Asymmetrical Length Adaptation in Airway Smooth Muscle: Possible Mechanisms

by

FARAH ALI

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Abstract

Airway smooth muscle (ASM) regulates flow resistance in the airways of the lung. Dysfunction of the smooth muscle is implicated in the exaggerated airway narrowing seen in asthma, possibly due to adaptation of ASM to excessively short lengths. In this study, we examined the behaviour of ASM in its adaptation to large changes in cell length and the underlying mechanisms for its ability to adapt and regain optimal contractility. Isometric force measured immediately after a length change revealed that the amount of decrease in force after the length change was very sensitive to the direction of the length change (more sensitive in release than in stretch), and relatively insensitive to the absolute lengths from/to which the muscle was stretched or released. Force decreased by 20.4±0.9 (%) when muscle length was doubled from an arbitrarily chosen reference length (L_{ref}, at which the muscle had been adapted); in the reversed direction with a halving of 2xL_{ref}, the decrease in force was 48.0±2.3 (%). Quantification of myosin filament density by electron microscopy revealed a similar asymmetry; a length increase from L_{ref} to 1.6xL_{ref} resulted in no significant decrease in filament density, but a length decrease from 1.6xL_{ref} to L_{ref} left only 81.0±3.3 (%) of the filaments intact (P<0.05). Velocity measurements after step changes in length revealed that velocity was proportional to muscle length, and the change in velocity was almost instantaneous after the length change (without full adaptation). We have developed a model to explain all the above results. It appears that length change leads to an immediate reconfiguration of the actin filament lattice so that the number of contractile units (appropriate to length) can be formed. Formation of myosin filaments within the actin filament lattice appears to be a separate process, which requires a longer time and tends to influence force and not velocity.
# Table of contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abstract</strong></td>
<td>ii</td>
</tr>
<tr>
<td><strong>Table of Contents</strong></td>
<td>iii</td>
</tr>
<tr>
<td><strong>List of Figures</strong></td>
<td>v</td>
</tr>
<tr>
<td><strong>List of Abbreviations</strong></td>
<td>vii</td>
</tr>
<tr>
<td><strong>Acknowledgements</strong></td>
<td>viii</td>
</tr>
<tr>
<td><strong>CHAPTER 1. Introduction</strong></td>
<td></td>
</tr>
<tr>
<td>1.1 General introduction to airway smooth muscle</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Cell structure of airway smooth muscle</td>
<td>2</td>
</tr>
<tr>
<td>1.3 Smooth muscle “sarcomeres”</td>
<td>4</td>
</tr>
<tr>
<td>1.4 Smooth muscle mechanics</td>
<td>7</td>
</tr>
<tr>
<td>1.5 Smooth muscle length adaptation</td>
<td>9</td>
</tr>
<tr>
<td>1.6 Mechanism of length adaptation</td>
<td>12</td>
</tr>
<tr>
<td>1.7 Length adaptation and disease</td>
<td>17</td>
</tr>
<tr>
<td>1.8 Hypotheses and specific aims</td>
<td>19</td>
</tr>
<tr>
<td><strong>CHAPTER 2. Materials and Methods</strong></td>
<td></td>
</tr>
<tr>
<td>2.1 Muscle strip preparation</td>
<td>23</td>
</tr>
<tr>
<td>2.2 Equilibration</td>
<td>24</td>
</tr>
<tr>
<td>2.3 Apparatus</td>
<td>26</td>
</tr>
<tr>
<td>2.4 Brief overview of experiment procedure</td>
<td>27</td>
</tr>
<tr>
<td>2.5 Change in force after quick-stretch or quick-release</td>
<td>30</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.6 Change in myosin filament density after quick-stretch or quick-release</td>
<td>32</td>
</tr>
<tr>
<td>2.7 Electron microscopy</td>
<td>33</td>
</tr>
<tr>
<td>2.8 Morphometric analysis</td>
<td>35</td>
</tr>
<tr>
<td>2.9 Change in velocity after quick-stretch or quick-release</td>
<td>36</td>
</tr>
<tr>
<td>2.10 Statistical Analysis</td>
<td>38</td>
</tr>
<tr>
<td><strong>CHAPTER 3. Results</strong></td>
<td></td>
</tr>
<tr>
<td>3.1 Change in force after quick-stretch or quick-release</td>
<td>39</td>
</tr>
<tr>
<td>3.2 Change in thick filament density after quick-stretch or quick-release</td>
<td>44</td>
</tr>
<tr>
<td>3.3 Change in velocity of shortening after quick-stretch or quick-release</td>
<td>51</td>
</tr>
<tr>
<td>3.4 Summary of results for a 60% length change</td>
<td>56</td>
</tr>
<tr>
<td><strong>CHAPTER 4. Discussion</strong></td>
<td></td>
</tr>
<tr>
<td>4.1 Result summary</td>
<td>57</td>
</tr>
<tr>
<td>4.2 Effects of quick-stretch and quick-release on force development</td>
<td>58</td>
</tr>
<tr>
<td>4.3 Effects of quick-stretch and quick-release on myosin filament density</td>
<td>62</td>
</tr>
<tr>
<td>4.4 Effects of quick-stretch and quick-release on velocity of shortening</td>
<td>64</td>
</tr>
<tr>
<td>4.5 The intermediate state after quick-stretch or quick-release</td>
<td>67</td>
</tr>
<tr>
<td>4.6 Alternative mechanisms</td>
<td>72</td>
</tr>
<tr>
<td>4.7 Physiological relevance</td>
<td>73</td>
</tr>
<tr>
<td><strong>References</strong></td>
<td>75</td>
</tr>
</tbody>
</table>
List of figures

| Figure 1-1. | Electron micrograph of a transverse section of a sheep tracheal smooth muscle cell | 3 |
| Figure 1-2. | Muscle contractile units | 6 |
| Figure 1-3. | Length-tension curves after length adaptation | 11 |
| Figure 1-4. | Contractile unit rearrangement in response to length adaptation of a two-fold length change | 16 |
| Figure 1-5. | Hypothesized model of immediate effects of length change on smooth muscle | 20 |

| Figure 2-1. | Experimental protocol: change in force after quick-stretch or quick-release | 29 |
| Figure 2-2. | Experimental protocol: change in myosin filament density after quick-stretch or quick-release | 29 |
| Figure 2-3. | Experimental protocol: change in velocity after quick-stretch or quick-release | 29 |

| Figure 3-1. | Change in force (kPa) upon quick-stretch or quick-release | 41 |
| Figure 3-2. | Relative change in force ($%F_{\text{max}}$) upon quick-stretch or quick-release | 42 |
| Figure 3-3. | Quick-stretch vs. quick-release | 43 |
| Figure 3-4. | Electron micrograph of a sheep trachealis muscle cell in transverse section, and experimental data | 46 |
| Figure 3-5. | Magnified portion of previous image | 47 |
Figure 3-6. Examples of electron micrographs from all experimental conditions

48

Figure 3-7. Thick filament density of four conditions sub-grouped by location along long axis of cell

49

Figure 3-8. Fractional change in force or thick filament density upon quick-stretch or quick-release

50

Figure 3-9. Velocity of shortening after quick-stretch and quick-release

54

Figure 3-10. Shortening velocities before length change, immediately after length change, and after adaptation to new length

55

Figure 3-11. Summary of results from quick-stretch or quick-release between L_{ref} and 1.6 L_{ref}

56

Figure 4-1. Partial adaptation during activation post-QR

61

Figure 4-2. A proposed model to explain the rearrangement of contractile units in series in response to length change

66

Figure 4-3. Modified model of structural changes after length change

71

Figure 4-4. Structural and functional outcomes at three states after a change in length from 1.5 L_{ref}

71
**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>ASM</td>
<td>Airway smooth muscle</td>
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<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EFS</td>
<td>Electrical field stimulation</td>
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<td>EM</td>
<td>Electronic microscopy</td>
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<td>( F_{\text{max}} )</td>
<td>Maximal force</td>
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<td>F-V</td>
<td>Force-velocity</td>
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<tr>
<td>kPa</td>
<td>Kilopascal</td>
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<tr>
<td>L-T</td>
<td>Length-tension</td>
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<td>( L_{\text{ref}} )</td>
<td>Reference length</td>
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<td>( L_{\text{in situ}} )</td>
<td><em>In situ</em> length</td>
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<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
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<td>( P )</td>
<td>( P ) value</td>
</tr>
<tr>
<td>PSS</td>
<td>Physiological saline solution</td>
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<td>QR</td>
<td>Quick-release</td>
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<td>QS</td>
<td>Quick-stretch</td>
</tr>
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<td>SE</td>
<td>Standard error of mean</td>
</tr>
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<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
</tbody>
</table>
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CHAPTER 1. Introduction

1.1 General introduction to airway smooth muscle

Vertebrates have two types of muscle, striated and smooth, the function of which is to generate force or motion. Striated muscle comprises skeletal muscle which is controlled by the somatic nervous system, and cardiac muscle which makes up the bulk of the heart. Smooth muscle is controlled by the autonomic nervous system and is usually found lining the lumen of hollow organs. Examples of these are the urinary bladder, stomach, gastrointestinal tract, uterus, iris of the eye, airways and blood vessels. Smooth muscle regulates the dynamic size and shape of the organ with which it is associated, thus dysfunction in smooth muscle contractility has been implicated in a variety of conditions such as hypertension, pre-term labour and diseases of exaggerated airway narrowing, such as seen in asthma and COPD.

The focus of this report is on airway smooth muscle (ASM) which controls the caliber of the trachea and airways of the lungs to regulate airflow. In mammalian trachea, smooth muscle bridges the dorsal side of the C-shaped cartilaginous rings that run in transverse orientation to the airway length. Further down the airways, smooth muscle surrounds the entire lumen and in 4th to 7th generation bronchi, ASM bundles become smaller and irregularly oriented (Daniel et al., 1986).
1.2 Cell structure of airway smooth muscle

Airway smooth muscle cells are spindle-shaped, about 250μm long and 3-5μm wide at the centre of the cell (Stephens, 2001). A cigar-shaped nucleus is located at the centre of the cell and organelles such as mitochondria, the Golgi apparatus and sarcoplasmic reticulum (SR) tend to cluster near the poles of the nucleus. The entire smooth muscle cell membrane (or sarcolemma) contains mutually exclusive areas of caveolae and dense plaques (Fig. 1-1). Caveolae are surface invaginations which greatly increase the cell surface area, allowing molecules, ions and liquid to rapidly pass from extracellular to intracellular space (Stephens, 2001). Intimately associated with SR, they may provide a conduit for calcium transport within a cell (Kuo et al., 2003a).

