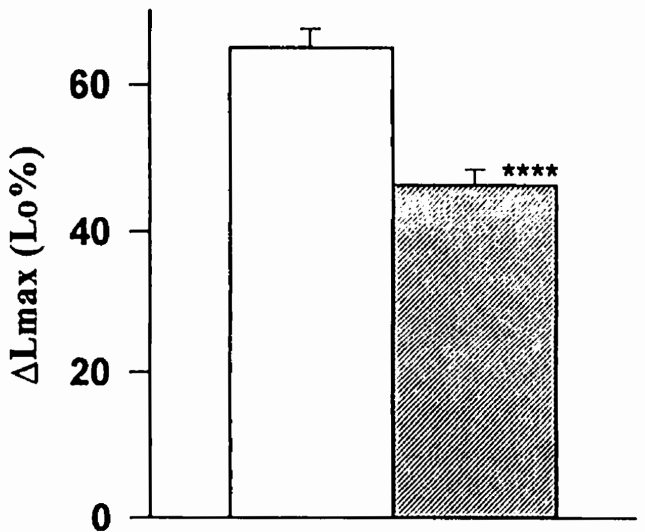
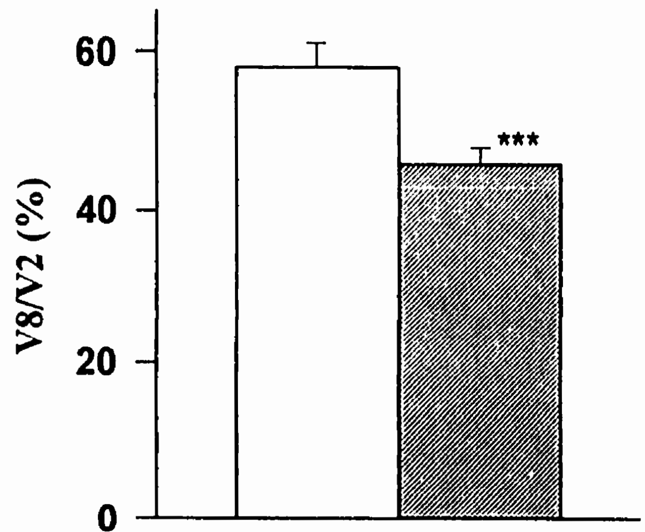

200
180
160
140
120
100
80
60
40
20
0
Isometric Tension (mN/mm²)
Extra-SM
Intra-SM

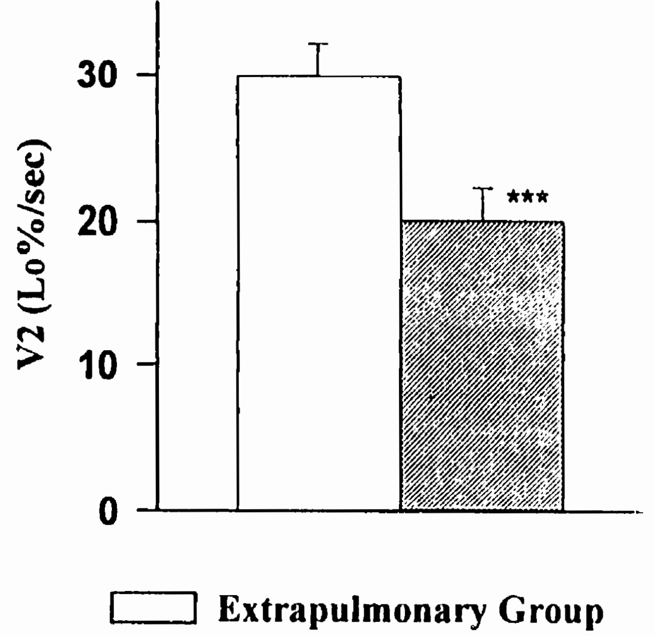
Figure 2. Shortening Properties of Extra- and Intrapulmonary

Canine Airway Smooth Muscles

Extrapulmonary airway smooth muscle showed significantly increased shortening properties compared with intrapulmonary airway smooth muscle. ΔLmax: maximal capacity of shortening; V2: maximal velocity of shortening at 2 second; V8: maximal velocity of shortening at 8 second; Lo: optimal length of contraction; ***$P<0.005$ (unpaired student t test); ****$P<0.001$ (unpaired student t test).


ΔLmax (Lo%)
60
40
20
0
****


V8/V2 (%)
60
40
20
0
***


V2 (Lo%/sec)
30
20
10
0
***

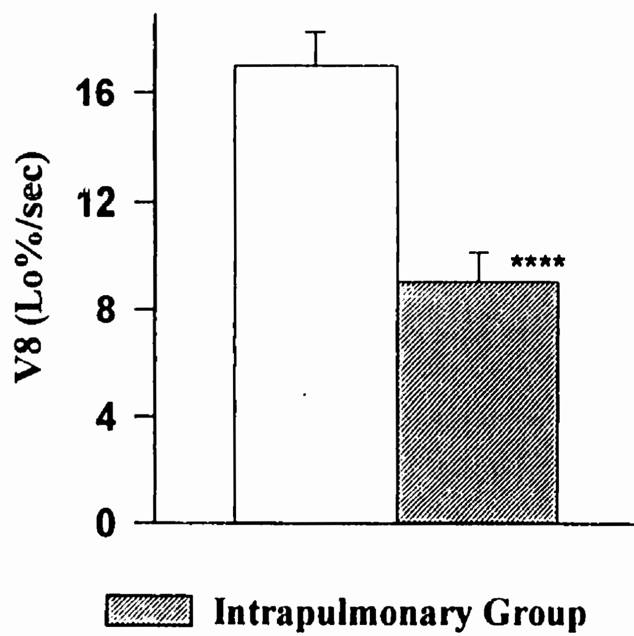
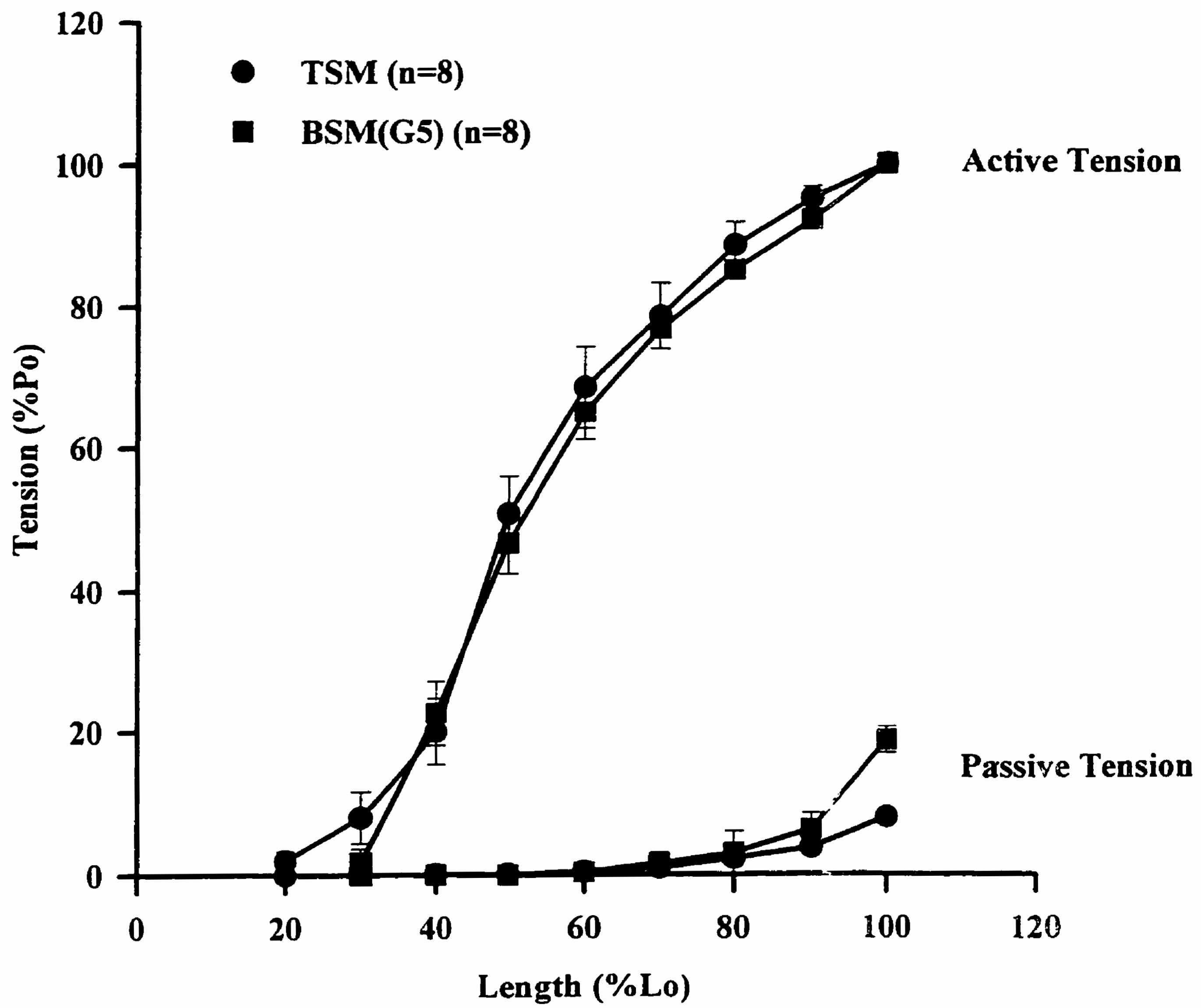

V8 (Lo%/sec)
16
12
8
4
0
****
Extrapulmonary Group
Intrapulmonary Group

Figure 3. Length-tension relationship for airway smooth muscle.

Means and standard errors are showed. There is no significant difference in active length-tension curves between tracheal (TSM) and bronchial smooth muscle (BSM). Po, maximal isometric tension; Lo, optimal length of contraction; n, number of samples.



TSM (n=8)
BSM(G5) (n=8)
Active Tension
Passive Tension
Tension (%Po)
Length (%Lo)
0
20
40
60
80
100
120

Figure 4. Zero load velocity-time course for airway smooth muscle.

Means and standard errors are shown. There is a significant difference between tracheal (TSM) and bronchial smooth muscle (BSM) ($P<0.05$, multiple-way of ANOVA). Lo, optimal length of contraction; n, number of samples.

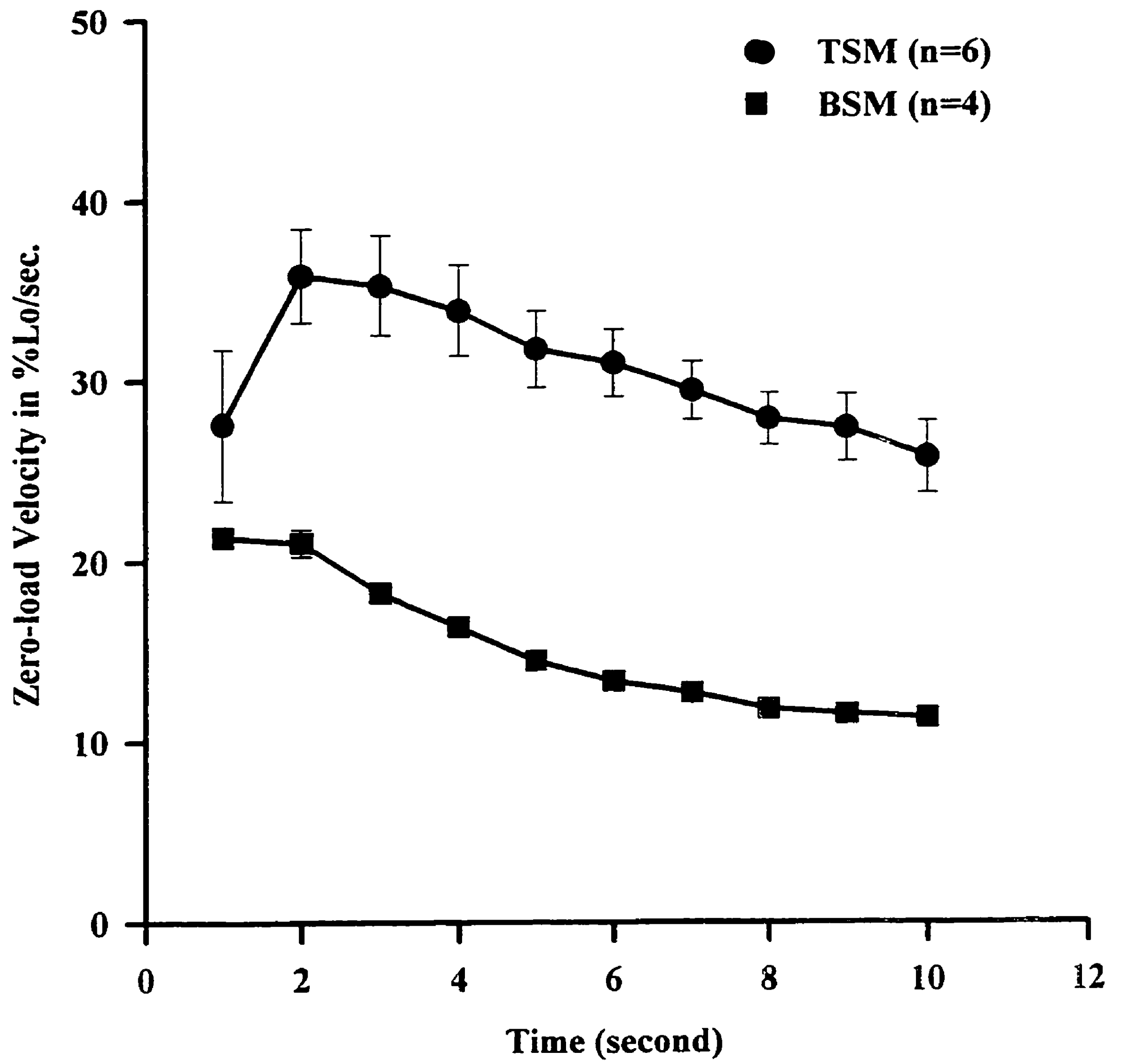

TSM (n=6)
BSM (n=4)
Zero-load Velocity in %L0/sec.
50
40
30
20
10
0
0
2
4
6
8
10
12
Time (second)

Figure 5. Protein Content of Total Smooth Muscle Myosin Heavy Chain for Airway Smooth Muscle

No significant difference is found in protein content of total smooth muscle myosin heavy chain between tracheal (TSM) and bronchial smooth muscle (BSM).

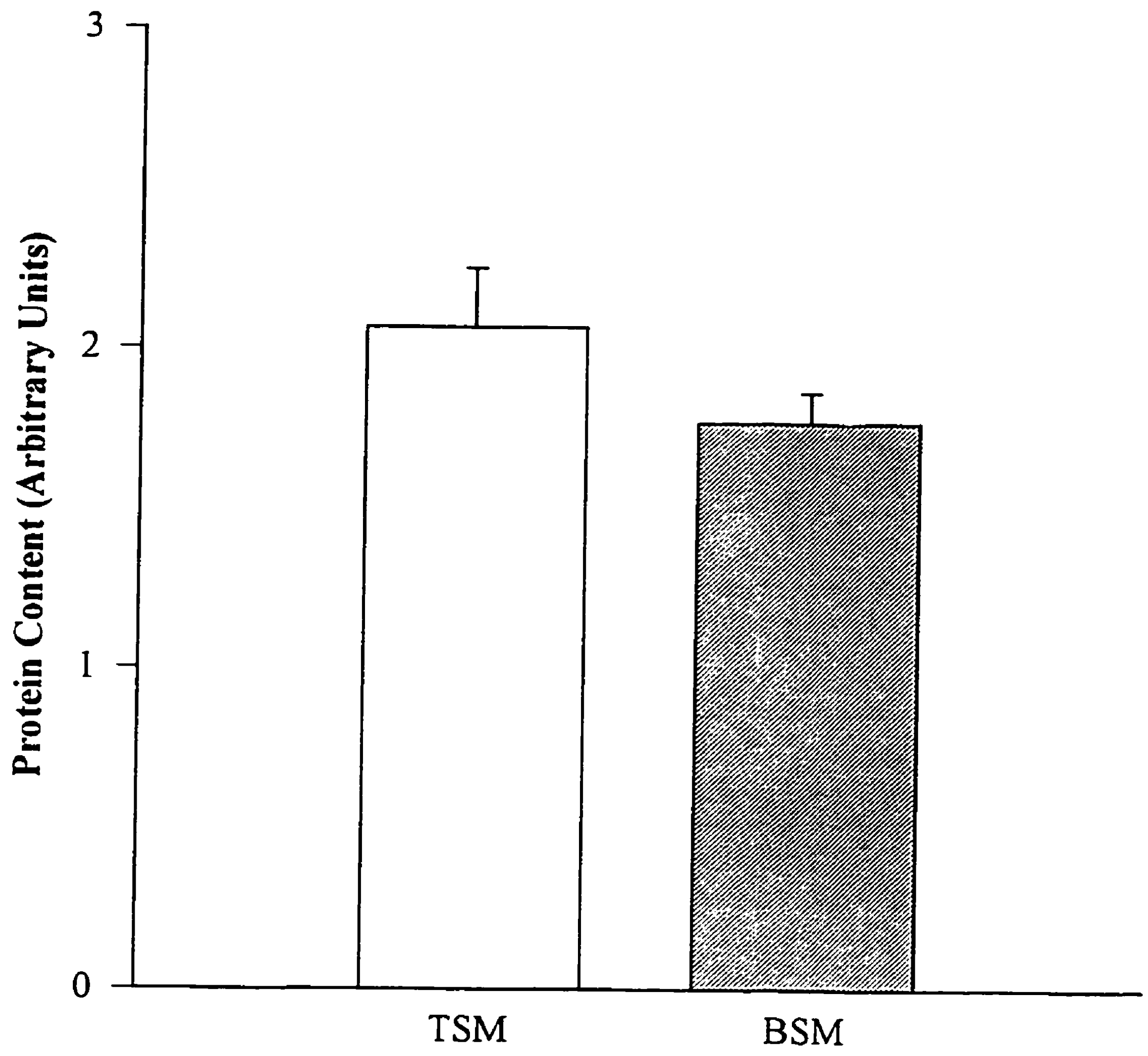
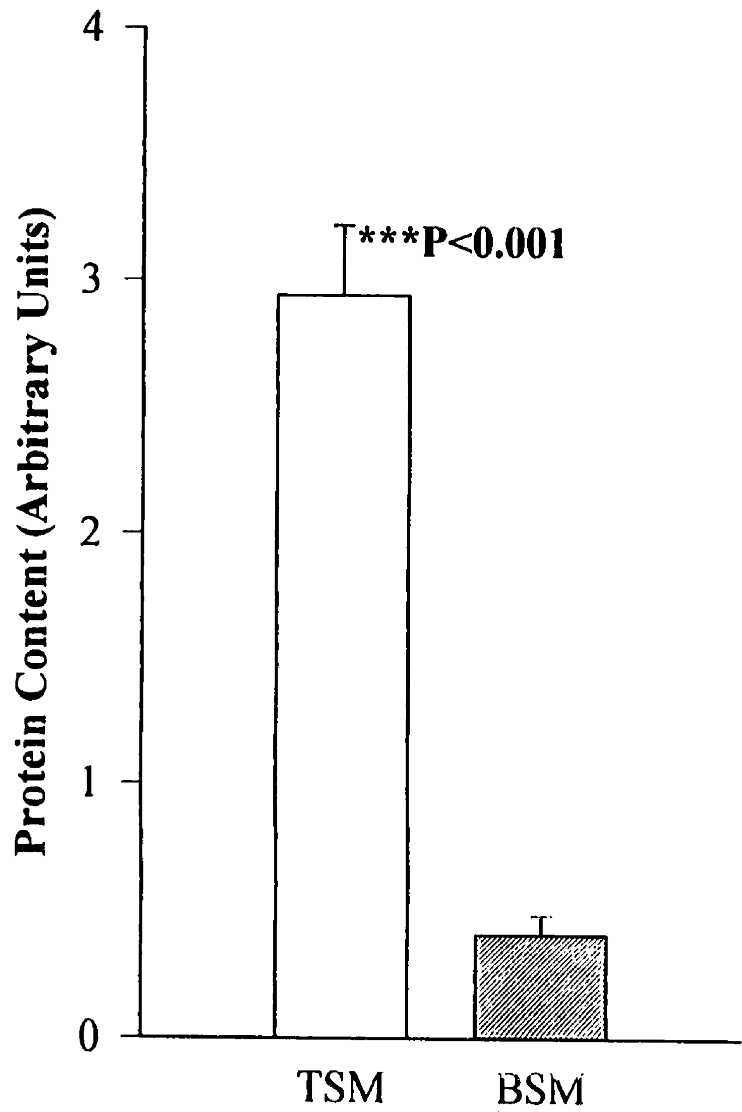

Protein Content (Arbitrary Units)
3
2
1
0
TSM
BSM

Figure 6. Protein Content in SM-α-actin and SM-MLCK of Airway Smooth Muscle

Tracheal smooth muscle showed significantly increased content of smooth muscle α-actin (SM-α-actin) and smooth muscle myosin light chain kinase (SM-MLCK) compared with bronchial smooth muscle (unpaired student t-test). Results obtained from Western-blot analysis employing specific protein antibodies.


Protein Content (Arbitrary Units)
0
1
2
3
4
***P<0.001
TSM
BSM
SM-α-actin

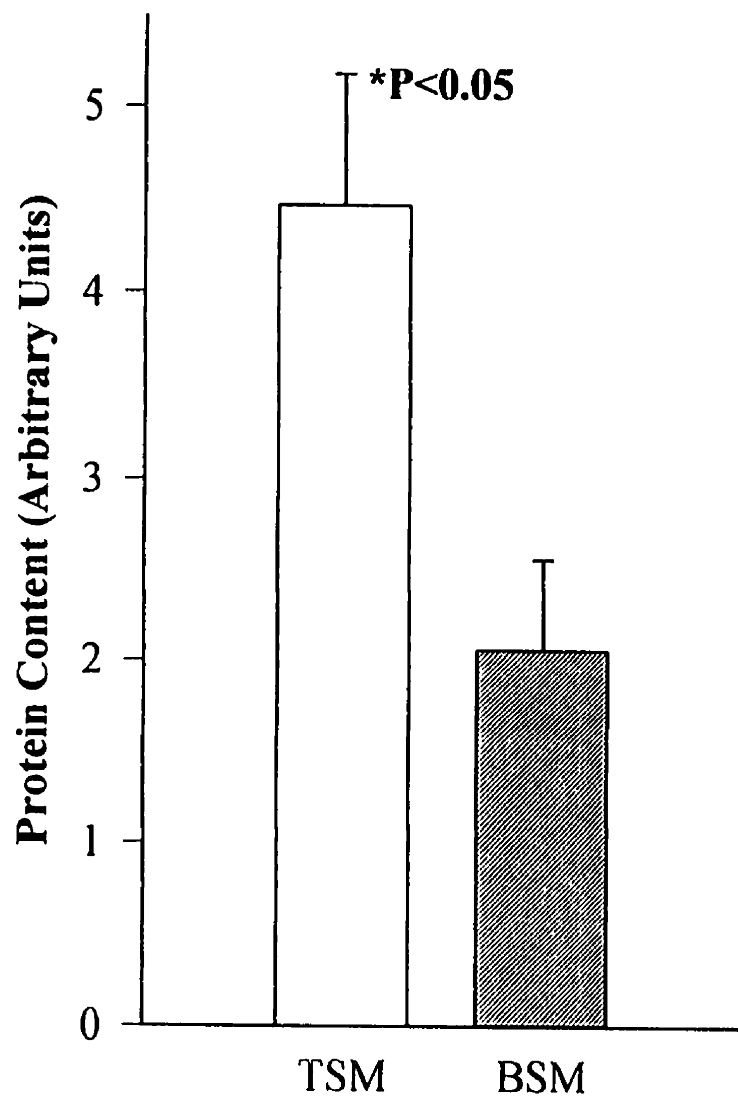
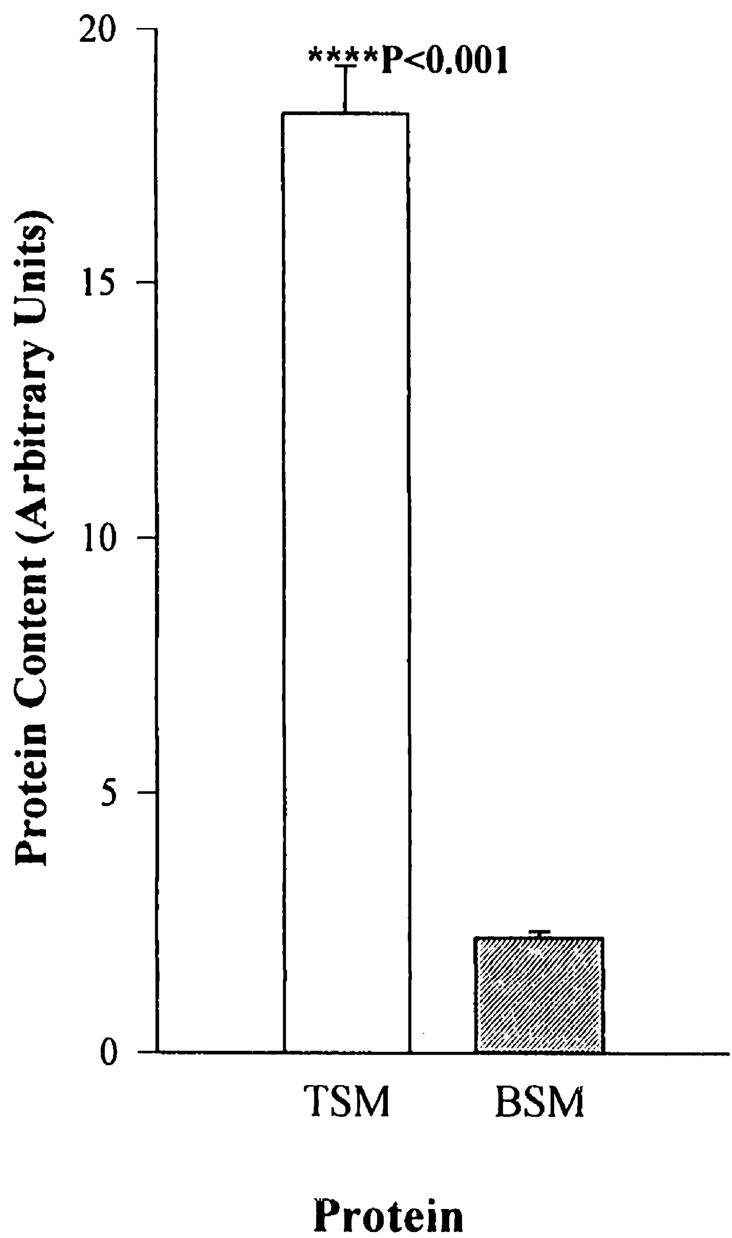
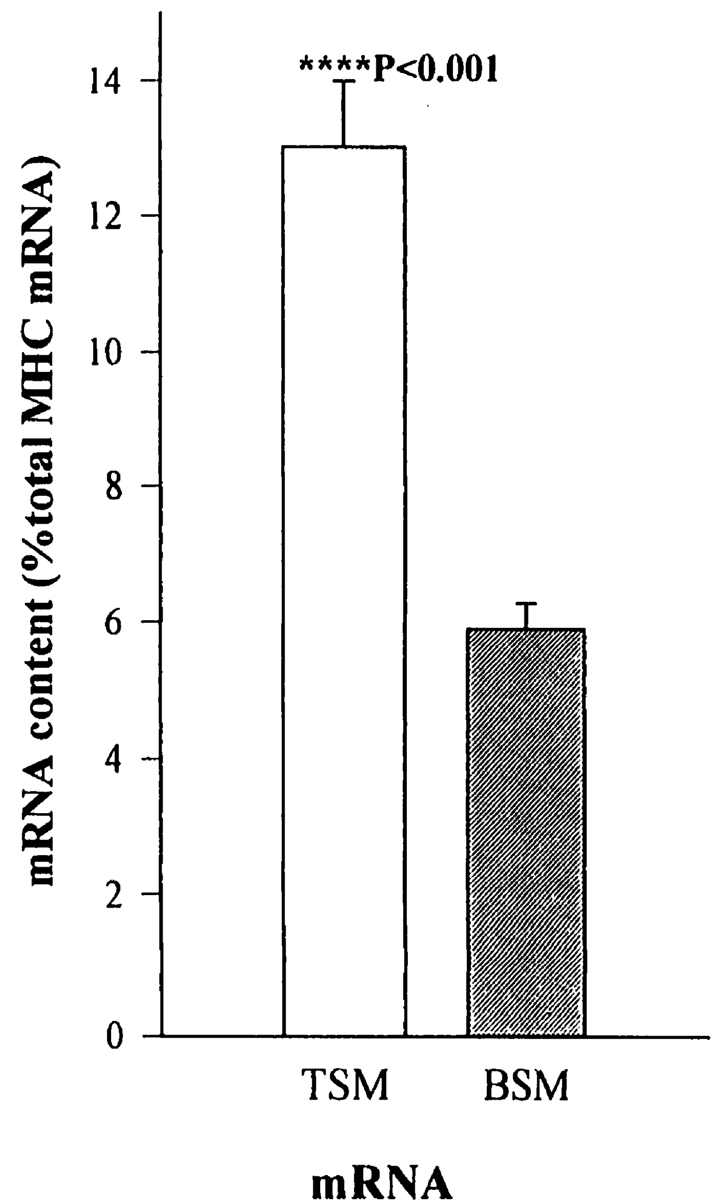

Protein Content (Arbitrary Units)
0
1
2
3
4
5
*P<0.05
TSM
BSM
SM-MLCK

Figure 7. Expression of N-Terminal Isoform

of Smooth Muscle Myosin Heavy Chain (SM-B) in Airway Smooth Muscle

Significantly increased expression of SM-B was found in tracheal smooth muscle (TSM) at both the protein and messenger levels compared with that in bronchial smooth muscle (BSM) (unpaired student t-test). Western-blot analysis was employed to measure the protein content; RT-PCR was used to analyze mRNA expression.


