Perturbed Equilibria of Myosin Binding in Airway Smooth Muscle and Its Implications in Airway Hyperresponsiveness and Asthma

by

David Shoichi Inouye

B.S., Mechanical Engineering (1989)
University of Hawaii at Manoa

and

M.S., Mechanical Engineering (1991)
Massachusetts Institute of Technology

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Signature of Author: ____________________________
Harvard – MIT division of Health Sciences and Technology
August 20, 1999

Certified by: ____________________________
Jeffrey J. Fredberg
Lecturer, Mechanical Engineering
Thesis Supervisor

Accepted by: ____________________________
Martha L. Gray
Professor of Electrical Engineering
Co-director, Harvard - MIT division of Health Sciences and Technology
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Abstract

In asthma, the key effector driving acute airway narrowing is thought to be airway smooth muscle (ASM); as the muscle surrounding the airways shortens, the airway lumen narrows. Airway hyperresponsiveness (AHR) - the excessive narrowing of the airways - is one of the cardinal features of asthma. Yet, the mechanism(s) regulating the airway lumenal radius, and perhaps the failure of these mechanisms to prevent excessive airway constriction, remains largely unexplained. This thesis shows that the regulation of ASM length corresponds to a dynamically equilibrated steady-state, not the static mechanical equilibrium that had been previously assumed. This dynamic steady state requires as an essential feature a continuous supply of external mechanical energy (derived from tidal lung inflations) that act to perturb the interactions of myosin with actin, drive the molecular state of the system far away from thermodynamic equilibrium, and bias the muscle toward lengthening. This mechanism leads naturally to the suggestion that excessive airway narrowing in asthma may be associated with the destabilization of that dynamic process and its resulting collapse back to static equilibrium. With this collapse the muscle undergoes a phase transition and virtually freezes at its static equilibrium length. This mechanism may help to elucidate several unexplained phenomena including the multi-factorial origins of AHR, how allergen sensitization leads to AHR, and the inability in asthma of deep inspiration to relax ASM.

Thesis Advisor: Jeffrey J. Fredberg, Ph.D.
Lecturer of Mechanical Engineering, MIT
Professor of Bioengineering, Harvard School of Public Health, Physiology Program

Thesis Committee: James P. Butler, Ph.D., Harvard School of Public Health
Chi-Ming Hai, Ph.D., Brown University
Thomas McMahon, Ph.D., Harvard University
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Chapter One

INTRODUCTION

1.1 WHAT IS ASTHMA?

Asthma is a word originating from the Greek noun "\( \alpha \sigma \theta \mu \alpha \)" meaning a hard drawn breath, a short breath, a labored breath, or panting [Klein'71]. The Greeks, from ancient writings, believed that asthma was due to an imbalance in the flow of "humors." The Chinese believed that asthma was due to an imbalance between the life forces of yin and yang [Sakula'88] [Marketos'82]. From the first written records of asthma in the third millennium BC to modern day, a multitude of mechanisms have been proposed for the etiology of asthma. Interestingly, asthma, until fairly recently, was believed to be a simple consequence of mast cell degranulation within the airways and the subsequent contraction of airway smooth muscle.

It has, however, become evident that asthma is not simply due to a process of mast cell degranulation and subsequent airway smooth muscle contraction. Rather, the emerging view is that asthma is a complex inflammatory syndrome of the airways involving many cell types and mediators [Drazen'98]. As more is discovered and illuminated on asthma and its complex biology, the single mechanism viewpoint of asthma is rapidly being replaced by the view that asthma is the final phenotypic expression of a multitude of distinct pathobiological processes [Drazen'98].

Today, \textit{asthma} means more than simply a hard drawn breath or a labored breath. It is a term used to describe a complex syndrome involving the triad of airway inflammation, airway hyperresponsiveness (defined below), and reversible airway obstruction [Drazen'98]. Its complex nature is reflected in the fact that while clinical, biological, and histopathological descriptions of asthma exist, a universal definition
remains elusive [Woolcock'97]. With that, a definitive answer to the question posed above “What is Asthma?” remains unresolved.

1.2 AIRWAY HYPERRESPONSIVENESS (AHR)

Airway hyperresponsiveness is defined as airways that narrow too easily and too much in response to a provoking stimulus [Woolcock'98]. It is a cardinal feature of asthma but its mechanism(s) remains largely unexplained [Fredberg'98]. Nevertheless, two features that characterize airway hyperresponsiveness in the asthmatic are increased sensitivity (leftward dose-response shift) to bronchoconstricting agonists and increased efficacy (increased maximal narrowing) of these constricting agonists. Figure 1.1 illustrates asthmatic airway hyperresponsiveness, as measured by a decrease in $FEV_1$ (Forced Expiratory Volume in one second). In this figure, the fall in $FEV_1$ ($\Delta FEV_1$) versus histamine dose shows the characteristic leftward shift and the increased maximal response for asthmatic subjects compared to non-asthmatic subjects. In the cases of the non-asthmatic and the mild asthmatic, a monotonic reduction in $FEV_1$ occurs with increasing histamine dose. For both subjects $\Delta FEV_1$ plateaus at high doses of histamine and $\Delta FEV_1$ is greater for the mild asthmatic compared to the non-asthmatic. Of interest, however, is the pronounced response of the moderate asthmatic where $\Delta FEV_1$ is unlimited, suggesting an abolition of the plateau [Woolcock'84] [Macklem'85].

While AHR is characterized by both increased sensitivity and efficacy to bronchoconstricting agonist, considerable interest is focused on the mechanism for the increased maximal narrowing of the airways for which several theories and models have been proposed. While the proposed models are capable of explaining many features of asthmatic AHR, there remains several important features and questions that each is unable to address. These models are described below.
1.3 THE CURRENT UNDERSTANDING OF AIRWAY HYPERRESPONSIVENESS

In the current understanding of AHR, the most widely accepted models are based on a mechanical balance of static forces, first developed by Moreno & colleagues [Moreno'86]. Crucial to this and related models is the assumption that static mechanical equilibrium conditions prevail within the airways. As such, the length to which ASM contracts is thought to be determined by a static equilibrium between ASM tension and the load against which it acts. ASM length, in turn, sets the degree of airway narrowing. The balance of static forces model provides a powerful tool for investigating factors that influence AHR [Wiggs'90] [Wiggs'92] [Yager'89] [Lambert'93] [Macklem'96]. Yet, despite its utility, there are several characteristics of AHR that the model cannot account for.

An important feature of AHR that does not fit into the framework of the classical model is the reduced bronchodilator response and the paradoxical bronchoconstricting effect of a deep inspiration (DI) in the asthmatic versus normal lungs [Fish'81] [Wheatley'89]. Why asthmatics with spontaneous bronchoconstriction respond paradoxically with more, not less, bronchoconstriction remains a mystery [Orehek'80] [Orehek'81].
Results pointing toward a new mechanism

The constrictor response to DI in asthmatics led Fish & colleagues to hypothesize that AHR is a result of an impaired ability of lung inflation to modify bronchomotor tone rather than the result of increased end-organ responsiveness to stimuli [Fish'81]. As a corollary to the hypothesis of Fish & colleagues, Skloot & colleagues hypothesized that if asthmatic airway obstruction was indeed due to an impairment in the ability of lung inflation to stretch airway smooth muscle, then normal subjects should respond like asthmatics if the bronchodilating effect of a DI was eliminated [Skloot'95]. Skloot & colleagues validated this hypothesis, showing that the prohibition of DI's in normal and asthmatic subjects during methacholine (MCh) challenge produced equivalent states of bronchoconstriction in both groups (as measured by the decrease in $FEV_1$). That is, non-asthmatics challenged with methacholine are equally prone to developing AHR if DI's are prohibited. Subsequent studies by Moore & colleagues have confirmed that this response is due to the prohibition of DI and not a consequence of reduced mean lung volume or transient excursions to RV [Moore'97].

While the results of Skloot et al. showed that the non-asthmatic lung is potentially as reactive as an asthmatic lung, under normal circumstances, the non-asthmatic rarely, if ever, becomes hyperreactive. The emergence of excessive airway narrowing with the loss of large tidal lung volume excursions suggests a bronchoprotective mechanism based on tidal lung volume excursions that, under normal circumstances, tends to prevent AHR from occurring in non-asthmatics. Complementary, these results suggest that this protective mechanism is impaired in the asthmatic. Indeed, animal studies support the notion that tidal lung inflation suppresses pharmacologically-induced AHR in canine and rabbit lungs [Gunst'90] [Warner'92] [Tepper'95] [Mehta'96] [Shen'97B]. Although several mechanisms might account for this bronchoprotective behavior, this thesis focuses on the effects of tidal stretch on ASM contractile behavior as a mechanism underlying changes in bronchomotor tone with lung inflation.

1.4 THESIS STATEMENT AND GOALS

This thesis focuses on airway smooth muscle and in particular its behavior under tidal stretch. Although the classical models of airway narrowing assume static equilibrium ASM behavior, the above
findings argue otherwise. The first goal of this thesis is then to determine the effects of tidal stretch, as occurs during tidal lung inflations, on ASM contractile behavior.

The second goal is then to establish a plausible mechanism underlying the effects of tidal stretch on ASM contractile behavior. In the pursuit of these goals, the central hypothesis of this thesis is proposed as:

Central hypothesis

Tidal stretch, as occurs during tidal breathing, is capable of perturbing airway smooth muscle length, tension, stiffness, and hysteresivity away from its static equilibrium steady-state values through the direct and disruptive action of stretch on the actin-myosin crossbridge.

In the current understanding of asthma and AHR, the classical models fail to explain the bronchodilatory effects of DI in the asthmatic lung. It is noted that one possibility for this failure is in the critical assumption of static equilibrium conditions, which may not pertain in the setting of tidal lung inflations. Indeed, the results of Fish, Skloot, and others investigators who have shown airway responses to DI maneuvers that do not fit the framework of the classical model support this assessment.

We breathe at a rate of about 12-20 times per minute and, with each breath, inflate and deflate our lungs by roughly 16% of our total lung volume. These tidal lung volume changes, through parenchymal tethering, impose a tidal strain on our airways ranging from 4 to 12% of muscle length [Fredberg’98]. Tidal strain, within this range, is known to exert substantial effects on the contractile behavior of ASM [Sasaki’79] [Gunst’83]. In keeping with the notion that tidal lung inflations can modify ASM contractile behavior, Molfino & colleagues showed that the cessation of tidal breaths in asthmatics, normals, and non-asthmatic lung transplant recipients resulted in decreased bronchial airway area [Molfino’93].

These findings suggest that the origin of the bronchodilatory effect of tidal lung inflation lies within airway smooth muscle itself. While several mechanisms for the stretch-inhibition of ASM tone have been proposed, a simple mechanism may lie at the level of the actin-myosin interaction. It is widely accepted that cyclic stretch depresses muscle tone by disrupting the actomyosin crossbridge in skeletal muscle [Zahalak’80] [Rack’74], but whether such a mechanism applies to ASM is less clear. The goal of this
thesis is then to establish the extent to which these effects are consistent with the ability of tidal stretch to disrupt the binding cycle of the actin-myosin crossbridge.