Dense plaques are similar to dense bodies, but are located along the sarcolemma as opposed to throughout the cell cytoplasm. Both are electron dense areas that serve as anchoring sites for actin and intermediate filaments (Kuo et al., 2003a; Small and Gimona, 1998). Dense plaques paired between adjacent cells create a desmosome-like intermediate junction, or a "hemidesmosome" if a single dense plaque is connected to the extracellular matrix (ECM). Hemidesmosomes are suggested to provide mechanical coupling between myofilaments and the ECM (Stephens, 2001), and to have a role in mechanotransduction (Gunst et al., 2003; Zhang and Gunst, 2006).
Fig. 1-1. Electron micrograph of a transverse section of a sheep tracheal smooth muscle cell. Nucleus (N), dense plaque (DP), dense body (DB), caveoli (C) are shown, along with an intermediate junction that is squared off. Scale bar represents 0.5μm.
1.3 Smooth muscle "sarcomeres"

A contractile unit (or sarcomere in striated muscle) is the basic functional unit of muscle. It generates force and turns microscopic motion into macroscopic shortening. In striated muscle, sarcomeres consist of thick filaments made of myosin, thin filaments made predominantly of actin, and thin filament anchoring structures called Z-disks (Fig. 1-2a) (Gordon et al., 1966). Contractile filaments are arranged in parallel to the long axis of a muscle bundle and in a repetitive fashion that gives striated muscle its "striated" appearance. Force and motion are generated by the ATP-dependent cyclical action of cross-bridges that form between actin filaments and the head region of myosin in nearby filaments, causing the thick and thin filaments to slide past each other (Huxley, 1957).

While the sliding filament theory of contraction is thought to be the mechanism for smooth muscle as well (Guilford and Warshaw, 1998), the exact structure of a contractile unit is as yet unknown. No doubt, this understanding has been delayed by the "smoothness" or lack of pattern in smooth muscle viewed under the microscope. Therefore information on smooth muscle contractile units has required a backward approach, using functional data and theoretical modeling to infer structure.

Herrera et al. (2005) have recently refined a model of a smooth muscle "sarcomere" (Fig 1-2b). In this model, thick filaments do not show the central bare zone characteristic of bipolar thick filaments in striated muscle. They are suggested instead to be row-polar or side-polar (Cooke et al., 1987; Hinssen et al., 1978; Small, 1977). Z-disks are not
present in smooth muscle but are suggested to be replaced by dense bands or bodies which, like Z-disks, are composed mainly of α-actinin (Small and Gimona, 1998; Stephens, 2001) and act as anchoring sites for thin filaments and intermediate filaments (Ashton et al., 1975; Small and Gimona, 1998). According to the model, thick filaments span the entire distance between dense bodies such that thick filaments completely overlap the thin filaments within the “sarcomere”. Though only two thin filaments are shown in the smooth muscle “sarcomere”, there are probably several others surrounding the thick filament.
Fig. 1-2. Muscle contractile units. (a) A striated muscle contractile unit. Cross-bridges are shown on a bi-polar thick filament. (b) A smooth muscle contractile unit. Cross-bridges are shown on a side-polar thick filament. For simplicity, this thick filament is shown to interact with two thin filaments of opposite polarity, but in fact, a thick filament could interact with a number of thin filaments that surround it. Images are modified from Lambert et al., 2004.
1.4 Smooth muscle mechanics

Muscle contractions in situ are auxotonic, meaning that the muscle contracts against a varying load. Under experimentally controlled conditions, muscle contraction is often isometric or isotonic. In the former, the length of the muscle remains constant by varying the external load to balance the tension generated by the muscle. In the latter, a constant external load is applied to the muscle such that the muscle will shorten when the force generated by the muscle exceeds the external load. Under these controlled loading conditions, analysis of muscle performance can be greatly simplified.

Upon contractile activation of a smooth muscle cell, calcium enters the cytoplasm through extracellular calcium channels and from intracellular stores (ie. sarcoplasmic reticulum). Calcium binds to calmodulin, causing a conformational change that allows calmodulin to activate the myosin light chain kinase (MLCK). Activated MLCK can phosphorylate the regulatory myosin light chain, turning on myosin's ATPase activity which allows cross-bridge cycling (i.e., cyclic interaction of myosin and actin). Phosphorylation of myosin also seems to play a role in stabilizing myosin thick filaments (Qi et al., 2002; Trybus and Lowey, 1984).

Total muscle force is generated by the summation of active and passive forces. Active forces is generated by energy dependent contractile structures such as thin and thick filaments. Passive force, or resting tension, is created by non-energy dependent structures within muscle cells and tissue such as the extracellular matrix and the
cytoskeleton. Force can be normalized by the cross-sectional area of the muscle; the normalized force is called stress.

Based on the cross-bridge, sliding filament theory of contraction, the active tension development of muscle is proportional to the amount of overlap between thick and thin filaments of appropriate orientation or polarity (Gordon et al., 1966). Thus force generation of a muscle is related to filament length. It also infers that when the muscle is stretched or shortened (actively or passively), filament overlap decreases and tension generation falls. This can be seen in a length-tension curve. A length-tension curve gives us information on the muscle’s ability to stiffen, support load and also about the muscle’s elasticity.

Another important measurement of muscle function, along with force generation, is the velocity at which a muscle shortens. A force-velocity curve can be constructed with isotonic contractions at a series of external loads. As the external load decreases relative to the maximal tension the muscle can generate, the velocity of muscle shortening increases because more cross-bridges can be devoted to shortening and less to counter the load. Force-velocity curves of both striated and smooth muscle can be fitted with Hill’s hyperbola (Hill, 1938) of the form \((F+a)(V+b) = b(F_{\text{max}}+a)\) where \(F\) is the isotonic load, \(F_{\text{max}}\) is the maximal isometric force, \(V\) is the shortening velocity, and \(a\) and \(b\) are Hill’s constants. By plotting Hill’s hyperbola, one can also find \(V_0\) of the muscle which is the extrapolated maximal velocity at zero load.
1.5 Smooth muscle length adaptation

During normal functioning, hollow organs such as the bladder, uterus and bowel undergo large changes in volume. Since smooth muscle lines the walls of these organs, it suggests that smooth muscle cells themselves must be functional through a large length range. In 1976, Uvelius reported that rabbit urinary bladder muscle could undergo a greater than 7-fold length change within which it retained its contractile ability (Uvelius, 1976). Since the change in muscle length could account for the overall change in volume of the bladder, this suggested that it was indeed a change in muscle cell length, and not an additional tissue enclosed within the muscle tissue, that occurred upon change in organ circumference. Length adaptation has also been shown in other types of smooth muscle such as swine carotid artery (Wingard et al., 1995), rat anococcygeus muscle (Gillis et al., 1988) and airway smooth muscle (Gunst et al., 1995; Gunst et al., 1993; Pratusevich et al., 1995) with differing amounts of length range.

A result of length adaptation is that the active length-tension curve of smooth muscle is not fixed (Fig. 1-3). A change in length will initially cause a drop in force development, however, during the period of adaptation, the entire length-tension curve will shift such that maximal force production ($F_{\text{max}}$) occurs at the new, adapted length. The passive length-tension curve also shifts during the period of adaptation such that the passive force will decrease after adaptation to a longer length, or increase after adaptation to a shorter length.
Smooth muscle length adaptation can be induced in a variety of ways. Once set at a fixed length, it can occur with a single contraction (Gunst and Wu, 2001; Seow et al., 2000), with a series of brief activations (Pratusevich et al., 1995) or with continuous submaximal activation for ten minutes (McParland et al., 2005). Length adaptation can also occur in relaxed muscle after a period of hours or days (Martinez-Lemus et al., 2004; Naghshin et al., 2003; Wang et al., 2001; Zeidan et al., 2000).

While skeletal muscle length adaptation has been shown to occur, it occurs only over long periods of time. Such is the case in chronic obstructive lung disease where patients develop a shortened diaphragm (Rochester and Braun, 1985; Sharp et al., 1974), but in general, its functional length range is only 10-20% (Gordon et al., 1966).
Fig. 1-3. **Length-tension curves after length adaptation.** Both the active and passive length tension curves of airway smooth muscle shift after a period of adaptation to a longer or shorter length, such that $F_{\text{max}}$ is produced over a large length range. (a) Closed circles represent trachealis strips that have been passively shortened, open circles represent control strips from the same animal. (b) Closed circles represent trachealis strips that have been passively lengthened, open circles represent control strips from the same animal. Reproduced from Wang et al., 2001.
1.6 Mechanism of length adaptation

Large changes in smooth muscle length cannot be produced by a fixed array of sliding filaments as found in striated muscle, thus the mechanism of smooth muscle length adaptation probably involves a structural reorganization of the cytoskeleton and contractile apparatus. A term to describe the ability to adapt and reform the filament lattice such that optimal overlap of thin and thick filaments (thus maximal force development) is achieved, was first coined by Ford et al. (1994) as plasticity of smooth muscle.