Protein Content (Arbitrary Units)
20
15
10
5
0
****P<0.001
TSM
BSM
Protein


mRNA content (%total MHC mRNA)
14
12
10
8
6
4
2
0
****P<0.001
TSM
BSM
mRNA

3.5 Discussion

Airway smooth muscle showed heterogeneity in mechanical properties from the trachea down to bronchial generation 6. Although there was no significant difference in maximum isometric tension (Po) between the generations, isotonic shortening mechanical parameters including ΔLmax and Vo showed significantly progressive decrease from trachea to bronchial generation 6. Results indicate that Po is a poor discriminator of functional airway smooth muscle heterogeneity. Isotonic shortening properties such as ΔLmax, and Vo are more important in characterizing altered airway smooth muscle behaviour. This was also supported by our findings in ragweed-sensitized canine airway smooth muscle where both ΔLmax and Vo increased without any changes in Po (Antonissen et al., 1979; Jiang et al., 1992). It is therefore quite clear, that shortening rather than force development plays an important role in regulations of airway resistance to airflow and the ratio of perfusion to ventilation (Stephens and Seow, 1988). Therefore studies of the isotonic shortening properties of airway smooth muscles are also more relevant to regulation of airflow *in vivo*.

The present study revealed that airway smooth muscle from trachea down to bronchial generation 6 could be divided into two groups: extrapulmonary and intrapulmonary. Smooth muscles from the intrapulmonary group showed homogeneous mechanical behaviour. Although they showed a trend of decrease in mechanical functions between generations, this was not found to be significant. Smooth muscles from the

extrapulmonary group showed some degree of nonhomogeneity in their mechanical behaviour, especially between the trachea and bronchial generation 2.

In the trachea and bronchial generation 1, supportive cartilage is present in the form of irregular, sometimes branching, crescentic rings, all of which are incomplete dorsally; the dorsal gaps are bridged with fibrous tissue and bands of smooth muscle. In bronchial generation 2, the cartilages are broken into small segments which are irregular in shape but, unlike the trachea, they are distributed around the entire circumference of the airway. The trachea and bronchial generation 1 are situated outside of the lung, while bronchial generation 2 is located at the interface between the extrapulmonary and intrapulmonary groups of the respiratory tree. However, intrapulmonary airways from generation 3 to 6 which show relative homogeneity in their structure, are all located in the lung and show no difference in the relative amounts of cartilage, connective tissue, and smooth muscle between the generations as determined by planimetry (Chitano et al., 1993). This indicates that the mechanical properties of airway smooth muscle are closely related to airway structure and topography.

The present study demonstrated that airway smooth muscle from extrapulmonary airways showed higher mechanical performance compared to that from intrapulmonary airways. The pattern of distribution in smooth muscle mechanical performances seems to fit the design of airway structure along the airway tree. In extrapulmonary airways, due to the existence of more abundant and stiffer cartilages that offer considerable resistance

to smooth muscle shortening, higher performance smooth muscles are needed for efficient regulation of airway size. In intrapulmonary airways, less abundant and softer cartilages exert relatively weak resistance to smooth muscle shortening. Therefore, it is unnecessary for them to have high performance muscle. Otherwise, excessive shortening of smooth muscle could occur, resulting in an increase in airway resistance and breathing difficulty as occurs in asthmatic patients where mechanical performance is increased. It implies that the lower mechanical performance of airway smooth muscles from intrapulmonary airways represents a strong self-limiting mechanism that prevents excessive shortening of smooth muscle. In addition to extra resistance such as exerted by cartilages and connective tissues, airway smooth muscle shortening is limited by its internal resistance which is believed to be partially contributed by smooth muscle slow-cycling cross-bridges (or latch-bridges) which retard the normal cross-bridges cycling (Seow and Stephens, 1986; Wang and Stephens, 1989; Warshaw et al., 1990). Therefore, the development of latch-bridges as revealed by lower V8 and V8/V2 found in smooth muscle from intrapulmonary airways may constitute one factor responsible for the self-limitation mechanism. Besides, the decrease in V2 may also contribute to this mechanism because most of the shortening (about 75%) in airway smooth muscle is completed within the first 3 seconds of contraction (Stephens et al., 1988).

Increased expressions of both sm-MLCK and SM-B in extrapulmonary smooth muscle may contribute to its increased shortening properties. Unlike skeletal myosin heavy chain isoenzymes, the activity of smooth muscle myosin heavy chain isoenzymes is

regulated by phosphorylation of the 20kD regulatory smooth muscle myosin light chain (MLC_{20}) via smooth muscle myosin light chain kinase. Increased protein expression or activity of smMLCK would result in an increased smooth muscle myosin Mg^{2+} ATPase activity, and subsequent increases of Vo and ΔLmax during smooth muscle activation. The importance of smMLCK in determining smooth muscle contractility was also supported by our previous studies in ragweed-pollen sensitized airway smooth muscle. Sensitized muscle showed increased contractility, but the only detected biochemical change of the muscle was the increase in protein expression of smMLCK, with consequent increases in phosphorylation level of MLC_{20} and myosin ATPase activity. More recently, Stephens and Jiang reported that in vitro motility of smooth muscle myosin head was positively related to the concentration of smMLCK in the system (1997). Four smooth muscle myosin heavy chain isoenzymes have been found to date (Kelley and Adelstein, 1994). The most recently-identified one, SM-B, proved to be predominant in determining smooth muscle contractility. Expression of an extra 7 amino acid insert in its N-terminal head region conferred an increased ATPase activity on SM-B resulting in increased velocity in translocation of actin filaments *in vitro*. The higher contractility of visceral smooth muscle compared with that of vascular smooth muscle was found to be related to its greater expression of SM-B (Kelley et al., 1993). A recent report by Chacko et al. further supported the importance of SM-B in determining smooth muscle contractility (DiSanto et al., 1997). They found that smooth muscle in small arteries resembled phasic visceral smooth muscle in its biochemical and biophysical properties, an increased expression of SM-B contributed to its increased ATPase activity and maximum velocity

of shortening compared with those of smooth muscle in large arteries.

In conclusion, airway smooth muscles showed a clear heterogeneity in their mechanical and biochemical properties along the airway tree. They showed progressive decreases in their mechanical performances from the trachea down to bronchial generation 6. The pattern of distribution of airway smooth muscle mechanical performance was closely related with airway structure and function. The increased expression of smMLCK and SM-B in extrapulmonary smooth muscle may contribute to its increased contractility. This also has important implications in the pathogenesis and treatment of asthma. The heterogeneity in mechanical properties of airway smooth muscle reported here relates to those determined by electrical stimulation. It is likely to be the same for acetylcholine or methacholine stimulation since electrical stimulation exerts 95% of its effect by acetylcholine release from the pre-synapstic terminals. It may well be different for agonists such as histamine or leukotrienes, since their receptor distribution down the airways may differ from that for acetylcholine (Leff, 1983).

Two groups of airway smooth muscle were identified based on their biophysical and biochemical properties in the present study. In the following studies to be reported in this thesis, tracheal smooth muscle was used to represent airway smooth muscle of the extrapulmonary group, and bronchial smooth muscle obtained from bronchus generation 3 to 6 was used to represent the intrapulmonary group.

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Chapter 4. Heterogeneity of Contractile Airway Smooth Muscle Cells

4.1 Background

Smooth muscles from different tissues or different sites of the same tissue show great differences in their pharmacological, contractile, and electrophysiological properties. Differences in expression of cell surface receptors, ion channels, and contractile and regulatory proteins are believed to be underlie the heterogeneity of smooth muscles (Somlyo and Somlyo, 1994; Daemen and De-Mey, 1995). Heterogeneous subpopulations of smooth muscle cells have been reported in varied tissues including vascular wall (Frid et al., 1994; Archer, 1996), airways (Halayko et al., 1997), urinary bladder (Lau and Chacko, 1996), and gastrointestinal tract (Liddell et al., 1993). Broadly, smooth muscle cells can be divided into two discrete phenotypes, contractile and non-contractile (Chamley-Campbell et al., 1979). The latter is also referred as "secretory" or "synthetic". Smooth muscle cells are believed to be highly plastic, and capable of phenotypic modulation between contractile and "synthetic" types depending on suitable environmental conditions. Subpopulations of cultured "synthetic" smooth muscle cells differing in sensitivity to specific growth factor-induced proliferation have been reported by Majack et al. (1996). Whether heterogeneity in contractility of airway smooth muscle cells also exists has not been reported.

We have recently reported that airway smooth muscle could be divided into two mechanically distinct groups: extrapulmonary *vs.* intrapulmonary (Ma et al., 1996). The reduced contractility of intrapulmonary smooth muscle were believed to be important in

preventing excessive narrowing of intrapulmonary airways during muscle contraction and maintaining optimal airflow. In addition, increased expression of smooth muscle myosin light chain kinase and the SM-B smooth muscle myosin heavy chain were identified in extrapulmonary smooth muscle (tracheal smooth muscle) (Ma and Stephens, 1997). Existence of an extra N-terminal insert conferred on SM-B increased ATPase activity and velocity of translocation of actin filaments *in vitro* compared with isoforms without that insert (Kelley et al., 1993). Contraction of smooth muscle is regulated by smooth muscle contractile and regulatory proteins. Differences in expression of these proteins would result in mechanically distinct phenotypes of smooth muscles. Therefore, mechanically distinct types of airway smooth muscle cells may exist along the airway tree.

This study was designed to characterize the morphological, contractile and biochemical properties of freshly-isolated airway smooth muscle cells. The major focus was delineation of heterogeneity of contractile airway smooth muscle cells, even at the same topographical locus in the airway.

4.2 Objectives

Delineation of heterogeneity of contractile airway smooth muscle cells and its significance in determining contractility of the airway smooth muscle strip.

4.3 Hypothesis

Airway smooth muscle consists of heterogeneous types of smooth muscle cells. Both contractile and non-contractile cells exist in airway smooth muscle. Contractile smooth muscle cells show heterogeneity in their morphological, biophysical and biochemical properties. Differences in proportions of different types of contractile cells contribute to mechanical differences between tracheal and bronchial smooth muscle.

4.4 Protocol

Airway smooth muscle cells were isolated from both trachea and 3rd to 6th generation of bronchi. Their general morphology was observed directly under a phase contrast inverted microscope. Cells were first grouped into contractile and non-contractile type based on the presence of contractile response. After measuring their relaxed length under an inverted microscope, the contractile cells were further grouped by their length

distribution employing "mixture" analysis. Heterogeneity of contractile cells was further analyzed with respect to their biophysical, and biochemical properties by measurements at single cell level. Finally, proportions of different types of contractile cells were calculated in tracheal and bronchial smooth muscle and compared.

4.5 Results

Preparation of contractile smooth muscle cells. Obtaining fully contractile smooth muscle cells was the most important initial step in characterization of the contractility of smooth muscle cells. Enzymatic isolation using a protocol previously used for our cultured smooth muscle cells (Halayko et al., 1996) did not yield normal contractile cells. Adaption of a method for the isolation of cells from tracheal smooth muscle for electrophysiological studies from Dr. Sims group proved to be successful in obtaining contractile smooth muscle cells. Addition of 10 mM taurine in the digestion solution was crucial, without it isolated cells never contracted. Why taurine plays such an important role in maintaining isolated smooth muscle cells contraction is not known. It produces extensive effects in living cells (Huxtable, 1992). One of the important actions of taurine is osmoregulation which may affect all aspects of cell function. It also regulates calcium movements, and works as a calcium buffer. However, it does not directly affect the contractility of smooth muscle (Croswell and Huxtable, 1987).

Morphological heterogeneity. Broadly, two populations of cells were isolated from canine airway smooth muscle (both trachea and bronchus). Half of all the cells appeared elongated with smooth and shining sarcolemmal membranes as seen under an inverted microscope (Fig. 8). Addition of relaxant agents such as atropine, isoproterenol, and norepinephrine did not result in further increase of their length indicating they were fully relaxed. These cells showed reversible contractile responses to acetylcholine, histamine,

KCl, and single-pulse electrical stimulation, and were categorized as cells belonging to a contractile group. Within this group, two statistically distinct types of cells were identified based on their resting length distribution employing "the MIX program". Type I cells had a mean length of 110 ±10 μm (SE); and type II had a length of 200 ± 20 μm (SE). Cells isolated from both tracheal and bronchial smooth muscle consisted of the same two types of cells, but there was a significant difference in the distribution of cells between tracheal and bronchial smooth muscle. Type I cells predominated in the trachea, and accounted for 86% of the total isolated contractile cells (Fig. 9). In the bronchus, 42% of the total elongated cells belongs to type I, while the remaining 58% belonged to type II (Fig. 10). The remaining half of cells were rounded in shape with a mean diameter of 5 to 10 μm, they did not show any contractile response. Therefore they were classified as non-contractile.

Mechanical heterogeneity. Single pulse electrical stimulation caused a reversible, but partial shortening contraction (about 75% of maximum) of freshly isolated contractile cells (Fig. 11). Under repeated (tonic) electrical stimulation, maximal contraction could be achieved, which was 25% greater than that with the single pulse. Different types of cells showed different responses to repeated electrical stimulation (Fig. 12). Figure 13 showed typical curves of single smooth muscle cell shortening. Under zero-loaded conditions, type I cells shortened maximally by 28 ± 3% of their resting length (Lcell); while within type II cells, the majority of cells (type IIA, 90% of total type II cells) shortened by 17 ± 2%, and the remainder 10% cells (type IIB) shortened by a remarkable 58 ± 6% (Fig. 14).

These cells also showed a significant difference in their maximum velocity of shortening (Vo). Type I cells shortened with Vo of 8 ± 0.6%Lcell/sec, type IIA of 4 ± 0.3%Lcell/sec, and type IIB of 10 ● 0.9% Lcell/sec (Fig. 14). The above results indicate that three types of smooth muscle cells exist in airway smooth muscle differing in their shortening properties.

Difference in expression of SM-B mRNA between type I and II cells. To further delineate the molecular basis of contractile differences, expression of smooth muscle myosin heavy chain SM-B mRNA was analyzed at the single cell level employing RT-PCR, and compared between different types of cells. Results showed that type I cells expressed SM-B at 34% ±1.3 of total smooth muscle heavy chain messenger, no SM-B mRNA was detected in type II cells (Fig. 15).

Figure 8. Typical Image of Freshly Isolated Tracheal Smooth Muscle Cells

Two populations of smooth muscle cells were identified in freshly-isolated airway smooth muscle cells. Half of the cells appeared elongated under the microscope, and showed a contractile response to various types of stimuli. The remaining half of cells were round, showed no contractile response. E, elongated cell; O, round cell.

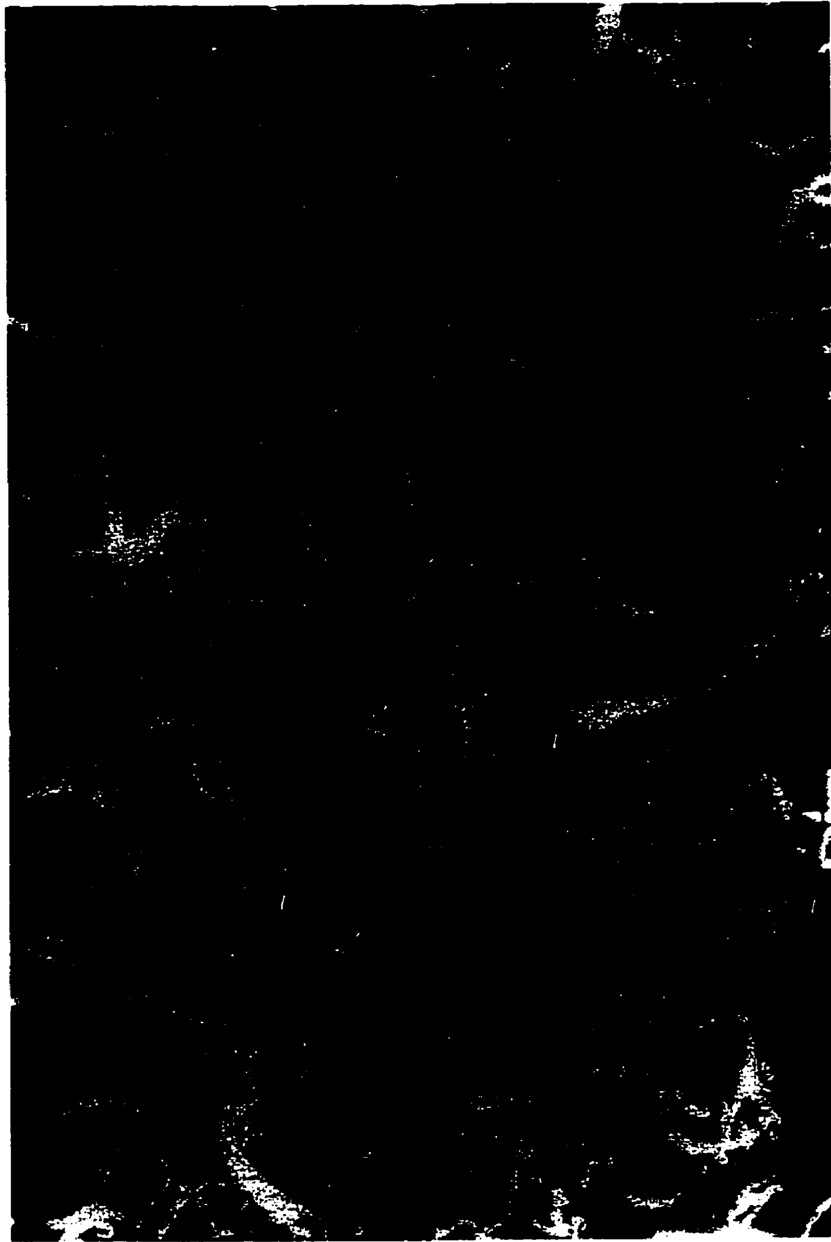
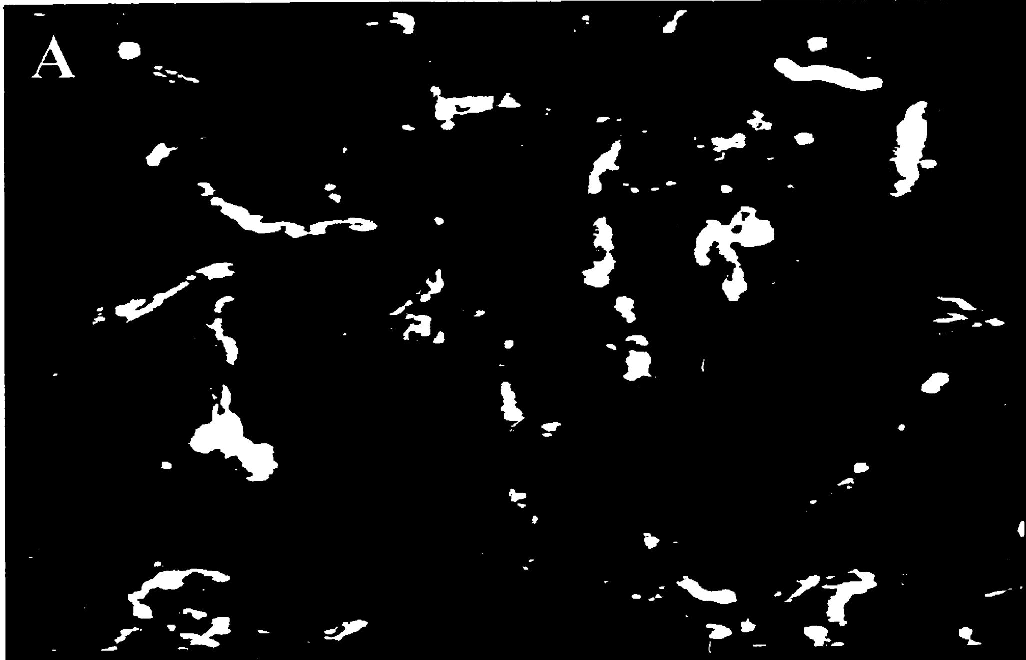


Figure 9. Length Distribution of Contractile Smooth Muscle Cells Freshly Isolated From Canine Trachea.

Panel A. Typical image of contractile airway smooth muscle cells freshly-isolated from canine trachea. Note type II cells are not seen in this figure. Due to their paucity, the sample did not pick them up.

Panel B. Histogram in length distribution of contractile cells. Two morphological distinct types of cells were identified: type I cells had a mean length of 113 ● 2 μm, accounted for 83.7 ± 3.6% of total contractile cells; type II cells had a mean length of 191 ● 9 μm, accounted for the remaining 16.3 ± 3.6% ("Mixture" analysis, Goodness of fit $P = 0.3739$).


A

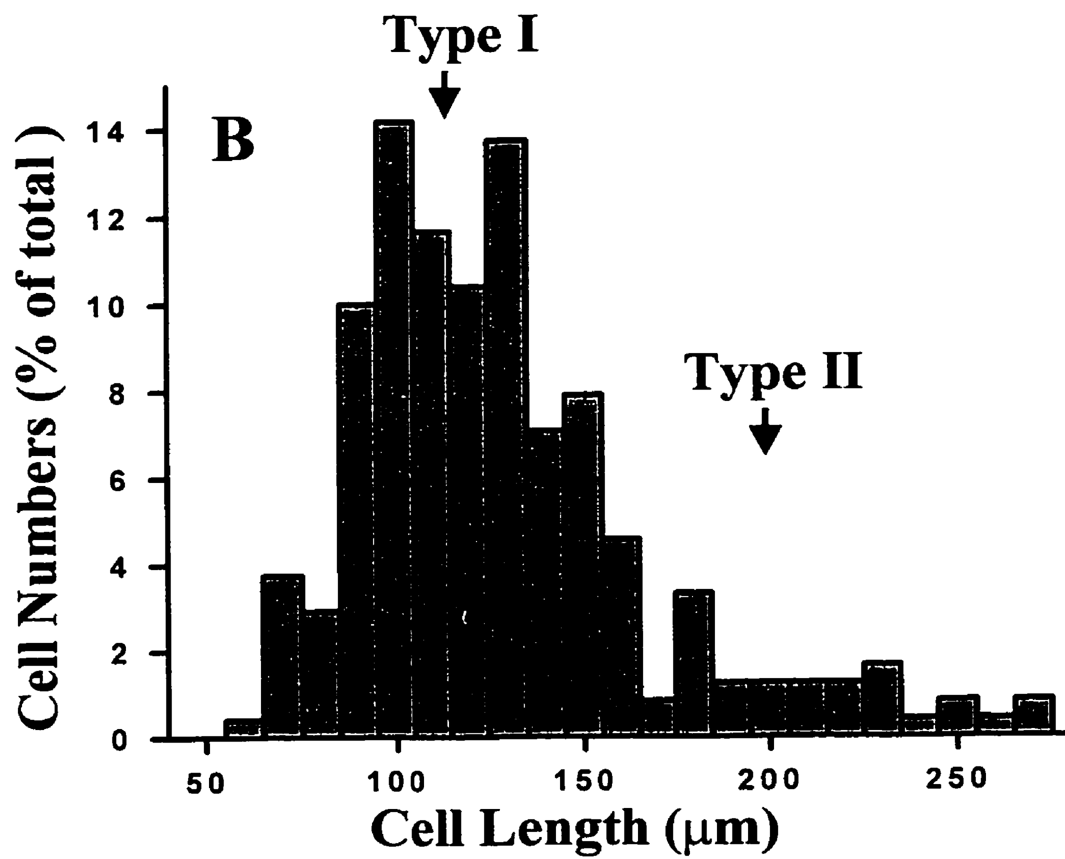
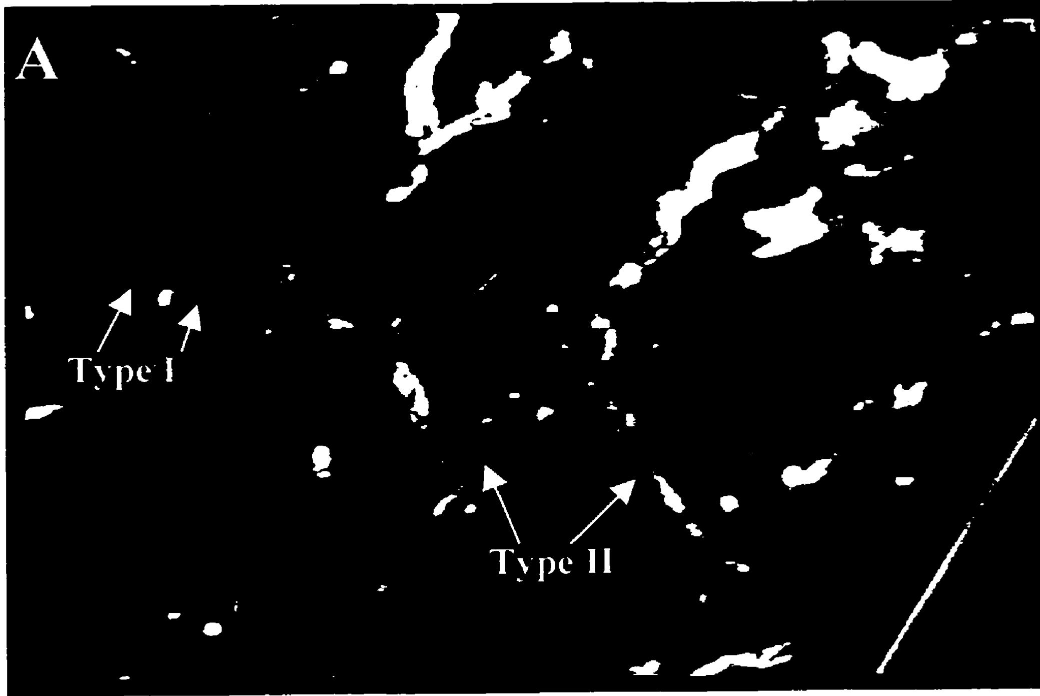

Type I
Type II
B
Cell Numbers (% of total)
14
12
10
8
6
4
2
0
50
100
150
200
250
Cell Length (μm)

Figure 10. Length Distribution of Contractile Smooth Muscle Cells

Freshly Isolated From Canine Bronchi.

