**Changes in Dense-body Structures in
Airway Smooth Muscle Adapted to Different Lengths**

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Abstract

Dense bodies in smooth muscle are thought to be the equivalents of Z-disks found in striated muscle. Using three-dimensional reconstruction of serial sections of electron micrographs of ASM, we have confirmed previous studies showing that dense-body (DB) aggregates inside smooth muscle cells resemble “stringy” structures lying in parallel with the myosin filaments. We found that the cable-like structure consists of DBs closely strung together by actin and intermediate filaments. This finding questions the conventional belief that DBs play the role of the Z-disks.

In this study we examined further the structural changes in the DB “cable” in ovine tracheal smooth muscle adapted to different lengths (length adaptation); specifically we examined the length ratio between the DB cable length (in three-dimensional space) and the segment length of the muscle cell in which the cable was embedded. This length ratio, or normalized DB-cable length, gave us a measurement of the “slackness” of the DB cables in the cell.

Length adaptation was a process in which ASM regained its force generating capacity after a change in the length of the muscle cell. The process involved brief (10 sec) electrical field stimulations once every 5 min applied to the muscle held isometrically. With a 50% muscle shortening, the average normalized DB-cable length increased by ~10%, the cables then straightened out slightly but significantly during length adaptation to the shortened length, decreasing the length by ~3% from the 10% increase. With a

50% stretch, the average normalized DB-cable length decreased by ~15% and ~27% in the length adapted and non-adapted states, respectively.

The most significant finding of this study is that passive tension in ASM cells is negatively correlated to the normalized DB-cable length (DB-cable/cell-segment length ratio). That is, higher passive tension is associated tauter DB cables. This suggests that the DB cables may be important in maintaining passive tension in smooth muscle cells. Understanding how DB cables adapt to length changes and how their structural integrity is modulated intracellularly is important for a better understanding of the roles ASM plays in the stiffening of airways and pathophysiology of asthma.

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List of Abbreviations

ASM	Airway Smooth Muscle
DB	Dense Body
COPD	Chronic Obstructive Pulmonary Disease
EFS	Electrical Field Stimulation
EM	Electronic Microscopy
F-V	Force-Velocity
L-T	Reference Length
MLCK	Myosin Light Chain Kinase
P	P value
PSS	Physiological Saline Solution
QR	Quick Release
QS	Quick Stretch
SE	Standard Error of Mean
SR	Sarcoplasmic Reticulum

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CHAPTER 1 Introduction

1.1 General introduction

1.1.1 Smooth muscle

Smooth muscle is widely distributed in our bodies and is responsible for the contractility of hollow organs, such as the gastrointestinal tract, the respiratory tract, the bladder, the uterus, and the large and small arteries and veins. Smooth muscle regulates organ functions by controlling the diameter and volume of the organ; dysfunction of smooth muscle can lead to serious diseases such as asthma and hypertension.

Unlike skeletal muscle, which has a highly organized sarcomeric structure for its contractile apparatus, smooth muscle lacks any visible regular and repeating filamentous structures that can be taken as evidence for contractile units (Gabella 1997). However, functional evidence suggests that the contractile apparatus of smooth muscle is also made of contractile units akin to the sarcomeres of striated muscle, and that the sliding-filament, crossbridge theory of contraction (Huxley and Hanson 1957) is also applicable to smooth muscle (Herrera et al. 2005). Despite the absence of sarcomeric structure, smooth muscle can develop isometric force per cross-sectional area equal to that of skeletal muscle. However, the speed of smooth muscle contraction is much slower (Gabella 1997). There is a variety of smooth muscle types with different membrane receptors and isoforms of contractile and cytoskeletal proteins. The ultrastructure of the various smooth muscles is nevertheless very similar and it is believed that the difference in mechanical properties among the different smooth muscles is only quantitative in nature. The focus of my

thesis research is on airway smooth muscle (ASM) and how one intracellular element, dense bodies, may modulate the airway smooth muscle contractile response.

1.1.2 Airway smooth muscle in the airway wall

The vertebrate trachea is the common airway through which respiratory airflow passes in and out of the lung. The trachea in human adults is a tube approximately 12 centimeter in length and 2.5 centimeters wide and is formed of four layers from lumen to the outside wall: a mucosa including an epithelial layer and lamina propria, a submucosa, a C-shaped fibrocartilage layer and an adventitia.

The mucosa consists of a pseudostratified, ciliated, columnar epithelial layer overlying an elastic lamina propria. The epithelial layer is composed of 3 cell types: 1) ciliated columnar epithelial cells, containing 200-300 cilia per cell at their apical surface, 2) goblet cells, with mucous droplets and 3) basal cells with basally located round nuclei. All cells rest on a prominent basement membrane. The basement membrane, on which the epithelia rest, is the thickest in the body. Beneath the epithelial layer and basement membrane is the lamina propria (LP). In the trachea the LP is thicker than it is in bronchi. The LP of the trachea has a superficial zone of loose connective tissue (CT), as well as a deeper area of dense CT, which includes a layer of longitudinally-oriented elastic fibers, the elastic lamina.

The submucosa is made of connective tissue which is rich in blood vessels and in glands. The submucosa contains extensive aggregations of mixed glands including the purely serous acini, purely mucous acini, and mixed acini. Ducts from these glands pass through the lamina propria to empty onto the epithelial surface. A layer of smooth muscle cells is also found in the

submucosa. The smooth muscle cells are connected to the surrounding layers of the airway wall by collagen and elastin fibers.

The layer containing the incomplete (C-shaped) rings of fibrocartilage consists of a series (16-20) of C-shaped cartilage rings which prevent the trachea from collapsing. The cartilage rings are surrounded by a band of dense connective tissue called perichondrium, which merges with the submucosa and the adventitia. The open end of each cartilage ring is directed posteriorly and is closed by a transverse band of smooth muscle (the trachealis). Contraction of this muscle reduces the tracheal diameter. The area between the rings is occupied by fibroelastic connective tissue.

The adventitia is a layer of connective tissue that binds the trachea to the adjacent structures in the neck and mediastinum. It contains blood vessels, nerves and lymphatics.

Around the centre of the chest, the trachea divides into two cartilage-ringed tubes called bronchi. This section of the respiratory system is also lined with ciliated epithelial cells. The bronchi enter the lungs and spread in a treelike fashion into smaller tubes called bronchial tubes. In the lower airways, smooth muscle is found circumferentially surrounding the lumen of the bronchi in a helix-antihelix pattern encasing the airway (Stephens 2001; Amrani and Panettieri 2002).