1.5 THESIS OVERVIEW

The next chapter introduces the actin and myosin interaction as the basic tension-generating unit of smooth muscle, the biophysics of this interaction as it relates to tissue level behavior, and the physiology of smooth muscle contraction. Each is necessary for the analysis and interpretation of data throughout this thesis. Chapter Three introduces quantitative models of the airway that are used to obtain estimates of how lung inflation transmits load fluctuations to airway smooth muscle. Chapter Four begins the experimental phase of this thesis with an investigation into the effect of tidal length fluctuations on airway smooth muscle tone. Through both experimental and quantitative methods, the results of Chapter Four establish the validity of the central hypothesis, showing that small tidal stretches can drive activated airway smooth muscle into dynamically-determined contractile states consistent with a perturbed equilibria of acto-myosin binding dynamics. Chapter Five continues the validation of the central hypothesis by investigating the effect of fluctuating load on muscle length. Chapter Six addresses the effect of load history on airway smooth muscle length. In this investigation, several of the alternative hypotheses are investigated. Chapters Seven investigates the effects of tidal stretch frequency on the regulation of ASM length. Chapter Eight addresses the functional implications of the experimental results of Chapters Four through Seven.
Chapter Two

AIRWAY SMOOTH MUSCLE: TISSUE BEHAVIOR AND MATHEMATICAL MODEL

2.1 INTRODUCTION

This chapter introduces the background concepts of airway smooth muscle contraction used in this thesis. Specifically, this chapter 1) begins with a brief background of the established mechanical behavior of ASM tissue, 2) continues with a rough description of the contractile mechanism of smooth muscle, and 3) introduces a mathematical model of airway smooth muscle contraction.

2.2 OVERVIEW OF MUSCLE CONTRACTION

Figure 2-1 presents a rough diagram of what is thought to be the main activation-contraction pathway of airway smooth muscle. Activation begins at the cell membrane, often via the binding of a contractile agonist (ACh) to its receptor. This agonist-receptor binding triggers, through the phospholipase-C pathway, a cascade of events (the production of IP₃, DAG, etc.) leading ultimately to an increase in the intracellular calcium level. As in striated muscle, calcium controls the binding cycle of airway smooth muscle myosin to actin. This binding is, however, mediated through regulatory proteins (calmodulin and myosin light chain kinase) different from those found in striated muscle. The binding cycle of myosin to actin is believed to be the fundamental force-generating unit of airway smooth muscle contraction.
**Figure 2 - 1:** The muscarinic activation contraction pathway of airway smooth muscle. In this pathway, acetylcholine (ACh) binds to its membrane associated receptor, activating phospholipase-C (PLC). Through several intermediate steps, PLC converts membrane lipid into inositol 1,4,5-triphosphate (IP₃). IP₃, in turn, induces the release of calcium from the sarcoplasmic reticulum (not shown). Calmodulin (CaM) binds to free calcium, which allows Ca²⁺ to bind to myosin light chain kinase (MLCK) forming the CaM-MLCK-Ca²⁺ complex. This complex then phosphorylates the regulatory light chain on myosin to begin the binding cycle of myosin to actin.

Within smooth muscle cells, the myosin-actin contractile unit is mechanically linked to the structures of the cell's cytoskeleton [Small'95]. Similarly, at a larger scale, individual smooth muscle cells are mechanically linked through intracellular bonds (desmosomes) and through connective and ground substance (collagen, elastin, proteoglycans, etc.). From the cytoskeletal scale to the tissue level, all components are mechanically coupled and this composite tissue or syncytium, in whole, confers the mechanical properties of smooth muscle tissue. While the overall structure is complex, smooth muscle tissue behavior can be simplified into a series and parallel arrangement of passive elastic and active contractile elements. The Voight model
(Figure 2-2) which consists of two elastic elements and one force-generating element is one such simplification [Stephens'93].

![Diagram of Voight model](image)

Figure 2 - 2: The Voight model of muscle tissue. In this model, the syncytium of airway smooth muscle is simplified into a lumped-parameter representation of two elastic elements and one tension-generating element. The parallel elastic component (PEC) determines to a large extent, the passive length-tension characteristics of the muscle. The series elastic component (SEC) confers to the model the characteristic of finite compliance between cells, myosin head stiffness, and possibly extensibility within the filaments of the contractile unit. The contractile unit (CE) represents the active (i.e. actin-myosin tension generating) component of the muscle.

2.3 SMOOTH MUSCLE TISSUE BEHAVIOR

This section introduces the mechanical behavior of airway smooth muscle tissue. Three response patterns of ASM that are referred to in this thesis are described. These responses are 1) The temporal response of muscle tension, stiffness, and hysteresivity to sustained maximal activation. 2) The length-tension relation for static equilibrium isometric and isotonic contraction in ASM, and 3) the tension-velocity response during isotonic shortening.

2.3.1 Temporal responses

When activated, ASM responds with a rise in tension that approaches a plateau (Figure 2-3a). For maximally activated muscle, tension reaches a plateau that does not increase with further activation. Similar to the tension response, activation results in a near-parallel increase in stiffness (Figure 2-3b) [Gunst'96]. Lastly, hysteresivity, an index of the mechanical energy dissipation of the muscle, responds in a biphasic manner, first increasing and then slowly decreasing to a non-zero asymptote as tension and stiffness increase to its maximal values (Figure 2-3c). In the majority of the cases, this thesis refers to the steady-state, static equilibrium (i.e. plateau) states of tension, stiffness, and hysteresivity.
Figure 2 - 3: The time response of tension (F), Stiffness (E), and hysteresivity (η) in response to maximal electric field stimulation. With the onset of stimulation, tension and stiffness rise asymptotically, in near parallel fashion. In contrast, hysteresivity changes biphasically with a sharp rising, a peak, and a slow decay. There is a second rise as electric field stimulation is ceased. From Fredberg et al., 1996. Friction in airway smooth muscle: mechanism, latch, and implications in asthma. J. Appl. Physiol. 81(6): 2703-2712.

2.3.2 Length-tension curves of smooth muscle tissue

Although there are recent report suggesting otherwise [Ford’94] [Pratusevich’95], it is usually assumed that there is a unique length-tension relation for both the passive and maximally-activated states of muscle. For relaxed and maximally activated states of muscle, the length-tension relations (passive or activated) define the minimum and maximum tensions that the muscle can achieve at a given length (Figure 2-4). Equally important is that the length-tension relation for maximally activated ASM also defines, for a given isotonic load, the shortest length to which ASM can contract [Stephens’ 79]. As a preview, it is noted that current models of the airway assume that this latter behavior pertains in vivo in the calculations of the final radius to which the airways would narrow. That is, the classical model of airway narrowing assumes that if given enough time, ASM will contract to it shortest length (as defined by the intersection of the load curve with the muscle’s length-tension curve.)
A second feature of the length-tension relations of ASM is that if the passive tension curve is then subtracted from the total tension curve, (to yield what is called the active tension curve) one finds that active tension first increases, then decreases as muscle length is increased from short to long lengths. The length at which active tension is maximal is defined as "optimal length," denoted by the symbol $L_o$ in this thesis.

When lightly loaded, smooth muscle can contract to 10-20% of $L_o$, while striated muscle contracts to only about 65% of $L_o$ [Stephens'93]. Moreover, smooth muscle generates the same stress as striated muscle but with five-fold less myosin [Stephens'93]. While the length dependence of tension suggests a similarity between smooth muscle and striated muscle structural organization (i.e., linear filament overlap), a definitive structural organization of ASM actin and myosin remains elusive [North'94].
2.3.3 Tension-velocity curves of smooth muscle tissue

The tension-velocity relations of smooth muscle, much like that of skeletal muscle, follows hyperbolic relation known as the Hill curve [Hill'38] (Figure 2-5). This curve is expressed as

\[(T + a)(v + b) = (T_o + a) \cdot b\]  \hspace{1cm} (2-1)

where \(v\) is the velocity of shortening, \(T\) is the muscle tension against which the muscle shortens, \(T_o\) is the optimal tension (as defined above), and \(a\) and \(b\) are empirical constants. While the Hill curve is qualitatively similar between smooth and striated muscle, the maximal velocity of shortening of smooth muscle is four to one hundred-fold slower than that of skeletal muscle [Stephens'93]. In addition, while the maximal velocity of shortening for striated muscle is relatively time invariant, it is time-dependent in ASM [Kamm'85] [Stephens'93]

![Figure 2-5: Tension (load)- velocity curve of ASM. From NL Stephens, H Jiang. 1995. Basic physiology of airway smooth muscle (Chapter 83, pg. 1094). In Asthma and Rhinitis. Busse WW, Holgate ST (Eds.). Blackwell Scientific, Boston.](image-url)
2.4 THE MECHANISM OF ASM CONTRACTION: THE MYOSIN ACTIN BINDING CYCLE

This section introduces the myosin to actin binding cycle, the sliding-filament theory of muscle contraction, and the interpretation of tissue level states (tension, stiffness, hysteresivity, and velocity of shortening) in the context of the myosin-actin interaction.

2.4.1 The myosin to actin binding cycle

How myosin converts the chemical energy of ATP into mechanical work remains an issue of intense research and debate [Thomas '93]. Nevertheless, one simple scheme that illustrates the general idea of the myosin-actin binding cycle is the classic textbook illustration which assumes a cycle of 1) myosin unattached to actin (in different stages of ATP hydrolysis), 2) myosin attached to actin in a weakly bound state, and 3) a subsequent "power stroke" in which a conformational change occurs while strongly bound to actin [Cooke '97] (Figure 2-6). Through such a scheme multiplied manifold, myosin molecules act in concert to contract and perform work at the tissue level. The sliding filament theory, a mathematical description of how the myosin-actin binding cycle generates tension and associated tissue properties of stiffness and hysteresivity, is introduced below.

![Diagram of the myosin-actin binding cycle](image)

Figure 2-6: Cartoon of power stroke.
2.4.2 Ensembles of crossbridges: the sliding filament theory

A mathematical description of how myosin molecules bind to actin and produce tension and/or muscle shortening was proposed by A. F. Huxley in 1957 [Huxley 57]. After more than forty years, the sliding-filament theory remains the gold standard muscle model and description of actin-myosin binding kinetics.

For a derivation of the sliding filament theory equations of motion, the reader is referred to the works by Huxley, McMahon, or Zahalak [Huxley 57] [McMahon 84] [Zahalak 80]. Under the assumptions of the sliding filament theory, the equations of motion for \( n(x,t) \), where \( n(x,t) \) is the spatial and temporal distribution of the fraction of actin binding sites along the thin (actin) filament occupied by a myosin, is given as

\[
\frac{\partial n(x,t)}{\partial t} - \nu \frac{\partial n(x,t)}{\partial t} = f(x) - [f(x) - g(x)] \cdot n(x,t)
\]  

(2-2)

where \( \nu \) is the relative velocity between the thick and thin filaments\(^1\), \( g(x) \) is the detachment rate of myosin from actin, and \( f(x) \) is the attachment rate for myosin to actin. Stated in words, this equations says that following a definite region on the thin filament (the convective derivative), the accumulation in time of crossbridges (i.e. myosins attached to actin) is the difference between the attachments and detachments. The spatial dependence of \( g(x) \) and \( f(x) \) (Figure 2-7) are defined as

\[
g(x) = g_1, \text{ for } x < 0
\]

(2-3)

\[
g(x) = g_1 \frac{x}{h}, \text{ for } x \geq 0
\]

and

\[
f(x) = 0, \text{ for } x < 0 \text{ and } x < h
\]

(2-4)

\(^1\) convention for \( \nu \) : positive during shortening.
\[ f(x) = f_1 \frac{x}{h}, \text{ for } 0 \leq x \leq h \]

\[ h = \text{ the range of attachment along the thin filament} \]

\[ \text{Attachment rate} \quad f(x) \]

\[ \text{Detachment rate} \quad g(x) \]

Figure 2-7: The spatial dependence of attachment and detachment rates as used in the sliding filament theory.

This partial differential equation can be solved for \( n(x,t) \) and the tissue level properties of tension \( T(t) \), stiffness \( K(t) \), and the maximal velocity of shortening \( v_{\text{max}} \) computed. More importantly, the sliding filament theory provides a mechanistic basis for interpreting the measurable mechanical properties of \( T(t) \), \( K(t) \), and \( v_{\text{max}} \) at the level of the actin-myosin interaction.