Panel A. Typical image of contractile airway smooth muscle cells freshly isolated from canine bronchi. Bronchial cells consist of a mixture of type I and II cells: type I cell has the mean length of 105 μm, and the type II cells show the mean length of 200 μm.

Panel B. Histogram in length distribution of contractile cells. Two morphological distinct types of cells were identified: type I cells had a mean length of 105 ± 3 μm, accounted for 42.1 ± 3.9% of total contractile cells; type II cells had a mean length of 200 ± 4 μm, accounted for the remaining 57.9 ± 3.9% ("Mixture" analysis, Goodness of fit P = 0.2867).


A
Type I
Type II

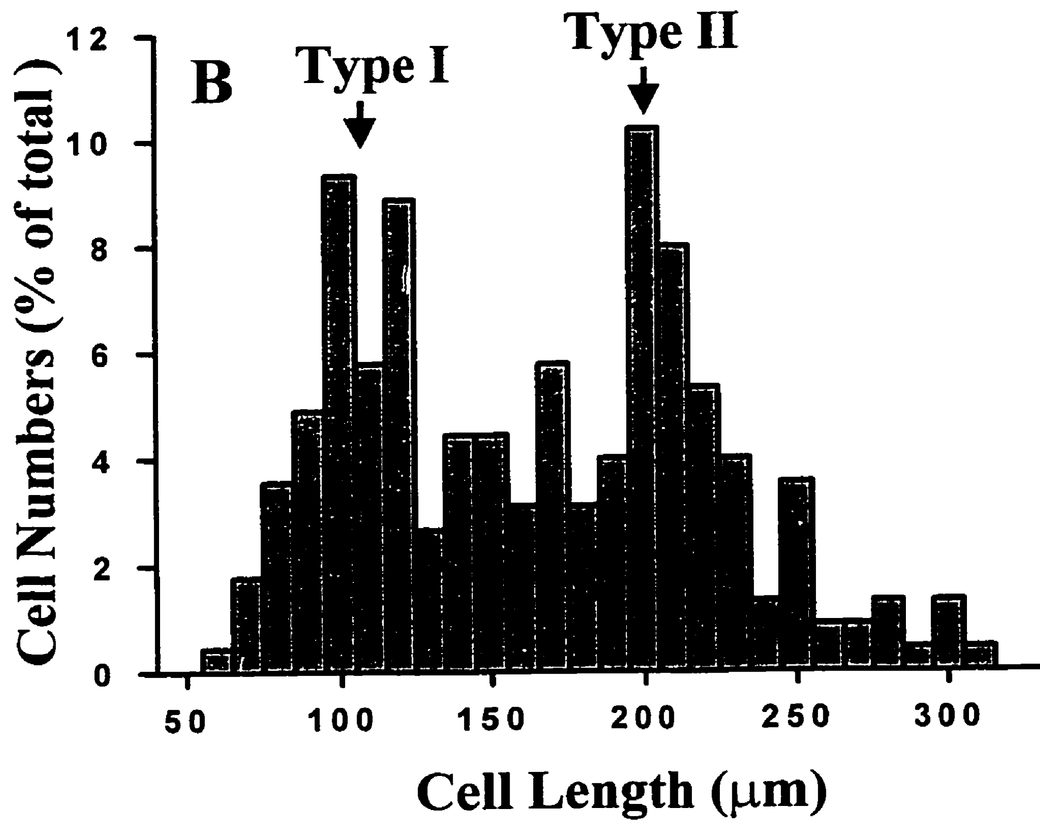

B
Type I
Type II
Cell Numbers (% of total)
0
2
4
6
8
10
12
50
100
150
200
250
300
Cell Length (μm)

Figure 11. Typical Images Shown Reversal of Contraction

of Freshly Isolated Canine Airway Smooth Muscle Cell

Freshly isolated canine airway smooth muscle cell showed a reversible contractile response to contractile stimulation. A, B, and C: images of a freshly isolated cell fully relaxed, partially contracted, and 10 min after withdrawal of stimulation, respectively. Cell was stimulated electrically. Dark spot near cell was one of the electrodes, the other was located at the other side of the cell.

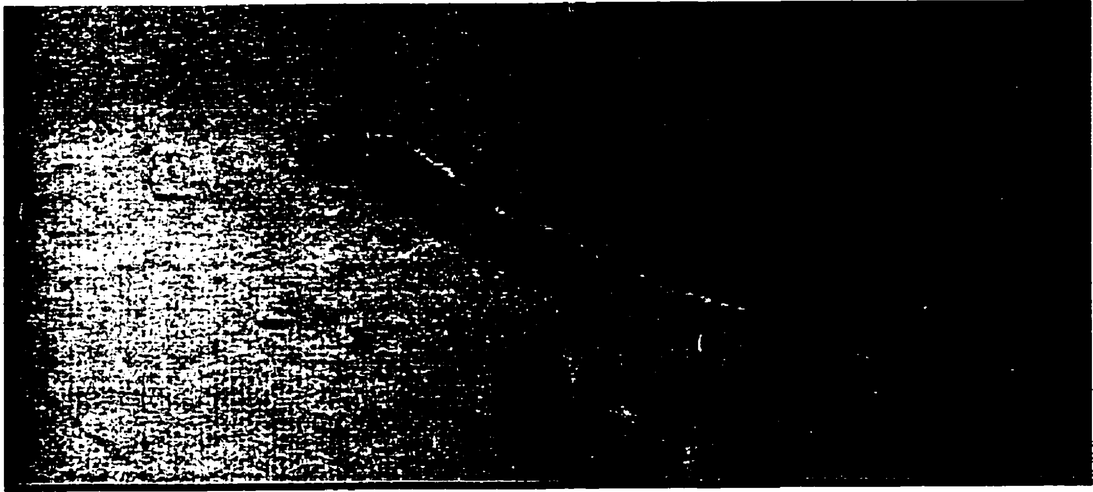


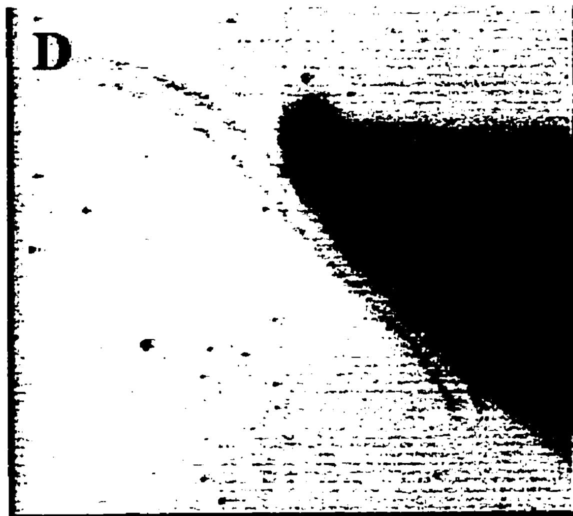
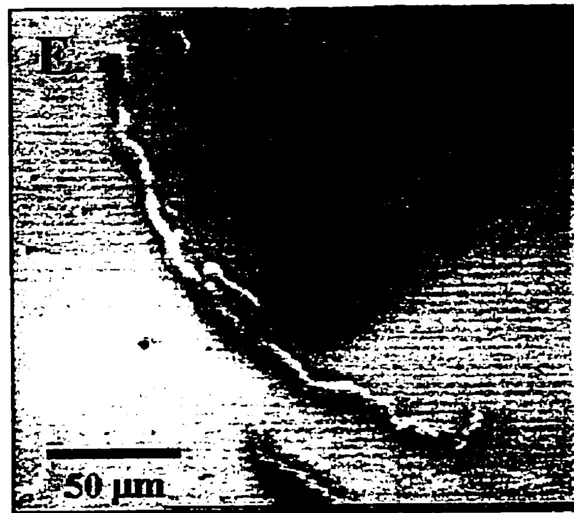
Figure 12. Illustration of Three Types

of Freshly Isolated Airway Smooth Muscle Cells.

Three morphologically and biophysically distinct types of contractile cells were identified from freshly isolated canine airway smooth muscle cells. Panel A and B: a type I cell before and after maximal electrical stimulation, respectively. Panel C and D: a type IIA cell before and after maximal electrical stimulation, respectively. Panel E and F: a type IIB cell before and after maximal electrical stimulation, respectively. The black spot in each panel was a spot electrode placed near the cell; the other electrode, which was not shown in the panel, was placed somewhere at the other side of the cell.

A

B

C

D

E
50 µm

F

Figure 13. Typical Curves of Unloaded Shortening

of Single Airway Smooth Muscle Cells.

Freshly isolated individual airway smooth muscle cells showed marked differences in their shortening properties. Cells were stimulated maximally with electrical stimulation at room temperature. Lcell, relaxed cell length.

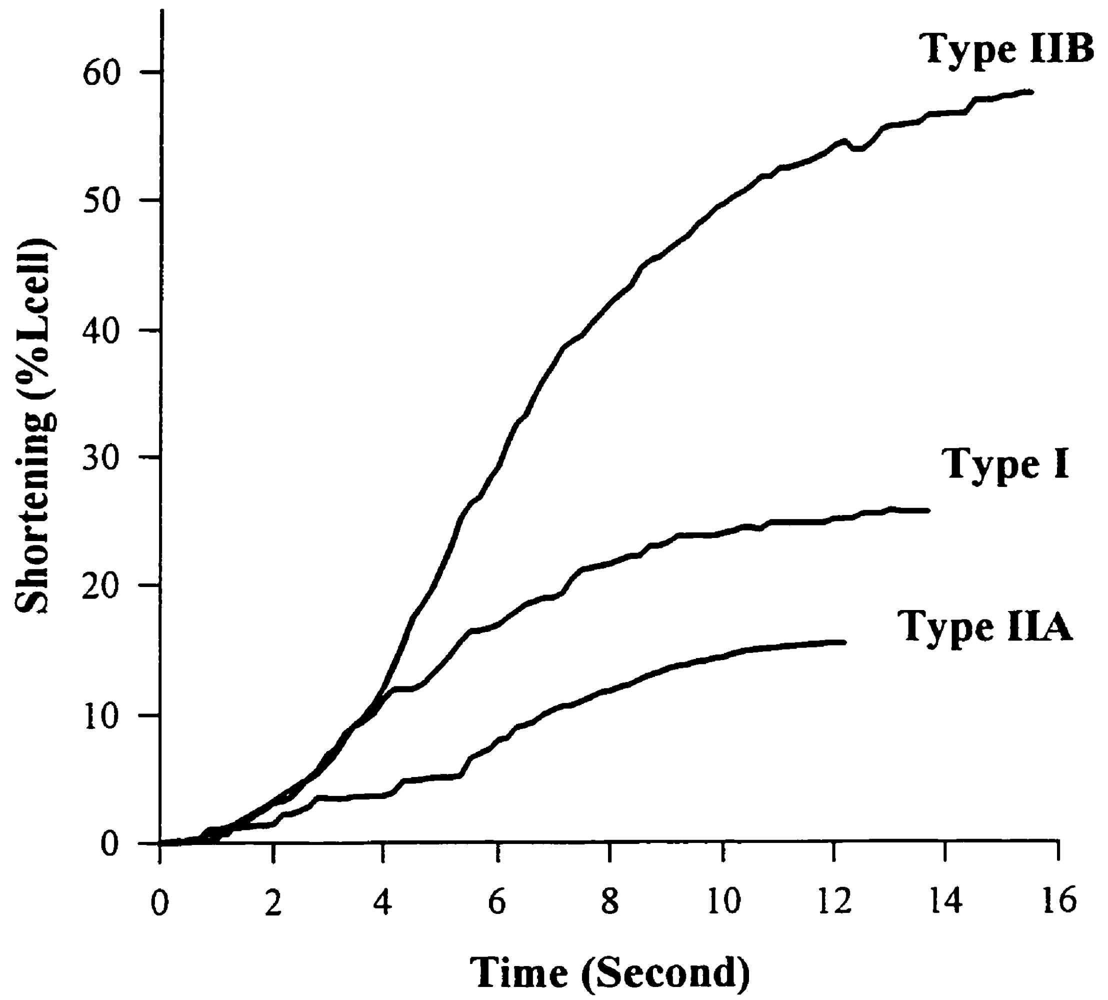

Type IIB
Type I
Type IIA
Shortening (%Lcell)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
Time (Second)

Figure 14. Shortening Properties of Freshly Isolated Canine Airway Smooth Muscle Cells.

Different types of contractile airway smooth muscle cells showed significant differences in their shortening properties. Type IIB cells demonstrated the greatest values in maximal capacity ($\Delta Lmax$) and velocity (Vo), while the type IIA cells showed the lowest values. Cells were electrically stimulated at room temperature. Lcell, relaxed cell length; ***$P<0.005$ (ANOVA).

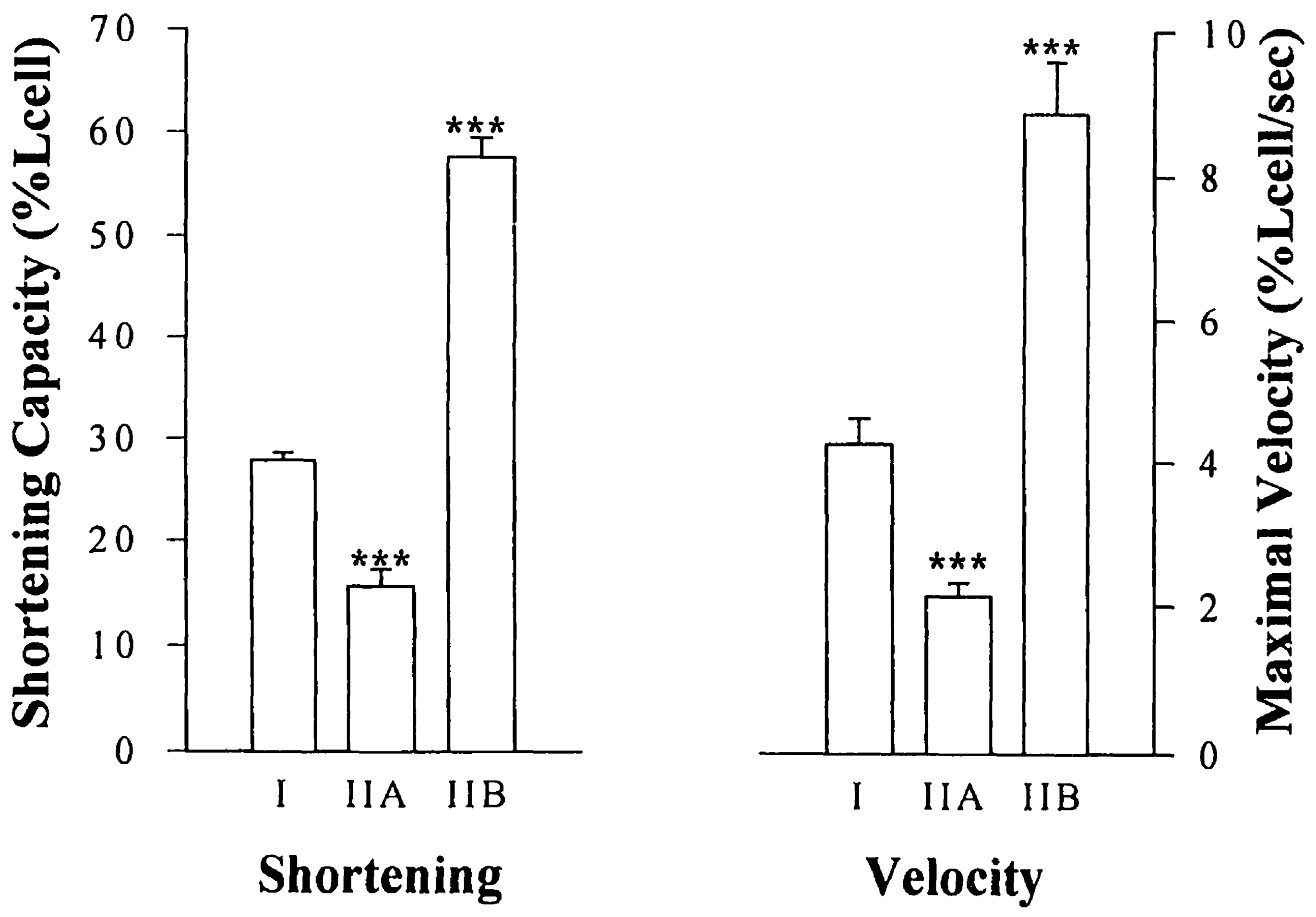
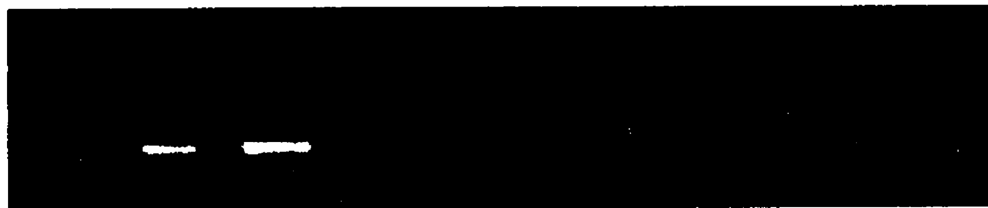

Shortening Capacity (%Lcell)
70
60
50
40
30
20
10
0
***
***
I
IIA
IIB
Shortening
Maximal Velocity (%Lcell/sec)
10
8
6
4
2
0
***
***
I
IIA
IIB
Velocity

Figure 15. Heterogeneity in Expression of SM-B

of Contractile Airway Smooth Muscle Cells

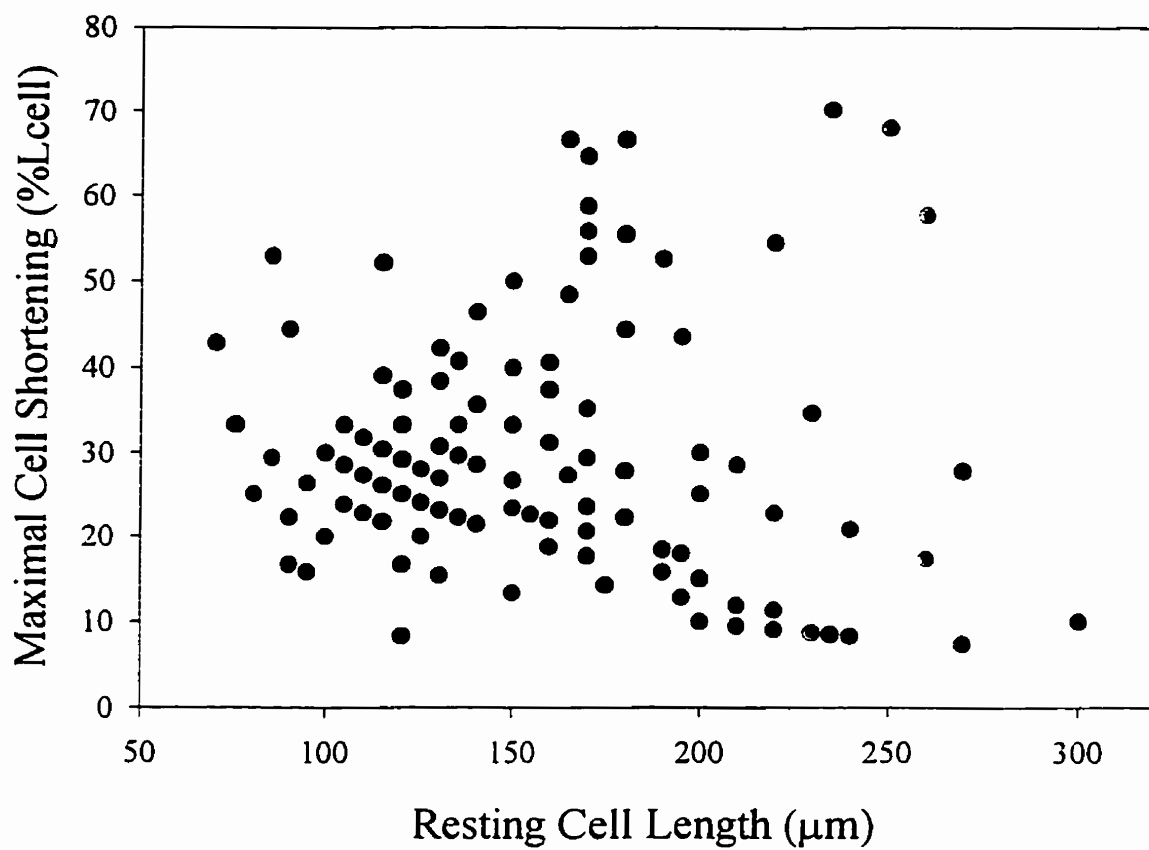
Expression of SM-B mRNA was detected in the amount of 34% ±1.3 of total smooth muscle heavy chain messenger in type I cells employing RT-PCR. No SM-B was found in type II cells including both type IIA and IIB. SM-B, smooth muscle myosin heavy chain isoform with a 7 amino acid insert in its NH_2-terminal region.



200 bp
100 bp
SM-B
SM-A
Type I
Type II

Figure 16. Resting Length-Shortening Capacity Relationship for Contractile Airway Smooth Muscle cells.

No linear relationship was demonstrated between resting cell length (Lcell) and maximal shortening capacity (ΔLmax) in freshly isolated contractile airway smooth muscle cells ($r=0.106$).



Maximal Cell Shortening (%Lcell)
80
70
60
50
40
30
20
10
0
50
100
150
200
250
300
Resting Cell Length (μm)

4.6 Discussion

Both contractile and non-contractile smooth muscle cells were isolated from airway smooth muscle. More interestingly, obvious heterogeneity in mechanical and biochemical properties was identified in contractile airway smooth muscle cells. At least three distinct types of contractile cells were identified in airway smooth muscle based on their morphological, mechanical and biochemical properties. To our understanding this is the first report of heterogeneity in contractility of airway smooth muscle cells. Heterogeneity of contractile smooth muscle cells has been reported in vascular smooth muscle. For example, Meer and Eddinger (1996) reported heterogeneity in expression of smooth muscle myosin heavy chain tail isoforms (SM1 and SM2) in carotid arterial smooth muscle cells; Archer et al. (1996) showed that electrophysiologically distinct myocytes existed in pulmonary vascular smooth muscle, and differences in distribution of different myocytes between pulmonary conduit and resistant arteries were present that contributed to differences in their responses to hypoxia. All these imply that contractility of smooth muscle *in situ* reflected at the tissue level represent the average effort of mixed populations of smooth muscle cells. Therefore, to further understand the molecular mechanism of smooth muscle contraction, one should focus on studies at the single cell level.

Enzymatic isolation of smooth muscle cells may cause partial contraction of individual cells, and subsequently contribute to the length difference between type I and

II cells. Cells in our preparation were proved to be fully relaxed. This is because: (1) while freshly-isolated resting cells show smooth and shining sarcolemmal membranes, contracted cells usually show obvious blebs around the sarcolemma (Fay et al., 1974); (2) addition of relaxant agents such as atropine, isoproterenol, and norepinephrine did not result in further elongation; (3) no linear relationship was found between resting length and shortening capacity in different types of cells (Fig 16); (4) if type I cells are partially contracted type II cells, we would expect to see reduced shortening capacity in these cells. Actually, the majority of type II cells (type IIA) showed a significantly lower shortening capacity than type I cells, while the mean length of type II cells almost doubled that of type I cells. Therefore enzymatic isolation of airway smooth muscle cells in our preparation does not contribute to the difference in mean lengths between type I and II cells. Type I cells are not partially contracted type II cells.

Previous studies in our laboratory demonstrated that airway smooth muscle from the trachea down to bronchial generation 6 could be divided into two groups: extrapulmonary including smooth muscles from trachea and bronchial generation 1, and 2, *vs* intrapulmonary including bronchial generation 3 to 6 (Ma et al., 1996). Extrapulmonary smooth muscle showed increased contractility compared with intrapulmonary. In this study, we further demonstrated that airway smooth muscle consisted of at least three types of smooth muscle cells differing in their contractility, furthermore there was a significant difference in proportions of these types of cells between extrapulmonary and intrapulmonary smooth muscles. The mechanical properties

of smooth muscle are not only dependent on the types of muscle cells present, but also in the arrangement of these cells. Therefore, it is difficult to directly apply these single cell data to explain differences in mechanical properties between extra- and intrapulmonary airway smooth muscles. The existence of increased numbers of type IIA cells in bronchial smooth muscle may be the major factor producing the reduced contractility of the bronchus. During muscle shortening, cells with lower shortening capacity, such as the type IIA cells, will exert an internal force and resist the further shortening of muscle.