To describe the cytoskeletal component of length adaptation, upon contractile activation, the cytoskeleton becomes a highly dynamic structure with properties comparable to those of a soft glassy material (Bursac et al., 2005; Gunst and Fredberg, 2003). In ASM, cytoskeletal proteins that regulate the structure and organization of the actin cytoskeleton are sensitive to mechanical and contractile stimuli, and contraction of the muscle at a short length results in a shorter, thicker array of cytoskeletal filaments compared to contraction at a longer length (Gunst et al., 1995; Gunst et al., 2003; Gunst and Wu, 2001). There is also evidence of polymerization of actin into thin filaments in response to contractile activation (Herrera et al., 2004; Mehta and Gunst, 1999) as well as to adaptation to a longer length (Herrera et al., 2004). Thus, the dynamic state of the cytoskeleton seems to be key in allowing smooth muscle cells to adapt their shape to accommodate external forces.
The contractile element seems to allow smooth muscle length adaptation via thick filament regulation. This occurs during contractile activation and length adaptation. The amount of myosin polymerization is substantially more than the amount of actin polymerization under the same conditions (Herrera et al., 2004), and thick filaments, unlike thin filaments, show a linear correlation to force production (Kuo et al., 2001). Structural evidence of thick filament evanescence, with thick filaments dissolving partially upon relaxation and reforming during activation was found in electron microscopic studies (Gillis et al., 1988; Herrera et al., 2002; Shoenberg, 1969), studies of birefringence of living tissue (Gillis et al., 1988; Godfraind-De Becker and Gillis, 1988a; Godfraind-De Becker and Gillis, 1988b; Xu et al., 1997) and by X-ray diffraction (Watanabe et al., 1993). Electron microscopic and birefringence studies also show evidence of an increase in thick filament density upon adaptation to a longer length (Herrera et al., 2004; Kuo et al., 2003b; Smolensky et al., 2005).

In 1994, Ford et al. proposed a model by which ASM adapts to an increase in length by plastic alterations that place more myosin thick filaments in series. Since actin thin filaments are in excess of thick filaments in smooth muscle [the ratio of thin to thick filaments is 20-30 to 1 (Herrera et al., 2004)], thick filaments are considered the limiting structure in a contractile unit and therefore an increase in thick filaments is assumed to be representative of an increase in contractile units. Assuming that the properties of individual contractile units are unchanged, an increase in thick filaments in series explains the increased velocity of shortening found at the longer adapted length. A 100%
increase in the length of the adapted muscle resulted in a 67% increase in shortening velocity (Pratusevich et al., 1995).

The model of Ford et al. (1994) was corroborated and further examined by Kuo et al. (2003b). After adapting a muscle strip to twice its original length Kuo et al. found no change in isometric force production, a 69.4% increase in shortening velocity and a 76.0% increase in myosin filament density. This was the first structural evidence of a change in thick filament mass with length adaptation. They also found that muscle power output and energy consumption as measured by ATPase rate had a similar dependence on muscle cell length; 35.4% and 34.6% respective increase for a 50% increase in cell length. These data support the notion that myosin polymerization plays an important role in length adaptation and that a pool of monomeric myosin exists such that thick filaments can form in a matter of seconds to minutes.

The data collected by Kuo et al. could still be explained by a variety of mechanisms, thus Herrera et al. (2005) narrowed down the possibilities by using additional ultrastructural and mechanical data. A schematic of the model that explained all the available data on length adaptation is shown in Fig. 1-4. According to this model, a doubling of the adapted cell length causes no net change in force production since the number of contractile units in parallel decreases by 20% (which would predict a 20% decrease in force) and the length of thick filaments increases by 20% (which would predict a 20% increase in force). It also predicts the observed 67% increase in shortening velocity as the number of contractile units in series increases by this amount, along with a similar
increase in power output, energy consumption, and thick filament density as found by Kuo et al. (2003b). It agrees with the finding that a 100% increase in adapted length caused a 34% increase in the number of dense bodies per cell volume and that maximally shortened muscle length has a linear relationship with the isotonic load imposed on the muscle.
Fig. 1-4. Contractile unit rearrangement in response to length adaptation of a two-fold length change. Left-hand muscle cell is half the length of the other. Model predictions are discussed in text. Thick filaments are shown as thick lines, thin filaments as thin lines and dense bodies as open ovals. Modified from Herrera et al., 2005.
1.7 Length adaptation and disease

Compared to normal airways, asthmatic airways have been shown to be more sensitive and constrict more in response to non-specific stimuli (King et al., 1999). The mechanism of this hyperresponsiveness, however, remains unclear. While chronic asthma is characterized by an increased thickening of all layers in the airway wall, computational modeling suggests that it is the increase in the ASM layer that contributes most to this non-specific hyperresponsiveness (Lambert et al., 2004). This has been corroborated by *in vitro* experiments using dynamically loaded smooth muscle strips to better mimic the *in vivo* effects of breathing and deep inspiration (Oliver et al., 2006). Thus, a major motivation for ASM research lies in its involvement in diseases of airway obstruction such as asthma.

The physiological function which requires ASM to undergo length adaptation is still unknown, however it may play a role in the pathology of airway diseases. For instance ASM plasticity may explain why asthmatics show less bronchodilation or bronchoprotective effects in response to a deep inspiration than control subjects (Scichilone et al., 2001; Wheatley et al., 1989). Chronic airway inflammation may decrease the diameter of the airway wall, thus resting length of ASM, resulting in ASM adaptation to this shorter length. This may hinder the muscle’s ability to undergo stretch-induced relaxation because of higher passive force (Wang et al., 2001), and may also allow the muscle to shorten more as was shown by Herrera et al. (2005) and McParland et al. (2005). In the study of Herrera et al. (2005), isolated porcine tracheal ASM strips
were shown to shorten more if they were adapted at a shorter length. The study by McParland et al. (2005) showed a 1.57-fold increase in shortening amount when bronchial smooth muscle was adapted at approximately half its original length (compared to adaptation at the original length) along with at 1.93-fold increase in developed force and 1.75-fold increase in rate of shortening. If this also occurs in vivo, it could play an important role in airway mechanics during or after an acute spontaneous asthma attack or any disease of chronic airway narrowing, such as COPD.
1.8 Hypotheses and specific aims

While there has been a growing amount of research into the functional and structural changes that occur when smooth muscle is adapted at different lengths, little is known about the changes occurring immediately after a large step-change in length. Thus, the purpose of this dissertation is to fill in some of these gaps.

Force generation, velocity of shortening, and thick filament density were evaluated shortly after quick-stretch (QS) or quick-release (QR) maneuvers in sheep tracheal smooth muscle strips. A QS/QR is a large lengthening or shortening step, respectively, which takes less than 2s and occurs 25s prior to electrical stimulation (allowing the passive viscoelastic tissue response to settle). After taking the desired measurements for the functional studies, the muscle is brought back to the adapted length as soon it returns to a relaxed state.

In regards to the immediate effects of a length change on a smooth muscle strip, we hypothesize the following:

1) A quick-stretch will result in a small drop in force and thick filament density.
2) A quick-release will result in a large and linear drop in force and thick filament density.
3) Both quick-stretch and quick-release will result in no change in shortening velocity.
The rationale for the hypotheses are as follows: Assuming that a length change does not result in a change in cross-bridge activity, there will be an asymmetrical change in the muscle's mechanical properties and ultrastructure in relation to the direction of length change, as illustrated in Fig. 1-5.

Fig. 1-5. Hypothesized model of immediate effects of length change on airway smooth muscle. Thin lines represent thin filaments, thick lines represent thick filaments, cross-bars on thick lines represent cross-bridges, filled circles represent dense bands or bodies. For simplicity only one row of contractile units is shown; in reality many exist in parallel in each cell.

The middle cell of Fig. 1-5 represents the contractile unit arrangement of muscle adapted at 1.5 $L_{\text{ref}}$. According to the model of contractile unit arrangement by Herrera et al. (2005), thick filaments span the entire distance between dense bodies such that there are no regions of non-overlap between myosin thick filaments and actin thin filaments. Though the model gives no information on the length of thin filaments relative to thick filaments (as the lengths of thick and thin filaments seem to be variable), we suggest that thin filaments are longer than thick filaments in the adapted state, thus there are segments of actin filament that are not interacting with thick filaments (not shown in Fig. 1-5). We also suggest that a length stretch or release causes cell membrane attachments of the thin
filament lattice to move further apart or closer together (respectively), pulling the dense bodies apart or allowing them to come closer together.

Hypothesis #1 is based on data from Herrera et al. (2005) where quick-stretches of 10% and 30% caused minor decreases in force (~10%). If the thin filaments are at least 33% longer than thick filaments, a stretch from $1.5 \text{L}_{\text{ref}}$ to $2 \text{L}_{\text{ref}}$ will cause no change in force as regions of thin filaments that previously did not interact with thick filaments become overlapped with thick filaments, resulting in no net change in thick and thin filament overlap. There may be some drop in force due to mechanical perturbation which has been shown to cause disassembly of myosin thick filaments (Kuo et al., 2001; Qi et al., 2002), however we assume this will be minimal due to the gentle length change over a 2-second period (see Materials and Methods).

Hypothesis #2 is also based on data from Herrera et al. (2005) where under non-adapted conditions the ability of the muscle to generate force declines linearly as the muscle shortens. In our model, a 33% release (from $1.5 \text{L}_{\text{ref}}$ to $\text{L}_{\text{ref}}$) causes a 33% loss of overlap between thick and thin filaments as thick filaments either dissolve when they run into adjacent thick filaments or are unable to interact with adjacent thin filaments because of the "wrong" polarity. As such, force drops in proportion to the loss of overlap.

If the changes in force are due to structural changes of the contractile apparatus as opposed to another factor affecting force, we would expect force and thick filament mass to be influenced in a similar way. Like force, a stretch should cause only a small
decrease in thick filament mass whereas a release should result in a large decrease, assuming thick filaments disassemble when they slide over dense bodies (this could prevent unwanted interaction of thick filaments with thin filaments of wrong polarity to generate negative force). Thick filament mass (the length of each filament multiplied by number of filaments) is quantified by the density of thick filaments in a transverse section of an electron micrograph of a smooth muscle cell.

While there have been no previous reports on velocity immediately following a length change, we speculate in hypothesis #3 that velocity will not change. We expect no immediate change in the number of contractile units in series upon stretch or release as this is the simplest case.

To examine these predictions, we will perform the following studies:

1) Measure the changes in force immediately after a quick-stretch or quick-release.
2) Measure the changes in thick filament density immediately after a quick-stretch or quick-release.
3) Measure the changes in shortening velocity immediately after a quick-stretch or quick-release.