Airway smooth muscle (ASM) plays key roles in maintaining airway tone. Dysfunction of ASM has been implicated in the pathogenesis of asthma. Adaptation of ASM to an inflamed airway environment could lead to ASM dysfunction (Seow and Pare 2007; Bosse et al. 2009).

1.2 General structure of smooth muscle

Smooth muscle cells are spindle shaped and usually organized in clusters, forming a mechanical syncytium (Kuo and Seow 2004). Smooth muscle cells from different tissues are classified as single-unit or multi-unit, based on the extent of the innervation of the cells have. In single unit smooth muscle the innervation is sparse, i.e., one single nerve innervates a large number of cells, whereas in multiunit smooth muscle each cell receives its own innervation. In a single-unit smooth muscle cell bundle, there are numerous communication junctions called gap junctions that allow activation to spread from cell to cell. Airway smooth muscle is considered to be neither purely single-unit nor multi-unit, but somewhere in between. Smooth muscle cells in general are smaller (2-10 μM in diameter) than skeletal muscle fibers (10-100 μM in diameter); the length of smooth muscle cells are variable, depending on their contractile state and the stretch applied by external forces. However, the cell length is typically 50-400 μm (Gabella 1997).

1.3 Ultrastructure of smooth muscle

1.3.1 Smooth muscle cell membrane

The smooth muscle cell (SMC) membrane, also called the “sarcolemma”, is an extensible lipid bilayer, part of which is developed into special structures such as the caveolae, cell-to-cell junctions and dense plaques. The SMC sarcolemma, like all other cell membranes, is designed to maintain an intracellular chemical environment that differs from the outside and receives and processes stimuli.

1.3.1.1 Caveolae

Caveolae are small invaginations (50–100 nm) of the plasma membrane which are vesicular or saccular. These flask-shaped structures are rich in proteins and lipids such as cholesterol and sphingolipids. They are believed to be involved in signal transduction (Anderson 1998). Caveolae possess microdomains specialized for cell-signaling and ion channels and are believed to help bring in calcium needed for contraction. In this sense, they are equivalent to the T-tubule system in skeletal muscle. They can increase the surface area and facilitate the transfer of calcium into the cytoplasm. Caveolae are often located close to the sarcoplasmic reticulum (SR) or mitochondria, and are believed to cooperate with SR to sequester calcium.

1.3.1.2 Gap-junctions

To work simultaneously, ASM cells are interconnected by “gap junctions”, which are specialized communication ports between the cells (see an example in Figure 1.1). The number of these junctions is especially high in single-unit type of smooth muscle. Gap junctions are regions of many paired connexon molecules that work like molecular pores or channels which connect across the intercellular space (25 nm - 30 nm). Gap junctions couple adjacent cells chemically and electrically, facilitating the spread of calcium or action potentials between ASM cells (Gabella, 1997).

Figure 1.1: Transverse section of an airway smooth muscle cells. The two opposing arrows point to a gap-junction between two cells. The inset is the magnified gap-junction. The single arrow points to a caveola.

1.3.2 Organelles

The nucleus of an ASM cell is the largest and most conspicuous organelle. The nuclear membrane is a double-layer membrane with pores, allowing the exit of large proteins and RNA molecules. In the nucleoplasm of the nucleus, there are areas rich in RNA and other areas containing the chromosomal DNA.

The endoplasmic reticulum is a network system localized in the cytoplasm of a cell and can be classified into two types: rough endoplasmic reticulum (RER) with small granules attached and smooth endoplasmic reticulum (SER) with no granules (see an example in Figure 1.2). The RER carries ribosomal RNA and the enzymes necessary for the synthesis of proteins. The SER participates in packaging the proteins into small vesicles and transporting them out of the cell. The SER in smooth muscle also acts as a storage site for calcium ions.

The Golgi apparatus is also involved in packaging proteins to be transported out of the cell. In addition, it may be the site for the biosynthesis of complex carbohydrate and other materials.

Lysosomes contain enzymes that degrade or hydrolyze other proteins so that the cell can reuse the products.

Mitochondria are specialized, oval-shaped cellular compartments that are present in clusters near the nucleus and in the periphery of smooth muscle cells (See Figure 1.2). Mitochondria are responsible for the aerobic (oxygen dependent) metabolism of the cell. They contain the enzymes required for the citric-acid cycle, ATP synthesis, and the oxidation of fatty acids. There is also

DNA in the mitochondria which are responsible for the synthesis of messenger RNA necessary to produce protein enzymes for use inside the organelle.

Figure 1.2: Organelles inside the airway smooth muscle: A: Nucleus, B: Mitochondrion, C: Smooth endoplasmic reticulum, D: Rough endoplasmic reticulum.

1.4 Cytoskeleton

1.4.1 Organization of filaments

Electron micrographs of smooth muscle cell reveal three different types of filaments inside the cell: thin filaments, thick filaments and intermediate filaments (Figure 1.3).

A thin filament (diameter of 5-8 nm) is made of the actin filament backbone plus tropomyosin, caldesmon and calponin. Actin filaments are formed by polymerization of monomeric G-actin (a globular, roughly 42-kDa protein). Thin filaments are one of the major components of the cytoskeleton and contractile apparatus. They are arranged parallel to the thick filaments. In transverse sections, each thick filament is surrounded by a group of thin filaments; the ratio of thin filaments to thick filaments is much higher in smooth muscle cell (~20:1) than in skeletal muscle (2:1) (Stephens 2001; Herrera et al. 2004). Thick and thin filaments are arranged in bundles and run parallel to the long axis of the cell. The exact length of the thin filaments in smooth muscle is not known.

Thick filaments (or myosin filaments) are made of a series of myosin dimers and their diameter ranges from 11-21 nm. A myosin dimer is made of two myosin heavy chains (MHCs) associated with two pairs of myosin light chains (MLCs). Vertebrate smooth muscle MHCs exist as two isoforms with molecular masses of 204 and 200 kDa (MHC204 and MHC200) that are generated from a single gene by alternative splicing of mRNA (Nagai et al. 1989). As essential and regulatory components, MLCs wrap around the neck region of each myosin heavy chain. The regulatory myosin light chains regulate the calcium-dependent activation of the muscle. The essential light chains are believed to have a structural role for the myosin molecule.

Intermediate filaments (IFs, 10 nm diameter) are part of the cytoskeleton in smooth muscle. The intermediate filament proteins contain type III proteins, desmin (55-kDa) and a minor component of vimentin. IFs have been shown to be associated with nuclear surface plasma membrane communication, interaction with cytoplasmic structures, maintenance of cell shape and support of the nucleus and other organelles. They are also found to be associated with dense bodies, but their exact function has not been established.