Results obtained from studies of tissue homogenates may result in erroneous interpretations of molecular mechanism(s) of smooth muscle contraction because of the heterogeneity of smooth muscle cells. Expression of SM-B was reported to be important in determining contractility of smooth muscle (Kelley et al., 1993; DiSanto et al., 1997). Our previous studies with tissue homogenates showed that tracheal smooth muscle expressed the SM-B isoform in amounts double those of bronchial smooth muscle (Ma and Stephens, 1997). And we consequently concluded that the increased expression of SM-B in tracheal smooth muscle contributed to its increased contractility compared with those of bronchial smooth muscles. The present studies revealed that two morphologically and biochemically distinct types (type I and II) of cells exist in airway smooth muscle; type I cells showed considerable amount of SM-B mRNA expression (34% ±1.3 of total smooth muscle myosin heavy chain isoforms) while type II cells did not show any expression of SM-B mRNA. Type I cells predominated in tracheal smooth muscle, but

only accounted for 46% of total contractile cells in bronchial smooth muscle. We can not directly use these data to calculate the relative amounts of SM-B expression in tracheal and bronchial smooth muscle due to the unavailability of information regarding the absolute amounts of SM-B in each type of cell. It is very reasonable to expect that tracheal smooth muscle would show increased amount of SM-B mRNA due to the increased numbers of type I cells, when it is compared with that in bronchial. In fact, previous studies at the tissue level had already demonstrated that tracheal smooth muscle possessed more SM-B than the bronchial smooth muscle, both at protein and messenger levels. Characterization of contractility of smooth muscle cells demonstrated that airway smooth muscle cells could be further divided into three types (type I, IIA and IIB). Surprisingly, Type IIB cells in which no expression of SM-B was detected showed the highest contractility in all these types of cells. This is contradictory to the notion of the importance of SM-B in determining the shortening properties of smooth muscle. The above evidence therefore indicates that results obtained from studies at the tissue level may not be suitable in providing cellular mechanisms regulating airway smooth muscle contraction. Currently we do not have a suitable explanation for the highest contractility of type IIB cells. Obviously, it is not SM-B. All these studies demonstrate that investigation of the molecular mechanisms of smooth muscle contraction should be carried out at the single cell level.

Smooth muscle myosin light chain kinase (smMLCK) is an important candidate that could be responsible for the variations in contractility of smooth muscle cells of

different types or origins. Smooth muscle MLCK is known to be a primary regulator of smooth muscle contraction through Ca^{2+}-CaM dependent phosphorylation of the 20-kDa regulatory myosin light chain (MLC_{20}). Changes in smMLCK content and activity can lead to alteration in level of MLC_{20} phosphorylation with concomitant alteration in contractility of smooth muscle during activation. The importance of smMLCK in regulating smooth muscle contractility was supported by our previous studies on ragweed-pollen sensitized canine airway smooth muscle, where an increased smooth muscle contractility was found to be closely correlated with the increased content and activity of smMLCK (Jiang et al., 1992). Stephens and Jiang (1997) recently reported, on the basis of a motility assay, that *in vitro* motility of the subfragment 1 of smooth muscle myosin increased with increases in smMLCK concentration presumably because of increased phosphorylation of MLC_{20}. This further demonstrated the significance of smMLCK in regulating smooth muscle contractility. In our arrested cell culture, increased expression of smMCLK was also found in hyper-contractile cells (Ma et al., 1998). The importance of smMLCK in determining contractility of isolated smooth muscle cells in current studies needs to be explored further.

Detection of the existence of different types of smooth muscle cells in airways will likely prove to be important in elucidating the pathogenesis of asthma, and in designing more effective therapy. Increased shortening capacity of airway smooth muscle and remodeling of the airway wall are two major factors causing the excessive increase of airway resistance in asthmatic patients. Differences in distribution of different types of

smooth muscle cells in different airways may be responsible for the nonhomogeneous distribution of bronchospasm during an acute asthma attack (Takishima et al., 1991), and the development of irregular smooth muscle hypertrophy and hyperplasia along the chronic asthmatic airways (Ebina et al., 1993). It seems that different type of cells play different roles in the pathogenesis of asthma.

In conclusion the current studies demonstrated that airway smooth muscle consists of different types of contractile smooth muscle cells. Differences in contractility of different types of cells could not be explained by different patterns of SM-B expression. Differences in magnitude of MLC_{20} phosphorylation and in proportions of different types of smooth muscle cells present in extrapulmonary and intrapulmonary airway smooth muscle may provide the major factors determining their mechanical heterogeneity.

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Chapter 5. Heterogeneous alterations in contractile properties of airway smooth muscle (cells) along the ragweed pollen-sensitized canine airways

5.1 Background

The significance of airway smooth muscle heterogeneity of the type just discussed, in pathogenesis of pulmonary diseases, especially asthma, has never been explored. Heterogeneity in the topography of the asthmatic attack was well-known. Bronchospasm predominated in central airways during the acute asthma attack (Takishima et al., 1991). In chronic asthma, non-homogeneous distributions of hypertrophy and hyperplasia of airway smooth muscle cells existed along the airway tree (Ebina et al., 1993). Elucidation of mechanism(s) underlying these phenomena would facilitate understanding of the pathogenesis of asthma and development of a new treatment.

Direct study of human asthmatic airway smooth muscle is limited by unavailability of adequate amounts of suitable samples. Sensitized animals, however, provide an alternative model for these studies. Our laboratory has previously demonstrated that ragweed-pollen sensitized canine airways possess hyperreactivity to specific and non-specific agonists, and the contractility of airway smooth muscle both from trachea and bronchus in these dogs was significantly increased (Jiang et al., 1992). However, no inflammation, hypertrophy or hyperplasia of smooth muscle cells were identified in these sensitized airways, which indicated that our canine model mimicked the early asthmatic response. Whether changes in contractility of sensitized smooth muscle along the airway tree are homogeneous or not, has never been identified. Due to the high degree of heterogeneity of airway smooth muscle cells, and remarkable differences between tracheal

and bronchial smooth muscle (Ma et al., 1997) we hypothesize that ragweed pollen-sensitization would produce different changes in contractility between tracheal and bronchial smooth muscle such that the different types of contractile cells would show different alternations in their contractility in response to ragweed pollen sensitization.

5.2 Objectives

Identification of whether ragweed pollen sensitization resulted in heterogeneous effects on contractility of airway smooth muscle and smooth muscle cells.

5.3 Hypothesis

1. Smooth muscles from trachea and bronchus generation 3 to 6 show different alterations in their contractility during ragweed pollen sensitization. Sensitization results in greater increases of contractility in bronchial smooth muscle than tracheal.

2. Different sensitization effects on mechanical properties of different types of contractile airway smooth muscle cells contribute to different sensitization effects on tracheal and bronchial smooth muscle.

5.4 Protocol

Airway smooth muscles were isolated from the trachea and bronchi (bronchial generation 3 to 6) of sensitized and litter-mate control dogs. The mechanical properties of these muscles were measured employing a electro-magnetic lever system (Brutsaert et

al., 1971). Amplitudes of maximal capacity and magnitudes of velocity of shortening were calculated from data obtained from sensitized tracheal and bronchial smooth muscles, and compared with each other to identify whether ragweed pollen sensitization generates a homogeneous effect on contractility of airway smooth muscles. Studies at tissue levels were carried out both in short- (6 to 8 months) and long-term (3 to 6 years) sensitized dogs. To further demonstrate whether different types of contractile airway smooth muscle cells respond differently to sensitization, airway smooth muscle cells were isolated from both sensitized and control airways, and their contractility was evaluated at the single cell level. These studies were only carried out in short-term sensitized dogs.

5.5 Results

Short term ragweed-pollen sensitization resulted in significant increases in maximal capacity and velocity of airway smooth muscles isolated from both trachea and central bronchi (Fig. 17, 18). Sensitized tracheal smooth muscle showed significant increases in maximal capacity and velocity of shortening compared with sensitized central bronchial muscles. No changes in isometric tension were found in either of these muscles. The increments in ΔLmax and Vo of sensitized bronchial smooth muscle were significantly greater than those of tracheal (Fig. 19).

Previous studies showed that there were at least three types of contractile smooth muscle cells present in airway smooth muscle. Each showed distinct morphological, biophysical, and biochemical properties. Current studies further demonstrated that all these cells possessed significantly increased ΔLmax and Vo in short-term sensitized airway smooth muscles compared to non-sensitized muscles (Fig. 20). However, obvious heterogenous changes in contractility were also identified among the different types of cells (Fig. 21). Type IIA cells showed the greatest increments in their contractility. They showed 30% and 25% increases in ΔLmax and Vo, respectively, compared with those of controls; while type I and type IIB cells showed increases in ΔLmax and Vo, of 20 and 25%, and 15 and 18% respectively.

Long term sensitization caused a different pattern of alteration in airway smooth

muscle contractility when compared with those in short term sensitized muscles. No significant differences in contractility were found in tracheal smooth muscle between long-term sensitized and control dogs, indicating that the mechanics in old sensitized dogs had regressed (Fig. 22). However, significant increases in ΔLmax and Vo were identified in long-term sensitized bronchial airway smooth muscle (Fig 23). In addition, bronchial smooth muscle from long-term sensitized dogs showed a significant increase in V8 that was not found in short-term sensitized dogs. No differences in mechanical properties of smooth muscle were found between short and long term control dogs (Fig. 24), indicating that the differences in mechanical changes between long- and short-term sensitized airway smooth muscles were not affected by the difference in ages of the two groups.

Figure 17. Contractility of Tracheal Smooth Muscle

Obtained from Short Term Ragweed Pollen Sensitized Dogs

Short term sensitization resulted in significant increases in shortening properties such as maximal capacity of shortening (ΔLmax) and velocity at 2 second (V2) of canine tracheal smooth muscle, without any changes in isometric tension (Po) and maximal velocity of shortening at 8 second (V8). Lo, optimal length of muscle for contraction. *$P<0.05$ (unpaired student t-test).

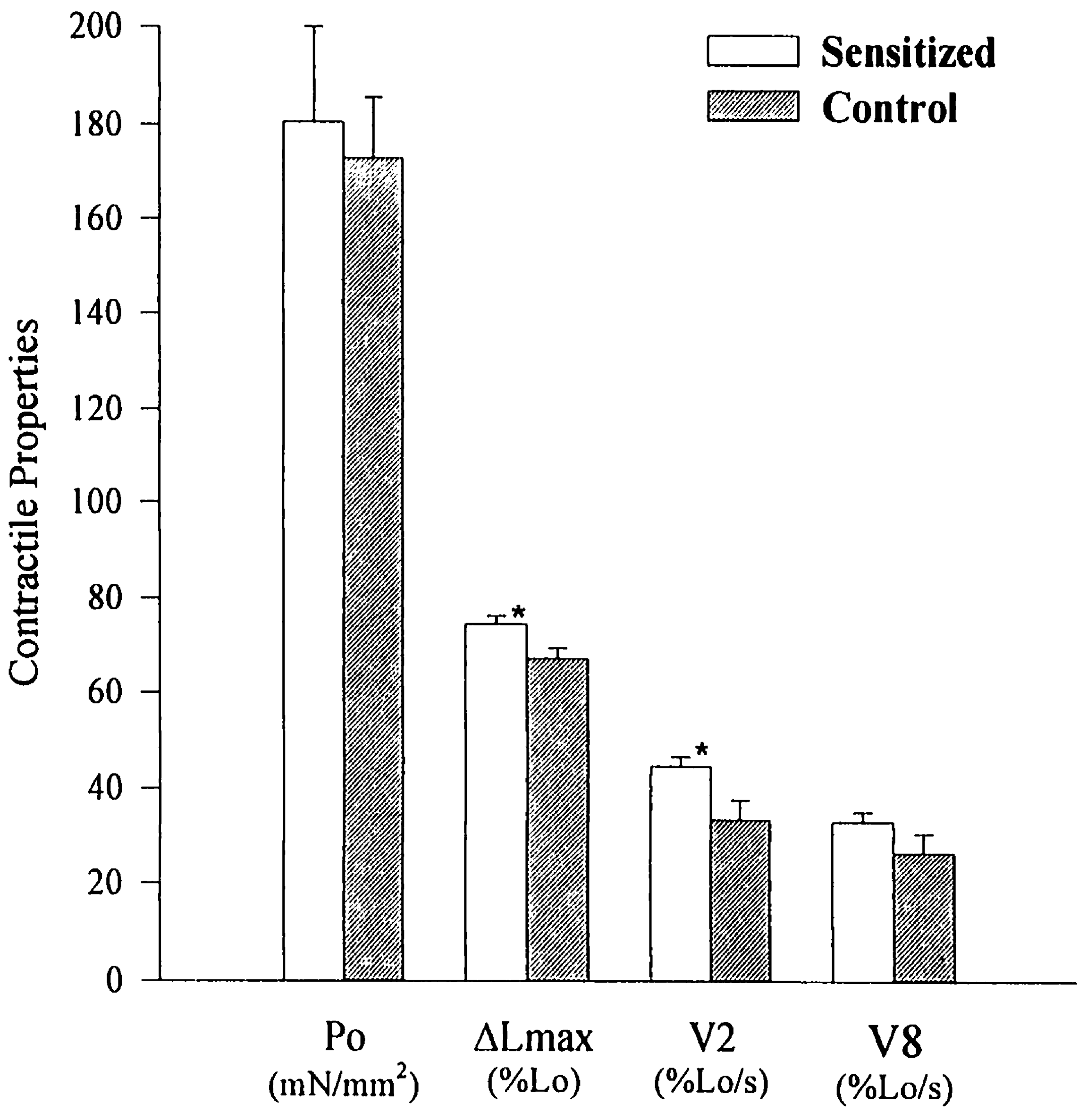

Sensitized
Control
Contractile Properties
200
180
160
140
120
100
80
60
40
20
0
*
*
Po
(mN/mm2)
ΔLmax
(%Lo)
V2
(%Lo/s)
V8
(%Lo/s)

Figure 18. Contractility of Bronchial Smooth Muscle

Obtained from Short Term Ragweed Pollen Sensitized Dogs

Short term sensitization resulted in significant increases in shortening properties such as maximal capacity of shortening ($\Delta Lmax$) and velocity at 2 second (V2) in canine bronchial smooth muscle, without any changes in isometric tension (Po) and maximal velocity of shortening at 8 second (V8). Lo, optimal length of contraction. *$P<0.05$, **$P<0.01$ (unpaired student t-test).

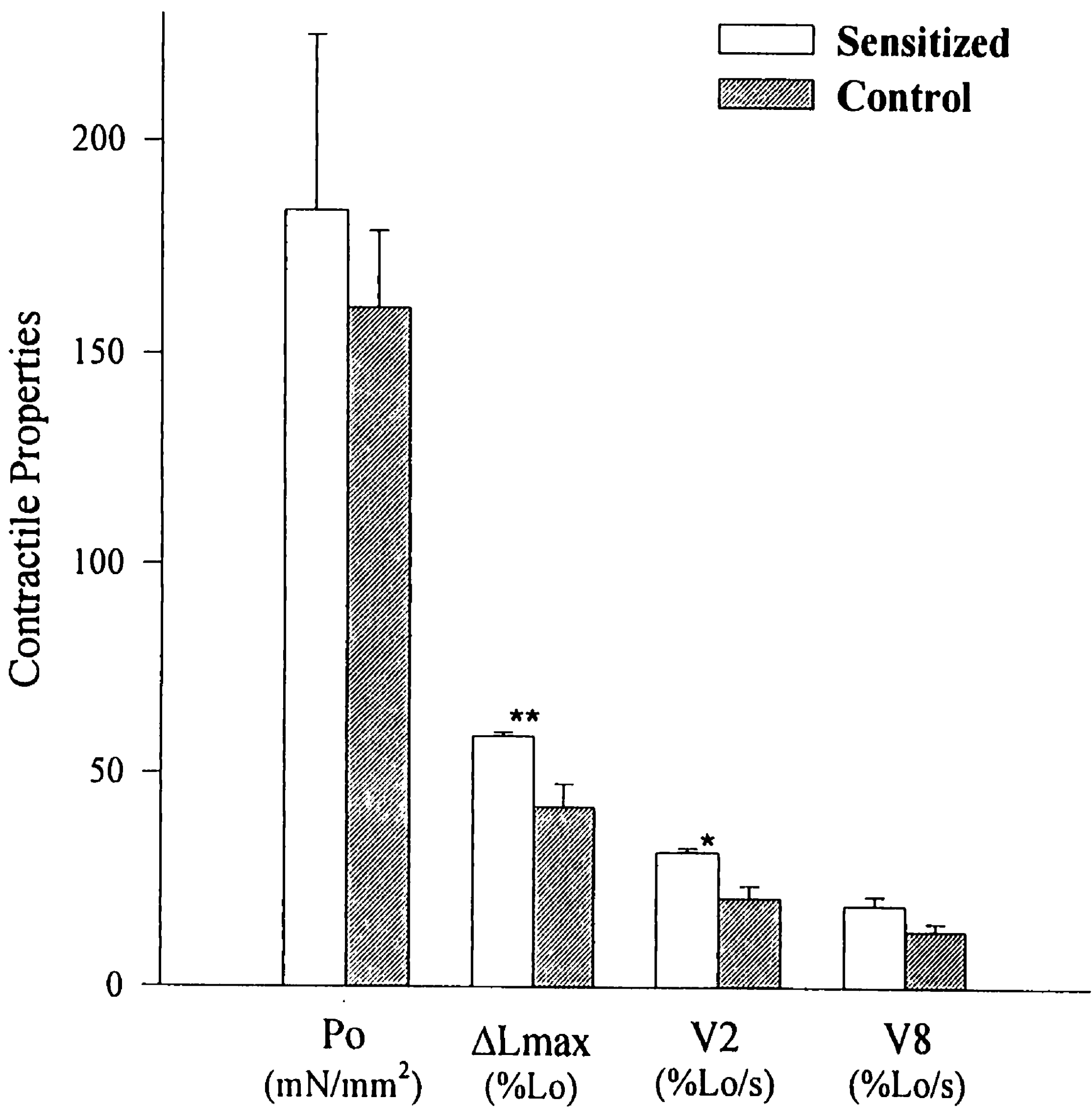
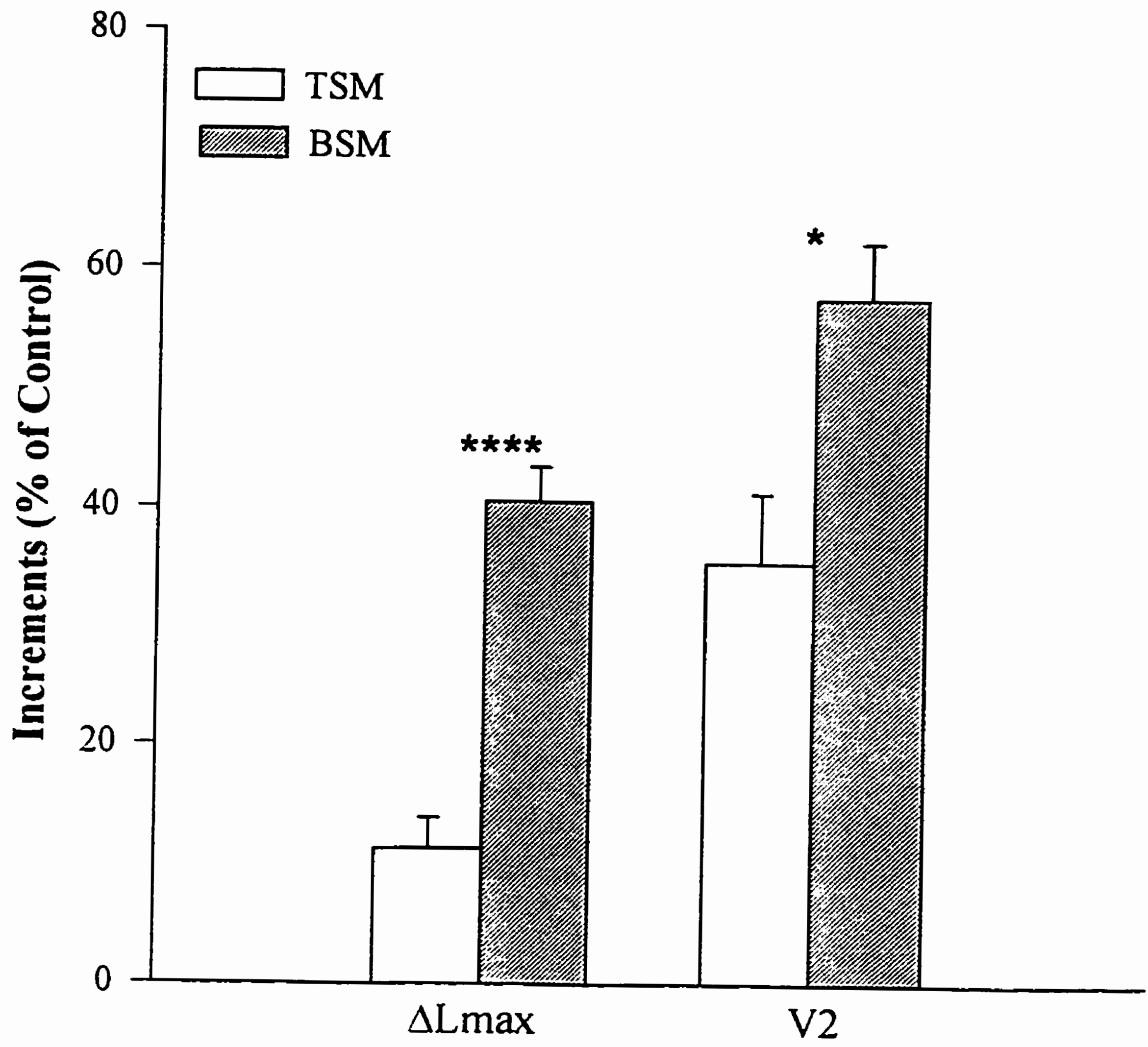

Sensitized
Control
Contractile Properties
200
150
100
50
0
**
*
Po
(mN/mm²)
ΔLmax
(%Lo)
V2
(%Lo/s)
V8
(%Lo/s)

Figure 19. Greater Increments in Contractility of Short-Term Sensitized Bronchial Smooth Muscle

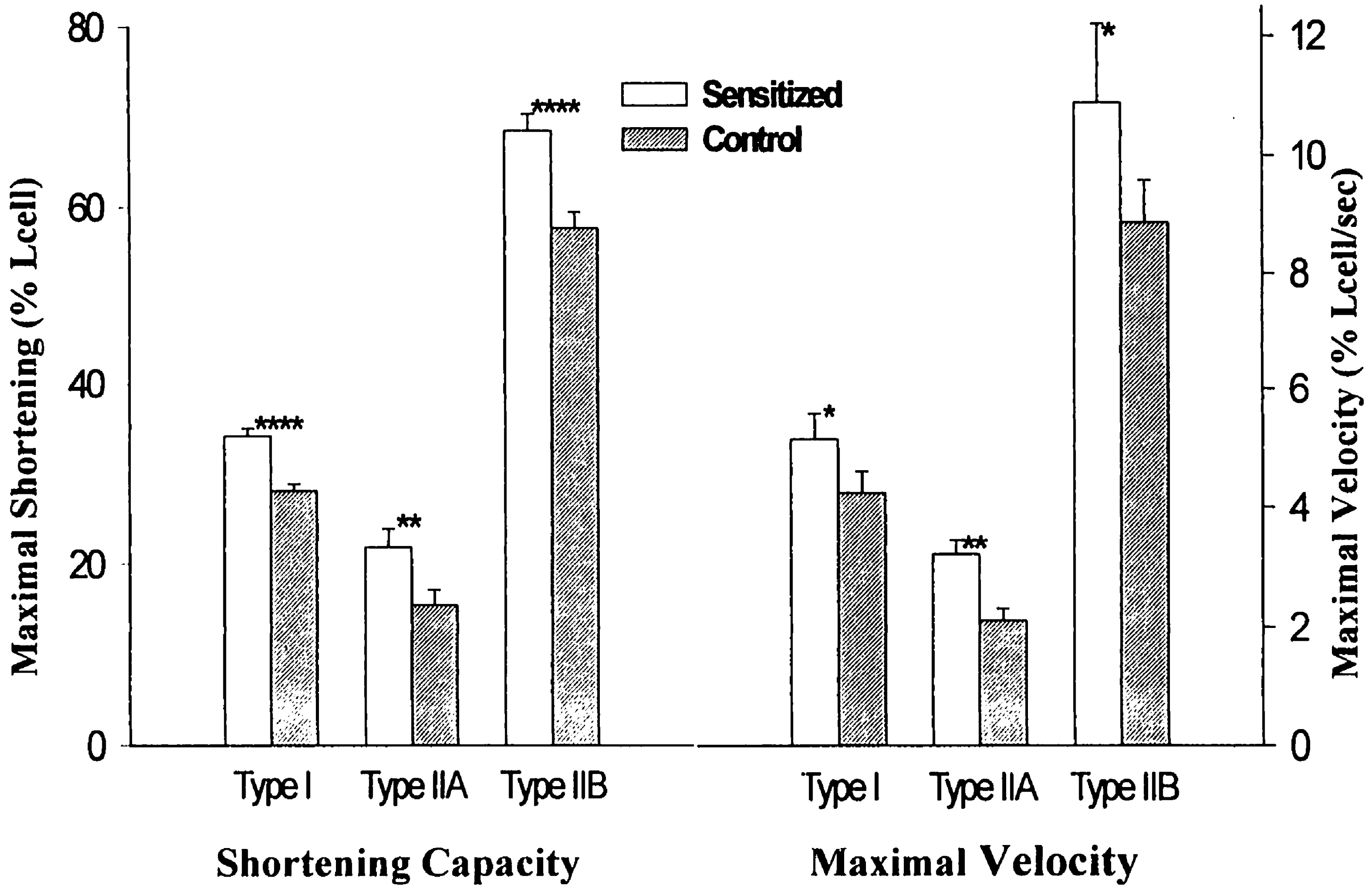
Short-term ragweed pollen sensitization resulted in increased contractility of canine airway smooth muscle, however, sensitized bronchial smooth muscle (BSM) showed greater increments in maximal shortening capacity (ΔL_{max}) and maximal velocity of shortening at 2 second (V2) when compared to those of sensitized tracheal smooth muscle. *$P<0.05$; ****$P<0.001$ (unpaired student t-test).



80
60
40
20
0
Increments (% of Control)
TSM
BSM
****
*
ΔLmax
V2

Figure 20. Enhanced Shortening Properties of Short-Term Sensitized Airway Smooth Muscle Cells.

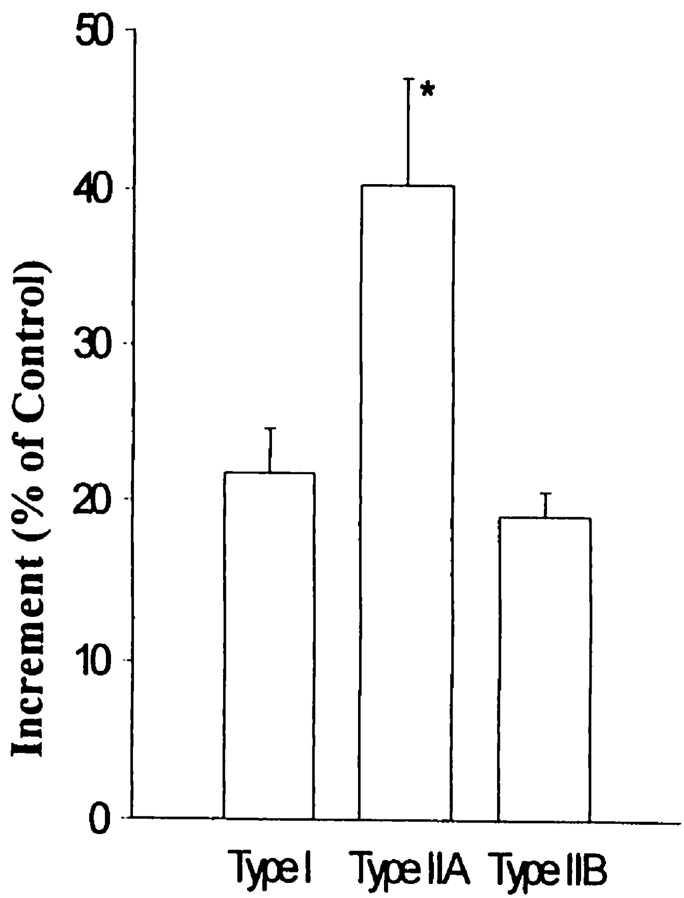
Three phenotypically distinct types of contractile airway smooth muscle existed in freshly isolated canine airway. All these types of cells showed increased maximal shortening capacity and velocity. Cells were stimulated electrically at room temperature. Lcell, resting cell length. **$P<0.01$; ****$P<0.001$ (ANOVA).