The first property is muscle stiffness \( K(t) \), which may be calculated from \( n(x,t) \) with the equation

\[ K(t) = k_{\text{myosin}} \cdot M_T \cdot \int_{-\infty}^{\infty} n(x,t) \cdot dx \tag{2-5} \]

where \( k_{\text{myosin}} \) is the stiffness of a single crossbridge and \( M_T \) is the total number of myosin molecules in parallel. Simply stated, this equation says that stiffness is equal to the stiffness per crossbridge \( k_{\text{myosin}} \) weighted sum of the total number of crossbridges (the integral multiplied by \( M_T \)). This equation then provides the basis for interpreting stiffness, a measurable property of muscle, as a rough index of the number of crossbridges [Zahalak '80].
The next property is muscle tension $T(t)$, which can be calculated from $n(x,t)$ with the equation

$$T(t) = k_{myosin} \cdot M \cdot \int_{-\infty}^{\infty} x \cdot n(x,t) \cdot dx$$ (2-6)

Stated in words, this equation says that muscle tension is the summation (the integral) of the force developed by each crossbridges, where each crossbridge contributes a tension of $k_{myosin} \cdot x$.

A third property considered is the maximal velocity of shortening, $\nu_{max}$. From the sliding filament theory equation of motion, $\nu_{max}$ is given in Huxley’57 as

$$\nu_{max} = 4 \cdot \frac{h}{s} \cdot (g_1 + f_1)$$ (2-7)

$$= 1.06 \cdot \frac{h}{s} \cdot g_2$$ (2-8)

The above equations, within the limits of the sliding filament theory, reveals that $\nu_{max}$ is intimately tied to the myosin attachment and detachment rates, as expressed in Equation 2-7. Alternatively, Equation 2-8 shows that $\nu_{max}$ is also linked to the $g_2$ component of the detachment rate, as used in the sliding filament theory equations above.

A final measurable property of muscle tissue, while not constrained by the sliding filament theory, is hysteresivity $\eta$, an index of the mechanical dissipation of muscle when externally imposed periodic stretch is applied [Fredberg’96]. An expression for $\eta$, as derived in Fredberg’96, is

$$\eta = \frac{\Phi}{U} = 2\pi \eta_o \cdot \frac{g_{app}}{\sigma}$$ (2-9)
where $\Phi$ is the total macroscopic mechanical energy dissipation per cycle, $U$ is the total internal energy content invested at peak tissue strain, $\eta_0$ is a scaling factor for the basal level of hysteresivity, $\kappa_{appr}$ is the apparent detachment rate of myosin from actin which corresponds to the original detachment rate $g(x)$ as described above, and $\sigma$ is the frequency of stretch (in radians). This equation then links $\eta$, a measurable property of muscle tissue, to the crossbridge cycling rate $g(x)$.

In summary, these equations provide a mechanistic basis for interpreting several measurable mechanical indices of ASM at the level of the actin-myosin interaction. $K(t)$, $v_{max}$, and $\eta$ provide a means of assessing roughly the number of attached myosins and the overall cycling rates of the myosin-actin binding cycle. These relations are used in the following chapters of this thesis in data interpretation.

2.5 THE INTEGRATION OF THE SLIDING FILAMENT THEORY WITH THE LATCH HYPOTHESIS: A SYNTHESIS FOR A QUANTITATIVE MODEL FOR ASM BEHAVIOR

A remarkable property of smooth muscle, when compared to other types of muscle, is its ability to maintain tonic contraction at a low rate of ATP consumption. For example, tonically contracted smooth muscle uses 300 times less ATP than striated muscle while maintaining equal levels of tension [Murphy'94]. This economy, compared with striated muscle, is believed to be attained through the ability of smooth muscle to regulate its myosin cycling rates [Hai'88] [Murphy'94].

The latch hypothesis of myosin cycling rate regulation was proposed to account for the reduced rate of ATP consumption while maintaining tonic tension [Hai'88]. The hypothesis asserts that myosin, once attached to actin, is able to drastically reduce its detachment rate, converting rapidly cycling crossbridges into slowly cycling "latch bridges," and thus decrease its rate of ATP consumption. This regulatory process eventually progresses to a steady state, and bridge dynamics are then said to have attained the "latch state" [Hai'89] [Murphy'94]. In the hypothesis, the formation of latch bridges is regulated through the phosphorylation and de-phosphorylation of the regulatory light chain of myosin (RLC) where phosphorylation increases the cycling rate and dephosphorylation reduces the cycling rate. While there remains some controversy of the regulatory mechanism of ASM contraction the latch
hypothesis, with its large body of supporting evidence, remains a major regulatory mechanism in smooth muscle contraction [Wingaard’94] [Murphy’94] [Horowitz’96].

The following section describes the equations governing the latch hypothesis. Additionally, the section describes the HHM theory (for “Huxley-Hai-Murphy”) in which the latch hypothesis and the sliding filament theory are combined to synthesize a quantitative model of ASM contraction.

_The latch hypothesis meets the sliding filament theory: a quantitative model of ASM contraction_

In latch hypothesis, as shown in Figure 2-8, myosin assumes one of four possible states. These states are defined as:

```
“M” = Myosin, de-phosphorylated and unattached,

“M_p” = Myosin, phosphorylated and unattached,

“M_p \cdot A” = Myosin, phosphorylated and attached, and

“M \cdot A” = Myosin, de-phosphorylated and attached.
```

The latch hypothesis proposes that transitions between the four states of myosin are governed by rate constants $k_i$ through $k_f$. In this scheme, $k_i$ & $k_s$ governs the phosphorylation of myosin while $k_f$ & $k_d$ governs the dephosphorylation of myosin. The rate constants $k_i$ & $k_s$ are associated with the attachment and detachment rates of myosin to actin while myosin is in the phosphorylated state. The final rate constant $k_f$, which may be considered the “latch constant,” governs the detachment rate of myosin from actin while it is in the dephosphorylated (“latch-bridge”) state.

In the latch hypothesis, as presented in 1988, the attachment and detachment rate constants $k_i$ & $k_s$ and $k_f$ were simplified to fixed numerical constants. While $k_i$, $k_s$, and $k_f$ are related to the $f(x)$ and $g(x)$ in the sliding filament theory, this simplification to spatially independent rates by Hai & Murphy reduced the equations of motion from a partial differential equation into an ordinary differential equation.
The HHM theory is a synthesis of these two schemes that recovers the lost spatial dimension by incorporating the regulatory scheme of the latch hypothesis while retaining the spatial myosin strain distribution of the sliding filament theory [Fredberg'99]. The equations of motion for this combined model are given in Fredberg'99 as

\[
\frac{\partial}{\partial t} \mathbf{n}(x, t) - \nu \frac{\partial}{\partial t} \mathbf{n}(x, t) = \mathbf{T}(x) \cdot \mathbf{n}(x, t) \tag{2-10}
\]

where

\[
\mathbf{n}(x, t) = \begin{bmatrix} M & M_p & AM_p & AM \end{bmatrix}^T
\]
and
\[
T(x) = \begin{bmatrix}
-k_1 & k_2 & 0 & g(x) \\
k_1 & -k_2 - g_p(x) & g_p(x) & 0 \\
0 & f_p(x) & -k_3 - g_p(x) & k_o \\
0 & 0 & k_3 & -k_o - g(x)
\end{bmatrix}
\]

Where the new variables \( f_p(x) \), \( g_p(x) \), and \( g(x) \) are equivalent to the rate constants \( k_3 \), \( k_1 \), and \( k_5 \). Moreover, the spatial distributions of these rate constants are of the form

\[
f_p(x) = \begin{cases} 
0 & x < 0 \\
f_{pl} \cdot x & 0 \leq x \leq h \\
0 & h < x
\end{cases}
\]

\[ (2-11) \]

\[
g_p(x) = \begin{cases} 
g_{p2} & x < 0 \\
g_{pl} \cdot x & 0 \leq x \leq h \\
(g_{pl} + g_{p3}) \cdot x & h < x
\end{cases}
\]

\[ (2-12) \]

\[
g_p(x) = \begin{cases} 
g_2 & x < 0 \\
g_1 \cdot x & 0 \leq x \leq h \\
(g_1 + g_3) \cdot x & h > x
\end{cases}
\]

\[ (2-13) \]

These equations are solved numerically and the solutions to this matrix partial differential equation are used in the quantitative investigation of the following chapters of this thesis. To account for the length-tension relations of ASM, changes in filament overlap with muscle length were set such that the static length-tension relationship was approximated by a straight line with muscle tension equal to zero at 15\%
L_e and equal to T_e at L_e. Further details of the parameters used in this model may be found in Fredberg' 99.

2.6 CONCLUSION

The above sections described the basic physiology of smooth muscle, from a simplified diagram of the muscarinic activation-contraction pathway to the tissue level behavior of ASM. Pertinent the central hypothesis of this thesis was the review of the steady-state, static equilibrium mechanical behavior of airway smooth muscle exemplified by its static equilibrium length-tension curves. This section introduced the static equilibrium behavior of both isometric and isotonic contraction where this behavior is assumed to also apply in the setting of tidal stretch.

In the description of the mechanical behavior of ASM, tension, stiffness, maximal velocity of shortening, and hysteresivity were linked mechanistically to events which occur at the level of the actin-myosin interaction. Importantly, this provided a means of interpreting changes in measurable mechanical properties of muscle tissue to molecular events at the level of the myosin bond. In particular, these equations showed that stiffness is a rough index of the number of attached myosin to actin, the maximal velocity of shortening is intimately tied to the cycling rates of myosin to actin. Hysteresivity, likewise, provides an alternative index into assessing the cycling rates of myosin to actin.

Finally, this chapter described the sliding filament theory, the latch hypothesis of myosin cycling rate regulation, and introduced the HHM theory. In the following chapters, the HHM theory is used as a quantitative tool to assess the relationship between tidal stretch and actomyosin dynamics.
Chapter Three

THE FORCES OF BREATHING: ESTIMATES OF IN VIVO ASM LOADS

3.1 OVERVIEW

This chapter addresses the tidal loads applied on airway smooth muscle during normal tidal and deep inspiratory breathing patterns. These tidal loads, in the general sense, are considered as a mean length or tension upon which a fluctuating component (of either length or tension) is superimposed. For both cases of tidal loading (length or tension), estimates of in vivo stretch are obtained using the airway recoil length-tension equations developed by Moreno, Lambert et al., and Macklem [Moreno'86] [Lambert'92] [Macklem'96]. Finally, these estimates of tidal loads are used to demonstrate the predictions of the classical airway models.

3.2 LUNG ELASTIC RECOIL FORCE - THE GOOD FORCE

Airway smooth muscle is capable of shortening to 20% of its initial length, and in doing so, provides more than enough shortening capacity to narrow an airway to closure [Macklem'85] [James'84] [Stephens'89]. Yet, airway narrowing is limited in the non-asthmatic, even when challenged with high doses of bronchoconstricting agents [Woolcock'84] [Ding'87]. One answer to this apparent puzzle is that the structures of the airway and the parenchyma, distended by transmural inflation pressure, provide an elastic recoil load that resists muscle shortening and thus maintains airway patency during ASM activation [Moreno'85]. Figure 3-1 demonstrates this interaction between ASM tension and recoil tension in a
straightened airway model. Elastic recoil tension, as conferred by parenchyma and passive airway structures, is represented by the spring element. In this tug of war configuration, airway smooth muscle does not contract freely but contracts against an elastic recoil load that impedes shortening. The characteristics of this elastic recoil load are discussed below.