1.4.2 Dense bodies and plaques

In electron micrographs, dense bodies and plaques are seen as electron dense foci in the membrane and within the cytoplasm of smooth muscle cells. The dense areas associated with the membrane are termed dense plaques, and those in the cytoplasm are referred to as dense bodies. Both dense plaques and dense bodies are believed to be the anchorage sites for the contractile and cytoskeletal filaments and are thought to be involved in force transmission within the cell and across the cell surface (Bagby et al. 1990).

Dense plaques are relatively narrow and long structures oriented along the long axis of the cell (McGuffee and Little 1992). Dense plaques are sometimes coupled to each other in adjacent cells at areas where actin and intermediate filaments can be found inside the cells. Basement membranes and other amorphous electron dense material can be found in the intercellular space between the dense plaques. The attachment plaques provide mechanical coupling between the adjacent smooth muscle cells (Gabella 1990).

α -Actinin is well known as a component of dense plaques (Geiger et al. 1981), vinculin and talin are also found in dense plaques (McGuffee et al. 1989). In addition, North et al (1994a) showed that calponin and β -actin are localized in both dense plaques and cytoplasmic dense bodies.

Cytoplasmic dense bodies are numerous in smooth muscle cells and occupy approximately 6% of the cell volume in renal arterial smooth muscle (McGuffee et al, 1991). Dense bodies are elongated structures that are distributed throughout the cytoplasm (Bond and Somlyo, 1982). Chains of dense bodies appear to be strung together by actin filaments and/or intermediate filaments and run parallel to the long axis of the cell (McGuffee and Little, 1992). North et al (1994b) also found that dense bodies are part of some long structures within chicken gizzard smooth muscle cells that contain β -actin and α -actinin.

A

Figure 1.3A: Electron micrograph of longitudinal section of airway smooth muscle. Inset: Triangle: dense body; single arrow: thick filament; double arrow: thin filament.

B

Figure 1.3B: Electron micrograph of transverse section of airway smooth muscle. Inset: Triangles: dense bodies; thick arrows: thick filaments; arrows: intermediate filaments; double arrows: thin filaments.

1.5 Smooth muscle “sarcomeres”

In striated muscle, sarcomeres are the basic contractile units and they form the cross striation. Sarcomeres are composed of different filaments: the thick filaments are connected to the M-line and the Z-disc by titin; the thin filaments are bound to nebulin; nebulin and titin give structural stability to the sarcomere. During contraction, the cross-bridge heads of thick filaments undergo repeated oar-like actions that enables the thick filaments to slide between thin filaments to generate force and length change (Guilford and Warshaw 1998).

In smooth muscle, the sliding filament mechanism of contraction is thought to be the contractile mechanism as well (Guilford and Warshaw 1998). However, the myofibrils of smooth muscle cells are not organized like sarcomeres and the exact structure of the contractile units, smooth muscle “sarcomeres”, is still unidentified. In conventional models of smooth muscle, the contractile filaments are arranged obliquely to the long axis of the cell and the thin filaments are depicted as being attached to the dense bodies so that the contractile unit resembles the sarcomere of striated muscle (Figure 1.4).

Figure 1.4: Schematic depiction of smooth muscle cells and the contractile units.

1.6 Contraction of smooth muscle

Smooth muscle is distributed in the walls of various hollow organs, performing the function of maintaining the shape, dimensions, and mechanical properties of the organs. Smooth muscle receives neural innervation from the autonomic nervous system, whereas skeletal muscle is under the control of the somatic nervous system. Nerve stimulation of smooth muscle causes membrane depolarization. Extra cellular calcium then enters smooth muscle cells through L-type calcium channels and initiates a chain of reactions that lead to contraction. Smooth muscle also contracts under the stimulation of hormones, autocrine/paracrine agents, and other local chemical signals, which may or may not cause membrane depolarization.

Unlike skeletal muscle, smooth muscle does not contain troponin (a calcium binding protein that activates thin filaments), but does contain the thin filament protein tropomyosin and other smooth muscle specific proteins: caldesmon and calponin. The exact roles for caldesmon and calponin in regulating smooth muscle contraction is not entirely clear. Contraction of smooth muscle is initiated by calcium activated phosphorylation of myosin rather than calcium binding to troponin (as in striated muscle). The intracellular calcium binds with calmodulin which then binds and activates myosin-light chain kinase (MLCK). The calcium-calmodulin-MLCK complex then phosphorylates the regulatory myosin light chain and initiates contraction.

Experimentally, smooth muscle is often made to contract isometrically or isotonically. In an isometric contraction the length of muscle is held constant, whereas in an isotonic contraction the muscle load is held constant. Smooth muscle contraction in vivo is rarely purely isometric or isotonic, that is, the muscle often shortens against a varying load. This type of contraction is called auxotonic.

1.7 Plasticity and length adaptation of smooth muscle

Smooth muscle cells (SMCs) possess remarkable phenotypic and mechanical plasticity which allows rapid adaptation to external environment (Halayko and Solway 2001). In smooth muscle, mechanical plasticity refers to the ability of muscle cells to rapidly alter the structural and functional properties in accordance with the physical conditions in the extracellular environment. Long term adaptation of the muscle often involves phenotypic plasticity, which alters either the quantity of protein or the type of protein comprising different subcellular components in response to any stimulus that disrupts normal homeostasis of the muscle.

Smooth muscle has the ability to function over a larger range of length than skeletal and cardiac muscle. This is important for the normal physiological function of hollow organs, where large volume changes are necessary. Uvelius (Uvelius 1976) reported that rabbit urinary bladder muscle can be increased in length by more than ~7 fold and retain its contractile ability over that length range. This change in muscle length accounts for the overall change in volume of the bladder, and it is also a reflection of changes in individual smooth muscle cell length. Structural plasticity over large ranges of length has also shown to be present in other types of smooth muscle such as swine carotid artery (Wingard et al. 1995), rat anococcygeus muscle (Gillis and Becker 1988) and airway smooth muscle (Gunst et al. 1995; Seow et al. 2000).

The process of plastic structural change in smooth muscle in response to changes in externally applied force is called length adaptation (Bai et al. 2004). A change in muscle length usually causes a decrease in the force produced by the muscle immediately after the length change. The force recovers if the muscle is allowed to adapt to the new length (i.e., length adaptation). Length adaptation therefore leads to shifts in the active length-tension (L-T) curve of smooth muscle. The passive L-T curve also shifts during the adaptation process (Wang et al. 2001).