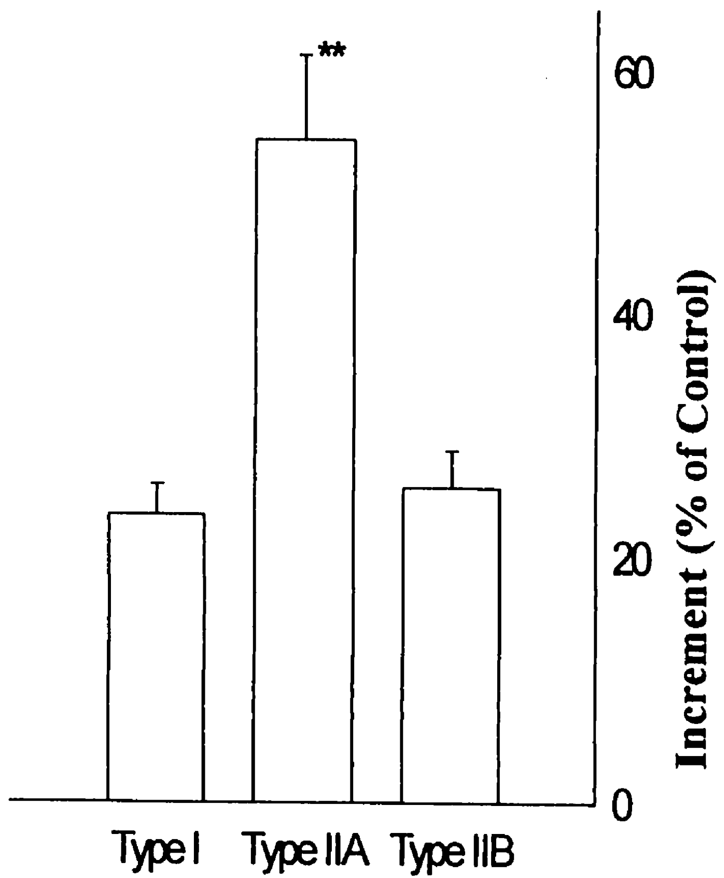
Maximal Shortening (% Lcell)
80
60
40
20
0
Sensitized
Control
****
**
****
*
**
*
Type I
Type IIA
Type IIB
Type I
Type IIA
Type IIB
Shortening Capacity
Maximal Velocity
12
10
8
6
4
2
0
Maximal Velocity (% Lcell/sec)

Figure 21. Heterogeneous Alterations in Shortening Properties of Short-Term Ragweed Pollen Sensitized Airway Smooth Muscle Cells.

Sensitized airway smooth muscle cells demonstrated increased contractility, however, type IIA cells showed the greatest increments in maximal capacity and velocity of shortening. *$P<0.05$; **$P<0.01$ (ANOVA, Duncan's new multiple range test).



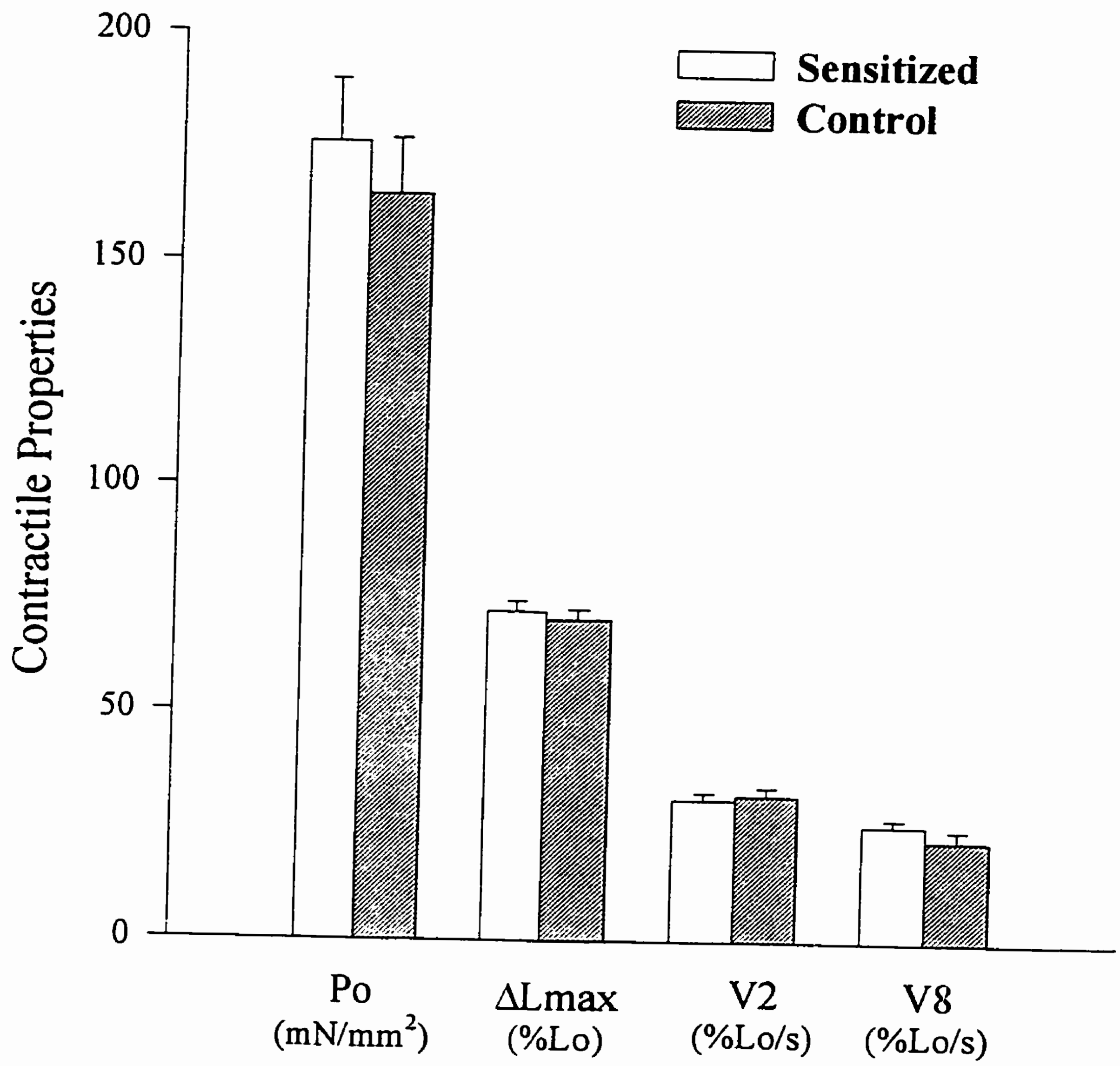
50
40
30
20
10
0
Increment (% of Control)
*
Type I
Type IIA
Type IIB
Maximal Shortening



60
40
20
0
Increment (% of Control)
**
Type I
Type IIA
Type IIB
Maximal Velocity

Figure 22. No Changes in Contractility of Long Term Sensitized Tracheal Smooth Muscle

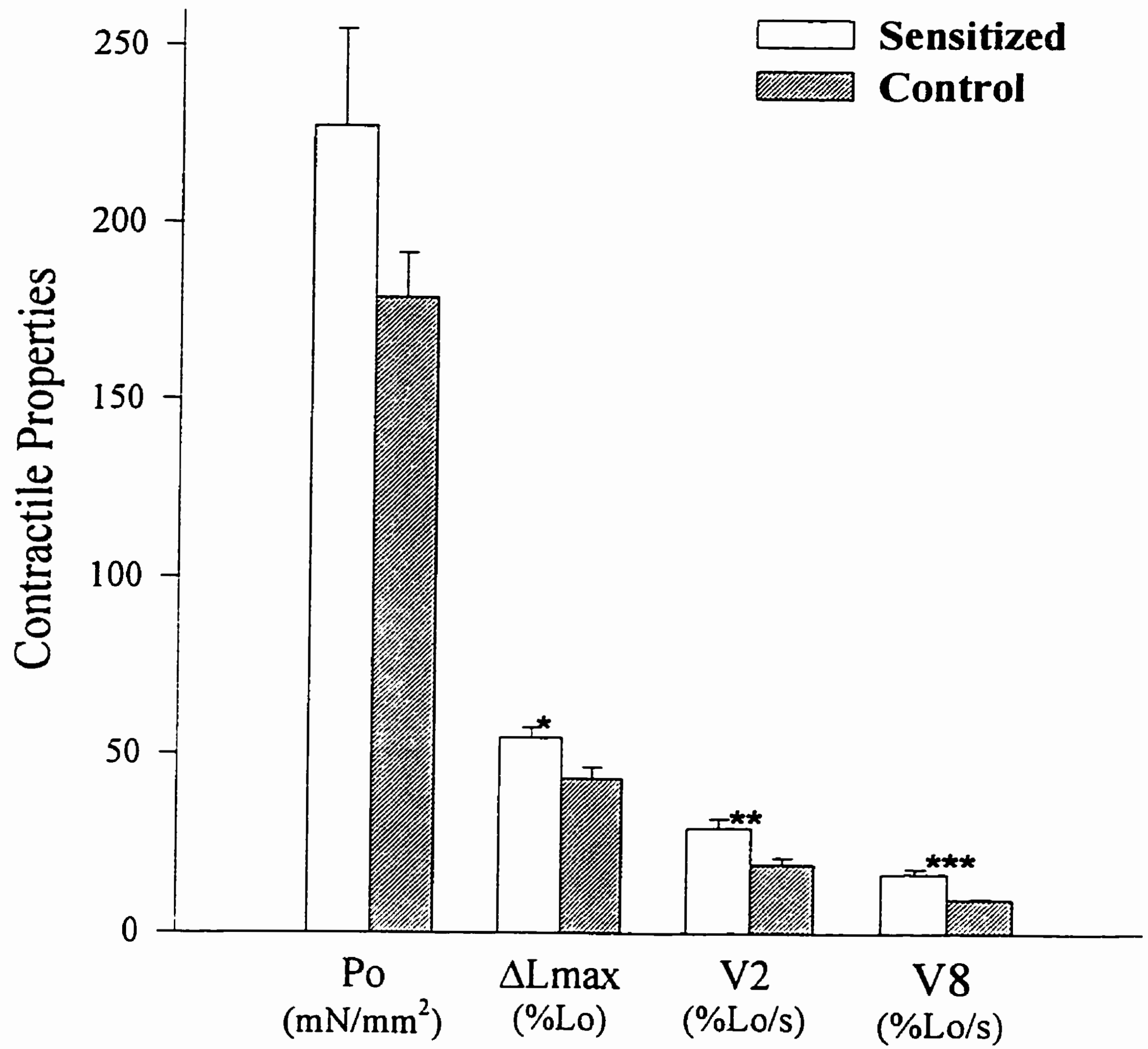
Tracheal smooth muscle obtained from long-term ragweed pollen sensitized dogs did not show significant changes in its contractility compared with control. Po, isometric tension; ΔLmax, maximal shortening capacity; V2 and V8, maximal shortening velocity at 2 and 8 second, respectively; were all statistically not different from each other. Lo, optimal length of contraction.



Sensitized
Control
Contractile Properties
200
150
100
50
0
Po
(mN/mm²)
ΔLmax
(%Lo)
V2
(%Lo/s)
V8
(%Lo/s)

Figure 23. Increased Shortening Properties of Long-Term Sensitized Bronchial Smooth Muscle.

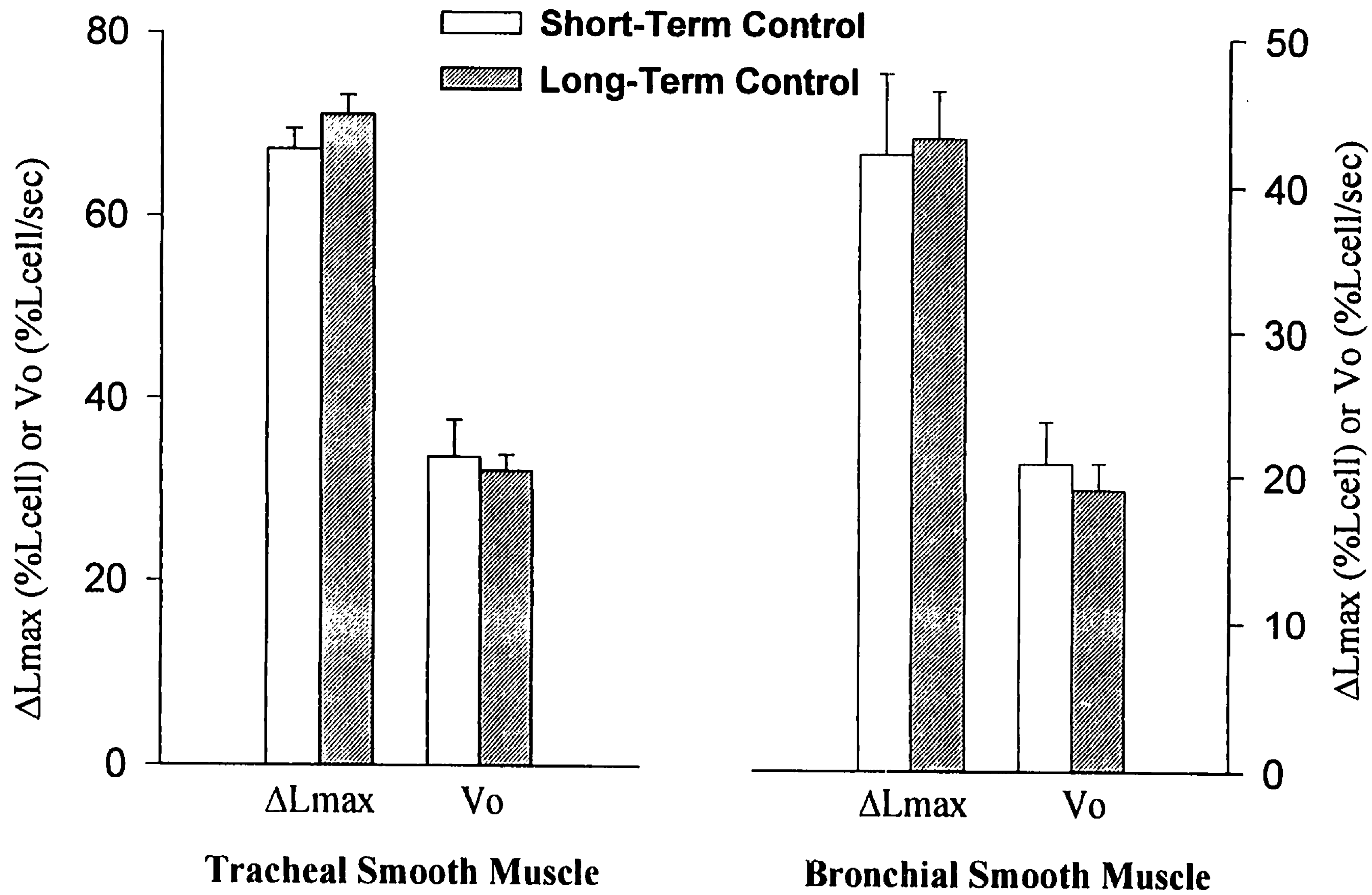
Bronchial smooth muscle dissected out from long-term ragweed pollen sensitized dogs demonstrated significantly enhanced shortening properties, such as maximal shortening capacity ($\Delta Lmax$), and maximal velocity of shortening at 2 (V2) and 8 second (V8). No obvious change in isometric tension (Po) was detected in sensitized bronchial smooth muscle. *$P<0.05$, **$P<0.01$, ***$P<0.005$ (unpaired student t-test).



Sensitized
Control
Contractile Properties
250
200
150
100
50
0
*
**
***
Po
(mN/mm²)
ΔLmax
(%Lo)
V2
(%Lo/s)
V8
(%Lo/s)

Figure 24. No Changes in Contractility between Short-Term and Long-Term Control Airway Smooth Muscles.

No significant differences in shortening properties were identified between airway smooth muscles obtained from short- and long-term sensitized dogs. ΔLmax, maximal shortening capacity; Vo, maximal velocity of shortening.


Short-Term Control
Long-Term Control
80
60
40
20
0
ΔLmax (%Lcell) or Vo (%Lcell/sec)
ΔLmax
Vo
Tracheal Smooth Muscle
50
40
30
20
10
0
ΔLmax (%Lcell) or Vo (%Lcell/sec)
ΔLmax
Vo
Bronchial Smooth Muscle

5.6 Discussion

Tracheal and bronchial smooth muscles showed heterogeneous responses to ragweed pollen sensitization. Short-term sensitization resulted in increased contractility in both tracheal and bronchial smooth muscle, however bronchial smooth muscle showed greater increments in $\Delta Lmax$ and Vo than tracheal. In long-term sensitized muscle, alterations of contractility were only seen in bronchial smooth muscle. Contractile parameters in both isometric and isotonic contraction increased in long-term sensitized bronchial smooth muscle. Short-term sensitization caused the increase of contractility of all contractile smooth muscle cells. Type IIA cells which showed the lowest contractility in normal airway muscle showed the greatest increment in sensitized muscle. This is the first demonstration of regional differences of airway smooth muscle in response to ragweed pollen-sensitization both at tissue and cellular levels. Whether these heterogenous effects are ragweed pollen specific or universal to other allergens remains to be further explored.

Previous studies showed that airway smooth muscle consisted of at least three morphologically, biophysically, and biochemically distinct contractile types of cells: type I, IIA, and IIB (Ma et al., 1997). Type I predominated in tracheal smooth muscle accounted for 86% of the total number of contractile cells. Type IIA cells accounted for almost half of total contractile cells in bronchial muscle. The present studies showed that short-term ragweed pollen sensitization most severely affected contractility of Type IIA

cells, because it caused increase of maximum shortening capacity by 49%, and increase of maximum velocity of shortening by 38%. The increments of ΔLmax in sensitized type I and IIB cells were 20% and 25% respectively, and of Vo, 22% and 30%. Biophysical studies of sensitized airway smooth muscle strips further demonstrated that ragweed pollen sensitization resulted in greater increases in contractility of bronchial smooth muscle than those of tracheal. Therefore, the heterogeneous effects of short-term sensitization on airway smooth muscle contractility may well be the result of heterogeneous effects of sensitization on the different types of contractile cells that are present in airway smooth muscle. The greatest increments of contractility of type IIA cells in sensitized airway smooth muscle and the increased numbers of these cells in bronchial smooth muscle contributed to the enhanced increments of contractility of sensitized bronchial smooth muscle. The molecular mechanism(s) of heterogeneous effects of sensitization on contractility of different types of contractile smooth muscle cells remained to be further explored.

The changes in contractility described above could be the result of alterations in intrinsic mechanical properties of muscle itself or of extracellular connective tissue (Bramley et al., 1994; Bramley et al., 1995). Biochemical analysis showed increases in protein content and total activity of smooth muscle myosin light chain kinase in sensitized muscle which suggests alterations in contractility of the muscle itself (Jiang et al., 1992). Our recent studies further demonstrated that extracellular matrix and intermediate filaments did not contribute to the increased contraction of sensitized muscle (Ma and

Stephens, 1997). Therefore, we are forced to hypothesize that sensitization results in alterations in intrinsic contractility of airway smooth muscle. The current results from single cell studies demonstrated that smooth muscle cells isolated from sensitized muscle possess increased Vo and ΔLmax, providing direct evidence to support that hypothesis.

With respect to location in the respiratory tree, attacks of asthma are known to be highly heterogeneous (Ebina et al., 1990; McFadden et al., 1977; Takishima et al., 1991). The present studies in the ragweed pollen-sensitized canine model demonstrated at least, that allergic heterogeneous bronchospasm could be the result of heterogeneity in airway smooth muscle contractility and differences in airway structure along the airway tree. As discussed in the previous section, due to the differences in composition of smooth muscle cells between tracheal and bronchial smooth muscle, ragweed pollen sensitization resulted in greater increases in contractility of bronchial smooth muscle than of tracheal. In the trachea, smooth muscle spanned only about one-third of the lumen and the contraction of smooth muscle was loaded by stiff cartilage rings which limited the shortening of muscle during contraction. While in the bronchus, smooth muscle surrounded the entire lumen, and was attached to cartilage plaques indirectly through compliant connective tissue. Bronchial smooth muscle therefore, was not heavily loaded like tracheal, and could shorten considerably during activation. In fact, shortening of bronchial smooth muscle could result in almost a complete closure of the airway lumen (Iizuka et al., 1992; Mitchell and Sparrow, 1994). Both of these factors may contribute to the heterogeneity in bronchospasm occurring in acute asthmatic attack.

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Chapter 6. Development of contractile and non-contractile phenotypes of smooth muscle cells in cultured tracheal smooth muscle cells subjected to prolonged serum deprivation

6.1 Background

Previous sections in this thesis demonstrated a clear heterogeneity in contractility of airway smooth muscle cells. Apart from contraction, smooth muscle cells are also capable of proliferation and phenotypic modulation. Current understanding of these properties comes from studies of smooth muscle cells in culture. Heterogeneity in proliferating properties of smooth muscle cells has been reported (Villaschi et al., 1994; Sartore et al., 1997). Some specific types of smooth muscle cells showed enhanced proliferative responses to mitogens.

Smooth muscle cells (SMC) exhibit a high degree of plasticity and do not undergo terminal differentiation (Owens, 1995; Thyberg, 1996). The principal function of mature SMC is contraction which depends on expression of a large number of different contractile and regulatory proteins present in precisely controlled concentrations (Horowitz et al., 1996). These cells undergo rapid phenotypic modulation in primary culture manifested by a marked decrease in the content of smooth muscle specific contractile and regulatory proteins, and increase of non-muscle proteins (Halayko et al., 1996; Shanahan et al., 1993). Retention of differentiated contractile and regulatory proteins and functional surface receptors were reported in cultured SMC (Rothman et al., 1992), and re-expression of smooth muscle marker proteins was identified in postconfluent cultures (Halayko et al., 1996), but re-development of contractility by proliferative smooth muscle cells has not been shown. Studies using magnetic twisting cytometry and atomic force microscopy

suggested that cultured smooth muscle cells retain the ability to stiffen in response to contractile agonists (Hubmayr et al., 1996; Jain et al., 1996). Ca^{2+} transients were also reported in cultured smooth muscle cells in response to a variety of contractile agonists (Li et al., 1994; Panettieri et al., 1989). However this indirect evidence does not prove the existence of shortening contraction in cultured smooth muscle cells. Increased stiffness of cultured cells as revealed by magnetic twisting cytometry and atomic force microscopy may well be the result of alteration of non-contraction related cytoskeletal structures. Dissociation of the Ca^{2+} signal from contraction may occur in cultured smooth muscle cells. Therefore, these reported properties of cultured smooth muscle cells can not be cited as clear evidence for the existence of contraction. Presently it is believed that smooth muscle cells in culture undergo dedifferentiation which only partially reverses at confluence. Functionally they remain very poorly contractile. Bowers and Dahm (1993) reported that the contractile phenotype of freshly isolated smooth muscle cells could be maintained in defined media where the proliferation of cells was minimal, but once the cells were cultured in proliferative media, their contractility was rapidly lost. Gunther et al. (1982) and Birukov et al. (1993) reported a transient maintenance of smooth muscle contractility in proliferative cultures, but again with increased duration of cell growth, contractility was lost.

Relatively little is known at this time about the development of differentiated smooth muscle cells, i.e. the phenotypic modulation of smooth muscle cells from a dedifferentiated type to differentiated type. This is due in part to the extreme plasticity

of this type of cell, and to limitations with respect to the inducibility and/or retention of the differentiated phenotype in cultures. Therefore, the very first step towards understanding smooth muscle differentiation is the establishment of a contractile smooth muscle cell culture.

Growth arrest may provide a practical way of inducing smooth muscle cell differentiation. Growth arrest of cultured myoblasts from skeletal muscle resulted in automatic expression of the MyoD family of transcription factors and consequent development of differentiated skeletal myocytes (Arnold and Braun, 1996). Insulin and IGFs are potentially capable of stimulating differentiation of skeletal muscle cells (Husmann et al., 1996). In cultured smooth muscle cells, induction of differentiation has not yet been achieved. In the post-confluence phase where the proliferation rate decreases considerably, cultured smooth muscle cells reaccumulate contractile proteins, indicating a partial restoration of contractility (Halayko et al., 1996). In primary culture, smooth muscle cells lose the expression of functional muscarinic receptors; growth arrest in the presence of insulin and/or IGF results in re-expression of these receptors (Yang et al., 1991). Confluent smooth muscle cell culture forms a typical pattern of hills-and-valleys (Chamley-Campbell et al., 1979). Elongated spindle-shaped cells, which represent differentiated, contractile smooth muscle cells, have been observed sometimes in growth arrested smooth muscle cell culture in the presence of ITS (insulin, transferrin, selenium). In this study, phenotypic modulation of primary cultured tracheal smooth muscle cells was examined during prolonged serum deprivation. Contractility of cultured tracheal smooth

muscle cells was estimated by direct measurement of zero-load shortening in "lifted" cells in response to contractile agonists and electrical stimulation.

Contractility of smooth muscle is regulated by a variety of contractile and regulatory proteins (Horowitz et al., 1996). Smooth muscle myosin light chain kinase is believed to be the major protein which regulates smooth muscle contraction. Recently, SM-B a new myosin heavy chain isoform (*vide supra*) was identified, and reported to be important in determining smooth muscle mechanical properties (Haase and Morano, 1996; Kelley and Adelstein, 1993). Other proteins such as smooth muscle α-actin, total smooth muscle myosin heavy chain, tropomyosin, caldesmon, and calponin are also important in determining smooth muscle cell contractility. Restoration of expression of these proteins in post-confluent cultures may be a necessary preparation for the recovery of cultured SMC contractility. Therefore, temporal changes in expression of these proteins were also examined during prolonged serum deprivation in airway smooth muscle cell (ASMC) cultures. Our results demonstrated that prolonged serum deprivation resulted in the emergence of two biophysically and biochemically distinct populations of cells in tracheal smooth muscle cell culture. One, which consisted of spindle-shaped cells, expressed considerably greater amounts of smooth muscle contractile and regulatory proteins than the second cell population. These cells remained elongated after being lifted from the plate, and demonstrated very similar morphology to that of freshly isolated contractile cells; they shortened in response to contractile agonists and electrical stimulation. Surprisingly, these cells showed the contractility that was even greater than that of freshly

isolated cells. These data indicate that a functional contractile phenotype can be induced in tracheal smooth muscle cell cultures through prolonged serum deprivation. The other. circular-shaped, expressed very little smooth muscle contractile and regulatory proteins, and showed no contractile response, representing a non-contractile phenotype.