Figure 3-1: A diagram of the airway. A) A diagram of an airway, its internal band of smooth muscle, its outer adventitial tissue, and the surrounding parenchyma. The arrows on the edges of the adventitia represent the parenchymal tensile forces. B) A free-body diagram of the airway-parenchyma interaction where transpulmonary pressure ($P_L$) across the airway is balanced by the active tension of ASM ($T_{ASM}$) and passive airway tissue tension ($T_{PA}$). C) An equivalent model of the airway in which transpulmonary forces, parenchymal distortion forces (not shown) and passive airway wall forces are lumped into a single elastic element (spring) connected in mechanical series with the active tension generating component of ASM. In this tug of war configuration, the elastic recoil tension is equal to the active tension of ASM.
3.3 THE LUNG IN TENSION - A MODEL OF THE ELASTIC LOAD

The inflated lung may be considered as a tensed pressure supported structure [Fredberg'98]. Transpulmonary pressure\(^1\), the pressure difference that maintains lung inflation, arises from the interaction of chest wall structure, inspiratory muscles, lung elasticity, and other factors. This pressure difference, in the form of a distending stress, is transmitted through all intrapulmonary structures where it is essential to proper lung functioning [Fredberg'98]. As the lung is inflated the elastic and connective tissue fibers of the lung parenchyma become tense. These parenchymal tissue stresses are then transmitted to the airways (Figure 3-1). Under normal circumstances, this distending stress maintains patency of the airways. This distending stress is also referred to as an elastic recoil stress as it is arises from the elastic recoil properties of lung tissue.

An estimate of the elastic recoil forces within the lung: the airway diameter-recoil load relation

Lung parenchyma, airway adventitia, and airway wall connective tissue confer an elastic recoil tension that counterbalances the constricting forces of activated airway smooth muscle and acts to distends the airways. Several investigators have developed quantitative airway models that estimate these recoil loads. This section presents a summary of these models, proposed initially by Moreno [Moreno'86]. The reader is referred to papers by Moreno, Lambert et al., Yager et al., Wiggs et al., or Macklem for details of the derivation [Lambert'93] [Yager'89] [Wiggs'90] [Macklem'96]. From the analysis, the length-tension relation of the airway may be expressed as

\[
T_{rec} = P_L \cdot R_{sm} - 1.4 \cdot P_L \left( R_{sm} - \frac{R_{sm}^2}{R_m^*} \right) - F_p(R_m) \tag{3-1}
\]

where

\[
T_{rec} = \text{muscle load.}
\]

---

\(^1\) Transpulmonary pressure is defined as the difference between airway opening and pleural pressures. This pressure difference is what drives inflation of the lung.
\[ P_L = \text{transpulmonary pressure.} \]

\[ R_m = \text{radius of the airway (defined by the radius of the airway smooth muscle band).} \]

\[ R'_m = \text{resting (passive) airway radius.} \]

\[ F_p \] is the passive elastic recoil of the airway wall tissues (scaled per unit axial length).

The equation shows that the recoil forces are composed of three distinct elastic components representing distending forces conferred by transpulmonary (inflation) pressure, parenchymal distortion, and passive elastic structures of the airway wall. In addition, the equation shows that these recoil forces are dependent upon the radius of the airway, which is assumed to be proportional to muscle length.

Figure 3-2 presents the muscle load versus airway radius for \( P_L \)'s of 3, 6, and 9 \( cmH_2O \). These three pressures represent the trough, mean, and peak pressures that might occur during normal tidal breathing. In addition, these curves represent the recoil-tension relation of a 2.2 mm outer diameter airway used by Macklem [Macklem '96]. A striking feature of these curves is the dependence of muscle load on radius. For the tension-radius curve at \( P_L = 6 \) \( cmH_2O \), as \( R_m \) is reduced from \( R_m = 0.80 \) mm, recoil tension increases from zero to a rough plateau at 0.45 g/cm (grams/cm). A further decrease in \( R_m \) results in a rapid rise in the elastic load as \( R_m \) approaches the closure radius of 0.44 mm. A second feature that can be appreciated from Figure 3-3 is the difference between the curves for \( P_L = 3 \) \( cmH_2O \) and \( P_L = 9 \) \( cmH_2O \). This difference represents the fluctuation in muscle load that would occur if \( P_L \) is cycled from 3 to 9 \( cmH_2O \). Quantitatively, \( P_L \)'s from 3 to 9 \( cmH_2O \) (the estimated swings in \( P_L \) for normal sized breaths of 600 ml about FRC) translates into a peak-to-trough recoil tension fluctuation of 0.46 g/cm (grams/cm) at small airway radii to 0.57 g/cm at large radii. Deep inspiration results in fluctuations that are even more pronounced. These fluctuations are not small. When these fluctuations are scaled to \( T_o \) for that airway\(^2 \), normal sized breaths corresponds to recoil tension fluctuations with a peak-to-trough variation of 21 - 26% of \( T_o \) (11 to 13% \( T_o \) in amplitude). In the case of deep inspirations, these

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\(^2\) The estimate for \( T_o \) is obtained by assuming an ASM thickness of 20 \( \mu m \) and a maximal stress generating capacity of 1.1 \( kg/cm^2 \) for canine tracheal muscle [Stephens '77]. The thickness of 20 \( \mu m \) is the mean thickness used in the airway model of Macklem [Macklem '96]. Additionally, the selection of 20 \( \mu m \) is supported by the noting that lung inflation near FRC (~6 \( cmH_2O \)) is unable to prevent excessive airway narrowing in non-asthmatics [Skloot '95].
fluctuations can easily exceed 50% of $T_e$ (30% $T_e$ in amplitude) and even reach 80 to 90% of $T_e$. These tidal fluctuations in the recoil load and its implication on airway lumen caliber are addressed below.

Figure 3-2: The muscle load-airway radius relation of an airway. The three curves shown are the muscle load ($T_{rec}$) versus airway radii ($R_{sm}$) relations, as expressed in Equation 3-1, at transpulmonary pressures of 3, 6, & 9 cm H$_2$O. The three curves represent the trough (dotted), mean (solid), and peak (dotted) transpulmonary pressures during normal tidal breathing. All curves show a strong non-linear dependence of $T_{rec}$ on $R_{sm}$, where $T_{rec}$ starts at zero for large $R_{sm}$, increasing to a quasi-plateau as $R_{sm}$ decreases, and sharply increase as $R_{sm}$ approaches the radius at which airway closure occurs. (Airway closure results from a non-zero mucosal thickness that is not explicitly shown. For the airway considered, closure occurs at $R_{sm}$ = 0.44 mm. This is indicated by the sharp increase in muscle load for each of the three curves.) Note that the difference between the curve for $P_L = 9$ cm H$_2$O and $P_L = 3$ cm H$_2$O represents the tidal fluctuation in muscle load that occurs during normal tidal breathing.

3.4 THE CLASSICAL AIRWAY MODEL AND ITS STATIC EQUILIBRIUM PREDICTIONS IN THE SETTING OF TIDAL STRETCH

In the classical model of airway narrowing, airway lumen caliber is believed to be set by a static equilibrium between the airway elastic recoil force and static ASM active tension. This is illustrated by the tug of war between airway elastic recoil force and ASM tension configuration of Figure 3-1. In addition, this tug of war can be expressed graphically by overlaying the curves that define the airway recoil-radius relation with the active tension curve of ASM (Figure 3-3). In this figure, the classical model and its
assumption of an equilibrium of static forces asserts that the final caliber that the airway tends to is determined by the point at which the two curves intersect. In the case of maximally activated ASM, the intersection point is believed to define the radius that the airway approaches during bronchospasm. Thus, at large lung volumes and hence large muscle loads, airway luminal caliber remains relatively large (point #1). In contrast, small lung volumes, which provide only a small recoil tension, allow the airways to constrict to a small radius (point #2).

Figure 3-3: A graphical interpretation of the classical model of airway narrowing. In the classical model of airway narrowing, airway smooth muscle length and thus airway caliber is believed to be determined by a balance of forces in which steady-state, static equilibrium airway smooth muscle active tension is counterbalanced by the forces of elastic recoil. Graphically, this may be interpreted as the point of intersection between the elastic recoil and the active tension curves. This point, and the associated length (or radius) defines the length to which maximally activated airway muscle will shorten to if given enough time. For a given load, this length then defines the smallest radius that the airway will tend to. For large $P_L$, the intersection point (point #1) defines the radius of the airway (R1). Likewise for small $P_L$ (point #2), the intersection defines the radius of the airway (R2) where R1 > R2.

The previous section, however, showed that the elastic recoil forces applied on airway smooth muscle are not fixed but fluctuate in response to tidal lung inflation. The classical models hold that static
equilibrium conditions prevail at every instant even under the setting of tidal stretch. Accordingly, airway caliber is set by a series of static equilibrium points, each determined by the intersection of the elastic recoil curve with the steady-state active tension curve of ASM (Figure 3-4).

![Diagram](image)

Figure 3-4: The classical model of airway narrowing in the setting of tidal loads. In the setting of tidal stretch (in either tension or length fluctuations forms), the classical model assumes that the fluctuations that occur with tidal breathing are sufficiently slow such that ASM is able to maintain its static equilibrium length-tension relation (i.e. quasi-static behavior.). In the case of tidal lung inflation, ASM accommodates its length (or tension) such that the static-equilibrium length-tension relation is traversed during tidal stretch.

From this, one sees that a fundamental assumption of the classical model is that ASM behavior, in the setting of tidal stretch, is set by its static length-tension relation. 3 (Figure 3-5). However, as mentioned in Chapter One, there exists a substantial body of evidence arguing against such behavior. Of note are the in vitro ASM studies by Sasaki & Hoppin and Gunst & colleagues who have shown that the application of

---

3 It then follows that for a tidal length fluctuation with a known time-averaged value, the resulting time-averaged response in tension should correspond to the length-tension point defined by its steady-state, static equilibrium length-tension curve. The same argument also applies to tidal fluctuations in tidal tension and the resulting time-averaged length.
small tidal length fluctuations, at rates equal or below those experienced in vivo, result in length-tension traces that are strikingly different from the static equilibrium length-tension curve of ASM [Sasaki'79] [Gunst'83]. The effects of small physiologic-range tidal length fluctuations on ASM contractile behavior are addressed in the next chapter.

Figure 3-5: The assumption of static equilibrium on ASM length-tension behavior asserts that the imposition of tidal length fluctuations results in a tension response that lies on or near its steady-state, static equilibrium length tension relation. Conversely in the case of imposed tidal tension fluctuations, the assumption asserts that airway smooth muscle length accommodates to tidal tension fluctuations by traversing a sequence of points defined by the steady-state static equilibrium length-tension relation. In both cases, the effect of tidal stretch is believed to shift ASM up and down its static equilibrium length-tension relation. For a load with a known cycle-averaged length, the cycle-averaged tension response is defined by the static equilibrium length tension relation. Likewise, for a known cycle-averaged tension load, cycle averaged muscle length is again defined by the static equilibrium length tension relation.

3.5 STATIC EQUILIBRIUM = AN ASTHMATIC NORMAL?

If the chosen airway model parameters described above represent that of a normal airway, the classical model of airway narrowing predicts that airway narrowing in the normal subject progresses to closure for lung inflation pressures less than 6 cm H₂O (Figure 3-6). This conclusion, which represents an
airway challenged while the lung is near FRC, is supported by the finding of Skloot, et al., who showed that if non-asthmatic subjects are challenged with methacholine and prohibited from taking deep inspirations from FRC, they develop a constrictor response indistinguishable from that of an asthmatic [Skloot’95]. Similarly, Gunst et al., have shown that airway closure occurs in canine lungs for transpulmonary pressures of 7.5 \text{ cm H}_2\text{O} or less [Gunst’88]. The airway model and the experimental findings are consistent as they show that lung inflation near FRC is unable to prevent excessive narrowing from occurring.

Figure 3-6: Elastic recoil curves and the ASM static equilibrium L-T relation as used in MacKem’95. Note that at FRC (P_L = 6 \text{ cm H}_2\text{O}) the intersection of the elastic recoil curve and the static equilibrium L-T curve occurs at point corresponding to 0.34\% T_o and 0.46 L_o on the L-T. Additionally, the length of this intersection point is where airway closure occurs.