1.8 Airway smooth muscle length adaptation and pulmonary disease

Length adaptation is a newly discovered phenomenon in which smooth muscle adapts and functions over a large length range. This normal smooth muscle plasticity is essential for normal function of many types of smooth muscle that requires large functional length range and these muscles include the ones that line the wall of organs such as urinary bladder, stomach, and uterus.

Although airway smooth muscle is not known for its need to function over a large length range, it has been found that it also has an extremely large functional length range (Pratusevich et al. 1995). With its ability to contract and control the diameter of the airways, ASM is a major regulating factor of airway resistance and plays an important role in maintaining normal lung function. Dysfunction of ASM, including alteration in its ability to adapt to length change, could lead to impaired lung functions. Adaptation to excessively short length could lead to airway obstruction and asthma symptoms.

1.9 Structural basis of length adaptation

As in striated muscle, the L-T curve in smooth muscle is also a reflection of the interaction between the contractile filaments (Herrera et al. 2005). There is evidence that the shift in the active L-T curve is due to rearrangement of contractile units in series that optimizes the overlap between the thick and thin filaments (Pratusevich et al. 1995). Kuo et al (2003) showed that thick filament formation and dissolution is associated with adaptation of ASM at long and short lengths, respectively, suggesting that addition and deletion of contractile units are part of the mechanism underlying length adaptation. These findings could explain changes in the active force generated by ASM during length adaptation; they however offer no insight into the mechanism responsible for shifting the passive L-T curve. The search for subcellular structures responsible for passive tension and its adaptation to length change is the focus of my thesis research.

The work done by McGuffee et al (McGuffee et al. 1991; McGuffee and Little, 1992) showed that dense bodies in arterial smooth muscle form "cable-like" structures lying parallel to the long axis of the cell, suggesting that these dense body structures may serve the purpose of maintaining passive tension. Definitive conclusions cannot be derived from these studies in this regard because passive tension was not measured in their studies and no measurements of dense body structures was carried out at different muscle lengths. Also in their studies, changes in the dense body structures due to length adaptation were not examined.

My thesis research focuses on elucidation of the dense body structures at different muscle lengths and under partially and fully adapted conditions. Previous studies from the Seow Laboratory confirmed that in ASM most dense bodies are strung together in series by thin and intermediate filaments and furthermore, they showed these dense-body cables are arranged in parallel to the myosin and actin filaments in the direction of force transmission (Herrera et al, 2004). The main goal of my thesis research is to determine whether this dense-body structure is responsible, at least partially, for the maintenance of passive tension in ASM cells.

CHAPTER 2 Hypothesis, specific aims, and rationale

2.1 Hypothesis

Dense-body cables in airway smooth muscle serve a role in maintaining cell integrity and passive tension, and the length adaptability of the dense body cable accounts for the shifts in the passive length-tension relationship of the airway smooth muscle cell.

2.2 Specific aims and rationale

To test our hypothesis, we performed experiments with the following specific aims:

1) Measurement of passive forces associated with different lengths in the fully and partially adapted states.

For each set of experiments, five muscle strips with the same initial resting length from a single sheep trachea were used. Muscle A was adapted by repeated electrical stimuli at its in situ length (or reference length, L_{ref}) and the passive tension was recorded after its active tension had reached a stable plateau value. The muscle was then fixed for electron microscopy (EM) for structural examination. Muscle B was adapted at the same reference length as Muscle A, but before it was fixed for EM examination, it was released to $0.5 L_{ref}$, followed by a quick (1 second) stimulation to straighten out the strip. The muscle was then fixed in the relaxed state. Both passive and active tension of the muscle were recorded during the maneuver up to the

moment it was fixed. Muscle C underwent the same maneuver as Muscle B except that it was fully adapted at the shortened length ($0.5 L_{ref}$) before it is fixed for EM. Muscle D was adapted first at the same reference length as Muscle A; it was then stretched to $1.5 L_{ref}$ and immediately fixed for EM. Passive tension was recorded during the length maneuver. Muscle E went through the same maneuver as Muscle D except that it was fully adapted to the stretched length ($1.5 L_{ref}$) before it was fixed for EM.

The above set of experiments was repeated with another 5 strips of trachealis from different sheep. These experiments provided us with measurements of passive tension associated with different muscle lengths ranging from 0.5 - $1.5 L_{ref}$, (a 3-fold difference in length) in partially and fully adapted states.

Rationale: The measurements allow us to correlate passive tension to the appearance of the dense-body cable in terms of its "slackness", i.e., the length ratio of DB-cable/cell-segment, or, the normalized length of a DB cable. From the correlation we are able to deduce whether the cable bears tension in the stretched cell. From previous work of our laboratory, we know that length adaptation leads to shifts in the passive L-T curve. The measurements allow us to confirm whether the same shifts occur in the sheep tachealis preparations used in this particular set of experiments, and also allow us to compare these findings directly with the results from the EM structural quantification.

2) Examination of the dense-body cable lengths at different muscle cell lengths in both adapted and partially adapted states.

For each of the 5 muscle preparations (A-E, described in Aim 1) fixed for EM, the 3-dimensional structure of dense bodies was reconstructed from serial 2-dimensional transverse EM thin (50 nm) sections. Approximately 100 thin sections were obtained, which allowed us to examine the structural details of dense-body cables in a $\sim 5\text{-}\mu\text{m}$ segment of an ASM cell, and also allowed us to measure the normalized length of DB cables (length ratio of DB-cable/cell-segment) from the 3-dimensional structural data. Specifically, multiple dense-body cables per cell were chosen and their lengths measured; the lengths were then averaged and divided by the cell segment length. The length ratio of DB-cable/cell-segment was the ratio of the length of the dense-body cable (in 3-dimensional space) divided by the segment length of the muscle cell in which the dense-body cable was embedded. The measurements were carried out in three cells to allow adequate statistical analysis.

Rationale: We assume that in a stretched muscle where passive tension is high, the dense-body cable will appear taut, whereas in a slack muscle where there is no passive tension, the dense-body cable will appear wavy. Once we obtain a numerical value for the length ratio of DB-cable/cell-segment (indication of the cable "slackness") of each muscle strip fixed under each of the experiment conditions, the value could be correlated to the passive tension measured under the same condition and statistical analysis can be performed, this allows us to determine whether the length ratio of DB-cable/cell-segment of the dense-body cables is inversely correlated to

passive tension in the muscle cell, and whether the dense-body cables could serve as tension-baring structures inside the muscle cell.