6.2 Objectives

Development of cultured differentiated tracheal smooth muscle cells and analysis of their phenotypic heterogeneity.

6.3 Hypothesis

Long term serum deprivation results in differentiation of cultured airway smooth muscle cells. Phenotypically distinct populations of cells exist in serum deprived smooth muscle cell culture.

6.4 Protocol

Airway smooth muscle cells were enzymatically isolated from dog trachea and cultured as stated above. Cells were then subjected to serum deprivation (growth arrest) for up to 20 days post-confluence. Phenotypic modulation was evaluated both biophysically and biochemically. Contractility of these cells was measured on dissociated cells at the single cell level, and biochemical properties were analyzed on homogenates obtained from cell culture. To further identify biochemical differences between different populations of cells in arrested cultures, immunostainning of smooth muscle contractile

and regulatory proteins was carried out.

6.5 Results

Characteristics of freshly-isolated tracheal smooth muscle cells. Broadly, two populations of cells were obtained from freshly isolated tracheal smooth muscle. Half of them appeared shining and rounded with diameters from 5 to 10 μm; no contractile responses were identified in these cells with different types of contractile agonists. The remainder were elongated and also possessed smooth and shining sarcolemma as seen under the microscope. They demonstrated very satisfactory reversible contractile responses to acetylcholine (Ach), histamine, high K^+, and electrical stimulation. The majority of these cells (86% of total elongated cells) had a mean length of 120 μm (type I cells). During culture only these rounded cells attached to the culture plate and replicated, while the elongated cells seldom attached and were washed away during the subsequent changes of culture medium. Cultured cells reached confluence at about 7 to 8 days in culture, formed a typical pattern of hills-and-valleys, and expressed smooth muscle α-actin and smooth muscle myosin heavy chain. Besides biophysical and cytokinetic differences between freshly isolated rounded and elongated cells, significant differences also existed in their biochemical properties. Elongated cells showed a significant greater expression in smooth muscle type protein compared to rounded cells per μg protein, such as smooth muscle α-actin, total smooth muscle myosin heavy chain, and SM-B.

Morphological characteristics of growth-arrested smooth muscle cells. Two distinct groups of cells appeared in post-confluent primary smooth muscle cell cultures during long-term

serum deprivation (Fig. 25): one group of cells appeared flat, circular, and bright under the inverted microscope; these comprised almost all the cells that were present before serum deprivation. The second group demonstrated the normal elongated spindle shape; they were aligned side-by side in bundles in most cases; they were dark, but possessed a shining sarcolemma. The cells in the second group began to appear after two days of serum deprivation, and increased in number as deprivation was prolonged. After 15 days of deprivation they comprised 28.5% ± 4.6 (SE) of all cells present, but occupied almost 40% of the total area of the culture dish. When cells were lifted using typsin/EDTA, those in the first group "balled up" much as non-arrested cells do; while those in the second group retracted slightly, but remained elongated. Their average length was similar to that of the freshly-isolated tracheal smooth muscle cells (110 ± 8 μm), i.e. akin to type I cells.

Contractility of elongated cells induced during serum deprivation. Elongated smooth muscle cells showed reversible contraction on stimulation with optimal doses of Ach (10^{-5} M), histamine (10^{-5} M), KCl (10 mM), and low level single pulse electrical stimulation (10 volts, 10 ms) (Fig. 26). Maximal contraction could be induced with repeated pulsed electrical stimulation. Figure 27 shows typical curves for the unloaded shortening of freshly isolated and the newly-induced contractile single tracheal smooth muscle cells, during repeated pulsed electrical stimulation. Surprisingly these newly-induced contractile cells evinced hyper-contractility to electrical stimulation (Fig. 28). Thus they shortened maximally by 50% of their original length at room temperature on repeated electrical stimulation, while freshly isolated cells only shortened by 27%. The former also

shortened faster with the maximal velocity of shortening (Vo) being 8 %Lcell/s, almost double the value of that of freshly isolated cells. In addition, arrested cells responded to electrical stimulation at lower current intensity than freshly isolated cells, indicating a higher sensitivity to electrical stimulation. The voltages inducing half-maximal contraction for freshly isolated and arrested contractile cells were 18 ± 3, and 11 ± 2 volts, respectively. These were significantly different ($P<0.05$). No contractile responses were found in flat circular cells.

Modulation of biochemical properties of cultured smooth muscle cells during serum deprivation. In correspondence with the time-dependent increase in the number of elongated spindle-shaped cells during serum deprivation, expression of contractile and regulatory proteins increased as revealed by western blot analysis (Fig. 29). After 15 days of arrest, total smooth muscle myosin heavy chain content per μg protein increased 10.8 ± 1.1 fold, smooth muscle α-actin content increased 5.9 ± 1.0 fold, and smooth muscle myosin light chain kinase increased by 62.9 ± 13.5 fold compared with contents of cells in non-arrested confluent cultures. The content of smooth muscle type myosin heavy chain reached the same level as in freshly-isolated cells. Surprisingly, the contents of smMLCK and α-actin were significantly (~30 and 2 times respectively) higher than those of freshly-isolated cells (Fig. 30). The expression of SM-B was not detected at any of the tested time points, either at protein or message level (Fig. 31).

Biochemical differences between flat and elongated cells in arrested culture. To further

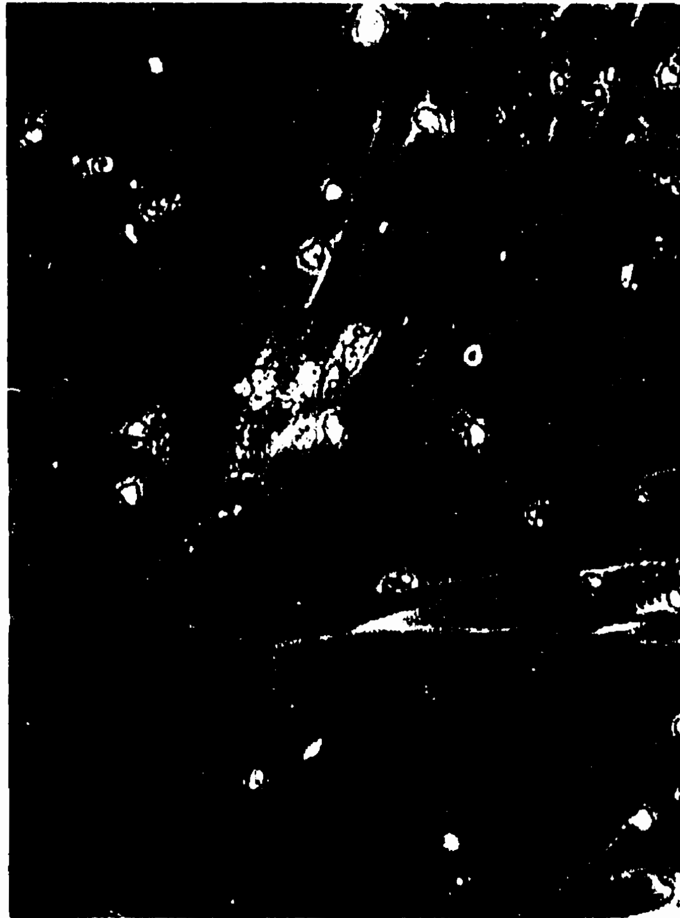
confirm that the newly-emerging spindle-shaped cells express more smooth muscle contractile and regulatory proteins, an immunocytochemical study was employed. Results of experiments employing specific antibodies showed that spindle-shaped cells stained intensively for smooth muscle type myosin heavy chain, α-actin, and myosin light chain kinase. However, the flat cells stained quite faintly with smooth muscle myosin light chain kinase and were almost negative to smooth muscle myosin heavy chain and α-actin staining (Fig. 32). In addition, these cells also stained strongly for non-muscle myosin light chain kinase and β-tubulin (Fig. 33). Centrally-located cigar-shaped nuclei were identified in spindle-shaped cells, but round nuclei were seen in flat ones (Fig. 33).

Figure 25. Induction of two distinct phenotypes of cells in primary airway smooth muscle cell culture under prolonged serum deprivation.

Panel A: phase-contrast micrograph of confluent, cultured cells, showing a relatively homogeneous population, with hills-and-valleys pattern.

Panel B: microscopy of 10 day arrested cultured cells, showing two distinct groups of cells: 1. flat-circular cells which appear bright; 2. spindle-shaped, elongated cells with phase lucent sarcolemma, which appear dark and are aligned side-by side in most cases.

Before Deprivation



10 Days After Deprivation

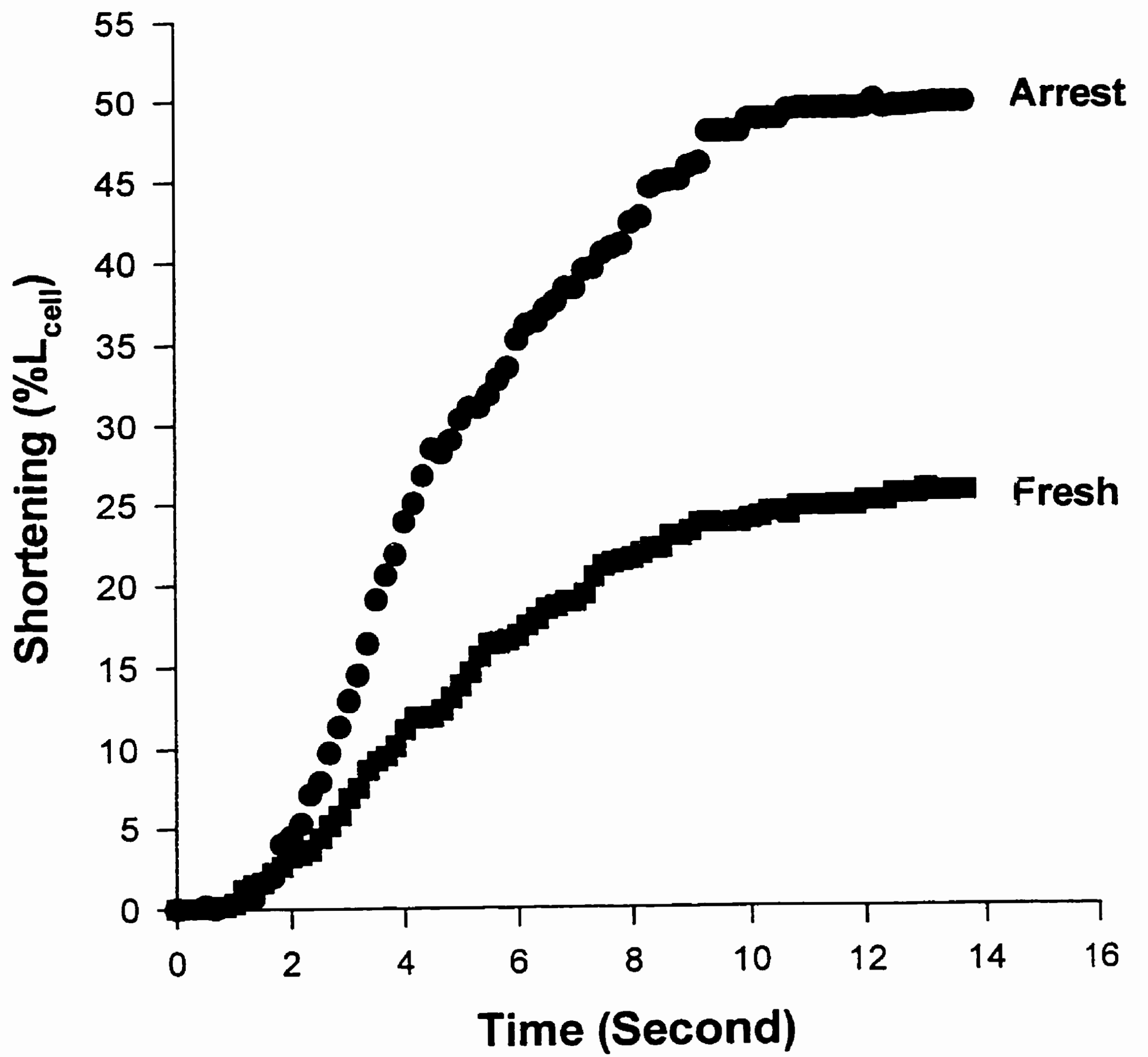
Fig 26. Typical images showing contraction and relaxation of

a single airway smooth muscle cell.

A, B, and C represent images of a freshly-isolated cell: fully relaxed, partially contracted, and 10 min after withdrawal of stimulation, respectively. D, E, and F represent images of a arrested contractile cell: fully relaxed, partially contracted and 10 min after withdrawal of stimulation, respectively. Cells were stimulated electrically. The dark spot near the cell was one of the electrodes, the other was located at the other side of the cell.

Fig 27. Typical curves of the unloaded shortening of single tracheal smooth muscle cells.

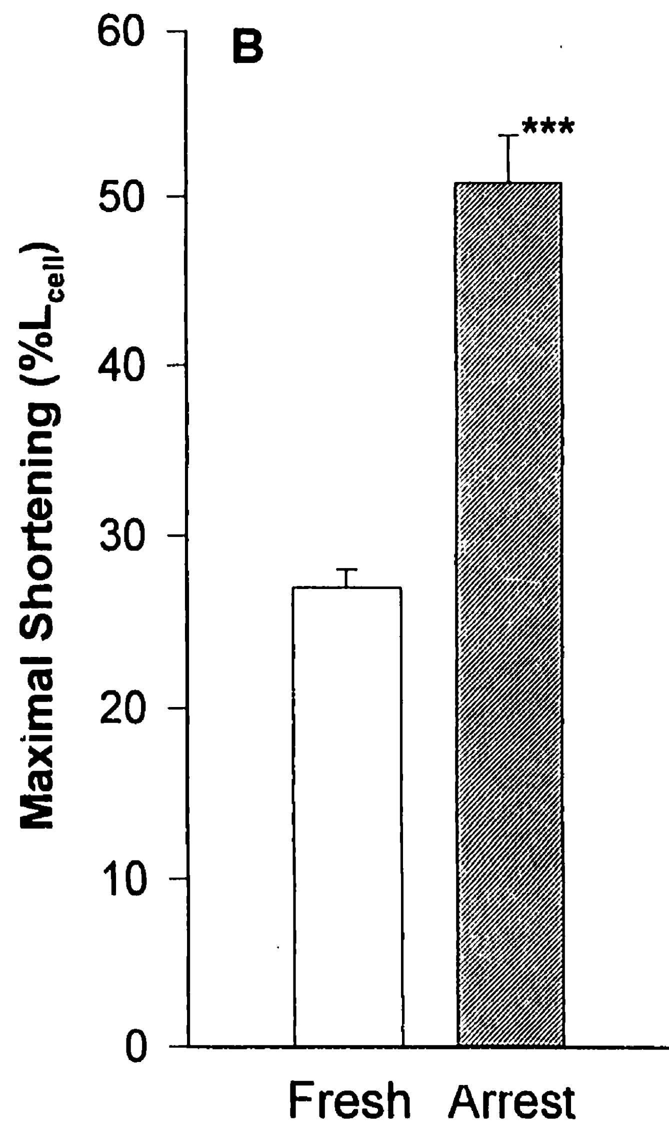
A greater contractility was found in arrested cells. Arrest, arrested contractile cells. Fresh, freshly-isolated contractile cells. Single cells were stimulated maximally with repeated electrical pulses at room temperature.



55
50
45
40
35
30
25
20
15
10
5
0
Shortening (%L_{cell})
0
2
4
6
8
10
12
14
16
Time (Second)
Arrest
Fresh

Fig 28. Comparison of mechanical properties of arrested contractile cells in tracheal smooth muscle cell cultures with those of freshly-isolated cells.

Significantly increased isotonic contractility (Vo and ΔLmax) was identified in arrested contractile cells. A: Maximal Velocity of Shortening (Vo). B: Maximal Shortening capacity (ΔLmax). ***P<0.005, compared with freshly isolated cells.


A
Maximal Velocity of Shortening (%L_cell/Sec)
10
8
6
4
2
0
***
Fresh
Arrest

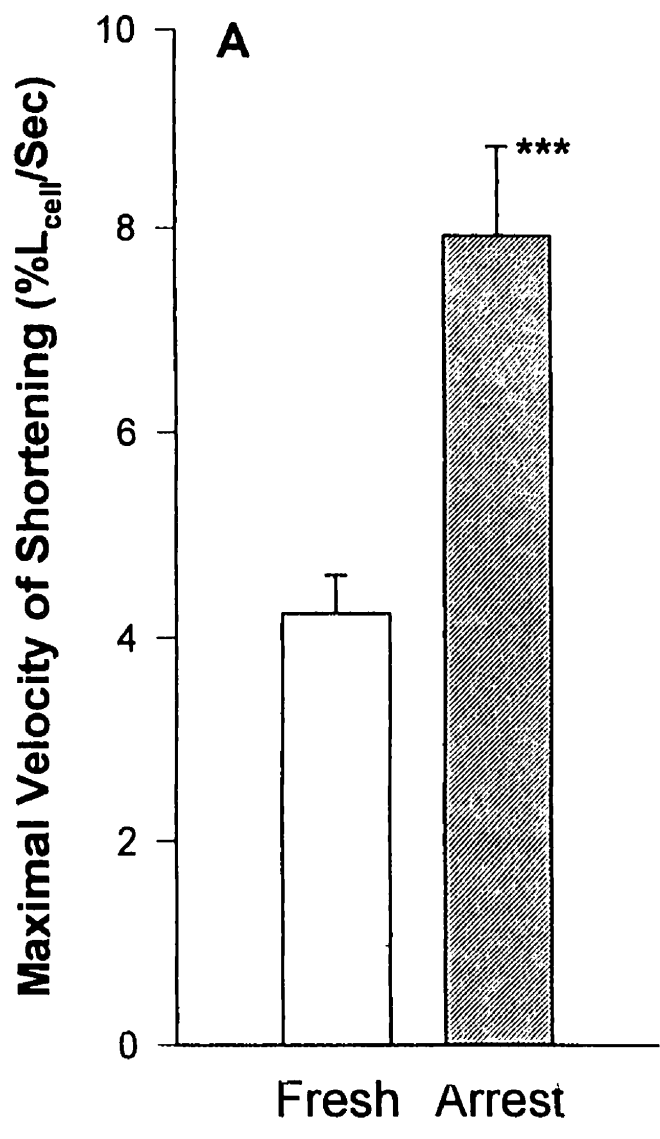
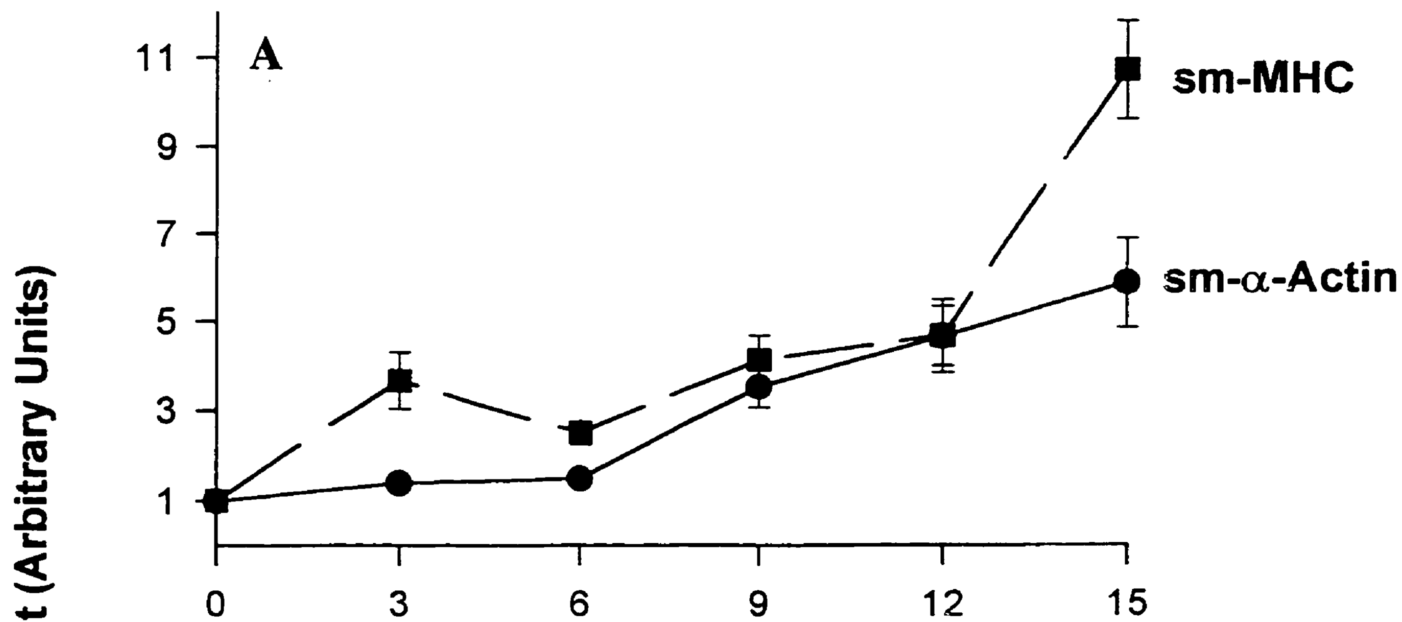

B
Maximal Shortening (%L_cell)
60
50
40
30
20
10
0
***
Fresh
Arrest

Fig 29. Temporal changes in contents of smooth muscle contractile and regulatory proteins in cultured tracheal smooth muscle cells during prolonged serum deprivation.

Progressive increases in the expression of these proteins in cultured smooth muscle cells were identified under prolonged serum deprivation. A: smooth muscle α-actin (sm-α-Actin) and smooth muscle total myosin heavy chain (sm-MHC). B: smooth muscle myosin light chain kinase (sm-MLCK).


A
sm-MHC
sm-α-Actin
11
9
7
5
3
1
0
3
6
9
12
15
Relative Protein Content (Arbitrary Units)

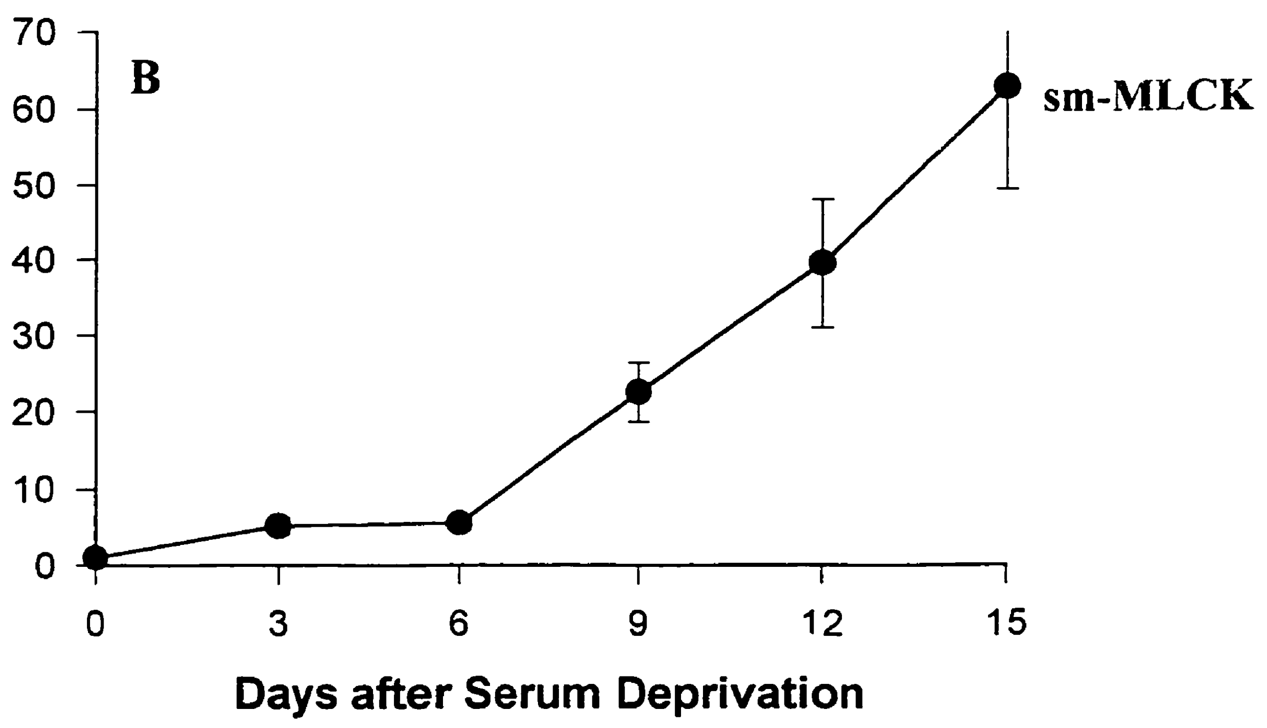

B
sm-MLCK
70
60
50
40
30
20
10
0
0
3
6
9
12
15
Days after Serum Deprivation

Fig 30. Comparison in contents of smooth muscle contractile and regulatory proteins among freshly-isolated, cultured confluent, and cultured, 15 day arrested tracheal smooth muscle cells.

Significant decrease of contractile proteins content in confluent cells, and restoration in arrested cells were found. Surprisingly, the content of smooth muscle α-actin and smooth muscle myosin light chain kinase in arrested cells were even significantly higher than those in freshly isolated cells. F: freshly-isolated cells. C: cultured confluent cells. A: cultured arrest cells. *$P<0.05$, ***$P<0.005$ compared with freshly-isolated cells.