3.6 SUMMARY

This chapter showed that the elastic recoil tensions conferred by the supporting tissue structures in the lung provide a load that maintains airway patency and in certain cases, protects the airways against unimpeded bronchoconstriction. Using a model of the elastic recoil tensions provided estimates of the loads
applied onto ASM in the setting of tidal breathing. In the case of normal tidal breathing, these loads were estimated at 11 to 13% of $T_o$ in amplitude. In the case of deep inspirations, the amplitude of the tidal recoil tension was estimated to exceed 30% $T_o$ and even approach 40 to 45% of $T_o$. Similarly, these tidal lung inflations, when translated to length fluctuations at the airway, are estimated to range from 2% to 30% of $L_o$.

The classical model of airway narrowing and its assumption of a prevailing static equilibrium were discussed. One essential assumption in this classical model was that ASM behavior, even in the setting of tidal stretch, was defined by its steady-state, static equilibrium length-tension relation. The implications of the static equilibrium assumption and the predictions of subsequent ASM behavior to imposed tidal fluctuations in length were illustrated. In the case of imposed tidal length fluctuations, the assumption of static equilibrium predicts that muscle tension remains on or near its static equilibrium length tension curve. Similarly, for an imposed tidal tension, the assumption of static equilibrium predicts that muscle length remains on or near its static equilibrium length tension curve. Tidal stretch, in the form of either imposed tidal length or tension fluctuations, therefore are assumed to have no effect other than shifting length or tension up and down its steady-state static equilibrium length-tension relation.

The next chapter tests the assumptions of static equilibrium, as made in the classical airway models, by investigating the effects of small physiologic-range tidal length fluctuations on ASM contractile behavior.
Chapter Four

STRETCH-MODULATION OF AIRWAY SMOOTH MUSCLE TONE

4.1. OVERVIEW

This chapter investigates the effect of small, physiologic-range tidal length fluctuations on airway smooth muscle contractile state, and tests the assumption that ASM state is defined by its static equilibrium steady-state isometric length-tension relation. The results will show that ASM state (tension, stiffness, and hysteresivity), under the influence of tidal length fluctuations, is not determined by its static equilibrium length-tension relation. Moreover, results obtained from the HHM theory support the central hypothesis that the perturbations from static equilibrium steady-state are mechanistically consistent with the direct and disruptive action of stretch on the actin-myosin crossbridge.

4.2. THE EFFECTS OF TIDAL STRETCH ON MUSCLE BEHAVIOR

If maximally activated skeletal muscle is forcibly lengthened, a sharp rise in muscle tension occurs. If the lengthening is sufficiently rapid, tension can increase to 2.5-fold above its isometric maximal tension ($T_e$) [Harry'90]. The converse effect is observed in rapid releases in length where a shortening length change of only 1.5% of its optimal length ($L_o$) is capable of reducing muscle tension by more than 80% [Huxley'71]. If sinusoidal length changes are applied to activated skeletal muscle, tension responds with fluctuating component but the mean tension decreases sharply over the first few cycles (Figure 4-1) [Rack'74]. This fall in tension becomes more pronounced with large amplitudes of stretch. Additionally, the decrease in tension is associated with changes equivalent to an increase in hysteresivity and a decrease in stiffness (increased work dissipation and a slower rate of peak-to-trough response in tension as the amplitude of length changes is increased.)
In activated smooth muscle, tidal length fluctuations also cause cycle-averaged tension to decrease from its isometric value and large hysteretic length-tension loops to form [Sasaki '79]. Similar studies have shown, likewise, that the imposition of length fluctuation ($0.5 L_o$ to $0.70 L_o$) decreased the cycle-averaged tension below that corresponding to the isometric steady state for the cycle-averaged length [Gunst '83].

Figure 4-1: The effect of tidal stretch on tetanized skeletal muscle (cat soleus muscle). A) The plateau in isometric tension is followed by a tidal stretches (11Hz). B) The same stretching protocol at a slightly lower level of activation. C) the effect of tidal stretch at 50 Hz on cat gastrocnemius muscle. Source: Rack PMH. 1974. The short-range stiffness of active mammalian muscle and its effect on mechanical properties, J. Physiol. 240: 331-350.

While the mechanism for these effects of tidal stretch is generally believed to be attributable to tidal stretch acting to disrupt established actin-myosin bonds (i.e. crossbridges.) [Rack '74] [Sasaki '79], recent studies have asserted that these effects cannot be accounted for using crossbridge mechanisms alone [Shen '97]. The hypothesis advanced in this thesis holds, as Rack and Sasaki & Hoppin have argued, that these effects are indeed consistent with actin-myosin crossbridge dynamics. Section shows the theoretical predictions of the HHM theory and the result that actin-myosin dynamics can indeed account for changes in tension that mirror the early observations of Rack and Sasaki & Hoppin.
4.3. MATERIALS & METHODS

In this investigation, bovine tracheal smooth muscle was used as a proxy for bronchial smooth muscle. Details of the tissue preparation and experimental apparatus are described below.

4.3.1. Tissue preparation

Bovine tracheae were obtained from a local slaughterhouse (Research 87), and a section of four to five rings in the central region were stored in cold-phosphate buffered saline for up to 24 hours before use. The inner mucosal layer, the outer connective tissue layer, and the adjoining cartilage rings were carefully dissected from the trachealis under Krebs solution (in mM: 118 NaCl, 4.59 KCl, 1.0 KH₂PO₄, 0.45 MgSO₄, 0.18 CaCl₂, 11.1 Glucose, 23.8, NaHCO₃; pH at 7.5-7.7) at room temperature, aerated with 95% O₂ - 5% CO₂. Strips of smooth muscle approximately 2x3x20 mm were carefully dissected out from the trachealis tissue. The ends of the muscle strips were attached to brass clips with cyanoacrylate glue (a.k.a. SuperGlue™), the muscle-clip assembly was attached to a straightened music steel wire (0.37 mm), and the assembly suspended in a 50-ml vertical tissue bath. The lower clip of the muscle-clip assembly was connected to a hook affixed to the bottom of the tissue bath. The music wire was attached to a combined force transducer/lever arm apparatus. The tissue bath was circulated with Krebs solution, aerated with 95% O₂ - 5% CO₂, and maintained at 37 °C with a surrounding water jacket. Each strip was allowed to equilibrate to the bath environment for 1 hour.

4.3.2. Apparatus

A schematic of the experimental apparatus is illustrated in Figure 4-2. Small length changes were imposed using a servo-controlled lever arm system (Cambridge Technologies 305B). The servo-controlled lever arm system was modified with the replacement of the lever arm with a cantilevered force transducer (Sensotec). The upper wire of the muscle-clip assembly was attached to the force transducer. Coarse length changes were made by a micropositioner (Ealing), on which the servo system was mounted.

Control and data acquisition of command and data signals were performed digitally, using a commercial computer program (LabView). The command signal from the digital-to-analog converter was
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filtered at 50 Hz. Length and tension signals were filtered at 25 Hz before analog to digital conversion. Filtering for both command and data signals was performed using analog, four-pole Butterworth filters (Ithaco). Command control signals and data acquisition were performed at a rate of 200 samples per second.

Figure 4-2: A schematic of the experimental set-up. Tidal length fluctuations are imposed by a servo controller. Muscle tension is measured with a transducer mounted at the tip of the servo actuator.

4.3.3. Experimental protocol

The muscle was first brought to optimal length ($L_o$) in the usual manner\(^1\). The experiment then proceeded as follows. A sinusoidal length fluctuation ($\delta L$) of amplitude 0.25% of $L_o$, at 0.2 Hz, was imposed on the muscle for 100 seconds. At $t = 100$ seconds, the muscle was then activated with acetylcholine to obtain a bath concentration of $10^{-4}$ M and allowed to contract “quasi-isometrically” (i.e. with the 0.25% length oscillation) for 600 seconds. At $t = 600$ seconds, $\delta L$ was increased to 0.5% of $L_o$ and maintained for 500 seconds. $\delta L$ was sequentially increased to 1, 2, 4, & 8% of $L_o$, with each amplitude maintained for 500 seconds. This stretching protocol is illustrated in Figure 4-3. Length and tension signals were recorded throughout the duration of the experiment. At the end of the 8%

\(^1\) Optimal length is defined as the length at which active tension (total minus passive tension) is maximal. Total tension was determined by the plateau value of tension for the given length. Passive tension represents the tension of relaxed muscle at that length.

\(^2\) Muscle states obtained for a strain amplitude of 0.25% $L_o$ is used to represent muscle under isometric conditions. This assertion is supported by pilot experiments that show isometric tension is not significantly altered with the imposition of strain amplitude < 0.5% of $L_o$. 

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oscillation, the oscillations were stopped and the bath was flushed. The muscle was allowed to rest for 15 minutes after which time the muscle was re-activated with $10^{-4}$ M acetylcholine and allowed to contract with a $\delta L$ of 0.25% $L_n$. The maximal mean tension at $\delta L = 0.25% L_n$ (which is assumed to represent the maximal isometric tension, $T_n$) was recorded. Muscle strips that did not reproduce peak tensions of at least 90% of the initial were excluded from the study.

![Graph showing tidal length fluctuation protocol](image)

Figure 4-3: The tidal length fluctuation protocol. The tidal length amplitude ($\delta L$) is increased two-fold at every stage beginning from 0.25% $L_n$ to 8% $L_n$. Note that the frequency is not to scale.

### 4.3.4. Mechanical measurements

Cycle-averaged tension, stiffness, and hysteresivity were calculated from the measured length and force signals as described by Fredberg [Fredberg’89]. The general form of the equations used in the calculation are the same as those defined in Chapter Two.

### 4.3.5. Additional studies

Several experiments were performed to assess the effects stretch-activated humoral and neurogenic pathways, and operating muscle length. They are discussed below.

**Inhibition of stretch-activated humoral and neurogenic pathways:** Stretch and other forms of mechanical perturbation are known to induce the synthesis and/or release relaxing factors [Shore’89]. These factors are known to be released through neurogenic or cyclooxygenase/prostaglandin pathways. In order to assess the possible role of these factors on changes in $T$, $K$, and $\eta$, paired strips of bovine trachealis were treated with vehicle (50 $\mu$l ethanol + 50 $\mu$l water) or with a combination of indomethacin ($10^{-6}$ M )
and tetrodotoxin (10⁻⁶ M) for 30 minutes before the start of the protocol. Indomethacin is an inhibitor of cyclooxygenase (COX-1 & COX-2), an enzyme required for prostaglandin synthesis. Tetrodotoxin (TTX) is sodium channel blocker than prevents depolarization of nerves.

**Effects of operating muscle length:** To assess the role of tidal stretch at operating muscle lengths different from \( L_o \), and to investigate whether the effect is present where muscle tension is less than maximal, the experimental protocol above (Section 4.3.3) is used at operating muscle lengths of 60, 80, & 100% of \( L_o \). Common strips were used to investigate the three operating lengths, with extended washout periods of 20 minutes. Strain oscillation amplitudes were based on the three operating lengths investigated.

4.3.6. Data analysis

Except where noted, cycle-averaged muscle states are presented as the mean ± standard deviation. In each case “N” represents the number of muscle strips used for each study. To account for the variability in peak tension and stiffness between muscle strips (primarily due to variability in muscle strip size), muscle tension and stiffness were normalized to peak isometric tension (represented by the cycle-averaged tension at a length oscillation of 0.25% \( L_o \)) and muscle stiffness was normalized by the stiffness at 0.25% \( L_o \). Hysteresivity was not normalized. Statistical analysis of the effect of strain oscillation was performed using a repeated-measures analysis of variance with a Bonferroni correction. The comparisons were made with the states obtained under strain oscillation at 0.25% \( L_o \), serving as control values.