All experiments will be done between two lengths ($L_{ref}, 1.6 \ L_{ref}$), and force measurements will also be taken between three lengths ($L_{ref}, 1.5 \ L_{ref}, 2 \ L_{ref}$).
CHAPTER 2. Materials and Methods

2.1 Muscle strip preparation

Sheep tracheas were obtained from a local abattoir (Pitt Meadows Halal Meats Ltd.). Upon sacrifice, tracheas were removed from the carcasses and immediately placed in 4°C physiological saline solution (PSS) (pH 7.4, contents in mM: 118 NaCl, 5 KCl, 1.2 NaH$_2$PO$_4$, 22.5 NaHCO$_3$, 2 MgSO$_4$, 2 CaCl$_2$, and 2g/l dextrose). Prior to experiment, a tracheal segment was removed from the trachea. The in situ length of tracheal smooth muscle bundle connecting the C-shaped cartilage ring was measured. Caution was taken to ensure that the smooth muscle was relaxed when the measurement was made. If the epithelial layer was thrown into large folds, indicating that the underlying smooth muscle was contracted, the trachea was not used for the experiment. Relaxed tracheal rings were then cut open on ventral side. Connective tissue and epithelial layer covering smooth muscle were removed. Muscle bundles of approximately 8 mm long, 1 mm wide, and 0.3 mm thick were dissected out and clipped on both ends with aluminum foil clips for attachment to the force/length transducer.
2.2 **Equilibration**

A muscle strip was connected in a tissue bath to two hooks - one stationary and one connected to the lever arm of a servo-controlled force/length transducer. PSS in tissue bath had previously been heated to 37°C, and pH stabilized by bubbling with carbogen gas (mixture of 95% O₂ and 5% CO₂). During the equilibration and experimental procedures, the muscle strip was activated every 5 minutes with a 12-second electrical field stimulation (EFS) and the PSS in the muscle bath was changed. Muscle equilibration was done before beginning any experiment to allow the muscle to recover from mechanical and metabolic perturbations caused by dissection, lack of perfusion and low temperature. Equilibration was considered complete when stimulations produced a stable maximal isometric force with low resting tension and took around 1.5 hours. During the equilibration period, an experimental reference length (L_{ref}) for the muscle strip was determined using the *in situ* length as a guide, i.e., the amount of stretch in muscle bath experienced by the muscle is approximately the same as that *in situ*.

It is important to note the difference between the adaptation and equilibration processes. In these experiments, equilibration and adaptation were both accomplished by 12-s isometric contractions at 5 minute intervals, but adaptation occurs only after equilibration. The main purpose of equilibration is to allow the muscle to recover from the trauma of dissection while the purpose of adaptation is to allow the muscle to recover from a length change. Thus during adaptation the muscle strip was set at a single length so that the structure of cytoskeleton and contractile apparatus could rearrange to
maximize contractility at that particular length (Kuo et al., 2003b; Pratusevich et al., 1995; Wang et al., 2001). Adaptation was considered complete when isometric force production stabilized at that length, and it usually took 0.5 hour.
2.3 Apparatus

The servo-controlled force/length lever system had a force resolution of 10 μN and a length resolution of 1 μm. The EFS was provided by a 60-Hz alternating current stimulator with platinum electrodes and a voltage of 20-30 V that produced stable, maximal response from the muscle. The analog signals were converted to digital signals by a National Instrument analog-to-digital converter and then recorded by a computer which also controlled the onset and duration of stimulation. This apparatus measured both isometric and isotonic contractions of the muscle, i.e., muscle contractions at a constant length or muscle shortening at a constant load, respectively. More details about the apparatus can be found in publications from the Seow laboratory (Wang et al, 2000, 2002; Kuo et al, 2003).
2.4 Brief overview of experiment procedure

The purpose of this study was to examine ultrastructural and functional changes in a strip of airway smooth muscle immediately after a length change. As discussed in Section 1.8, this was outlined in three specific aims:

1) Measure the changes in force immediately after a quick-stretch or quick-release.
2) Measure the changes in thick filament density immediately after a quick-stretch or quick-release.
3) Measure the changes in shortening velocity immediately after a quick-stretch or quick-release.

For aim #1, quick-stretches (QS) and quick-releases (QR) were performed between three lengths ($L_{ref}$, 1.5 $L_{ref}$ and 2 $L_{ref}$). More specifically, the muscle was adapted at one of these preset lengths then quick-stretched or quick-released to another pre-determined length. The muscle was then adapted at another length and a QS or QR was performed from this new length. All possible length permutations were studied. The force generated by the first contraction after QS or QR was recorded and compared to the force produced at the adapted lengths. (Fig. 2-1)

For aim #2, QS and QR maneuvers were performed between two lengths ($L_{ref}$ and 1.6 $L_{ref}$). After adaptation at $L_{ref}$ or 1.6 $L_{ref}$, a QS or QR to the opposite length was applied, force was recorded and the muscle strip was fixed for transmission electron microscopy.
A control muscle strip that had undergone the same treatment, but without the QS or QR step, was fixed simultaneously. The control strip for QS was fully adapted at $L_{\text{ref}}$, and the control strip for QR was fully adapted at 1.6 $L_{\text{ref}}$. (Fig. 2-2)

For aim #3, the force-velocity curves of a muscle strip adapted at $L_{\text{ref}}$ and 1.6 $L_{\text{ref}}$ were determined, and at each adapted length, a QS or QR was performed to the opposite length. Force and velocity were recorded after the QS or QR, and the positions of the F-V points following the QS or QR were compared to the F-V curves at the adapted lengths. (Fig. 2-3)
Fig. 2-1. **Experimental protocol: change in force after quick-stretch or quick-release.** After equilibration, a single muscle strip was subjected to the following length manipulations. The order of length change was alternated. Arrows pointing right represent quick-stretches, those pointing left represent quick-releases.

<table>
<thead>
<tr>
<th>Pair 1</th>
<th>Pair 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Adapt at $L_{\text{ref}}$</td>
<td>• Adapt at $1.6 , L_{\text{ref}}$</td>
</tr>
<tr>
<td>• Adapt at $L_{\text{ref}}$ then <strong>quick-stretched</strong> to $1.6 , L_{\text{ref}}$</td>
<td>• Adapt at $1.6 , L_{\text{ref}}$ then <strong>quick-released</strong> to $L_{\text{ref}}$</td>
</tr>
</tbody>
</table>

Fig. 2-2. **Experimental protocol: change in myosin filament density after quick-stretch or quick-release.** After equilibration, two pairs of muscle strips from the same trachea were subjected to the following treatments (1 treatment per strip) before force was recorded and strips were fixed for electron microscopy. Pairs 1 and 2 were reversible in order.

<table>
<thead>
<tr>
<th>Part 1</th>
<th>Part 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Adapt at $L_{\text{ref}}$</td>
<td>• Adapt at $1.6 , L_{\text{ref}}$</td>
</tr>
<tr>
<td>• Determine F-V curve at $L_{\text{ref}}$</td>
<td>• Determine F-V curve at $1.6 , L_{\text{ref}}$</td>
</tr>
<tr>
<td>• QS to $1.6 , L_{\text{ref}}$</td>
<td>• QR to $L_{\text{ref}}$</td>
</tr>
<tr>
<td>• Plot F-V point of QS</td>
<td>• Plot F-V point of QR</td>
</tr>
</tbody>
</table>

Fig. 2-3. **Experimental protocol: change in velocity after quick-stretch or quick-release.** After equilibration, a single muscle strip was subjected to the following steps. Parts 1 and 2 were reversible in order.
2.5 Change in force after quick-stretch or quick-release

After equilibration, the shortest and longest muscle lengths that produced nearly maximal isometric force ($F_{\text{max}}$) were identified. Muscle length was recorded during the plateau of an isometric contraction. $F_{\text{max}}$ was identified by adapting the muscle at a length ~1 mm less than in situ length. The muscle was then shortened by small increments until the maximal isometric force produced at that shortened length was around 90% of $F_{\text{max}}$ after adequate time for adaptation. This corresponded to the top of the ascending limb of the adapted length-tension curve and was taken as a reference length ($L_{\text{ref}}$). By lengthening the muscle in increments, the longest muscle length that produced >90% of $F_{\text{max}}$ and <1.5 mN of resting tension after adaptation was also identified. This low resting tension was to ensure that passive elastic components did not contribute excessively to the internal load during a quick length change. A muscle strip was deemed suitable for our experiment if the long length was twice $L_{\text{ref}}$ or greater.

The muscle strip was adapted at 1 of 3 lengths: $L_{\text{ref}}$, 1.5 $L_{\text{ref}}$, or 2 $L_{\text{ref}}$. At each adapted length the muscle was manually quick-stretched (QS) or quick-released (QR) to the other experimental lengths. QS/QR is a large lengthening or shortening step, respectively, which takes less than 2 s and occurs 25 s prior to electrical stimulation (this was to allow the passive viscoelastic tissue response to settle). The force produced by the muscle at this quick-stretched or quick-released length was recorded and the muscle was brought back to adapted length within 25 seconds of stimulation ending (when the muscle had
relaxed). Muscle recovery at the adapted length was achieved by stimulating the muscle electrically every 5 minutes until force production stabilized, usually reaching the $F_{\text{max}}$ it had attained prior to the length step, and for a minimum of 3 stimuli. The order of adapted lengths alternated for every experiment to control for the effects of time or sequence. The experiment was repeated with 10 strips from 3 animals. (Fig. 2-1).

The force produced after QS or QR was expressed as a percentage of the force when the muscle was adapted at the QS or QR length. For example, the force produced after a quick-release to $L_{\text{ref}}$ would be normalized by the maximal force of the muscle adapted at $L_{\text{ref}}$. This was to account for slight differences in maximal force at different lengths since the plateau of the length-tension curve of smooth muscle is not perfectly horizontal (Pratusevich et al., 1995).

An experiment example is as follows. At each length (adapted, quick-stretched/released) the force was recorded. Muscle was adapted at $1.5 \, L_{\text{ref}}$, quick-released to $L_{\text{ref}}$, stimulated by EFS then brought back to $1.5 \, L_{\text{ref}}$ within 25 s after stimulation. Force recovered at $1.5 \, L_{\text{ref}}$ before a quick-stretch and stimulation at $2 \, L_{\text{ref}}$, then it was adapted to $2 \, L_{\text{ref}}$, quick-released to $1.5 \, L_{\text{ref}}$ and $L_{\text{ref}}$, and finally adapted at $L_{\text{ref}}$, then quick-stretched to $1.5 \, L_{\text{ref}}$ and $2 \, L_{\text{ref}}$. 