CHAPTER 3 Materials and methods

3.1 Tissue sample

Sheep tracheas collected from a local abattoir (Pitt Meadow Halal Meats Ltd.) were used in the experiments. The tracheas were removed from the animals immediately after death and stored in physiological saline solution (PSS) at 4°C (pH 7.4, 118 mM NaCl, 5 mM KCl, 1.2 mM NaH₂PO₄, 22.5 mM NaHCO₃, 2 mM MgSO₄, 2 mM CaCl₂, 2g/l dextrose).

3.2 Sample preparation

Prior to experiment, a tracheal ring (~8 mm in width) was removed from the whole trachea. Before the tracheal ring was cut open, the *in situ* length of the tracheal smooth muscle that connects the C-shaped cartilage was measured. If the epithelial layer showed large wrinkles (indicating that the underlying smooth muscle was not relaxed and that the tissue might have been damaged), the trachea was not used for the experiment.

The tracheal ring was cut open on the ventral side through the middle of the C-shaped cartilage ring. The epithelial layer, connective tissue and cartilage were completely removed and a rectangular piece of smooth muscle was obtained (~8 × ~10 × ~0.2 mm in dimension). From each trachea six strips were dissected and clipped on both ends with aluminum foil clips for attachment to the force/length transducer. Care was taken to make sure that the resting lengths of all 6 strips were the same.

3.3 Equilibration of the muscle preparations

The dissected and clipped muscle strip was mounted to the experimental apparatus; one end of the strip was connected to a stationary hook at the bottom of the muscle bath, the other end was connected to the lever arm of a servo-controlled force/length transducer. The PSS in the bath was preheated to 37 °C and kept at that temperature throughout the experiment. Before the muscle was chemically fixed for electron microscopy (EM), it was equilibrated (preconditioned) in the PSS at 37 °C for 1 hour. During the equilibration period, the muscle was stimulated by electrical field stimulation (EFS) for 10 seconds and this was repeated every 5 minutes.

A reference length of the muscle (L_{ref}) was established by stretching the muscle to approximately the *in situ* length ($\pm 5\%$). The passive force in the muscle strip was about 1.0 mN at that length. The EFS was provided by a 60-Hz alternative current stimulator with platinum electrodes inside the bath. The muscle was considered equilibrated when it developed a stable maximal active force. The maximal isometric force (F_{max}) of each muscle at L_{ref} was recorded; the strip was then ready for the next experimental step.

Six muscle strips (of identical initial length) from the same trachea were used for each set of experiments. Two such sets of experiments were carried out for my thesis research. The first strip was fixed at its *in situ* length (L_{ref}) and was used as a reference. The second strip was fixed after it was passively shortened to $0.5 L_{ref}$. Brief EFS (1 sec) was applied to the shortened and slackened strip to make it straight before it was fixed for EM. The third strip was fixed at $0.5 L_{ref}$ after the muscle had been fully adapted to the shortened length. The fourth strip was fixed

immediately after the muscle had been stretched to $1.5 L_{ref}$. The fifth strip was fixed after the muscle had been fully adapted at $1.5 L_{ref}$. The sixth strip was not fixed for EM but was used to obtain the length-tension relationship of the muscle adapted to the 3 lengths (0.5, 1.0, and $1.5 L_{ref}$).

3.4 Chemical fixation for EM

Primary fixation (15 min) was carried out while the muscle strips were still attached to the apparatus. The fixing solution was prepared by mixing 1% paraformaldehyde, 2.5% glutaraldehyde, and 2% tannic acid in 0.1 M sodium cacodylate buffer. The solution was preheated to 37°C . After the primary fixation, the muscle strip was removed from the apparatus and cut into small blocks with $\sim 2 \times \sim 0.5 \times \sim 0.2$ mm in dimension in cold fixation buffer. The small blocks were then put in the same fixative for 2 hours at 4°C on a shaker.

Secondary fixation was carried out on the same day of the experiment. The tissue blocks were transferred to 2% Osmium buffer for 1.5 hours at 4°C on the shaker, followed by three washes with distilled water (10 minutes per wash). The tissue blocks were then stored in 1.25% NaHCO_3 solution overnight.

3.5 Block staining and dehydration

In the process of *en bloc* staining, the muscle blocks were treated with saturated 1% uranyl acetate for 1 hour at room temperature followed by 3 washes with distilled water. The muscle blocks were then dehydrated with increasing concentrations of ethanol (50%, 70%, 80%, 90%, 95%, 10 minutes for each concentration). Ethanol (100%) and propylene oxide were used (3x10 minutes then 1x15 minutes, respectively) for the final dehydration.

3.6 Embedding and molding

After dehydration, the tissue blocks were treated with an increasing ratio of mixes of resin (TAAB 812 mix) over propylene oxide (propylene oxide: resin = 2:1 then 1:2), and finished with pure resin. The tissue blocks were stored in pure resin overnight on a shaker. On the second day, the blocks were embedded in pure resin in molds and placed in an oven at 60°C for 8-10 hours.

3.7 Sectioning

Resin blocks containing muscle tissue was sectioned into 50-nm thin sections using an ultramicrotome with a diamond knife. All the thin sections were picked up serially and placed on pre-coated single slot cooper grids (1x2 mm). The sections were further stained with 1% uranyl acetate for 4 minutes followed by lead citrate treatment for 3 minutes.

3.8 Electron microscopy

Images of the cross sections of the muscle cells were obtained with an electron microscope (Philips / FEI Tecnai 12 TEM, Software version 2.1.8). For 3-dimensional reconstruction of ultrastructure, images from about 100 cell cross-sections for each of the 5 groups of tissues (fixed under the 5 conditions described above) were collected. All the images were taken with a digital camera (Gatan 792, Software 3.11.2) at a magnification of 37,000X. To capture the whole cross-section of a single cell it often required taking multiple images of different parts of the cell cross-section and merging the parts in Photoshop®.

3.9 Alignment and tracing of EM images

For 3-dimensional reconstruction of dense-body cables, approximately 100 transverse, serial sections were used for each experimental condition, and the same reconstruction was repeated 3 times. Therefore, a total of 1500 (5 conditions x 100 thin sections x 3 repeats) thin EM sections were used. A total of 3 cells (one from the first trachea, and 2 from the second) were chosen. The selection criteria were 1) that all of the ~100 consecutive sections were of good image quality; and 2) the sections did not contain the nucleus, because the large area occupied by the nucleus would reduce the number of dense bodies available for measurement.

The serial sections in each of the 5 groups were aligned using Adobe Photoshop®. The alignment was needed because each one of the serial EM images was taken from different sections, and these sections had slightly different orientations. Intracellular organelles and some

cell features, but not dense bodies (e.g., mitochondria, endoplasmic reticulum, caveolae, and microtubules) were used as markers for the alignment. Usually 3 or more markers from each of the adjacent sections were aligned to allow orientation of the image to be determined. See Figure 3.1 for an illustration.