4.4. EXPERIMENTAL RESULTS

Tension and length traces of a representative muscle strip are shown in Figure 4-4. The first 100 seconds of the time course shows the passive response of the muscle to a strain oscillation amplitude (\( \delta L \)) of 0.25% \( L_o \). At \( t = 100 \) seconds, the addition of acetylcholine induced a rapid increase in muscle tension and the peak-to-trough fluctuations in tension (\( \Delta T \)). The increases in tension and tension
fluctuations approached a plateau over the next 500 seconds. At $t = 600$ seconds, the increase of strain amplitude to $\delta L = 0.5\%$ resulted in a proportional increase in $\Delta T$ and a slight decrease in cycle-averaged muscle tension ($\bar{T}_{\text{cycle}}$). Similarly, the increase of strain amplitude to $\delta L = 1\%$ at $t = 1100$ seconds, resulted in a nearly proportional increase in $\Delta T$ and further decrease in $\bar{T}_{\text{cycle}}$. However, as $\varepsilon$ was increased to 2% of $L_o$, the resulting increase in $\Delta T$ lagged noticeably the increase in $\delta L$. Moreover, the decrease in $\bar{T}_{\text{cycle}}$ was considerable, declining over the first several strain cycles and gradually reaching a new and lower steady-state value. This trend continued as $\varepsilon$ was increased to 4 and 8% of $L_o$.

In addition, for $\delta L$ at 4 and 8% of $L_o$, peak tensions decreased to a level near that obtained "quasi-isometric" steady-state at $t = 600s$. Trough tensions approached tensions obtained for passive, unactivated muscle.

Figure 4-4: Representative time response of tension with the length change protocol. For small length fluctuation amplitudes, mean tension remains constant and the amplitude of tension increases nearly proportionately to the increase in length amplitude. As tidal length fluctuation is increased, the mean tension begins to fall, and does so over the first few cycles of the new amplitude. At the largest amplitude, the mean tension falls to less that 50% of its initial tension.
4.4.1. The effects of tidal stretch at $L_s$: Length-tension loops

As illustrated in Figure 4-4, the imposition of tidal strain oscillation resulted in a rapid re-equilibration of muscle tension to a new steady-state pattern over the first few cycles. Figure 4-5 shows the steady-state length-tension loops for the tidal length fluctuation protocol. For values of $\delta L = 0.5\% L_s$, or less, the loops were nearly elliptical in shape, not very hysteretic (i.e. small loop area), possessed steep chord slopes, maintained near constant mean tensions, and increased in height in proportion to the...
imposed strain amplitude. However, when $\varepsilon$ was increased to values of $1\%L_n$ or more, the loops lost their elliptical shape and became more banana-shaped. Moreover, the loops became more hysteretic, the chord slopes decreased, average tensions decreased, and the heights increased slower than the increases in strain amplitude. For $\delta L$ at $4 \& 8\%L_n$, peak tensions decreased to near $\bar{T}_{cycle}$ obtained for the small values of $\delta L$. Trough tension for the largest strain amplitude of $\delta L = 8\%L_n$ decreased to values near that obtained for passive, unactivated muscle. Peak and trough tensions for each loop versus strain amplitude (Figure 4-6) show that as the amplitude of length fluctuation is increased, peak tension increases, and reaches a yield tension near 27% above $T_o$. As $\delta L$ is further increased beyond 1% $L_n$, the peak tension begins to decline, and decreases to a level near To at $\delta L = 8\%L_n$. Trough tension decreases as the amplitude of length fluctuation is increased. For $\delta L = 8\%L_n$, trough tensions decreases to near zero tension.

Figure 4-6: Peak, trough, and mean (dotted line) tensions during tidal length fluctuations over all strips. Note that for small fluctuations, the peak and trough tension deviated equally from the mean tension. The peak tension, however, reached a maximum near 1% $L_n$ where the maximum tension is roughly 27% over $T_o$. As the tidal length amplitude was further increased, the peak tension decreased. Trough tension also decreased at a faster rate.
4.4.2. The effects of tidal stretch at $L_n$: velocity-tension loops

In identical fashion to the length-tension loops presented above, Figure 4-7 illustrates the steady-state tension patterns re-plotted in tension versus velocity format. Similar to the results above, for values of $\delta L = 0.5\%$ or less, the loops were nearly elliptical in shape. However as $\delta L$ was increased to $1\% L_n$ or greater, the loops began to lose their symmetric shape and developed an egg-like shape. Figure 4-8 presents the same tension-velocity loops as Figure 4-8, with a representative Hill curve for canine ASM superimposed. Note that the tension-velocity loops do not follow the Hill curve.
4.4.3. Time course of tissue states

To quantify the height, slope, and fatness of the loops as they varied in time, stiffness (quantifying height and slope) and hysteresivity (quantifying fatness) were calculated on a loop-by-loop basis. The time-course of these tissues states, as well as $T_{\text{cycle}}$, are shown in Figure 4-9. The first 20 loops (equivalent to the first 100 seconds for $f = 0.2$Hz) show the passive response of the muscle to $\delta L = 0.25\%L_n$. The addition of acetylcholine induced near parallel and monotonic increases in $T_{\text{cycle}}$ and $K$. In contrast, $\eta$ exhibited a biphasic time course, rapidly increasing at the onset of stimulation and then slowly decreasing in asymptotic fashion. During force development, the time course of $\eta$ was markedly dissociated from those of $T_{\text{cycle}}$ or $K$. As $\delta L$ was increased to $0.5\%L_n$, changes in $T_{\text{cycle}}$, $K$, and $\eta$ were minimal. However, as $\delta L$ was increased to $1\%$, $T_{\text{cycle}}$ and $K$ noticeably decreased over the first several loops and reached new and lower steady state values. $\eta$ increased with the increase in $\delta L$. These trends continued for $T_{\text{cycle}}$ and $K$ through $\delta L = 8\%L_n$. $\eta$, however, reversed its trend at $\delta L = 8\%L_n$,
decreasing slightly as $\delta L$ was increased from 4% $L_n$ to 8% $L_n$. The effects of tidal strain amplitude on steady-state values of $\bar{T}_{cycle}$, $K$, and $\eta$ are illustrated in Figure 4-10 and listed in Table 4-1.

Figure 4-9: A representative time course of tissue states. The top graph shows cycle-averaged tension normalized by $T_o$ versus time. The middle graph shows muscle stiffness normalized by the final stiffness at $\delta L = 0.25% L_n$. The lower graph shows the time course of hysteresivity during the experimental protocol. As strain amplitude is increased at each stage, tension and stiffness decrease rapidly over the first few cycles. Hysteresivity increases in a stepwise like fashion as stain amplitude is increased. At the highest amplitude of $\delta L = 8\%$, hysteresivity decreased.

Figure 4-10: Steady state tension, stiffness, and hysteresivity values. Steady-state values of each state are obtained by taking the final values of each state of each of the strain amplitude states. These values are then incorporated into the calculations of means and standard deviation. Again, tension and stiffness values are normalized to their respective reference values. These results show that tension and stiffness decrease as strain amplitude ($\delta L$) is increased from 0.25% $L_n$ to 8% $L_n$. Hysteresivity responds to an increase in strain amplitude in a bimodal fashion where hysteresivity first increases then decreases with increasing stain amplitude. *: $p < 0.005$, †: $p < 10^{-6}$. 

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Table 4-1: Steady-state cycle averaged tension (\(T_{\text{cycle}}\)), stiffness (\(K\)), and hysteresivity (\(\eta\)), at different strain amplitudes (\(\delta L\)) as shown in Figure 4-11. N=9, *: \(p < 0.005\), †: \(p < 10^{-6}\).

<table>
<thead>
<tr>
<th>(\delta L)</th>
<th>(\overline{T_{\text{cycle}}})</th>
<th>(K)</th>
<th>(\eta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25%</td>
<td>1</td>
<td>1</td>
<td>0.1850±0.0209</td>
</tr>
<tr>
<td>0.50%</td>
<td>1.0181±0.0276(^{NS})</td>
<td>0.9882±0.0297(^{NS})</td>
<td>0.1884±0.0162(^{NS})</td>
</tr>
<tr>
<td>1%</td>
<td>0.9467±0.0576(^*)</td>
<td>0.8400±0.0545(^*)</td>
<td>0.2237±0.0232(^*)</td>
</tr>
<tr>
<td>2%</td>
<td>0.7499±0.0714(^†)</td>
<td>0.5621±0.0645(^†)</td>
<td>0.2990±0.0335(^†)</td>
</tr>
<tr>
<td>4%</td>
<td>0.5570±0.0584(^†)</td>
<td>0.3221±0.0456(^†)</td>
<td>0.3539±0.0424(^†)</td>
</tr>
<tr>
<td>8%</td>
<td>0.4206±0.0578(^†)</td>
<td>0.1736±0.0327(^†)</td>
<td>0.3225±0.0453(^†)</td>
</tr>
</tbody>
</table>

4.4.4. The effect of pharmacological blockade with indomethacin and tetrodotoxin

The effect of tidal stretch on activated muscle strains that were treated with tetrodotoxin and indomethacin were not different from the effects observed in strips that were treated with vehicle. There was a tendency for \(\eta\) of the strips treated with tetrodotoxin and indomethacin to be greater, but this tendency was not significant. The results of this study are shown in Figure 4-11.

Figure 4-11: The effect of tetrodotoxin and indomethacin on muscle state response to sinusoidal length changes. Tetrodotoxin and indomethacin test results (solid line) and vehicle control (no INDO or TTX, dotted line) shows the characteristic changes in ASM tissue state as the amplitude of length fluctuation is increased. The tension and stiffness responses are nearly indistinguishable from each other. Hysteresivity is higher for the test case compared to control but this difference ins not statistically significant. N=5 for both control and test.
4.4.5. The effect of tidal strains at operating lengths less than $L_o$

The effects of tidal strain oscillation, centered at operating lengths different from $L_o$, on cycle averaged tension are shown in Figure 4-12. The results show that the effects of tidal strain on $\bar{T}_{\text{cycle}}, K_x$, and $\eta$ at different operating lengths are virtually the same as those obtained at $L_o$. Namely, an increase in tidal strain amplitude resulted in a decrease in cycle-averaged muscle tension and stiffness, and a bimodal change in hysteresivity. For a given strain amplitude as a percentage of the operating length, the effect of tidal strain amplitude was similar at low strain amplitudes and diverged slightly as strain amplitude was increased for cycle averaged tension and hysteresivity. The fall in stiffness, however, remained essentially unchanged at different baseline lengths.

![Graphs showing the effects of tidal strain on tension, stiffness, and hysteresivity at different operating lengths](image)

Figure 4-12: Effect of operating length on tension, stiffness, and hysteresivity. A) At length less than $L_o$, tidal strain amplitude resulted in slightly less decrease in tension when compared to the results at $L_o$. B) Decreasing the operating length from $L_o$ to 0.8 $L_o$ or 0.6 $L_o$, however, did not result in changes in the stiffness response to tidal strain amplitude. C) For low tidal strain amplitude, changes in operating length appear to alter hysteresivity only slightly with lower length resulting in a slightly higher value of hysteresivity. However, as the tidal strain amplitude is increased, there is a departure in the responses where lower lengths result in higher values of hysteresivity.