31
2.6 Change in myosin filament density after quick-stretch or quick-release

This experiment was performed with two pairs of adjacent muscle strips from a single trachea. As previously described, the longest and shortest (L_{ref}) muscle lengths that produced near maximal force were found for each strip. The important lengths for this experiment were L_{ref} and 1.6 L_{ref} thus the strips were deemed suitable if each had a longest length (which maintained F_{max}) greater or equal to 1.6 L_{ref} and if F_{max} and L_{ref} were similar for strips in a pair. Force was recorded and strips were fixed for electron microscopy at four points: adapted at L_{ref}, adapted at L_{ref} then QS to 1.6 L_{ref}, adapted at 1.6 L_{ref}, and adapted at 1.6 L_{ref} then QR to L_{ref} (Fig. 2.2). Fixation was done 50 seconds after the final electrical stimulation, once the muscle had returned to its relaxed state. Three animals were used for this experiment.
2.7 Electron microscopy

Strips were fixed while they were still attached to the experiment apparatus for 15 minutes with a 37°C fixative solution of 2% gluteraldehyde, 2% formaldehyde and 2% tannic acid, 0.1M Na-cacodylate buffer, pH 7.3. Care was taken not to disturb the strips during this initial fixation. Strips were then removed from tissue bath, cut into 6-8 pieces so they measured less than 1 mm in length, and immersion fixed in fresh fixative for 2 hours. Samples were rinsed three times with 0.1 M Na-cacodylate buffer (pH 7.3), 10 minutes per rinse. Secondary fixation was done for 2 hours with 1% osmium tetroxide in buffer (diluted from 2% osmium tetroxide on day of use). Samples were again rinsed with buffer 3 times, 10 minutes per rinse, and finally stored overnight at 4°C in fresh buffer. Except for the initial fixation and overnight storage, all steps previously described were performed on ice, on a shaker in fumehood. The following day, samples were rinsed 3 times for 10 minutes in distilled water, then en bloc stained with 1% uranyl acetate for 1 hour. Dehydrations were performed with 10 minutes each of 50%, 75%, 80%, 90%, 95% ethanol, then 3 times in each 100% ethanol and propylene oxide. Infiltration with freshly mixed resin (TAAB 812 mix, medium hardness) was done by diluting with propylene oxide in a ratio of 1:3 then 3:1 for 30 minutes per dilution. Infiltration with pure resin was done for 1 hour, then overnight in fresh resin. All steps of day 2 were done on a shaker in fumehood. Finally, tissue samples were placed in labeled moulds and baked in resin at 60°C overnight.
Blocks were sectioned on a Leica EM UC6 ultramicrotome with a diamond knife at 50-70 nm thickness and sections placed on 400-mesh copper grids. Grids were stained with 1% uranyl acetate for 4 minutes and Reynolds lead-citrate for 3 minutes before being viewed under a FEI Technai 12 transmission electron microscope. For each experimental group (example of one group is "animal 3, quick-stretched from L_{ref}"), a minimum of two blocks were sectioned and multiple grids per block were imaged.

In each group, images of 15 cells in cross-section were collected; 5 cells smaller than 2.5µm in diameter, 5 cells larger than 2.5µm in diameter with no nucleus, and 5 cells with nucleus (usually larger than 2.5µm in diameter). This ensured that we gathered an equal amounts of information from different areas of the cell; near tapered cell end, near central cell segment, and at central segment with nucleus, respectively. Images were taken with a digital camera (Gatan BioScan, Model 792) at a magnification of 37 000X, so to capture the cross-section of a single cell often required taking multiple images and reconstructing the whole cell cross-section in Photoshop®.
2.8 Morphometric analysis

Image-Pro Plus software (version 4.0) was used to find thick filament density in cell cross-sections. Density is equal to mass divided by volume, and since cell volume was assumed to be independent of cell length, thick filament density is an indirect measure of the mass or content of thick filaments in a smooth muscle cell (Kuo et al., 2003b). Thick filaments were counted in a cell cross-section using the "manual tag" function. The cytoplasmic area of a cell cross-section was found by subtracting the area taken up by the nucleus and organelles from the area of the entire cell. Thick filament density was calculated by dividing the number of thick filaments in a cross-section by the cytoplasmic area to give a value in #/μm². Thick filament density was compared within pairs.

Samples were "masked" on day 2 of EM preparation protocol and remained masked until after morphometric data had been analyzed. Thus, the group identity of the samples (adapted at L_{ref} / QS / adapted at 1.6 L_{ref} / QR) were hidden from the experimenter until morphometric analysis was completed. This ensured unbiased counting of thick filaments.
2.9 Change in velocity after quick-stretch or quick-release

After equilibration, the shortest \(L_{\text{ref}}\) and longest muscle lengths that produced near maximal force were identified as in Section 2.5. This experiment was performed at two lengths, \(L_{\text{ref}}\) and 1.6 \(L_{\text{ref}}\), thus the strip was deemed suitable for experiment if the longest length was greater or equal to 1.6 \(L_{\text{ref}}\). Starting length of \(L_{\text{ref}}\) or 1.6 \(L_{\text{ref}}\) was alternated, with two experiments starting at \(L_{\text{ref}}\) and two at 1.6 \(L_{\text{ref}}\) for a total of four experiments or four animals.

The force-velocity (F-V) curve at \(L_{\text{ref}}\) and 1.6 \(L_{\text{ref}}\) was determined for each tissue. This was accomplished by measuring the maximal shortening velocity during isotonic contractions at \(\sim 75\%\), \(50\%\), \(30\%\), \(20\%\) and \(15\%\) of maximal isometric force. [\(F_{\text{max}}\) was determined prior to every isotonic shortening.] The F-V points at \(L_{\text{ref}}\) were fit to Hill’s hyperbola (Hill, 1938) which is defined by the equation \((F+a)(V+b) = b(F_{\text{max}}+a)\) where \(F\) is the isotonic load, \(F_{\text{max}}\) is the maximal isometric force, \(V\) is the shortening velocity, and \(a\) and \(b\) are Hill’s constants. To find the change in velocity from \(L_{\text{ref}}\) to 1.6 \(L_{\text{ref}}\), F-V points at 1.6 \(L_{\text{ref}}\) were fitted by scaling up the F-V curve at \(L_{\text{ref}}\). SigmaPlot® software (Version 8.02) was used to plot the curves.

After making the F-V curve at each length, the muscle was twice quick-stretched/released to the opposite length; once to measure the force produced after the QS/QR, and after re-adapting the muscle to the original length, the second time with the isotonic load set between 12%-24% of the force found at the length step. Although changes in velocity
become more sensitive as \(\%F_{\text{max}}\) decreases (i.e., the slope of the F-V curve increases in absolute value), the velocity of QS and QR was measured between 12-24\% F_{\text{max}} to avoid instability of the force/length transducer at values below 10\% F_{\text{max}}. Velocity at the quick-stretched/released lengths were examined and compared to the velocity of the muscle at the adapted lengths. (Fig. 2-3)
2.10 Statistical analysis

In all experiments, data from each animal was averaged before averaging the means from different animals. Unless otherwise noted, data is shown as means ± standard error of mean (SE). Significant difference was determined using the Student’s paired t-test, significance being $P<0.05$. 
3.1 Change in force after quick-stretch or quick-release

The purpose of this study was to examine the effects of a large step-change in length on force generation in sheep tracheal muscle. This was done by subjecting a single muscle strip to quick-stretches and quick-releases from three adapted lengths (L_{ref}, 1.5 L_{ref}, and 2 L_{ref}) and measuring isometric force generation immediately after the length-step. The average $F_{\text{max}}$ for the three animals in this group was 129.6±5.3 kPa. Fig. 3-1 shows the changes in force immediately after a quick-stretch or quick-release in kPa. Fig. 3-2 shows changes in force in relative terms such that the force produced immediately after a QS or QR is normalized by the maximal adapted force ($F_{\text{max}}$) produced at that QS/QR length. In other words the QS/QR force was normalized by the force of the muscle when adapted to the QS/QR length. This normalization simplifies results by accounting for slight differences in $F_{\text{max}}$ at different lengths.

Results of these experiments suggest that a quick-release causes a larger decrease in force production than a quick-stretch, i.e., that shortening an ASM strip causes more disruption than stretching it. Fig. 3-3 shows the $P$-values of a Student’s paired t-test when the relative force change of a quick-stretch was compared to that of a quick-release of a similar length step. Significant difference ($P<0.05$) between shortening and lengthening was seen especially when the change in length was large (i.e., 100%). The directional effect also seemed to be relatively insensitive to the absolute length to/from which it was
shortened. This was demonstrated at the intermediate length: the decrease in force from 1.5 $L_{ref}$ to 2 $L_{ref}$ was 8.75±3.41% and from 1.5 $L_{ref}$ to $L_{ref}$ was 20.44±0.36% ($P=0.07$).

From a structural point of view, force generation is proportional to the number of contractile units in parallel or a loss of thick and thin filament overlap. Results of this study suggest that a QR causes a greater loss of these units than a QS, assuming other factors influencing muscle force remain the same. This will be further investigated in the next group of experiments presented in this dissertation, using the density of myosin (thick) filaments in a cell cross-section as a measure of contractile unit mass (Section 3.2).
Fig. 3-1. Change in force (kPa) upon quick-stretch or quick-release. A single muscle strip was subjected to quick-stretches and quick-releases (as shown by grey arrows) from three adapted lengths. Isometric force generation was measured immediately after the length-step. Data shown are means ± SE.
Fig. 3-2. Relative change in force ($\%F_{\text{max}}$) upon quick-stretch or quick-release.