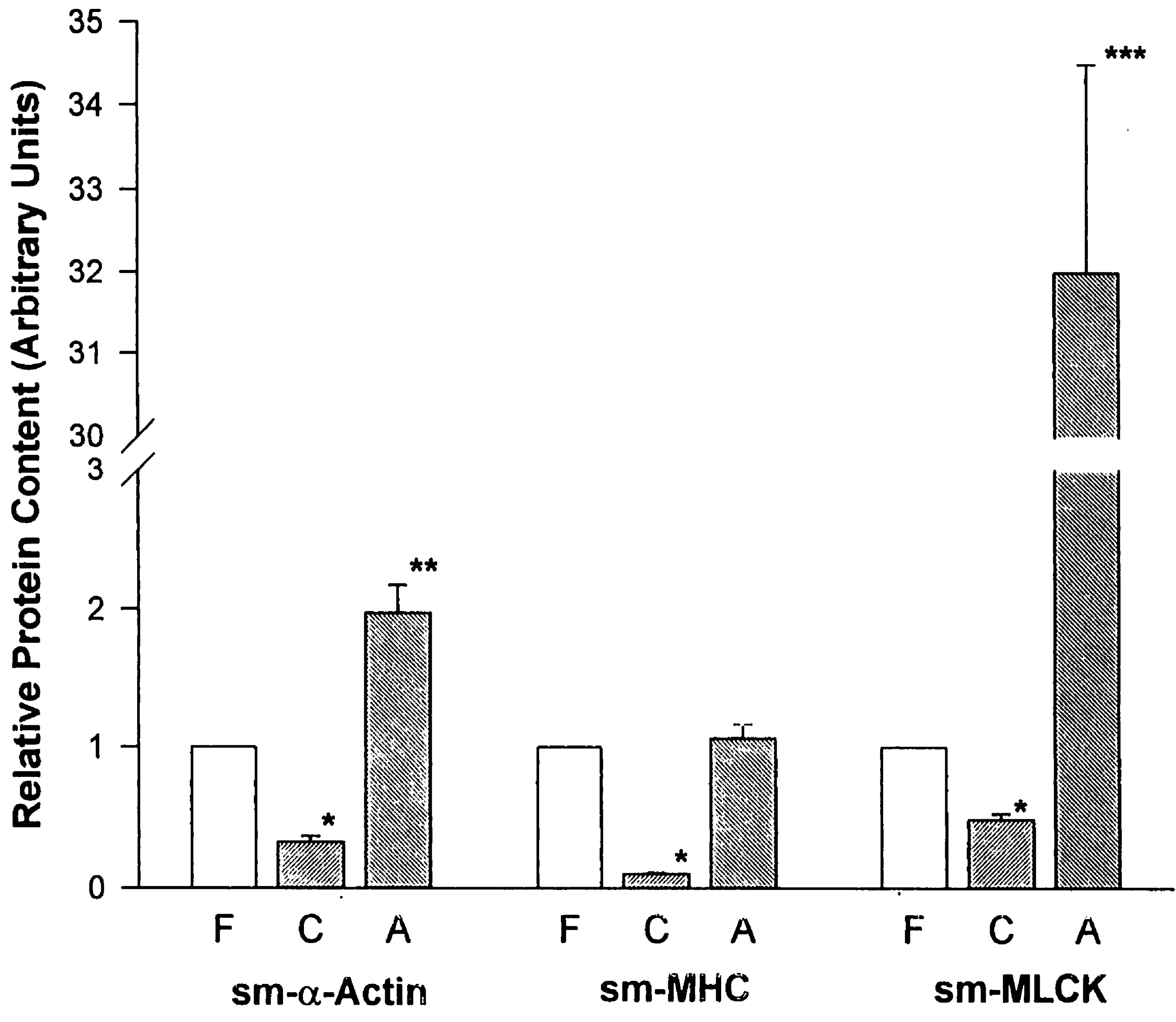

Relative Protein Content (Arbitrary Units)
35
34
33
32
31
30
3
2
1
0
***
**
*
*
*
F
C
A
F
C
A
F
C
A
sm-α-Actin
sm-MHC
sm-MLCK

Fig 31. Messenger expression of smooth muscle myosin head isoform (SM-B) in tracheal smooth muscle cell culture during prolonged serum deprivation.

No SM-B mRNA was detected in cultured cells from confluence to 15 days of serum deprivation using RT-PCR. F: freshly-isolated cells. Mr: molecular marker.

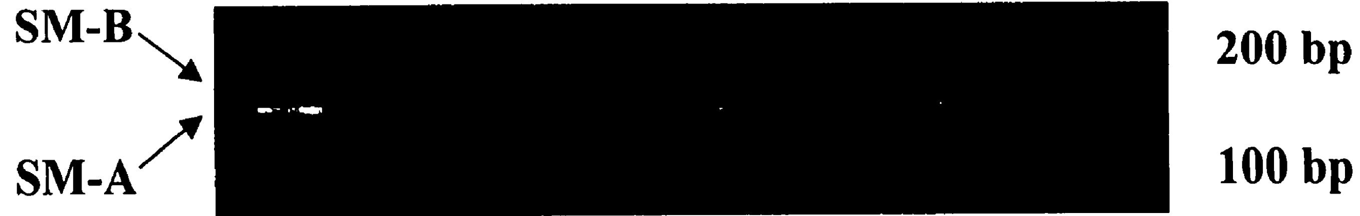
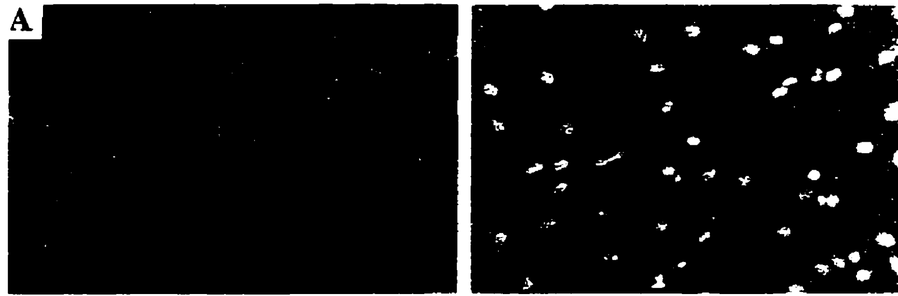

SM-B
SM-A
200 bp
100 bp
15
12
9
6
3
0
F
Mr
Days of Serum Deprivation

Fig 32. Fluorecent graphs of cultured, smooth muscle contractile cells 10 days after serum deprivation immunostained for contractile proteins

Elongated spindle-shaped contractile cells stained intensively for smooth muscle contractile type and non-contractile type proteins. Flat, circular, non-contractile cells stained relatively weakly for non-muscle myosin light chain kinase, but almost negatively for smooth muscle myosin heavy chain and smooth muscle α-actin. Centrally-located cigar-shaped nuclei were identified in serum deprivation-induced contractile cells, while relatively round nuclei were found in circular non-contractile cells. A: smooth muscle myosin heavy chain. B: smooth muscle α-actin. C: smooth muscle myosin light chain kinase. Corresponding nuclear stained cells are presented towards the right of protein stained cells.


A

B

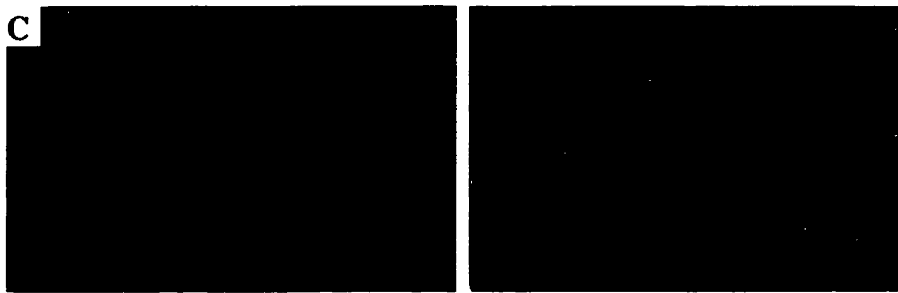
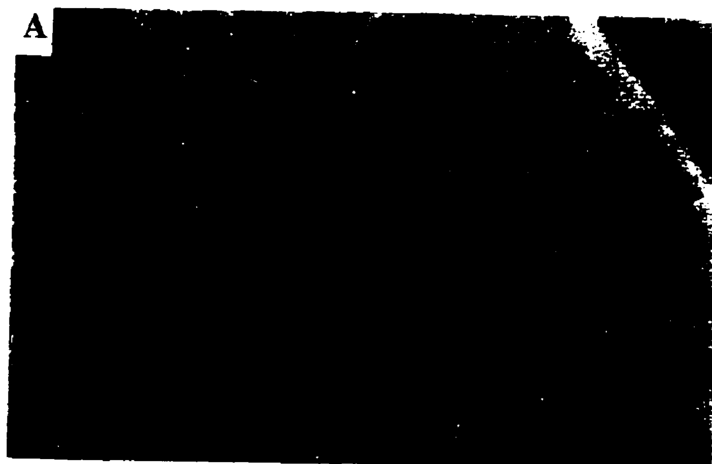

C

Fig 33. Microscopy of cultured tracheal smooth muscle cells after 10 day serum deprivation immunostained with a non-contractile type protein specific antibody.

Elongated spindle-shaped contractile cells stained intensively for these non-contractile type proteins. Flat, circular, non-contractile cells stained relatively weakly for non-muscle myosin light chain kinase, and β-tubulin. A: non-muscle light chain kinase. B: β-Tubulin.



6.6 Discussion

The present studies demonstrated that two distinct populations of cells, ie. contractile and non-contractile, developed in tracheal smooth muscle cell culture under prolonged serum deprivation. More interestingly, serum deprivation resulted in a hyper-contractile phenotype of smooth muscle cells, in spite of the fact that SM-B, the isoform reported to be responsible for conferring maximal shortening velocity, was considerably down-regulated in those cultures. This provides two important pieces of information in smooth muscle research. Firstly, differentiated, contractile smooth muscle cells can be induced from dedifferentiated smooth muscle cells in culture through long-term serum deprivation in the presence of insulin, transferrin, and selenium; this may provide a model for the study of smooth muscle development and differentiation. Secondly, contrary to expectation, expression of SM-B may not be the most important factor in determining smooth muscle contractility.

The concept of plasticity of the smooth muscle cell is widely-accepted and considered to be a necessary part of the SMC differentiation program that evolved because it confers a survival advantage to the organism (Schwartz et al., 1986). It is well-established that the smooth muscle cell can change its phenotype from contractile to synthetic , and *vice-versa,* in response to environmental influences (Owens, 1995). The mature contractile smooth muscle cell was reported to undergo a rapid modulation of its phenotype to the immature synthetic type during culture in the presence of serum

(Halayko et al., 1996; Shanahan et al., 1993). This was demonstrated by a considerable decrease in smooth muscle-specific contractile and regulatory proteins, and an increase in non-muscle type proteins. Direct evidence for reversal of modulation of cultured smooth muscle cells, i.e., re-development of contractility, has never been demonstrated, although re-accumulation of smooth muscle myosin heavy chain was found in post-confluent cultures (this is the biochemical concomitant of the contractile phenotype) (Halayko et al., 1996). Recovery of contractile responsiveness was reported in cultured smooth muscle cells (Li et al., 1994; Panettieri et al., 1989). However increased amplitude of the intracellular Ca^{2+} transient and/or myosin light chain phosphorylation in response to contractile agonists were used as indices of contractility. These are indirect methods for inferring contractility and have limited usefulness. Our results demonstrated that mature contractile smooth muscle cells could be induced in post-confluent primary cultures under long term serum deprivation. After "lifting", these cells retained their normal spindle shape, and shortened isotonically in response to contractile agonists such as Ach, histamine, KCl, and electrical stimulation. This also indicates that appropriate receptors were present. The data demonstrate that cultured airway smooth muscle cells (synthetic phenotype) retain the capability of reversing their phenotype, and undergo differentiation to a contractile phenotype as a result of serum deprivation. Our studies thus provide novel direct evidence that under appropriate conditions cultured, non-contractile smooth muscle cells developed into a contractile phenotype.

Molecular mechanisms that control the differentiation program of smooth muscle

cell have not yet been identified. The smooth muscle cell is believed to be remarkably plastic in that it can undergo rapid and reversible changes of its phenotype in response to a variety of different stimuli. Consistent with this property, differentiation of smooth muscle cells appears to be highly dependent on environmental influences (Owens et al., 1996). Our results demonstrated that the differentiated phenotype of cultured airway smooth muscle cells could be induced and maintained as the result of serum deprivation. This implicates the importance of growth arrest in determining smooth muscle cell differentiation and may provide a model to study differentiation of the smooth muscle cell. The re-development of normal contractility of cultured smooth muscle cells was accompanied by re-appearance of smooth muscle contractile, structural and regulatory proteins such as smooth muscle myosin heavy chain, α-actin, and smooth muscle type myosin light chain kinase, and morphological reversion to a normal spindle shape. Our data also showed that the content of non-muscle myosin light chain kinase and β-tubulin remained elevated in these contractile cells. This provided an additional difference between arrested contractile and freshly-isolated cells. This suggests that down-regulation of non-muscle type proteins may not be required in induction of contractile type of cells from cultured non-contractile smooth muscle cells under serum deprivation. Communication among cells may also be important in differentiation under serum deprivation, because induced contractile cells were found in most cases formed in bundle-like clusters aligned in parallel. Instead of invoking communication between cells, the grouping of the contractile cells in bundles can be also be attributed to their forming a colony that originated from a common precursor. We currently cannot distinguish these

two possibilities.

Induction of a contractile phenotype in cultured tracheal smooth muscle cells was found to be accompanied by considerably increased expression of smooth muscle α-actin, smooth muscle myosin heavy chain, and smooth muscle myosin light chain kinase during prolonged serum deprivation. Surprisingly, we did not find corresponding changes in the expression of smooth muscle myosin heavy chain isoform SM-B. Actually, we did not detect any expression at all of SM-B of either protein or message in cultured tracheal smooth muscle cells before and during serum deprivation. But it was prominently detected in freshly-isolated tracheal smooth muscle cells. Expression of SM-B has been reported important in determining smooth muscle mechanical properties (White et al., 1993). It confers a cycling velocity on the muscle which is three times faster than those of the other isoforms (Kelley et al., 1993). Our finding seems contradictory to this notion. The newly-induced spindle-shaped cultured tracheal smooth muscle cells showed elevated contractility as a result of arrest, but did not express SM-B, while freshly-isolated cells did express SM-B isoform, but showed lower contractility, indicating that proteins other than SM-B are responsible for the super-contractility of newly induced contractile cells. Dissociation of the content of SM-B expression from contractility of smooth muscle was also found by other investigators. Haase and Morano (1996) reported a decrease of SM-B expression, while the maximal shortening velocity of smooth muscle increased in pregnant rat myometrium fibers. Siegman et al. (1997) recently found no correlation between the amount of SM-B and shortening velocity in mouse megacolon. The latter pointed out that

to compare velocities regulated by the different myosin heavy chain isoforms, full phosphorylation of the 20 kDa myosin light chain must be ensured, and the complement of MLC_{20} and MLC_{17} must be the same. When this was carried out using ATPγS no difference in velocity due to the different isozymes was seen. The correlation reported by others between Vo and myosin heavy chain isozyme is therefore not the effect of the isozyme itself.

Smooth muscle myosin light chain kinase (smMLCK) is an important candidate that could be responsible for the correlation between Vo and myosin heavy chain isozyme activity. Considerable increase in smMLCK content was found in newly-induced contractile cells. After 15 days serum deprivation, smMLCK content in cultured cells increased 62.9 fold compared with cells in non-arrested confluent culture. Our previous studies showed that the content of smooth muscle myosin light chain kinase in confluent tracheal smooth muscle cell cultures decreased by 50% compared with that of freshly-isolated cells (Halayko et al., 1996). Arrested cells expressed smMLCK at a content 30 times that of freshly isolated cells; this may contribute to the increased contractility of arrested cells. Smooth muscle MLCK is known to be a primary regulator of smooth muscle contraction through Ca^{2+}-CaM dependent phosphorylation of the 20 kDa regulatory myosin light chain (MLC_{20}). Increased smMLCK content and activity would lead to increase of MLC_{20} phosphorylation with concomitant increase in velocity during smooth muscle activation (Somlyo and Somlyo, 1994). The importance of smMLCK in regulating smooth muscle contractility was supported by our previous studies on ragweed pollen-

sensitized canine airway smooth muscle, where an increased smooth muscle contractility was found to be closely correlated with the increased content and activity of smMLCK (Jiang et al., 1992). Stephens and Jiang (1997) recently reported on the basis of a motility assay, that *in vitro* motility of the smooth muscle myosin head increased with increase of smMLCK concentration which further demonstrated the significance of smMLCK in regulating smooth muscle contractility. Increased expression of smooth muscle α-actin may also contribute to the increased contractility, by providing more attachment sites for myosin heads.

Serum deprivation-induced contractile airway smooth muscle cells were found to be hyper-reactive and hyper-sensitive to contractile stimuli. Although the mechanisms underlying this phenomenon are unknown, it is easy to see that they could contribute to allergic bronchospasm. Inflammation and airway wall thickening, due to infiltration with leukocytes and macrophages, epithelium denudation, subepithelial fibrosis, and medial smooth muscle hypertrophy and hyperplasia, are pathological features of asthmatic airways (Ebina, 1993). Inflammatory mediators such as histamine, leukotrienes, and prostaglandins which exist in asthmatic airways, are all potent mitogens (Hall and Kotlikoff, 1995). Release of these inflammatory mediators during an asthma attack may result in proliferation of airway smooth muscle cells. Growth arrest (as occurred under serum deprivation of cultured cells) will then occur *in vivo* in between consecutive asthma attacks. This may result in generation of hyper-contractile and hyper-sensitive smooth muscle cells. Excessive shortening of the smooth muscle, and excessive narrowing of the

airway lumen will then occur during the subsequent asthma attack. This was supported by a recent report by Bramley et al. (1994) in which considerable increase in shortening capacity of human asthmatic airway smooth muscle was found. Therefore, the *in vitro* model system we report here may be useful for the study of mechanical properties of asthmatic airway smooth muscle cells. A similar phenomenon may also exist in hypertensive vascular smooth muscle cells, where hypertrophy and hyperplasia of smooth muscle cells also occur.

Serum deprivation resulted in two phenotypically distinct populations of cells in cultured tracheal smooth muscle. Two similar populations were also found in cells freshly-isolated from tracheal smooth muscle. Further studies are required to identify relationships between arrest-induced and freshly-isolated cells. It seems fairly certain that contractile cells in arrested culture definitely did not originate from freshly-isolated contractile cells. Cultured smooth muscle cells were derived almost certainly from a rounded, non-contractile population of freshly isolated cells. The freshly isolated elongated, contractile cells seldom attached to the culture plate or replicated. Similar findings were also reported by others. Recent reports from Chacko's and Stenmark's groups, in cultured smooth muscle cells, stated that not all smooth muscle cells were equal in their proliferative properties; only the circular, rounded smooth muscle cells showed high proliferative response to mitogens (Lau and Chacko, 1996; Dempsey et al., 1997). It may well be that in some specific culture medium, and specifically conditioned matrix, these freshly-isolated contractile smooth muscle cells could attach and grow. Therefore, it is possible

that both contractile and non-contractile cells in our arrested culture came from the non-contractile freshly-isolated population of cells. It is possible that further heterogeneous subpopulations of cells (at least two subpopulations) exist in the freshly-isolated non-contractile population of smooth muscle cells. Each may respond differently to serum deprivation and subsequently serve as progenitor of contractile or non-contractile cells in arrested cultures.

Both contractile and non-contractile cells existed in arrested smooth muscle cultures and in differentiated, mature airway smooth muscle *in vivo*. The function of contractile cells is clear, the functions of non-contractile cells are not known. It is possible that they mainly contribute the proliferative response to mitogens, and subsequently to the development of new contractile cells. It has been reported that there are heterogeneous cells in vascular smooth muscle, but that only one sub-type (the stellate) responds to growth factors (Wohrley et al., 1995).

In conclusion, our data demonstrate that serum deprivation resulted in development of both contractile and non-contractile smooth muscle cells in tracheal smooth muscle cell culture. These newly-induced contractile smooth muscle cells possess even greater contractility than that of freshly-isolated tracheal smooth muscle cells. This model could provide a tool for studies to determine the relationship between smooth muscle differentiation and contraction. The development of hyper-reactivity in cultured smooth muscle cells, stemming from growth arrest as a result of serum deprivation represents a

novel finding. The responsible mechanisms need to be investigated. The existence of cellular heterogeneity also requires that these mechanisms be delineated for the different sub-populations of cells.

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Chapter 7. Altered contractile phenotypes of asthmatic bronchial smooth muscle cells

7.1 Background

Perhaps the greatest importance of airway smooth muscle research is its application to the elucidation of the pathogenesis of allergic bronchospasm. The high prevalence and increasing mortality of asthma has directed more and more research effort towards this issue. Because of the variety of triggers that can precipitate an attack of asthma, it is felt that the origin of asthmatic bronchospasm must lie in the final effector, viz., the airway smooth muscle. Whether the contractility of human asthmatic airway smooth muscle is intrinsically altered has never been demonstrated, apart from a single case in which tissue was obtained post-mortem (Bramley et al., 1994). The reason for this, of course is that it is extremely difficult to obtain suitable and adequate material from human asthmatic cases. A recently developed technique by Dr. Michel Laviolette of Hospital of Ste Foy, Quebec (double bronchoscopic biopsy) provides a practical way of obtaining bronchial sample consisting of epithelium with a small number of smooth muscle cells. This in turn makes it possible to enzymatically isolate viable single smooth muscle cells from these samples for mechanical studies. The double biopsy consists of making the first bronchial biopsy through the epithelium and adjacent lamina propria. Through that hole, a second biopsy is made which almost always procures bronchial smooth muscle cells.

It needs to be pointed out that current thinking in the field is that asthma is primarily and predominantly a chronic inflammatory disease. However the fact that $\beta 2$ adrenergic agonists can usually relieve the respiratory distress of the asthmatic attack

suggests that contraction of airway smooth muscle plays an important role. The argument that the airway smooth muscle is the seat of the first change in the mechanical properties of the airways is supported by findings obtained from our canine model of ragweed pollen-induced allergic bronchospasm (Jiang et al., 1992). These animals have been sensitized but never challenged and histological studies have shown no evidence of inflammation. Furthermore bronchospasm can occur in the absence of any increased inflammation in exercise-induced asthma where bronchoalveolar lavage (BAL) revealed no significant elevation in levels of inflammatory mediators or/and cells (Broide et al., 1990; Jarjour and Calhoun, 1992). Liu et al. reported, in studying the immediate and late asthmatic responses, that the 5-minute bronchoconstrictor response to antigen challenge was not associated with increases in cell number in BAL fluid nor in airway permeability, indicating that airway smooth muscle contraction plays a predominant role in the immediate asthmatic response (1991). It is for these reasons we have developed the hypothesis that asthma, in its early phase, is primarily a disease of airway smooth muscle. Later on, inflammation develops along with hypertrophy and hyperplasia of the airway smooth muscle, all of which reduce ventilation by virtue of a geometric factor.

This project was designed with its focus on investigation of the contractility of single asthmatic airway smooth muscle cells. Since adequate amounts of airway smooth muscle cannot be obtained from living asthmatic subjects, study of single cells obtained by endobronchial biopsy was the only remaining option. Viable cells were isolated from endobronchial biopsy samples obtained from asthmatic airways, and their contractility was

analyzed and compared with those of cells obtained from non-asthmatic subjects. The study provided the first direct evidence that human asthmatic airway smooth muscle cells possess elevated intrinsic contractility. Most studies in asthma research use, as a control group (Thomson et al., 1996), surgical specimens obtained from seemingly healthy airways in lobes resected for neoplasm. To validate whether such tissues are suitable controls in studying asthmatic airway smooth muscle contractility, airway smooth muscle cells were also isolated from them, and their contractility compared with those of asthmatic and non-asthmatic subjects.

7.2 Objectives

Measurement of contractility of human asthmatic bronchial smooth muscle cells.

7.3 Hypothesis

Airway smooth muscle cells from asthmatic subjects possess enhanced contractility compared with cells from healthy siblings.

7.4 Protocol

Asthmatic and non-asthmatic human bronchial samples were obtained via double endobronchial biopsy. Smooth muscle cells were isolated enzymatically, and their contractility was evaluated at the single cell level and compared between asthmatic and non-asthmatic cells.

7.5 Results

Characteristics of subjects. Subjects' characteristics are presented in table 2. Normal subjects had normal spirometry parameters and PC20 values. One had a positive skin reaction to ragweed. All asthmatics were atopic. Three asthmatics had normal FEV1 and were using a $\beta 2$ agonist occasionally; their PC20 was measured at the time of evaluation. Two had unstable asthma with a FEV1 $< 70\%$ of predicted and were taking inhaled $\beta 2$ agonist 4 to 10 times a day; on the evaluation day, their FEV1 improved significantly with this medication from 2.17 to 2.89 liter and 1.92 to 2.48 liter respectively. In these two subjects, PC20 was not measured at the time of the study but later when their asthma had improved after the study with the regular use of inhaled steroids.

Contractility of asthmatic airway smooth muscle cells. Freshly isolated airway smooth muscle cells appeared elongated with a smooth and shining sarcolemma when viewed with an inverted phase contrast microscope. These cells showed a reversible contraction in response to low doses of Ach, histamine, high K^-, and low level single pulse electrical field stimulation. Maximal contraction (shortening) could be induced with repeated pulses of electrical stimulation. It was about 15% greater than the response to a single pulse. Cells isolated from biopsy samples did not show obvious heterogeneity in their morphological and mechanical properties. Cells obtained from surgical material showed similar tendency of heterogeneity as that of canine tissue. However, conclusive information about heterogeneity of human airway smooth muscle cells needs more

systematic studies. Smooth muscle cells isolated from airway biopsies had a mean length of 46 ± 5 μm (SE) at relaxation. A significant difference was identified in contractility among asthmatic, non-asthmatic, and surgical non-asthmatic airway smooth muscle cells (Fig. 34). Asthmatic airway smooth muscle cells showed significantly increased maximum velocity (Vo) and capacity (ΔLmax) of unloaded shortening compared with those of non-asthmatic biopsies. No differences were found in either Vo or ΔLmax between asthmatic and surgical non-asthmatic, and non-asthmatic and surgical non-asthmatic groups.

Table 2. Characteristics of Study Subjects

Subject	Allergy mm**	Age y	Sex	FVC* l (%)	FEV1* l (%)	PC20 mg/ml§
Normal						
MD	Rw3	27	M	5.54 (112)	5.29 (126)	>128
DF	none	20	M	7.46 (141)	6.74 (146)	>256
VF	none	26	F	4.34 (113)	3.88 (119)	>256
SP	none	20	M	5.08 (94)	4.47 (96)	38.6
RD	none	27	M	7.17 (128)	5.20 (111)	>128
Asthmatics						
GS	Du4 Du-mite6	23	F	3.92 (100)	3.39 (101)	2.57
PL	C8, D6 Rw8, Du4	39	M	3.45 (70)	2.17 (56)	1.8¶
RG	C4, D4 Du4, Rw3	33	M	3.25 (78)	5.16 (103)	0.29
HT	C4, D8 Du5	36	F	3.22 (89)	1.92 (64)	4¶
CD	C4, D5 Du5, M8	24	M	5.95 (112)	4.32 (96)	0.53

* forced vital capacity (FVC) and forced expiratory volume in one second (FEV1), expressed in liters and (%) of predicted.