4.5. THE EFFECT OF TIDAL LENGTH FLUCTUATIONS AS PREDICTED BY THE HHM THEORY

The HHM theory may be used to predict the effects of tidal length fluctuations on a system that incorporates crossbridge mechanisms only. In this test, the HHM theory, as described in Chapter Three, is used to compute the steady-state tension, stiffness, and hysteresivity response to tidal length fluctuations. Figure 4-13 shows the results of the HHM theory predictions. The predictions show that tidal length fluctuations (0.2Hz) centered about optimal length ($L_o$) are capable of decreasing tension
and stiffness, and increasing hysteresivity. Moreover, at tidal length amplitude ($\delta L$) of 4% $L_o$, the theory predicts roughly a 55% decrease in tension (From 100% $T_o$ to 44% $T_o$), an 80% decrease in stiffness (to 21% of the stiffness obtained at $\delta L = 0.25\% L_o$), and a near three-fold increase in hysteresivity from baseline (0.18 to 0.50). These predictions argue strongly against the assertions of Gunst & colleagues. Indeed, crossbridge mechanisms alone are capable of producing substantial changes in tension, stiffness, and hysteresivity on a purely theoretical basis.

![Graph showing the effect of tidal length fluctuation on tension, stiffness, and hysteresivity](image)

**Figure 4-13:** Steady-state HHM theory results for the effect of tidal length fluctuation on tension ($T$), stiffness ($K$) and hysteresivity ($\eta$). Tension and stiffness control values ($T_o$ and $K_o$ are steady-state tension and stiffness at $\delta L = 0.25\% L_o$.) The HHM theory results are represented as the solid, dashed, and dotted lines ($T$, $K$, and $\eta$, respectively.). The points on the graph represent the experimental results obtained through a second set of experiments using the identical protocol described in Section 2.3.3. Note: Slight differences in experimental values for $T$, $K$, and $\eta$ occurred between the different set of experiments even though the same protocol was used.

### 4.6. DISCUSSION

The principle finding of this study was that airway smooth muscle states (tension, stiffness, and hysteresivity) could depart dramatically from those associated with maximally activated, isometric steady-state with the imposition of tidal length oscillations. Moreover, these dynamically determined contractile states could be maintained for as long as the tidal stretches were sustained. These states were
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classified by a graded stretch-effect relation where the greater the tidal stretch amplitude, the greater were the departures from their values measured in conditions approximating the isometric steady state. The provocative stretch amplitude causing cycle-averaged muscle tension ($\bar{T}_{\text{cycle}}$) and muscle stiffness ($K$) to fall by half, or hysteresivity ($\eta$) to double, was noted 4%. Because muscle stiffness is a rough reflection of the numbers of actin-myosin interactions, and muscle hysteresivity is a rough reflection of the rate of turnover of those interactions1 [Fredberg’96], these results suggest that tidal stretch decreases the numbers of actin-myosin interactions and increases their turnover rate.

There were several differences between the protocol used here, that of Sasaki & Hoppin and Gunst & colleagues, that make detailed comparison difficult, but as in these previous studies the data reported here also show that the imposition of length fluctuations depress the mean tension. With regard to hysteresis, the above results, for all but the largest strain amplitude, show that hysteresivity increased as the amplitude of strain was increased. This is in agreement with the findings of Rack & Westbury for skeletal muscle and Gunst & colleagues for smooth muscle [Rack’74] [Gunst’83]. Although the activated state of ASM has generally been considered to be a high hysteresis state based on the studies of Sasaki & Hoppin, the above studies and the recent results of Gunst and colleagues show that length fluctuations have a graded stretch-effect on hysteresis where low amplitudes of stretch result in low values of hysteresis and larger amplitudes of stretch increase hysteresis [Sasaki’79] [Gunst’83]. The difference between the results of Sasaki & Hoppin and the current study is reconciled by the fact that the studies of Sasaki & Hoppin correspond to tidal stretch amplitudes of 5% $L_n$, which is associated with larger values of hysteresivity.

**Mechanism: From extracellular signaling to the contractile apparatus**

The results of the control studies show that the effect tidal length fluctuations occurred at all lengths tested. The dependencies of $\bar{T}_{\text{cycle}}$, $K$, and $\eta$ upon tidal stretch amplitude were uninfluenced by the treatment of the tissues with TTX and INDO, thus ruling out the importance of relaxation and/or deactivation mechanisms based upon stretch activated neural pathways or prostanoid release. Falling activation through acetylcholine degradation is also ruled out by time-controls that show sustained tension for the duration of the experimental protocol.

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1 As discussed in Chapter Two.
A related mechanism warranting consideration is stretch-induced changes in activation level. It has been shown that mechanical stretch on some types of smooth muscle is capable of modulating muscle contraction [Kirber ‘88] [Kulik ‘91]. Yoo & colleagues have shown the presence of a mechanosensitive mechanism capable of decreasing the level of muscarinic activation when the muscle is subjected to decrease in length [Yoo ‘94]. This mechanism appears to lie at the level of phospholipase C, decreasing its activity and thus measurable phosphotidylinositol turnover. Although a likely and important mechanism regulating smooth muscle contractility in vivo, mechanisms based at the level of the cell membrane cannot account entirely for the response of smooth muscle to tidal length fluctuations. In studies using Triton-X skinned smooth muscle preparations, the removal of the cell membrane and its associated proteins did not qualitatively alter the dependencies of $\bar{T}_{\text{cycle}}$, $K$, and $\eta$ upon tidal stretch amplitude. Specifically, the imposition of tidal length fluctuations at 0.331 Hz with an amplitude of 4% of $L_o$, resulted in a greater than 50% decrease in $\bar{T}_{\text{cycle}}$ and $K$, and a greater than twofold increase in $\eta$ [Fredberg ‘97]. Moreover, for these skinned preparations, activation is controlled directly through the bathing solution calcium concentration. Taken together, these results suggests that the mechanism responsible for the effect of tidal length fluctuations on muscle state lie below the level of the membrane and also below the level of intracellular calcium, to the level of cell mechanical structure or myosin-actin binding.

Another mechanism warranting consideration is the plasticity of the contractile elements/filaments within the cell as either a reorganization of the contractile elements or stretch-induced remodeling of the cytoskeleton. Pratusevich and colleagues have demonstrated mechanical behavior suggestive of the idea of the reorganization of the contractile elements within a smooth muscle cell over serial activation events [Pratusevich ‘95]. In addition, Gunst and colleagues have also demonstrated mechanical behavior suggestive of the idea that the contractile filaments within smooth muscle can plastically interact, with a remodeling of the focal adhesion complex and/or the cytoskeletal network occurring during the course of force development [Gunst ‘95]. Remodeling of these types are important and cannot be ruled out as mechanisms contributing to the responses reported here. However, the rapidity, the magnitude, and the nature of the observed responses suggest that stretch-induced plastic remodeling cannot be the primary mechanism accounting for the observations reported here. First, when tidal stretch amplitude was suddenly increased, the effects on tension, stiffness, and hysteresivity were
essentially completed within two to three tidal stretches, or less than 15 seconds. Whereas the remodeling events reported previously by Pratusevich and colleagues revealed themselves only over longer duration. Second, the onset of tidal stretches led to decrements of force and stiffness that were not only much faster but also much larger than those associated with remodeling events that have been reported previously. Finally, even if plastic remodeling of the cytoskeleton were able to account for stretch-induced changes in force and stiffness, both the temporal evolution of the hysteresivity and the stretch-induced change in the hysteresivity would remain unaccounted for and would the require their own ad hoc explanations.

This brings us to the mechanism at the level of the crossbridge, which could come into play in several ways. The first consideration is how muscle length modulates the maximum number of actin-myosin interactions that can be attained in the isometric steady-state; changes in the number of interaction with changes of muscle length determine the shape of the static force-length characteristic and set the optimal length, $L_0$. The range of length excursions used in this study represent deviations from $L_0$ of 8% or less. Over this length range, the static force length characteristic of smooth muscle predicts changes in muscle tension no greater than 10% of $T_n$ at the extremes of the length excursion. Moreover, the imposition of length fluctuations at a length less that $L_n$ did not qualitatively change the response. Therefore the shapes of the dynamic force-length loops and the large force decrements that occurred with the onset of tidal stretches cannot be attributed to traversing the plateau or descending limb of the static-force length characteristic.

Another consideration is the effect arising from heterogeneity of contractile strength (as quantified by maximum stress) between individual cells or between the contractile units within cells. To this effect, two mechanisms which may play a role during repeated shortening and lengthening are the depressive effect of shortening on isometric stress development as proposed by Becker & Murphy and a phenomena known as “sarcomere popping” as proposed by Julian & Morgan [Becker’88] [Julian’79] [Morgan’90]. Becker & Murphy showed that a decrease in length from 1.07 $L_n$ to 1.0 $L_n$ after isometric contraction of skeletal muscle strips was capable of depressing steady-state tension recovery by 4.7 to 8.3% of $T_n$ [Becker’87]. This depression in muscle tension cannot, however, account for the near 50% depression in $T_n$ when activated muscle is subject to a strain oscillation of 4% $L_n$. 

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An alternate mechanism arising from strength heterogeneity is through “sarcomere popping” as proposed by Talbot & Morgan [Talbot'96]. In this mechanism an inherent strength heterogeneity between contractile units connected in series leads to weaker contractile units being extended (“popped”) to a length beyond its optimal tension. Sarcomere popping is, thus, a mechanism sensitive to the changes in muscle tension. The hypothesis that sarcomere popping can account for the observed fall in tension postulates that exposure to large tensions, as occurs transiently during the initial few length cycles, “pops” weaker contractile units. If operating length is decreased, the contractile units shorten further away from their optimal length and overall muscle tension decreases accordingly. Sarcomere popping then predicts that the effect of tidal length fluctuations should diminish as each sarcomere is exposed to lower peak tensions and is further away from optimal length (where popping is most likely to occur). The experimental results, however, show that tidal length fluctuations, even when operating at lengths less than \(L_o\) (where the isometric tension is only 40% of \(T_o\)), remain effective at decreasing substantially tension and stiffness and increasing hysteresivity (Refer to Figure 4-12). These results suggest that sarcomere popping, and other mechanism based on strength heterogeneity of the contractile units, are unlikely as dominant mechanisms in the observed effects of tidal length fluctuation.

Lastly, is the direct effect of tidal stretch on bridge dynamics. It is generally held to be true for all types of muscle that the isometric steady-state the rate of crossbridge cycling is constant and that if steady state is disturbed sufficiently, then crossbridge dynamics will be altered. In striated muscle, it is well established that the inhibition of force and stiffness that occurs with the onset of sinusoidal stretches is attributable to the direct effect of tidal stretch upon bridge dynamics [Rack'74] [Zahalak'80]. While the case is less clear in smooth muscle, [Sasaki'79], predictions of the HHM theory support the hypothesis that tidal stretch can modify ASM tension through the action of stretch on the actin-myosin binding cycle. The experimental results support the hypothesis that tidal stretch acts to perturb the myosin-actin binding equilibrium of airway smooth muscle from that associated with static, steady-state conditions into one which is dynamically-determined.

\footnote{The term “pop” meaning lengthened beyond optimal tension generating capacity and onto the descending length-tension limb for that contractile element.}
4.7. SUMMARY

The data presented here suggest that tidal lung inflations have the potential to modulate profoundly the behavior of maximally contracted airway smooth muscle. The experimental result showed that the imposition of small tidal length changes drove ASM states away from its steady-state isometric length-tension relation to a new state characterized by decreased tension and stiffness, and increased hysteresivity. Moreover, these changes occurred in a threshold like manner where sufficiently large amplitudes drove ASM state away from isotonic steady-state values but smaller amplitudes did not.

The changes in tension, stiffness, and hysteresivity that occurred with tidal length fluctuations were consistent with mechanism of a direct and disruptive action of tidal stretch on the actin-myosin crossbridge. The predictions of the HHM theory showed that indeed, tidal stretch acting at the level of the actin-myosin bond and binding cycle could manifest in substantial tissue level changes. Moreover, these tissue level changes in tension, stiffness, and hysteresivity mirrored the experimentally observed effect of tidal length changes.