Data from Fig. 3-1 is normalized to the maximal force ($F_{\text{max}}$) produced at each QS/QR length. Data shown are means ± SE.
Quick-stretch | Quick-release | P-value
---|---|---
$L_{ref}$ to $1.5 \ L_{ref}$ | $1.5 \ L_{ref}$ to $L_{ref}$ | 0.01
$L_{ref}$ to $2 \ L_{ref}$ | $2 \ L_{ref}$ to $L_{ref}$ | <0.01
$1.5 \ L_{ref}$ to $2 \ L_{ref}$ | $2 \ L_{ref}$ to $1.5 \ L_{ref}$ | 0.73
$1.5 \ L_{ref}$ to $2 \ L_{ref}$ | $1.5 \ L_{ref}$ to $L_{ref}$ | 0.07

**Fig. 3-3. Quick-stretch vs. quick-release.** The change in relative force due to a similar length change but in different directions (stretch vs. release) is examined via a Student’s paired t-test.
3.2 Change in thick filament density after quick-stretch or quick-release

Four strips of trachealis from a single animal were fixed at four conditions; adapted at \( L_{\text{ref}} \), adapted at \( L_{\text{ref}} \) then QS to 1.6 \( L_{\text{ref}} \), adapted at 1.6 \( L_{\text{ref}} \), and adapted at 1.6 \( L_{\text{ref}} \) then QR to \( L_{\text{ref}} \). This allowed the examination of the immediate effects of length change on myosin thick filament assembly and disassembly, and verification of their effects on force generation. To look for structural changes in the filament lattice, thick filament density in cell cross-sections was measured by electron microscopy. Force was measured just prior to muscle fixation. Three animals were used for this study.

Fig. 3-4 is an example of an electron micrograph of a sheep trachealis smooth muscle cell in transverse section. Included is a detailed list of the data obtained from this cell, such as a breakdown of cellular areas. Note that these data were obtained under "masked" conditions, thus the experimenter was without knowledge of the experimental group the sample belonged to. Fig. 3-5 is a magnified portion of Fig. 3-4 with dense-bodies and thick, thin and intermediate filaments pointed out. Thick filaments were identified by their non-uniform cross-section of about 12-20 nm in “diameter”, thin filaments by their 6-7 nm diameter and approximately circular shape, and intermediate filaments by their perfectly round shape and consistent 10 nm diameter. Fig. 3-6 shows examples of electron micrographs from each experimental group and includes the thick filament density of each cell.
A summary of results from all three animals is shown in Figs. 3-7 and 3-8. Fig. 3-7 shows the thick filament density in every experimental group, sub-grouped by the area of the cell at which the filaments were counted. Fig. 3-8 shows the average results of changes in thick filament density and compares them to changes in force upon QS or QR. These data indicate that myosin filament density does not decrease significantly from control upon QS, but does decrease significantly from control upon QR. The filament density drops by 19.0±3.2% for a 60% decrease in length. The decrease in force is similar to what would be expected from the first study of this dissertation, with QR again causing a greater loss in force than a QS (Section 3.1). QS caused a decrease in force of 5.0±2.5% and QR caused a decrease in force of 17.2±5.3%. Since the change in force shows a similar trend to the change in density, we can extrapolate to suggest that the changes in force seen between the three different lengths in Section 3.1 study may also be caused by a change in thick filament density. However to be sure, experiments measuring thick filament density at these three lengths would be necessary.
Fig. 3-4. Electron micrograph of a sheep trachealis muscle cell in transverse section, and experimental data. QS group, sectioned through central segment of cell, animal 3. Total cell area, 7.283μm²; area of nucleus, 4.252μm²; area of mitochondria, caveolae and other organelles, 1.505μm²; number of thick filaments, 136. Therefore cytoplasmic area of cell is 1.546 μm² and thick filament density is 89.12/μm². Scale bar represents 0.5μm.
Fig. 3-5. Magnified portion of previous image. Bottom right-hand corner of Fig. 3-4. Arrowheads show dense bodies, large arrows point to thick filaments surrounded by thin filaments, small arrows point to intermediate filaments. Scale bar represents 0.5μm.
Fig. 3-6. Examples of electron micrographs from all experimental conditions.

(a) Adapted at L_{ref}, sectioned near tapered cell end as evidenced by the size, animal 3. Thick filament density is 90.36/μm². Magnification 37000x. (b) QS, sectioned near central segment as evidenced by the size, animal 2. Thick filament density is 51.52/μm². Magnification 23000x. (c) Adapted at 1.6 L_{ref}, sectioned near central segment as evidenced by the centrally clustered mitochondria, animal 1. Thick filament density is 60.25/μm². Magnification 23000x. (d) QR, sectioned near the central cell segment, animal 1. Thick filament density is 42.82/μm². Magnification 23000x. All scale bars represent 0.5μm.
Fig. 3-7. **Thick filament density of four conditions sub-grouped by location along long axis of cell.** Each solid-coloured bar shows averaged data of 5 cells per animal, from 3 animals, thus 15 cells per bar. Striped bars represent overall average of other 3 bars of the group, thus 45 cells each. Thick filament density is in number/μm². Data shown are means ± SE.
Fig. 3-8. Fractional change in force or thick filament density upon quick-stretch or quick-release. Control bars represent pre-QS or pre-QR values (i.e., adapted at $L_{\text{ref}}$ or $1.6 \ L_{\text{ref}}$ respectively). Fractional change in force is calculated from single muscle strips. Fractional change in filament density is calculated from paired muscle strips from the same trachea. $P$-values show results of Student's paired t-test against their respective controls. Data shown are means ± SE.
3.3 Change in velocity of shortening after quick-stretch or quick-release

In this group of experiments, we examined the acute effects of a large length-step on the shortening velocity of trachealis muscle. A single strip of muscle was adapted at two lengths, $L_{ref}$ and $1.6 \times L_{ref}$. At each adapted length, force-velocity curves were plotted by subjecting the strip to isotonic contractions against loads of 10-75% $F_{max}$. At each adapted length, the strip was also subjected to two quick-stretch or quick-release maneuvers to the alternate length, and immediately stimulated. The first maneuver was to measure the force ($F_{max}$) generated after QS/QR, and the second was to find the velocity of shortening by contracting against an isotonic load of 12-24% of the found $F_{max}$. Thus, each QS or QR had a F-V point associated with it and the position of these points relative to the F-V curves at adapted lengths was examined.

These experiments were carried out using four tracheas yielding an average $F_{max}$ of $162.2 \pm 14.3$ kPa. Averaged F-V points are shown in Fig. 3-9, along with curves fitted to these points. Filled circles represent F-V points from strips that were adapted at $L_{ref}$ and open circles represent F-V points adapted at $1.6 \times L_{ref}$. Solid line is Hill’s hyperbola that best fits all points adapted at $L_{ref}$ (thus all filled symbols) and the dashed line represents the curve fitted to $L_{ref}$ scaled vertically by a factor of 1.42. This was the scaling factor that most closely fit the points adapted at $1.6 \times L_{ref}$, and is also the average scaling factor ($\pm 0.05$) for all four experiments. It suggests that a 60% increase in adapted length causes a 42% increase in shortening velocity. This is close to what would be expected. Pratusevich et al. (1995) showed a 67% increase in velocity after a 100% increase in
adapted length in canine trachea, and Kuo et al. (2003b) and Herrera et al. (2005) showed a 69% and 64% velocity increase after a similar length change in porcine trachea.

The average change in velocity immediately after QS and QR are shown as red squares in Fig. 3-9; the filled square represents the change after stretch and the open square represents the change after release. The results of these experiments suggest that a quick length-step causes an immediate change in shortening velocity in airway smooth muscle, such that the velocity becomes identical to the velocity at the adapted QS/QR length. For example, when a strip is adapted at $L_{ref}$ and quick-stretched to 1.6 $L_{ref}$, the velocity is closer to that of a strip adapted at 1.6 $L_{ref}$ than it is to a strip that has been adapted at $L_{ref}$, and vice-versa.

The average velocity after QS and QR are presented as bar graphs in Fig. 3-10. Shortening velocities immediately after QS at loads between 18-24% $F_{max}$ (average 21.2±1.2%) was found to be 0.265±0.011 $L_{ref}$/s (middle bar of Fig. 3-10a). The expected velocity of strips adapted at $L_{ref}$ and 1.6 $L_{ref}$ were calculated at the same load by substituting the %$F_{max}$ of the QS into the equations of the fitted curves of each experiment. The average expected velocity of a strip adapted at $L_{ref}$ was 0.157±0.009 $L_{ref}$/s and that of a strip adapted at 1.6 $L_{ref}$ was 0.228±0.015 $L_{ref}$/s (respectively, left and right bars of Fig. 3-10a). Therefore even though the strip had been adapted at the lower length, upon QS the velocity immediately increased to 72.2±9.4% above the value measured at this lower length and even 20.4±5.6% above the velocity measured at the longer adapted length. [We suggest that the velocity after QS went beyond the velocity at
1.6 \text{L}_{\text{ref}} \text{ due to an artifact of elastic recoil. This would have caused an increase in the passive tension upon quick-stretch. If it had been corrected for, it would have shifted the QS point further left on the F-V curve and closer to the expected velocity at 1.6 \text{L}_{\text{ref}}.}

The results of QR experiments were similar. The average velocity following a QR was found at an average \%F_{\text{max}} \text{ of 15.3±1.2}. This velocity value was 0.197±0.005 \text{L}_{\text{ref}}/\text{s}. The velocities when adapted at \text{L}_{\text{ref}} \text{ and 1.6 \text{L}_{\text{ref}}} \text{ (found at \%F_{\text{max}} \text{ between 12-18\% by the method described above}) was found to be 0.196±0.013 and 0.284±0.022 \text{L}_{\text{ref}}/\text{s respectively (Fig. 3-10b). Thus, there was a 25.1\%±4.5 drop in velocity after QR from 1.6 \text{L}_{\text{ref}} \text{ and no significant difference between the velocity after QR and after adaptation to \text{L}_{\text{ref}}. In other words, the velocity upon QR immediately dropped to the velocity of the strip when adapted that shorter length. The expected velocities are higher in the case of QR than in QS because they were calculated at a slightly lower, but not significantly different, \%F_{\text{max}} \text{ and therefore further left along the adapted F-V curves.}

Since velocity was shown to be related to the number of contractile units in series (Lambert et al., 2004), the results shown here suggest that a length change has an immediate impact on contractile unit placement. In the case of muscle shortening, contractile units are immediately taken out of series and require no additional change (in terms of number) during the adaptation process. In the case of muscle lengthening, contractile units are immediately added in series. A proposed model of how this occurs is presented in the discussion (Section 4.5).
Average F-V and QS/QR points