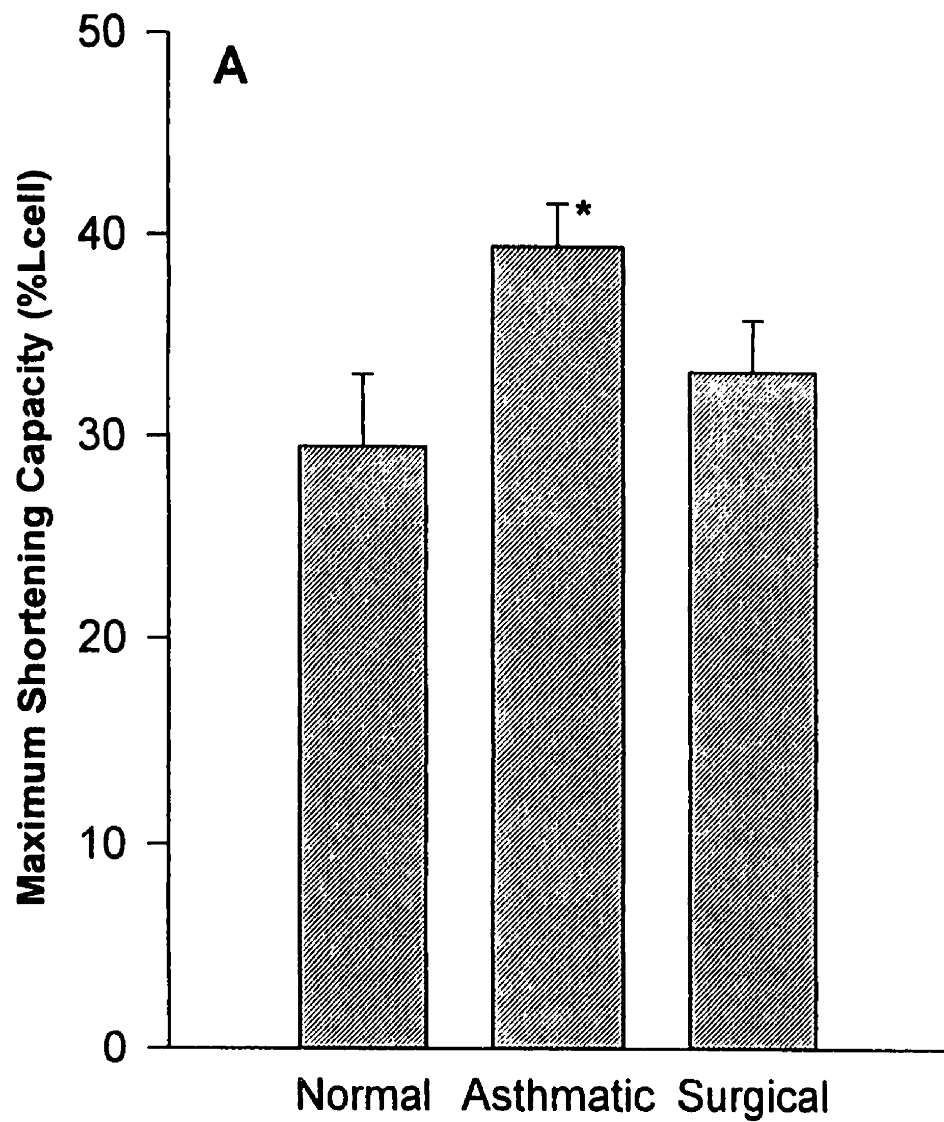
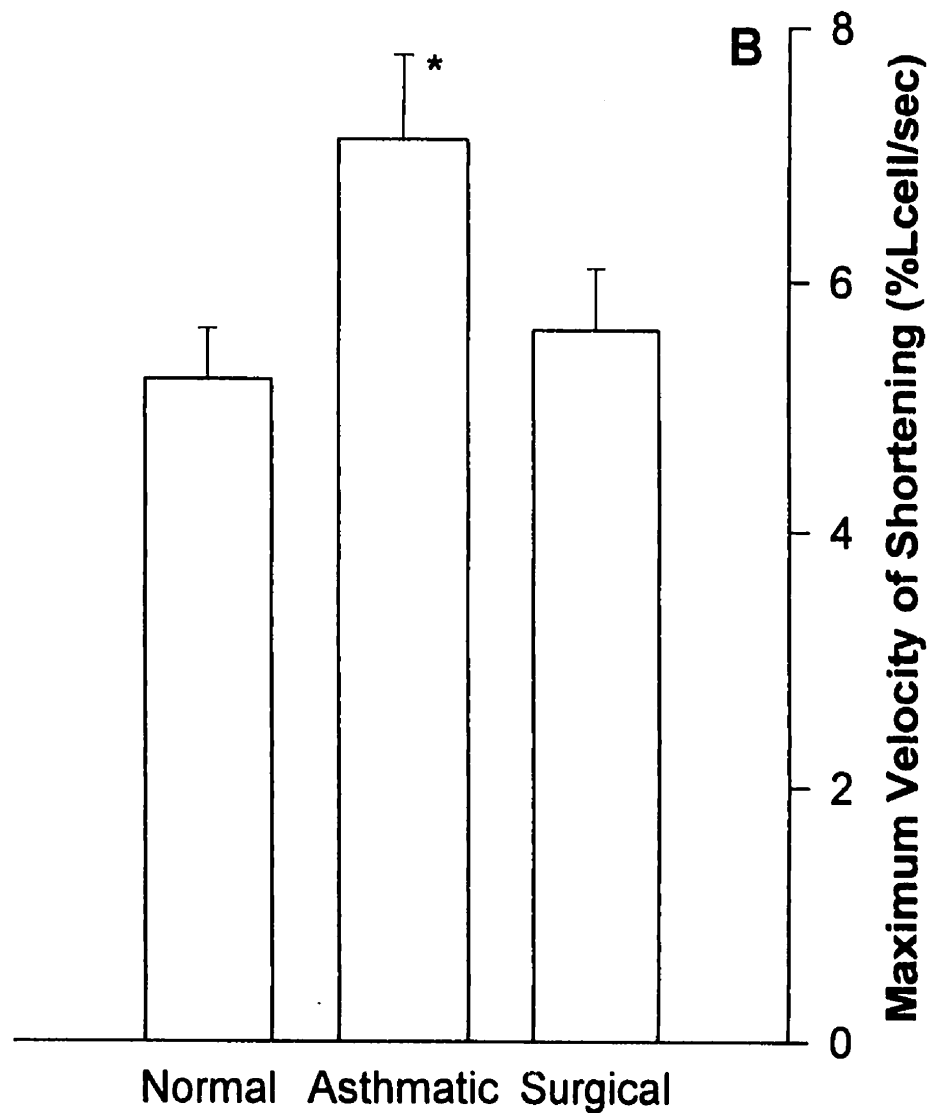
§ concentration of methacholine inducing a 20% fall in FEV1.

** mean wheal diameter of skin response read at 10 min; Rw: ragweed, D: dog, C: cat, Du: dust, M: mold.

¶ measured after regular use of inhaled steroids to stabilize their asthma.

Figure 34. Mechanical properties of single human airway smooth muscle cells.

Significantly increased contractility (Vo and ΔLmax) was identified in asthmatic airway smooth muscle cells. A: Maximal Shortening Capacity (ΔLmax). B: Maximal Velocity of Shortening (Vo). *P<0.05 compared with non-asthmatic controls (normal). Asthmatic: Cells isolated from bronchoscopic asthmatic airway biopsies. Normal: Cells isolated from bronchoscopic normal airway biopsies. Surgical: Cells isolated from surgical airway material.


A
Maximum Shortening Capacity (%Lcell)
50
40
30
20
10
0
*
Normal
Asthmatic
Surgical

B
Maximum Velocity of Shortening (%Lcell/sec)
8
6
4
2
0
*
Normal
Asthmatic
Surgical

7.6 Discussion

The novel findings of current studies provide direct evidence that asthmatic airway smooth muscle possessed enhanced intrinsic contractility, which indicates that studies of single airway smooth muscle cells would facilitate elucidation of the pathogenic changes underlying asthma. Due to the cellular heterogeneity of smooth muscle, the external behaviour of smooth muscle at tissue level only represents the average output of a mixture of populations of cells (Lau and Chacko, 1996; Archer, 1996; Frid et al., 1994). Furthermore, the amount and properties of extracellular connective tissue in smooth muscle strips would also affect their mechanical properties extensively (Bramley et al., 1994; Ma and Stephens, 1997). Increased amount or elasticity of connective tissue would result in increased resistance to smooth muscle shortening. Therefore, results obtained from studies at the tissue level may not be extrapolated to what is occurring at the cellular level. The advantage of the current study is the direct measurement of contractility at single cell level. Therefore, it provides direct information about the intrinsic contractility of the smooth muscle cell itself.

Our studies demonstrated that asthmatic airway smooth muscle cells shortened maximally by 39.5 ± 2.1 %Lcell, which was one third more than that of non-asthmatics (29.5 ± 3.6 %Lcell). This one-third increase of $\Delta Lmax$ was pathophysiologically and statistically significant. If this occurred *in vivo*, it would result in an 81 fold increase in airway resistance as predicted by Poiseuille's law, and that would be more than enough

to account for the paroxysmal dyspnea during an asthma attack. That involvement of smooth muscle contraction is an outstanding feature of the asthma attack, is supported by the fact that $\beta 2$ adrenergic agonists (powerful smooth muscle relaxant) can almost always rapidly relieve the respiratory distress. Airway hyperreactivity and inflammation are the two most prominent features of asthmatic airways. Mediators released from mast cells during the allergic reaction, or inflammatory mediators released from inflammatory cells are all powerful agonists for smooth muscle contraction, which would lead to excessive narrowing of the airway lumen during an asthma attack.

Changes in the contractility of smooth muscle may represent the earliest pathogenic alterations of asthma. Currently we do not have any direct evidence to support this. It is almost impossible to identify asthmatic patients who do not have airway inflammation. In clinical practice by the time the asthmatic patient gets to the clinic, usually a matter of days, airway inflammation has already developed. Our laboratory has worked on a ragweed pollen-sensitized canine model of asthma for more than 30 years. These animals show both specific and non-specific airway hyperreactivity. No inflammation, hypertrophy or hyperplasia were found in their airways. Therefore, this model mimics early asthma. Just as in asthmatic airway smooth muscle cells, increased contractility of airway smooth muscle cells was also identified in sensitized canine airways. This evidence from the two species indicates that changes in contractility of airway smooth muscle could occur without the development of inflammation. Though admittedly a minority opinion (Skloot et al., 1995), we feel the very earliest manifestation of asthma is an alteration in airway

smooth muscle contractility. This likely precedes development of symptoms and of inflammation.

The molecular mechanisms of increased contractility in asthmatic airway smooth muscle are currently unknown. The major difficulty is the difficulty in obtaining enough material for biochemical studies. Currently, we are developing techniques for biochemical studies at a single cell level. Our studies on sensitized airway smooth muscle indicate that increased expression in protein content of smooth muscle type myosin light chain kinase (smMLCK) contributed to its increased contractility (Jiang, 1992). Contractility of smooth muscle is mainly regulated by smMLCK. Unlike regulation of skeletal myosin heavy chain isoenzymes, which is via Ca^{2+}-calmodulin and troponin C, the activity of smooth muscle myosin heavy chain isoenzymes is regulated by phosphorylation of the 20kD regulatory smooth muscle myosin light chain (MLC_{20}) via smooth muscle myosin light chain kinase. Increased protein expression or activity of smMLCK results in increased smooth muscle actin-activated myosin Mg^{2+} ATPase activity, and subsequently increases of velocity and capacity of smooth muscle shortening during smooth muscle activation. Stephens and Jiang recently reported that the *in vitro* motility of actin filaments by smooth muscle myosin heads was positively related in a dose-response manner, to the concentration of smMLCK in the system (1997). Increased smMLCK content has also been reported by others in passive sensitized human airway smooth muscle (Ammit et al., 1997). Differences in distribution of smooth muscle myosin heavy chain isoenzymes would also affect smooth muscle contractility. Four smooth muscle myosin heavy chain

isoenzymes have been found to date, ie. two alternatively spliced C-terminal and two alternatively spliced N-terminal isoenzymes. The most recently identified one, SM-B, was proved to be important in determining smooth muscle contractility. Expression of an extra 7 amino acid insert in its N-terminal head region conferred on this enzyme an increased ATPase activity and velocity in translation of actin filaments *in vitro* (Kelley et al., 1993). However, in our sensitized airway smooth muscle no increase in SM-B expression was found (Stephens and Jiang, 1997). This dissociation surprised us, however dissociation of SM-B expression from smooth muscle contractility has also been reported recently by several researchers (Haase and Morano,1996; Siegman et al., 1997; Ma, et al., 1998). Other proteins such as calponin, caldesmon and the essential myosin light chain are also important in regulation of smooth muscle contraction. Their functions in sensitized muscle need further investigation.

Previous studies demonstrated a clear heterogeneity of airway smooth muscle cells in their morphological, mechanical, and biochemical properties in canine airways (Ma et al., 1997). It is surprising that cells isolated from human bronchial biopsies did not show as marked structural heterogeneity. This is most probably because of the limitation of our mode of sampling. Endobronchial biopsy obtains smooth muscle from only superficial layers of the muscle and that too from very limited areas of airways. Another limitation of this study in identifying smooth muscle cell heterogeneity lies in the difficulty in obtaining adequate numbers of cells from each sample. Usually only 5 to 10 cells were isolated from 6 - 8 punch biopsies obtained from each patient. Therefore, heterogeneity

in airway smooth muscle cells may also exist in human but cannot be demonstrated here due to the paucity of isolated cells. In fact, cells obtained from surgical materials showed some tendency to heterogeneity although this was not statistically significant. Though the amount of surgical material was much greater than the amount obtained from bronchial biopsy, it was still inadequate, as usually only one bronchial ring could be obtained from each patient. In all instances the resections were carried out for neoplastic disease and understandably the bulk of the tissue had to be sent for histological diagnosis. These limitations in sampling will not affect our results relating to the contractility of asthmatic airway smooth muscle cells. Although different populations of smooth muscle cells may exist in human airway, our studies do, at least, demonstrate that one specific population of cells showed increased contractility in asthmatic airways. What happens to other populations of cells, if such do exist, we do not know. However, studies in our canine model have shown that sensitization resulted in an increase of contractility of all the three different phenotypes of contractile smooth muscle cells that we have reported.

Most studies of asthmatic airway smooth muscle use surgical, non-asthmatic specimens as normal control. Whether smooth muscle cells from such controls are normal in their contractility has never been addressed. Although no significant differences in Vo and ΔLmax between non-asthmatic and surgical non-asthmatic airway smooth muscle cells were found, the mean values of ΔLmax cells from surgical non-asthmatic patients were 13% higher than that of non-asthmatics. Furthermore, if we use the surgical group as normal control, statistically significant differences disappear between contractility of

airway smooth muscle cells from asthmatics and controls. Therefore cognizance must be taken of the care at that smooth muscles from such surgical non-asthmatic airways may not be normal in their mechanical properties. In fact, these surgical airways are mostly obtained from cancer patients, most of whom are heavy smokers. It is most probable that these airways, including their smooth muscle are abnormal. Therefore, smooth muscles from such a group are not good controls in asthma studies, particularly those dealing with contractility of airway smooth muscle.

In conclusion, the present studies demonstrate that human asthmatic airway smooth muscle cells possess increased intrinsic contractility compared with those of non-asthmatic. The molecular mechanisms of such alterations are unknown. It is very likely that changes in smMLCK total content and activity occur in asthmatic airway smooth muscle, and contribute to its increased contractility. Such changes have been reported by us for sensitized canine airway smooth muscle (Jiang et al., 1992). Future studies will focus on identification of these mechanisms.

7.7 References

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Chapter 8. Conclusions

This study demonstrates a clear heterogeneity of airway smooth muscle at both the tissue and single cell levels. At the tissue level two mechanically distinct groups of smooth muscle exist in canine airway from the trachea to bronchial generation 6. The extrapulmonary group consisting of smooth muscles from trachea and bronchial generation 1 and 2, possesses enhanced contractility; the intrapulmonary group containing smooth muscles from bronchial generation 3 to 6, showed reduced contractility. At the single cell level, three phenotypically distinct types exist in canine airway smooth muscle; each possesses a unique morphological, biophysical and biochemical phenotype. Type I cells with a mean length of 110 μm account for 84% of total freshly isolated contractile cells in extrapulmonary airway smooth muscle, type II cells with a mean length of 200 μm, account for 54% of total in intrapulmonary muscle. Within type II cells, mechanical subtypes of cells are further identified: type IIA and IIB. Type IIA accounts for 90% of total type II cells. The contractility of these cells is: type IIB > type I > type IIA. Differences in proportions of different types of cells between extra and intrapulmonary airway smooth muscle may contribute to their mechanical differences at the tissue level. Therefore, measurements of mechanical properties obtained from smooth muscle strips only reflect the average output of a mixture of populations of contractile cells; they may not be extrapolated to explain molecular mechanisms of smooth muscle contraction. Bronchial smooth muscles show significant differences in their contractility and composition of contractile cells from those of the trachea. Thus results obtained from tracheal smooth muscle may not be extrapolated to bronchial; this is especially important in the study of asthma, where the asthmatic attack predominates in central or peripheral

bronchi.

Isolation of contractile airway smooth muscle cells and measurement of their contractility at a single cell level enabled the evaluation of intrinsic contractility of sensitized airway smooth muscle cells. Ragweed pollen sensitization does not homogeneously affect the mechanical properties of airway smooth muscle along the airway tree. It results in increases in contractility of both extra- and intrapulmonary airway smooth muscle. Intrapulmonary airway smooth muscles, however, show a greater increase in their contractility than extrapulmonary. Sensitization also affects contractility of all three types of smooth muscle cells. However, type IIA cells show the greatest increase in their contractility. These novel findings imply that heterogeneity of airway smooth muscle cells plays an important role in the pathogenesis of asthma, and also suggest that treatments for asthma may be best designed to down-regulate type IIA cell contractility in particular.

Serum deprivation results in development of differentiated, contractile smooth muscle cells from dedifferentiated cultured tracheal smooth muscle cells. This novel finding may provide a practical *in vitro* model in investigation of smooth muscle development and differentiation. This is the first demonstration of induction of a contractile phenotype in cultured smooth muscle cells. The superiority of this type of study is the employment of direct measurement of smooth muscle cell contraction. No ambiguity therefore exists in determining whether the arrested cells are really contractile

or not. Apart from the contractile phenotype of cells, non-contractile phenotypes of cells also exist in arrested smooth muscle cell cultures. Another exciting finding of these arrested contractile smooth muscle cells is their greater contractility when compared with freshly-isolated tracheal smooth muscle cells. Development of these enhanced contractile cells may also occur in chronic asthmatic airways, where smooth muscle cell proliferation is initiated by allergic and/or inflammatory mediators released during an asthma attack, and growth arrest is subsequently achieved as the attack subsides. Therefore, this arrested culture system will also be useful in studies of the pathogenesis of asthma.

The most valuable finding of this study concerned with asthma research is the identification of increased contractility of bronchial smooth muscle cells isolated from endobronchial biopsies from living asthmatics. This novel finding solves a long-lasting puzzle relating to the contractility of asthmatic airway smooth muscle. It further indicates that the study of airway smooth muscle could facilitate understanding not only of smooth muscle contraction, but also of the pathogenesis of asthma.

Increased expression in protein content of smooth muscle myosin light chain kinase is correlated with increased contractility of tracheal smooth muscle and "arrested" contractile smooth muscle cells. No positive relationships are found between the content of SM-B expression and airway smooth muscle (cell) contractility. This questions the importance of SM-B in determining contractility of smooth muscle. We conclude that contractility of smooth muscle is determined by the level of activation of myosin ATPase

activity by smooth muscle myosin light chain kinase, but not the endogenous activity of the myosin isozyme itself. Similar to what occurred in sensitized canine airway smooth muscle, the increased contraction of human asthmatic bronchial smooth muscle may well be determined by an increased expression of smooth muscle myosin light chain kinase.

Table 3. Heterogeneity of Airway Smooth Muscle at Tissue Level

	Extrapulmonary Airway Smooth Muscle	Intrapulmonary Airway Smooth Muscle	P Value
Contractility:			
Po (mN/mm^2)	167.8 ± 11.5	162.1 ± 10.4	NS
ΔLmax (%Lo)	65.0 ± 2.6	46.5 ± 2.3	****
V2 (%Lo/sec)	29.9 ± 2.3	20.1 ± 2.1	***
V8 (%Lo/sec)	17.1 ± 1.3	9.1 ± 1.1	***
Cell Composition: (% of total contractile cells)			
Type I	83.7 ± 3.6	42.1 ± 3.9	**
Type II	16.3 ± 3.6	57.9 ± 3.9	**
Contractility Increase after Ragweed Pollen-Sensitization (% of normal value):			
1. Short Term Sensitization			
Po	4.05 ± 5.12	14.29 ± 16.50	NS
ΔLmax	11.33 ± 2.54	40.55 ± 2.89	****
Vo	35.45 ± 5.80	57.52 ± 4.80	*
2. Long Term Sensitization			
Po	6.68 ± 8.46	26.79 ± 26.79	NS
ΔLmax	2.48 ± 3.07	27.29 ± 6.66	***
Vo	-3.38 ± 5.35	53.32 ± 14.07	****
Protein Content (arbitrary unit):			
smMHC	2.06 ± 0.18	1.77 ± 0.10	NS
SM-B	18.33 ± 0.96	2.16 ± 0.11	****
sm-α-actin	2.94 ± 0.28	0.41 ± 0.08	***
sm-MLCK	4.48 ± 0.70	2.06 ± 0.50	*

Values are means ± SE. NS, not statistically significant.

Table 4. Heterogeneity of Airway Smooth Muscle at Cellular Level

	Type I	Type IIA	Type IIB	P Value
Length (μm)	113 ± 2	200 ± 4[a]	200 ± 4[a]	*
ΔLmax (%Lcell)	27.88 ± 0.80	15.54 ± 1.65	57.69 ± 1.88	*
Vo (%Lcell/sec)	4.24 ± 0.36	2.11 ± 0.19	8.86 ± 0.72	*
SM-B Expression	Yes	No	No	
Short-term Sensitization Effects (Increase in % of nommal):				
ΔLmax	21.81 ± 2.92[b]	40.32 ± 6.71	19.02 ±1.59[b]	*
Vo	23.21 ± 2.51[c]	54.32 ± 6.85	25.42 ± 3.20[c]	*

Values are means ± SE. *P<0.05, one way ANOVA. Values donoted by same letter are not statistically different (Duncant's new multiple-range test).

Table 5. Heterogeneity of Arrested Tracheal Smooth Muscle Cells in Culture

	Elongated Cells	Circular Cells
Proportion (% of total cells)	28.5 ± 4.6	71.5 ± 10.3
Length (μm)	109 ± 10	Not Determined
Contractility:		
ΔLmax (%Lcell)	50.87 ± 2.79***	Not Detectable
Vo (%Lcell/sec)	7.93 ± 0.89***	Not Detectable
Protein Expression:		
sm-α-actin	High	Not Detectable
smMHC	High	Not Detectable
SM-B	Not Detectable	Not Detectable
smMLCK	High	Low
nmMLCK	High	Low
β-tubulin	High	Low

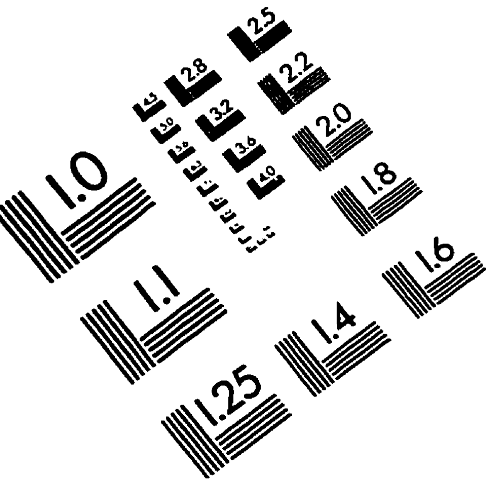
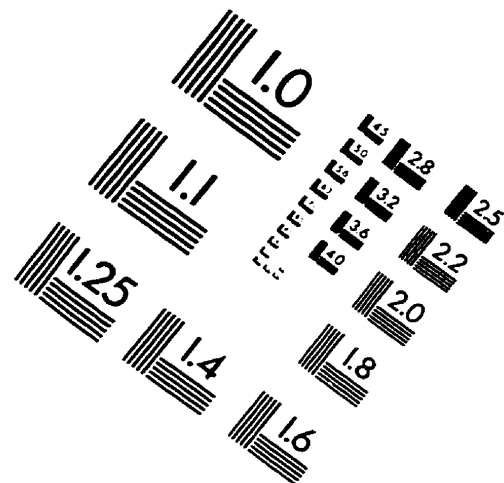
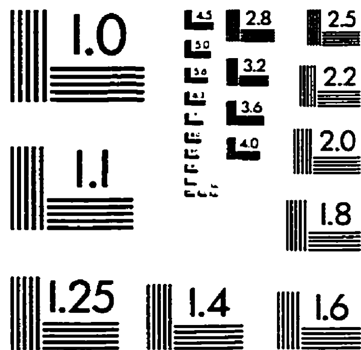
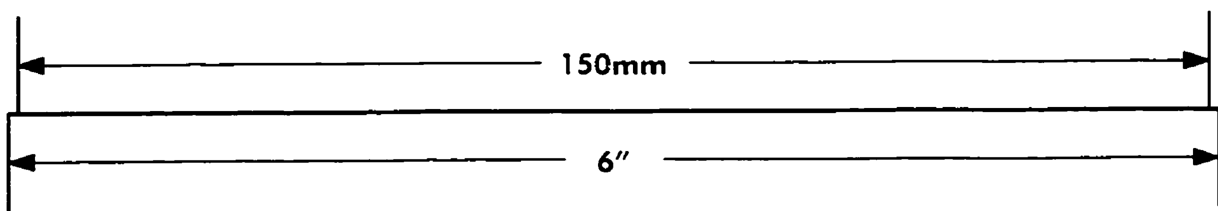
Values are means ± SE. ***P<0.005, unpaired student t-test, compared with the values of freshly-isolated tracheal smooth muscle cells (type I cells in table 4).

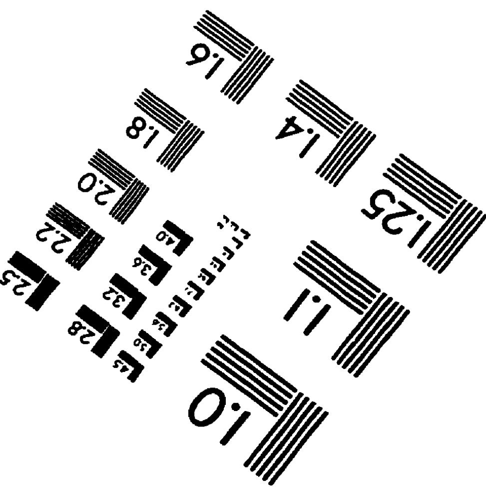
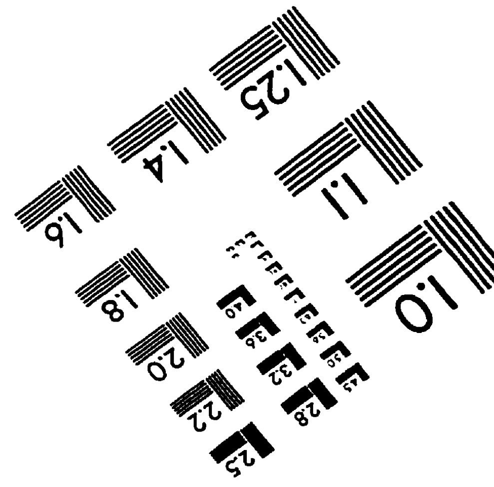
Table 6. Contractility of SMC from Human Airway

	Normal Airway	Asthmatic Airway	Surgical Normal	P Value
ΔLmax (%Lcell)	29.50 ± 3.55^{a}	39.47 ± 2.05^{b}	$33.19 \pm 2.72^{a,b}$	*
Vo (%Lcell/sec)	5.21 ± 0.41^{c}	7.10 ± 0.68^{d}	$5.63 \pm 0.50^{c,d}$	*

Values are means ± SE. *P<0.05, one way ANOVA. Values donoted by same letter are not statistically different (Duncant's new multiple-range test).

IMAGE EVALUATION TEST TARGET (QA-3)

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