A

Figure 3.1: Alignment of serial sections of ASM cells. *A. Electronic micrograph of ASM cell in transverse section with some labeled markers: 1- microtubule; 2- caveola; 3- mitochondrion; 4- endoplasmic reticulum. B. Example showing how organelles in an ASM cell (such as those shown in A) were used as markers for aligning the serial sections before 3-D reconstruction. The two illustrated sections shown in B are consecutive sections and they usually are very similar in terms of the shape of cell perimeter, positions of the organelles, etc. This is because they are only 50 nm apart. However, the images are not identical, and minor rotation of the images is often required to align the markers (organelles).*

After the alignment of the serial EM images, organelles and cell elements (nucleus, mitochondria, myosin filaments, dense bodies, and dense plaques) of each cell cross-section were traced with different colors, and then the background image of the cell was removed to reveal only the traced structures. (See Figure 3.2 for an example).

Figure 3.2: Traced organelles and cell elements in an ASM cell cross-section indicated with different colors. The traced image was obtained from EM section shown in Figure3.1A.

3.10 Determination of the length of dense-body cables

The normalized DB cable length (indicated by the length ratio of DB-cable/cell-segment) was used to indicate whether the cables were under tension. The actual 3-dimensional length of dense-body cables was calculated using Image J software (version 4.0). For each cell, five to six dense bodies were chosen for tracing. Some of the dense bodies did not go through the whole muscle segment. At least three dense-body cables were traced through the whole serial sections representing $\sim 5 \mu\text{m}$ of cell segment. The centroid of each dense body was measured using Image J. In the 3-dimensional analysis, we defined the transverse section of the cell as the X-Y plane, and the Z-axis was parallel to the long axis of the cell (and perpendicular to the X-Y plane). Each dense body (centroid) measured was given an X-Y coordinate with reference to the centroid on the first section (i.e., $X=Y=0$). It was assumed that the distance (D) between the centroids of two adjacent dense bodies in section i and $i+1$ of the same cable was the length of the dense-body cable in the i th section (Figure 3.3).

Figure 3.3: Depiction of 3-dimensional relations among traced dense bodies and the distance between 2 centroids of adjacent (in series) dense bodies. A dense-body cable was traced through 3 serial sections represented by the solid-, dash-, and dotted-lined ovals.

It follows that the length of dense-body cable in section i is: $D_i = \sqrt{L^2 + d_i^2}$, where L was the section thickness (50 nm) and d_i was the projected distance on the X-Y plane between the centroids of the two dense bodies in sequence in section i and $i+1$, and was calculated as:

$$d_i = \sqrt{|X_{i+1} - X_i|^2 + |Y_{i+1} - Y_i|^2}$$

The total length of the dense-body cable in the cell segment was the sum of the D s calculated for all serial sections (~ 100 in numbers). The ratio of the dense-body cable length to the length of the muscle segment (50 nm multiplied by the number of sections) indicates the slackness of dense body cables.

3.11 Measurement of passive tension in ASM at different lengths

After equilibration and adaptation to L_{ref} , the muscle length was changed from L_{ref} to $0.5 L_{ref}$ or $1.5 L_{ref}$ and fixed for EM examination. As described above, a total of 5 muscle strips were fixed, each under a different condition, for each set of experiments. The resting tension of these muscle strips was recorded just before the muscles were chemically fixed. In addition, a 6th strip of muscle from the same trachea for each set of experiments was used in the measurement of length-tension properties without being fixed. This was carried out to make sure that the length adaptation in the muscle was reversible. As described above, each of the 5 strips for EM examination were fixed at different lengths and states of adaptation, therefore reversibility of length adaptation could not be tested. For the 6th strip, after full adaptation at L_{ref} , the muscle was manually stretched to $1.5 L_{ref}$ in about 1 second, and the passive force was recorded before, during and after the stretch. The muscle strip was then adapted at the stretched length with repeated EFS stimulation at 5-min intervals until the active muscle force reached a steady, maximal plateau. The muscle was then released back to L_{ref} . After adaptation at L_{ref} , it was released to $0.5 L_{ref}$. The muscle was then adapted to the shortened length. After full adaptation at the short length, the muscle was stretched back to L_{ref} and re-adapted. The changes in both active and passive forces were measured during the length maneuver. The measurements confirmed that length adaptation in the sheep trachealis preparations was fully reversible, and that stretching or shortening the muscle to the set lengths did not cause irreversible damage to the muscle. Data obtained in these measurements from the 6th muscle strip were grouped with those from the 5 strips for plotting the passive tension with each experiment condition.

3.12 Statistical analysis

Two tracheas from two animals (sheep) were used in this study. A total of 3 sets of serial sections (~100 for each set) were obtained from 3 cells, one from the first trachea and 2 from the second. For each cell, 3 dense-body cables were reconstructed. Unless otherwise stated, data are shown as means $\pm$ standard error of means (SE). Statistical difference was considered significant at $P < 0.05$ using one-way repeated measures ANOVA followed by Tukey's *a posteriori*-test.

CHAPTER 4 Results

To test our hypotheses as outlined in Chapter 2, we applied interventions (length change and adaptation) to our muscle preparations and observed the changes in ultrastructure of the muscle cells, as well as changes in passive tension in the muscle strips.

4.1 Changes in passive tension at different muscle lengths and adaptation states

As described in the Methods, 4 interventions were applied to separate muscle preparations before the tissues were fixed for EM examination. Passive tensions of these preparations at the time of fixing were recorded and are plotted in Figure 4.1. Included in the plot are also data from one muscle strip that was not fixed for EM.

Figure 4.1: Passive tension of muscle strips fixed under different conditions. *A. Adapted at L_{ref} (Control). B. Released from the control condition to $0.5 L_{ref}$ and partially adapted at the shortened length. C. Released from the control condition to $0.5 L_{ref}$ and fully adapted at the shortened length. D. Immediately after stretch from the control condition to $1.5 L_{ref}$. E. Fully adapted at $1.5 L_{ref}$ after stretch from control condition.*

Results from One-Way repeated measures ANOVA on the data shown in Figure 4.1 are listed in Table 4.1. Muscle strips from the same trachea were studied under the 5 conditions (control plus 4 interventions, A-E) in a single experiment; three such experiments were carried out to generate the data plotted in Figure 4.1. In two of these experiments the muscle strips were fixed for EM examination.

Table 4.1: One-Way ANOVA and Tukey's test on passive tensions at different muscle lengths and adaptation states.