Fig. 3-9. Velocity of shortening after quick-stretch and quick-release. Filled circles represent velocity measured from trachealis strips that were adapted at $L_{\text{ref}}$ and open circles represent velocity when same strips were adapted at 1.6 $L_{\text{ref}}$. Solid line is Hill’s hyperbola that best fits all points adapted at $L_{\text{ref}}$ and dashed line represents the curve fitted to $L_{\text{ref}}$ scaled vertically by a factor of 1.42. QS and QR points are shown as squares; the filled square represents velocity immediately after a QS and the open square represents velocity immediately after a QR.
Fig. 3-10. Shortening velocities before length change, immediately after length change, and after adaptation to new length. Middle bars show actual measured velocity (L_ref/s) after QS or QR. Velocity values from remaining bars were calculated from curves fitted to F-V measurements of length adapted muscle at the same relative load (%F_{max}) as QS or QR measurement was taken. In both cases, QS or QR velocity was closer to that observed when adapted at the stretched or released length. All values are significantly different (example is marked by asterisk) except V after QR and expected V at L_ref (P-value is shown). (a) Velocity comparison after QS, i.e., velocity at 21.2±1.2 %F_{max} (b) Velocity comparison after QR, i.e., velocity at 15.3±1.2 %F_{max}.
3.4 Summary of results for a 60% length change

The results of a length change between \( L_{ref} \) and \( 1.6L_{ref} \) are summarized in Fig. 3-11. This highlights airway smooth muscle's asymmetrical response to length change, shortening being more disruptive to force and filament density than lengthening, and also shows the correlation between force and myosin filament density. Finally, it shows that velocity changes immediately and significantly upon a 60% length change.

|                         | Quick-stretch \((L_{ref} \text{ to } 1.6 \ L_{ref})\) | Quick-release \((1.6 \ L_{ref} \text{ to } L_{ref})\) |
|-------------------------|-----------------------------------------------------|
| force                   | 0.95 ± 0.02                                         | 0.83 ± 0.05                                       |
| myosin filament density | 0.98 ± 0.01                                         | 0.81 ± 0.03 *                                     |
| velocity                | 1.72 ± 0.09 *                                       | 0.75 ± 0.04 *                                     |

Fig. 3-11. Summary of relative change of quick-stretch or quick-release from control \((L_{ref} \text{ and } 1.6 \ L_{ref} \text{ respectively})\). *Statistically significant difference from control value (Student's t-test, \(P<0.05\)). †Statistically significant difference from control value (Student’s t-test, \(P<0.10\))
CHAPTER 4. Discussion

4.1 Result summary

In examining the mechanical behaviour and ultrastructure of airway smooth muscle immediately following a change in length, we have found that these are affected asymmetrically. A passive shortening of the muscle results in a larger drop in force and thick filament density than an equivalent stretching of the muscle. For example, a 60% length increase causes no significant drop in force, unlike the 17.0±5.0% drop in force with a similar length decrease. Changes in force are reflected by changes in myosin filament density, suggesting that the mechanical responses observed are due to structural changes within the muscle as opposed to other factors that affect force. The response of velocity to length change was opposite to what was hypothesized. While we expected to see no change in velocity of shortening within the first contraction after a length step, in fact the velocity rapidly changed to its value if adapted at the QS or QR length.

As will be argued in the following sections we suggest that the results from this study occur after thin filament lattice adaptation is complete, but while thick filament polymerization is as yet incomplete. Therefore the changes in velocity and force, and thus adaptation of the thin filament lattice and polymerization of thick filaments within the lattice, are regulated by separate mechanisms and occur at different speeds.
4.2 Effects of quick-stretch and quick-release on force development

As hypothesized, quick-release resulted in a larger drop in force compared to a quick-stretch of a similar length change. However the decrease in force due to QR was not linear, nor as much as was expected. These observations came from two studies that measured force directly after changes between 2 or 3 lengths. Possible mechanisms underlying the observations are stipulated below.

Stretch can cause a loss of force by two mechanisms; 1) mechanical disruption which causes thick filament disassembly in relaxed muscle (Qi et al., 2002) and 2) stretch pulls filaments apart, reducing thin and thick filament overlap. The contribution of the second mechanism depends on the thin filament length relative to the thick filament length, and the amount of stretch. If thin filaments are 50% longer than thick filaments, this mechanism would not be important in a stretch of 50% or less. However there is no quantified relationship between the amount of length change and the amount of filament disassembly, nor do we know the ratio of thin to thick filament length. Thus we cannot differentiate whether stretch causes a reduction in force due to one or both mechanisms.

Release also affects force due to mechanical disruption, but there appears to be another mechanism which makes release much more disruptive than stretch. We suggest that this additional mechanism stems from the structure of the contractile unit of smooth muscle as described by Herrera et al. (2005). A lack of non-overlap areas of thick and thin
filaments in the contractile units of adapted muscle necessitates that shortening of muscle leads to a reduction of the overlap zone, and hence force production.

The drop in force associated with QR is not as much as was expected from data by Herrera et al. (2005), nor is it linear with respect to the magnitude of length release. By superimposing data from Section 3.1 on the L-T curve from Herrera et al., the difference in predicted and actual results can be quantified (Fig 4-1). This difference is attributed to a small amount of adaptation that occurs in our specimen during the contractile activation following QR. Thus, the specimen is neither fully adapted to its pre-release length, in which case its L-T points would be along the predicted (dashed) line, nor is it fully adapted to its QR length, in which case it would contract at 100% F\text{max} (dotted line). The specimen recovered by 20.3±3.2% of isometric force after a 25% QR (2 L\text{ref} to 1.5 L\text{ref}), 21.7±0.4% after a 33% QR (1.5 L\text{ref} to L\text{ref}) and 15.3±2.3% after a 50% QR (2 L\text{ref} to L\text{ref}). The force recovery therefore generally seems to be less with greater length step of quick-release.

Because of the rapidity of the length adaptation process, designing an experiment that disallows reorganization of the sub-cellular structures (i.e., that is capable of observing the \textit{immediate} effects of length change) but takes functional measurements is not always possible. Herrera et al. (2005) circumvented this issue in their QR experiments by measuring the maximally shortened muscle length at isotonic loads between 10-90% F\text{max}, taking advantage of the fact that there is little time for adaptation during continuous active muscle shortening. The protocol for the present experiments involves bi-
directional changes in length. Since imposing a large stretch on an activated muscle can cause damage to the muscle, we have adopted a protocol of changing muscle length during the relaxed state. Because one of the primary objectives is to determine the symmetry of adaptation in stretched and shortened muscle, length changes in both directions were carried out in the relaxed state to ensure that any asymmetry observed was due to direction of length change and not other factors such as state of activation. One shortcoming of this approach is that partial adaptation occurs between the time of length change and the time isometric force is measured, as shown in Fig. 4-1.

The current QS results are comparable to the data presented in Herrera et al. (2005) probably because both groups performed these experiments in a similar way. Herrera et al. found that a 10% QS caused a 6% decrease in the force associated with that stretch, and a 30% QS caused ~10% decrease in force. In the current data, a QS of 33% (from $1.5 \text{ L}_{\text{ref}}$ to $2 \text{ L}_{\text{ref}}$) caused a drop in force of $8.7\pm3.4\%$. These data may also have come from muscle that had undergone partial adaptation to the new length, however for the purpose of the model presented later, we assume for simplicity that QS causes only this minor drop in force with or without adaptation having occurred.
Fig. 4-1. **Partial adaptation during activation post-QR.** Dashed line shows length-force relationship of ASM strip fully adapted only at $L_{\text{in situ}}$ (equivalent to $L_{\text{ref}}$) and not adapted at the shortened lengths (solid symbols with error bars. From Herrera et al., 2005). Open circles show length-force results from Section 3.1 normalized to $L_{\text{in situ}}$ and assumed to be only partially adapted to a particular length (see text). The difference in % isometric force of open circles and reference line represents the % isometric force recovery in the current experiments. For example, the % force recovery after a 50% QR (shown by bracket) corresponds to 15.3±2.3%. If ASM strip was fully adapted at 0.5 $L_{\text{in situ}}$, the % isometric force would be 100%, as indicated by the dotted horizontal line.
4.3 Effects of quick-stretch and quick-release on myosin filament density

In this group of experiments we looked at the effects of a 60% length change on force production and myosin filament density, and found that the changes in force were reflected in the changes in density (Figs. 3-8 and 3-11). This provides strong evidence that the change in force we observed were indeed caused by a change in the structure of the contractile apparatus as opposed to other variables that influences force. [More discussion on factors influencing force will be presented later.] The correlation between force and myosin filament density also implies that myosin filaments have undergone partial adaptation (i.e., re-polymerization) along with force.

Ultrastructural data from this study shows that myosin polymerization in response to a length change is not complete by the first contraction after the length change. Based on data by Kuo et al. (2003b), a 60% increase in length should result in a 33.6% increase in myosin filament density after adaptation to the stretched length, but the present results showed that no additional myosin polymerization occurred immediately after the 60% stretch. Length adaptation in the opposite direction should cause myosin depolymerization of thick filaments, and while some depolymerization had occurred after the initial contraction at the short length, the extent of depolymerization was less than expected. Adaptation from $1.6L_{\text{ref}}$ to $L_{\text{ref}}$ should cause myosin filament density to decrease by 25% (Kuo et al, 2003), however it only decreased by 19.0%. This could be due to a less-than-expected degree of depolymerization with the length change, or due to some recovery after the length change.
To have a better correlation between force and myosin density, it would have been preferable to measure myosin filament density in the activated state. This could also have reduced the effects of length adaptation as the time after stimulation at the new length would have been reduced. However to fix a strip in the activated state requires the plateau of contraction to be maintained. This cannot be accomplished by electrical field stimulation (EFS), which is how all the previous measurements were made, but by stimulation with acetylcholine (Ach). Since acetylcholine also causes a higher level of muscle activation than EFS, fixing the tissue in the activated state would have made it difficult to compare our previous force measurements by nullifying the baseline force as measured by EFS. We could not compare force measurements within a strip, or with force measurements made in our other studies. Therefore fixing in a relaxed condition was a useful compromise.