One-Way ANOVA

Treatment Name	N	Mean	Std Dev	SEM
A	6	0.860	0.392	0.160
B	3	0.293	0.0945	0.0546
C	3	0.373	0.146	0.0841
D	3	20.067	3.450	1.992
E	3	9.943	0.690	0.398

F=88.5; P<0.01.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	P	P<0.050
D vs. B	19.773	<0.001	Yes
D vs. C	19.693	<0.001	Yes
D vs. A	19.023	<0.001	Yes
D vs. E	10.123	0.002	Yes
E vs. B	9.650	0.002	Yes
E vs. C	9.570	0.002	Yes
E vs. A	8.900	0.001	Yes
A vs. B	0.750	0.975	No
A vs. C	0.670	0.984	No
C vs. B	0.0800	1.000	No

Note: The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P = <0.001$). Not all groups are different from each other though, as indicated by the Tukey's Test. The experiment conditions (or interventions) A to E corresponds to the conditions indicated in Figure 4.1. At the 0.05 level, the means are significantly different. N indicates the number of muscle strips used. Two animals were used for these experiments.

4.2 Reconstruction of 3-dimensional structure of dense body aggregates

To determine the length of the dense body cables, 3-dimensional reconstruction of $\sim 5\text{-}\mu\text{m}$ segments from ASM cells was carried out. Each of the $\sim 5\text{-}\mu\text{m}$ segments contained approximately 100 serial thin sections (50 nm). EM images were taken from each of the thin sections, and the 3-dimensional pictures of the cable-like dense-body aggregates were obtained as described in Methods. An example of the pictures is shown in Figure 4.2.

In general, the dense body aggregates examined in the 3-dimensional cell segment were aligned with the long axis of the cell, which was parallel to the axis of force transmission in the cells. The software Velocity TM was used for the 3-D reconstruction.

Figure 4.2: *Examples of 3-dimensional reconstruction of dense-body cables (with other intracellular components removed for clarity). A. Muscle strip fixed at L_{ref} , fully adapted (Control). Grid size 300x300 nm. B. Muscle strip fixed at $0.5 L_{ref}$, partially adapted. Grid size 300x300 nm. C. Muscle strip fixed at $0.5 L_{ref}$, fully adapted. Grid size 200x200 nm. D. Muscle strip fixed at $1.5 L_{ref}$, not adapted. Grid size 200x200 nm. E. Muscle strip fixed at $1.5 L_{ref}$, fully adapted. Grid size 300x300 nm.*

4.3 Lengths of the dense-body cables under different experiment conditions

The normalized lengths of the dense-body cables (i.e., the length ratio of DB-cable/cell-segment) were calculated as outlined in Methods. The results are plotted in Figure 4.3. These results are from 3 separate sets of measurements with 5 conditions (A-E) in each set. Therefore a total of 15 cell segments were serially sectioned to obtain the 3-dimensional images and measurements. The lengths of these cell segments are listed in Table 4.2. Table 4.3 lists the normalized length of DB cables and the results of a One-Way ANOVA and Tukey's Test.

Figure 4.3: Normalized lengths of the dense-body (DB) cables measured under different conditions. See text for definition of the normalized length of DB cable. The conditions A to E are described in Figure 4.2.

Table 4.2 Length of the ASM cell segments used in 3-D reconstruction

Intervention	Experiment #1 Segment Length (μm)	Experiment #2 Segment Length (μm)	Experiment #3 Segment Length (μm)
A	5.45	5.3	5.2
B	5.45	5.3	5.2
C	5.45	5.3	4.8
D	5.25	5.5	5.1
E	5.45	5.5	5.0

Note: Cells in experiment #1 & #2 are from one animal, cells from experiment #3 are from a different animal.

Table 4.3 One-Way ANOVA on the normalized length of dense-body cables and Tukey's Test for pairwise difference among the different groups

One-Way ANOVA

Treatment Name	N	Mean	Std Dev	SEM
A	3	1.754	0.0682	0.0394
B	3	1.917	0.140	0.0811
C	3	1.878	0.174	0.101
D	3	1.284	0.140	0.0809
E	3	1.489	0.112	0.0646

F=12.7; P=0.02.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	P	P<0.05
B vs. D	0.633	0.003	Yes
B vs. E	0.428	0.024	Yes
B vs. A	0.164	0.575	No
B vs. C	0.0393	0.995	No
C vs. D	0.594	0.004	Yes
C vs. E	0.389	0.040	Yes
C vs. A	0.124	0.773	No
A vs. D	0.469	0.015	Yes
A vs. E	0.265	0.191	No
E vs. D	0.205	0.384	No

Note: The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P = 0.002$). The experiment conditions (A-E) are described in Figure 4.2. The N number (3) indicates the number of cells examined. Data from the same cell were first averaged; the means of these averages were then used for the statistical analysis. Two animals were used for these experiments.

4.4 Correlation between passive tension and the normalized dense-body cable length

In Figure 4.4, the mean values of passive tensions associated with each of the test conditions (A-E) are plotted against the normalized lengths of the DB cables measured under the same conditions. The plot shows that the normalized DB cable length (an estimate of cable slackness) is significantly reduced at high passive tension. Note that the correlation is not as good at low passive tension.

Figure 4.4: Correlation between passive tension and the normalized DB cable length. The means values plotted are from Figs. 4.1 and 4.2. The goodness of fit (r^2) is 0.94; the correlation is statistically significant ($p=0.007$).

CHAPTER 5 Discussion

The most significant finding of my thesis research is that shown in Figure 4.4, that is, passive tension in ASM cells is negatively correlated to the normalized DB cable length in the cells. The results suggest that the dense-body cables may be important in maintaining passive tension in smooth muscle cells. The results also challenge the common assumption that dense bodies play the role of Z-disks found in skeletal muscle.

5.1 New roles for dense bodies in smooth muscle?

The conventional belief that dense bodies are the Z-disks equivalent in smooth muscle originated from studies by Bond et al (Bond and Somlyo 1982) where dense bodies were shown to be the anchorage sites for actin filaments. Furthermore, the polarity of the actin filaments was shown to be opposite on the opposite sides of a dense body. In these studies, however, only the 2 dimensional structure of the cell architecture was examined. In their 2-dimensional description, the dense bodies were discrete elements and appeared to be randomly distributed in the cell's cytoplasm (Bond and Somlyo 1982). In 3-dimensional description of ASM cell architecture, McGuffee and colleagues (McGuffee et al, 1991; McGuffee and Little, 1992) showed that the dense bodies were arranged in series, lying parallel to the long axis and the cell. This was confirmed by North et al (1994b) using immuno-labeling of β -actin and α -actinin. Results from our laboratory (Herrera and Seow, 2004) also revealed the cable-like structure of dense-body aggregates and for the first time Herrera and Seow (2004) showed that the cable-like DB

structures run in parallel with the myosin filaments which in turn run in parallel with the long axis of the cell in the direction of force transmission (Figure 5.1).

Figure 5.1: 3-D reconstruction of a dense-body cable (red) and myosin filaments (blue) from an ASM cell segment. Grid size: 300 x 300 nm. (From Herrera and Seow, 2004; not published, permission from the authors).

North et al (1994b) postulated that the long DB structures in smooth muscle serve the same function as Z-disks in striated muscle. But for that to work it requires that myosin and actin filaments lie in an oblique angle to the long axis of the cell, which is contradictory to the evidence (shown Figure 5.1) indicating that both the DB cables and the contractile filaments lie in parallel to the long axis of the cell. This type of arrangement (shown in Figure 5.1) does not support the theory that dense bodies are the Z-disks equivalents, and it raised the question of what exactly the roles are played by the dense bodies.

5.2 Dense-body cables as structures bearing passive tension?

The long cable-like structure of dense bodies suggests that they may be important in supporting passive tension in smooth muscle cells. In an activated smooth muscle, tension is mostly borne by the contractile filaments, i.e., the thick and thin filaments. In a relaxed muscle, the thick and thin filaments can slide past each other freely, and are not able to support tension. However, in the relaxed state, we know that isolated single smooth muscle cells are able to bear tension and resist stretch (Warshaw and Fay 1983). There must be intracellular structures in smooth muscle that are capable of supporting passive tension. Could the dense-body cables be the structures? Or at least be part of the structures. The results shown in Figure 4.4 are the first demonstration that the configuration of the DB cables is correlated with the degree of passive tension. Stretching the cell elongates and straightens the cables with an associated increase in passive tension. Note that the normalized lengths of the cables have a poor correlation with passive tension when the tension is low (less than 1 mN or 3% of maximal active tension). This can be explained by the fact that the passive tension cannot go below zero, whereas the cables can be quite wavy especially after the muscle has been acutely shortened. We did observe that some cables did not go through the entire length of the 5- μ m cell segment. It is possible that some DB cables do not attach themselves to any intracellular structure, and are "freely floating" in the cytoplasm. It is also possible that the discontinued DB cables are connected to other cell structures such as another DB cable or dense plaque by actin filaments and/or intermediate filaments. Unfortunately we were not able to follow these filaments in our 3-dimensional reconstruction. However, that fact that at least some DB cables can be straightened out by stretching the muscle bundle suggests that at least some of them are physically connected to the outside world.

5.3 The effect of length adaptation

The adaptation protocol used in our experiments revealed another remarkable property of the dense-body cable structure, and that is, its ability to quickly adjust its length with the change in muscle length. As mentioned above, the fact that the cables can be straightened out with a stretch applied to the muscle cell bundle suggests that the cables span the length of the muscle cell and are connected to the adjacent cells and extracellular matrix. With a 50% shortening, if the cable length stayed the same, we would expect to see doubling of the cable length in a segment that was previously twice as long (before the muscle was released from L_{ref} to $0.5 L_{ref}$), that is, the normalized length of DB cables under the conditions of B and C would double that under the condition of A. That of course was not the case as shown in Figure 4.3. The normalized DB cable length in the case of B and C on average increased by 7-10%, nowhere near the 100% expected if the cable length were to stay the same. The partial and full length adaptation associated with cases B and C respectively therefore has resulted in a large shortening of the cable length. It is not known how the DB cables accomplish these large changes in structure. We also have no information regarding the changes in the dimensions of individual dense bodies after a length change and after length adaptation. The information we do have suggests strongly that the structure of the DB cables is highly dynamic.

5.4 Are the dense-body cables responsible for the shifts in the passive length-tension curve in airway smooth muscle?

As described in Introduction, both the active and passive forces in airway smooth muscle are altered in the process of length adaptation. As a result, the passive and active length-tension curves are shifted. Previous research from our laboratory has revealed that the shifts in the active length-tension curve may be due to addition and deletion of contractile units in series in the ASM cells (Kuo et al. 2003). The shifts in the passive length-tension curve, however, have never been explained. Results from my thesis research suggest that the length-adjustable nature of the DB cables may underlie the shifts in the passive length-tension curve in ASM during length adaptation (Figure 4.3). Of course, there are other candidates that cannot be ruled out. For example, the cytoskeletal network of filaments not including the dense-body cables. And for a multi-cellular muscle preparation, the extracellular matrix (ECM) likely plays an important role in the maintenance of passive tension, due to connectivity between ECM elements and DB cables via dense plaques (Bramley et al, 1994; 1995). The results presented in Figure 4.3, however, suggest that the dense-body cables likely have some role in bearing passive tension in a relaxed muscle. These cables likely play an even bigger role in the shifts of the passive length-tension curve, because, being intracellular structures, their length could be altered relatively quickly by the intracellular machinery, whereas for extracellular matrix to plastically alter its structure, long term adaptation may be required.

5.5 Why is it important to understand the source and mechanism of passive tension in ASM?

A significant contributor to airway stiffness is the tension in the ASM; it does not matter whether the tension is active or passive. In a relaxed airway, airway stiffness perhaps stems from the passive tension in the ASM. It is known that changes in lung volume associated with tidal breathing and deep inspiration stretch the ASM periodically (Salerno et al. 1999; Krishnan et al. 2008) and cause the muscle to produce less active force in a stretch-amplitude-dependent manner (Wang, Pare et al. 2000). Length perturbation associated with lung volume fluctuation therefore has a potent bronchodilator effect. The higher the passive tension in the ASM, the less will be the strain of the muscle due to tidal breathing and deep inspiration. This will lead to a vicious cycle of diminishing bronchodilator effect due to breathing, and it has been hypothesized that a "frozen" state (high stiffness) of ASM as a result of diminishing length perturbation on ASM could lead to airway hyperresponsiveness (Gunst and Fredberg 2003). It is well known that asthmatics in remission still possess airway hyperresponsiveness. It is possible that in the asthmatics, the passive length-tension relationship of their ASM is shifted to the left (to a shorter length), so that even without stimulation and generation of active force, their airways are stiff and more resistant to stretch. Part of this high resistance may originate from the dense-body cables whose lengths have been shortened due to adaptation of the muscle at short lengths. Understanding how DB cables adapt to length changes and how their structural integrity is modulated intracellularly will therefore lead to a better understanding of the roles that ASM plays in the pathogenesis of asthma.

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