Although it would have been expected by previous reports, we saw no increase in thick filament density of muscle strips adapted and fixed at $L_{ref}$ compared to strips at $1.6L_{ref}$ (Herrera et al., 2004; Kuo et al., 2003b). This could be because strips adapted at $L_{ref}$ and $1.6L_{ref}$ were not paired in this study. They were controls for QS and QR groups, respectively, therefore they were paired with their experimental equivalent. Pairs may have differed if their absolute lengths were not identical. They may also have differed if structural changes occurred due to time or treatment, as one pair of dissected strips was placed at $4^\circ$C while waiting for the other to be fixed. The order of QS and QR experiments was alternated to control for treatment differences between pairs.
4.4 Effects of quick-stretch and quick-release on velocity of shortening

While the results of the force and density experiments did not stray too far from what was expected, the immediate changes in velocity upon length change was contrary to what was hypothesized. Shortening velocity immediately changed to the value it would be after adaptation to its new length. However, if our force results have been affected by some length adaptation (Section 4.1), it would also seem likely that the velocity results have been similarly affected. We propose that the rearrangement of contractile units in series is actually complete by the first contraction after a length step and that no further rearrangement occurs during the adaptation process.

The idea that reorganization of contractile units in series is much faster than a change in thick filament number or length does not seem unreasonable; thin filaments are much less structurally labile and in vast abundance over thick filaments in a smooth muscle cell (Herrera et al., 2004). It is possible that thin filaments are pre-arranged into a lattice which is easily rearranged, maybe via regulation by intermediate filaments (Fig. 4-2), whereas thick filaments, which require re-polymerization after mechanical perturbation or contractile unit shortening, require more time to adapt to a length change. Intermediate filaments are a good candidate for rearranging the actin filament lattice because they are abundant in smooth muscle, associate with dense bodies and dense bands, and have previously been shown to affect mechanical properties of smooth muscle (Small and Gimona, 1998). There is also an increasingly popular notion that thin filaments (or thin filament associated proteins such as caldesmon) provide a framework
upon which myosin monomers can polymerize into thick filaments (Gunst and Tang, 2000; Katayama et al., 1995; Kudryashov et al., 2002; Seow, 2005; Small et al., 1992). This suggests that the thin filament lattice must be in place before myosin polymerization can occur, also backing up the idea that alterations in the thin filament lattice are faster than those of the thick filament lattice.
Fig. 4-2. A proposed model to explain the rearrangement of contractile units in series in response to length change. Intermediate filaments (shown as thin grey non-straight lines) are attached to dense bodies in sequence such that stretching the cell brings additional contractile units into the series with little disruption of existing units.
4.5 The intermediate state after quick-stretch or quick-release

We believe that the current results may not reflect the most immediate effects of a length change, i.e., when the muscle has not yet undergone any adaptation to its new length. Our findings may instead reflect a state of partial adaptation of the muscle to its new length. At this intermediate state, the rearrangement of contractile units in series is complete, but the repolymerization of thick filaments is not. We have modified our hypothesized model of structural rearrangements occurring upon length change to include this intermediate state (Fig. 4-3). Mechanical behaviour (force and velocity) and structural outcomes (myosin filament density) of each of the three steps after length change shown in our model are described in Fig. 4-4.

There are most likely several steps that occur during length adaptation, but we highlight three in our modified model. The first step after length change is identical to that hypothesized in Section 1.8 (Fig. 1-5). We believe this is the outcome of length change from 1.5 L_{ref} if no adaptation to the new length has occurred. The force, velocity and filament density associated with this state are shown with a dotted line in Fig. 4-4. The second step after the length change would be the intermediate state that we observed in our protocol by the first contraction after length change. This is represented by a pink line in Fig. 4-4. Finally, full adaptation to the new length is shown as the last step of the length change and is represented as a solid black line in Fig. 4-4.
The first step after length change is expected to look as such for the reasons discussed in the Section 1.8. Briefly, the expected force after QS or QR comes from data by Herrera et al. (2005) where a 33% QR caused a 40% decrease in force (shown in Fig. 4-1) and a 30% QS caused a ~10% drop in force. Myosin filament density shows the same relative response as force since the current and previous data show that they are correlated. To simplify the modified model by showing only one row of contractile units, the decrease in force is shown as a decrease in thick filament length (but in fact, the decrease in force and density could be accomplished by either a decrease in thick filament length, a decrease in contractile units in parallel, or a combination of both). The velocity of shortening while the muscle has not undergone any adaptation to the new length has yet to be shown, but we speculate it is as described in the graph (discussed in Section 1.8 and earlier in this section), and is represented in the model by no change in the number of contractile units in series.

The intermediate state is expected to look as such because of the data presented in this dissertation. The force data is shown as in Fig. 3-2, and the filament density data is again assumed to match the force by changing thick filament length in the model. As velocity at this intermediate state was shown to be identical to the value at its newly adapted length (Section 3.3), the velocity graph shows the pink line following the black line, and the model shows that the number of contractile units in series is the same as after full adaptation to the new length.
The structural manifestations of a QS at the intermediate state (compared to the pre-stretched state) would be the addition of contractile units in series to accommodate the increase in velocity. There would also be a concomitant decrease in filament overlap of the original contractile units to account for the small drop in force and myosin filament density. At the intermediate state, a 33% stretch (i.e. from 1.5 $L_{ref}$ to 2 $L_{ref}$) would result in a 22% increase in velocity and contractile units in series, since a length doubling causes a 67% velocity increase (Herrera et al., 2005; Kuo et al., 2003b; Pratusevich et al., 1995). This is shown in our model as a ~22% increase in the number of contractile units in series. According to data from Section 3.1, this same 33% stretch would cause a force decrease of 8.8% ± 5.9 (Fig. 3-2) which is shown in our modified model as a thick filament shortening of 10% due to mechanical perturbation (as opposed to a loss of available thin filament length).

Upon QR, this intermediate state is caused by both a decrease in contractile units in series as well as a reduction in the overlap of thick and thin filaments as compared to the pre-release state. The thick filaments in the intermediate state are slightly longer than those immediately after the quick-release to account for the partial force recovery seen in the graph of Fig. 4-1. However, they still require additional polymerization before adaptation is complete. In the intermediate state, a QR of 33% (i.e. from 1.5 $L_{ref}$ to $L_{ref}$) would result in a 22% decrease in the number of contractile units in series according to the previous model (Herrera et al., 2005; Kuo et al., 2003b; Pratusevich et al., 1995), and a 20.4% ± 0.6 decrease in force according to data from the present study (Fig. 3-2) shown by a 20% reduction in thick filament length.
The final state of length change, i.e., full adaptation, is shown as described by Pratusevich et al. (1995), Kuo et al. (2003b) and Herrera et al. (2005). Again, they suggested that a 100% increase in adapted length caused no change in force generation, a 67% increase in velocity and a 67% increase in myosin filament density. Therefore, a length change of 33% (i.e. from 1.5 \( L_{\text{ref}} \) to 2 \( L_{\text{ref}} \) or 1.5 \( L_{\text{ref}} \) to \( L_{\text{ref}} \)) would result in no change in contractile unit length, a 22% change in velocity and contractile units in series and a 22% change in myosin filament mass. Fig. 4-3 shows the structural arrangement after adaptation to a new length as presented by Kuo et al. (2003b).
Fig. 4-3. Modified model of structural changes after length change. Top panel shows effects of a length decrease. Bottom panel shows effects of a length increase.

Fig. 4-4. Structural and functional outcomes at three states after a change in length from 1.5 L_{ref}. Values are reflective of model in Fig. 4-3. Dotted line with diamonds represent outcomes after length change when muscle is still fully adapted to original length (1.5 L_{ref}), grey line with squares represent the findings in this study, i.e., the intermediate state, and solid black line with triangles represent full adaptation to the new length.
4.6 Alternative mechanisms

Other possible interpretations of the immediate change in velocity upon length change could be related to changes in calcium levels or MLCK activity. An increase in calcium levels or MLCK activity would cause an increase in myosin phosphorylation and therefore more rapid cross-bridge cycling. This would cause an increase in velocity of muscle shortening and could therefore account for the changes observed after stretch. Similarly, a decrease in MLCK activity or calcium levels could cause the decreased velocity after release. More experiments would be needed to determine whether these interpretations are valid, but our model assumes that they are not. The current theories regarding calcium and MLCK regulation cannot explain the structural changes observed in the present study. The changes in myosin filament density observed suggest the force and velocity alterations seen after a length change is likely due to restructuring of contractile apparatus at the contractile filament level.
4.7 Physiological relevance

The purpose of this dissertation is to examine the structural and functional state of airway smooth muscle after a length change but before full adaptation to the new length. This intermediate state is perhaps more physiologically relevant than the fully adapted state which can only be observed under static conditions. Under *in vivo* conditions, the airways are continuously perturbed by the action of breathing because of the tethering of lung parenchyma to the airway wall. The perpetual oscillatory strain on the airway smooth muscle disallows the muscle to adapt fully to any particular length. The present findings have provided us a clearer picture of the dynamic state of mechanical performance of airway smooth muscle during adaptation. If maintaining airway patency is the goal, then reduction of active muscle force is a positive outcome of length oscillation. The asymmetry of force decrease observed in this study also points out another positive aspect of airway smooth muscle behavior, that is, greater force loss is associated with shortening compared with that associated with lengthening. Occlusion of the airways is more likely to occur if force is not diminished at short lengths. Yet another positive aspect of muscle behavior is the immediate reduction in shortening velocity upon reduction in muscle length, even before the muscle force has a chance to recover. A reduction in muscle length is presumably associated with a narrowed airway. A greater shortening velocity in a narrowed airway would increase the likelihood of airway closure, whereas in a distended airway the velocity of shortening may not be as crucial. Considering the fact that airway smooth muscle is capable of shortening by an amount equivalent to >80% of its original length (Herrera et al., 2005), it is perhaps not difficult
to understand that the dynamic *in vivo* environment where airway smooth muscle reside and how the muscle react to the externally imposed forces all worked together to prevent excessive shortening of the muscle. It is not difficult either to imagine that this delicate balance could be disturbed under pathological conditions leading to airway obstruction.
References