The ability to perturb the ASM state, through the disruptive effect of tidal stretch on the actin-myosin binding cycle, then suggests an important role of this interaction in excessive airway narrowing. The ability to escape steady-state isometric conditions with tidal lung inflation, versus failing to do so, may be a major mechanism distinguishing the behavior of activated ASM in the normal lung from that in the spontaneously obstructed asthmatic lung. While abnormal ASM behavior (stronger, more sensitive to agonist, faster, less likely to relax, etc.) may contribute to the development of asthmatic AHR, the above interaction suggests that abnormality of the muscle is not a prerequisite for excessive airway narrowing. Rather, the mechanism suggests that an abnormal coupling between tidal lung inflation and airway smooth muscle stretch may be a major determinant of excessive airway narrowing. That is, tidal lung volume changes, for whatever reason, are unable to impose sufficient tidal stretch on ASM to prevent a collapse to steady-state conditions. This then leads to the speculation that any tissue or condition involved in reducing the transmission of tidal lung volume changes to ASM stretch can potentially allow airway smooth muscle to either attain is full isometric maximal tension and in doing so shorten to its static equilibrium length. Excessive airway narrowing may then indeed be a consequence of an impaired ability to dilate the airway with tidal lung volume changes. This chapter suggests that this impaired
ability may arise from an inability of stretch to mechanically drive the binding equilibrium of myosin and actin away from its static equilibrium state.

Within this investigation, it is noted that while imposing tidal length changes provides an answer to the effects of stretch on muscle tension, this method cannot answer directly the question of how tidal stretch influences muscle length. The next chapter addresses this shortcoming through the inverse question of how tidal tension fluctuations influence cycle-averaged muscle length, through the application of sinusoidal tension fluctuations on contracted airway smooth muscle.
Chapter Five

FLUCTUATION-DRIVEN MUSCLE LENGTHENING

5.1. OVERVIEW

This Chapter addressed how tidal force fluctuations affect muscle length. This chapter shows that tidal force fluctuations lengthen ASM from its static equilibrium isotonic length to one substantially longer.

5.2. TIDAL LUNG INFLATIONS AND THE TIDAL LOAD GENERATED ON THE AIRWAYS

In Chapter Three, the tidal loads on airway smooth muscle were estimated at approximately 11 to 13% $T_o$ in amplitude for normal sized breaths\(^1\) and 30% $T_o$ in amplitude for a deep inspiratory sigh. Nevertheless, the classical theory of airway narrowing assumes that airway smooth muscle length is set by a mechanical balance of static forces. With the addition of a fluctuating load, the theory then assumes that static equilibrium conditions prevail on an instant by instant basis such that airway smooth muscle length is determined by a sequence of steady-state static equilibrium states, all lying on the static length-tension relation of the muscle. However, the result of Chapter Four showed that under the influence of tidal length fluctuations, as those which occur during tidal breathing, airway smooth muscle tension did not remain near its isometric tension but decreased markedly as the amplitude of length fluctuations was

\[^1\] Where normal sized breaths of 600 ml about FRC are estimated to correspond with $P_e$ swings from 3 to 9 cmH$_2$O.
increased. This chapter then tests the assumption of the classical theory that in the setting of tidal force fluctuations, airway smooth muscle length is set by a mechanical balance of static forces.

5.3. EXPERIMENTAL MATERIALS & METHODS

In this investigation, bovine tracheal smooth muscle was used as a proxy for bronchial smooth muscle. Details of the tissue preparation and experimental apparatus are described below.

5.3.1. Tissue preparation and experimental apparatus

The muscle preparation method and the experimental apparatus used in this series of experiments were identical to that described in Chapter Four. The reader is referred to 4.3.1 for details. One modification made to the existing apparatus was the addition of a servo control loop to apply a sinusoidal tension load. The control and data acquisition was performed digitally, using a computer program (LabView). The command signal from the digital-to-analog converter was filtered at 50 Hz. Length and force signals were filtered at 25 Hz before analog to digital conversion. Filtering for both command and data signals was performed using analog, four-pole Butterworth filters (Ithaco). Data control and acquisition were performed at a rate of 100 samples per second.

The time varying load pattern on the muscle was controlled using an internal PID control algorithm in which the load on the muscle was controlled by the rapid adjustment of muscle length. This method was capable of tracking of the prescribed time-varying loading pattern to within 0.5% of $T_o$.

5.3.2. Experimental protocol

The muscle was brought to optimal length using electric field stimulation for 30 seconds every five minutes. Passive tension (defined as the tension for unactivated muscle) immediately prior to stimulation and peak tension during stimulation were recorded. The difference between the peak total tension and the passive tension, which we define as the active tension, was calculated and recorded. The muscle was allowed to relax for 120 seconds before increasing muscle length by one millimeter. Muscle
length was incremented until a plateau in active tension was reached. (Plateau was defined as an increase in active tension less than 1% of the current active tension.) This length is defined as the optimal length, $L_o$. The muscle length was then calculated and recorded.

Due to strip variation in the response to electric field stimulation, peak isometric tension was determined using acetylcholine stimulation at a bath concentration of $10^{-4}$ M for fifteen minutes. The bath was then flushed and refilled with fresh solution and the muscle was allowed to relax.

The experiment proceeded as follows. The muscle was contracted isometrically to a tension threshold of $0.32T_o$ with an added offset to compensate for passive, connective tissue tension. This offset varied from strip to strip, but was roughly 3% $T_o$. When muscle tension reached the target tension, the contraction mode was then shifted immediately from isometric to isotonic ($T_{iso}$) where the load was clamped at $0.32T_o$. Isotonic contraction conditions were maintained for 120 minutes. At $t = 120$ minutes, a sinusoidal force fluctuation of 0.2Hz was superimposed on the target load ($0.32T_o$). The loading pattern followed the sequence of force amplitudes ($\delta T'$) of 4, 8, 16, 24, 32 & 8% of $T_o$, with each amplitude maintained for the following durations: 60 minutes for $\delta T = 4$, 8, and 16%$T_o$, 90 minutes for $\delta T = 24%T_o$, 120 minutes for $\delta T = 32%T_o$, and 120 minutes for $\delta T = 8%T_o$ (Figure 5-1). At the end of the loading protocol, the bath was flushed, and the muscle length was returned to $L_o$. The end condition of the muscle strip was tested using $10^{-4}$ M acetylcholine and allowing isometric tension development for 15 minutes. The maximal tension was recorded. To minimize the confounding effects of that damaged or fatigued muscle might exert on the experimental results, muscle strips that did not redevelop more than 85% of $T_o$ (as determined at the beginning of the experiment) were excluded from the study.

5.3.3. Mechanical measurements

Mechanical measurements followed that described in chapter Four to which the reader is referred.
5.3.4. Control experiments

Additional experiments were performed to assess the effects of time, isotonic load set point, and history. The details are discussed below.

*Time effects:* Time control experiments were executed in similar fashion to the experiments as outlined above in the Section 5.3.2 (Experimental Protocol). However, instead of a sequential increase in force amplitude $\delta T$, the amplitude was maintained constant at $\delta T = 4\%$ of $T_u$ (to be denoted as "$\delta T = 4\% T_u$") This force amplitude was maintained for the entire duration of the experiment. Force and length signals were filtered, digitized, recorded, and processed in an identical manner.

5.3.5. Data analysis

Cycle-averaged tension, stiffness, and hysteresivity are calculated using the method described by Fredberg [Fredberg'89]. The time course of muscle length change was obtained by averaging the fluctuations in muscle length over each cycle. Except where noted, cycle-averaged muscle states are presented as the mean ± standard deviation. In each case "N" represents the number of muscle strips used.
for each study. To compensate for variability between muscle strip, muscle length is presented normalized to $L_0$ and force is normalized to peak isometric tension (represented by the cycle-averaged tension for the length oscillation of 0.25% $L_0$). Stiffness is normalized to the stiffness at $\delta T = 4\% T_0$. Statistical analysis of the effect of force oscillation on cycle averaged muscle length ($L_{\text{ave}}$), normalized stiffness ($K$), and hysteresivity ($\eta$), was performed using a repeated-measures analysis of variance with a multiple comparison Bonferroni correction for p-values. Muscle length for $\delta T = 0\% T_0$, and stiffness and hysteresivity at $\delta T = 4\% T_0$, were used as reference values for the comparisons.

5.4. QUANTITATIVE METHODS—HHM THEORY FOR MUSCLES SUBJECTED TO FORCE FLUCTUATIONS

The HHM theory was used to investigate the effects of tidal force fluctuations on actin-myosin dynamics and on the resulting tissue level states of length, stiffness, and hysteresivity. The model parameters remained identical to those described in Chapter Four. A loading sequence identical to that used in the experimental protocol was used and the computation time for each force amplitude pattern was extended until muscle length reached a steady-state value. Stiffness, hysteresivity, and strain amplitude were calculated from the time and length responses using the identical method detailed in Section 4.2.4.

5.5. RESULTS-EXPERIMENTAL

A representative time course of the muscle length response to the fluctuating force is shown in Figure 5-2. The cyclic fluctuations in length have been suppressed by averaging length over each cycle. With the addition of $10^{-4}$ M acetylcholine, muscle tension rose to the isometric threshold of $0.32 T_0$ within several seconds. At the instant muscle tension reached the isometric threshold, the mode of contraction was switched from isometric to isotonic (where the isotonic load was clamped at 0.32 $T_0$) and the muscle shortened. Muscle shortening continued for more than 60 minutes, contracting from $L_0$ to a final steady-state length of about 50% of $L_0$ after 120 minutes. At $t=120$ minutes, the superimposition of the $\delta T = 4\% T_0$ force fluctuation amplitude produced small strain fluctuations in muscle length (not

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shown) but the cycle averaged muscle length (\( \bar{L}_{\text{cycle}} \)) remained contracted near its isotonic steady-state length of 50% of \( L_o \). After 60 minutes, the force amplitude was increased to 8% \( T_o \), and produced a proportional increase in the strain fluctuations (again not shown) but \( \bar{L}_{\text{cycle}} \) remained near its isotonic steady-state length ("static equilibrium length") of 0.50 \( L_o \). For force fluctuations of amplitude 8% \( T_o \) or less, \( \bar{L}_{\text{cycle}} \) remained near the length determined by its steady state, isotonic length-tension relation.

![Graph showing muscle length response to tidal force fluctuation](image)

Figure 5-2: Representative muscle length response to tidal force fluctuation. The cycle-averaged tension was maintained at 32% of \( T_o \) throughout the duration of the entire experiment. The numbers at the top of the graph represent the amplitude of tidal force fluctuations as a percent of \( T_o \). Small fluctuations in length that occurred in response to the applied tidal force fluctuations were suppressed by averaging length over each cycle.

However, as the amplitude of force fluctuation was increased to 16% \( T_o \) or greater, the muscle lengthened from its static equilibrium length. Moreover, this change in length occurred despite the maintenance of a constant cycle averaged force of 32% of \( T_o \). Specifically, for a force fluctuation of amplitude 16% \( T_o \), \( \bar{L}_{\text{cycle}} \) increased from 0.50 \( L_o \) to 0.59 \( L_o \). The resulting strain amplitude increased slightly faster than the proportional increase in force amplitude. The time required to reach the new steady-state length took place on a scale of several minutes, reaching the new length after roughly 15 minutes of force fluctuation at the amplitude of 16% \( T_o \). As the amplitude of force fluctuation was further increased to 24 and 32% of \( T_o \), \( \bar{L}_{\text{cycle}} \) increased to lengths of 0.67 \( L_o \) and 0.79 \( L_o \), respectively. Similarly, the amplitude of the resulting strain fluctuations increased out of proportion to the increases in force amplitude. In both cases of 24% \( T_o \) and 32% \( T_o \), force amplitude, the time evolution of muscle length required occurred through a slower time scale on the order of 30 to 60 minutes.

As the force amplitude was returned to 8% of \( T_o \) after exposure to the sequence of increasing force amplitudes, the muscle re-shortened and reached a steady-state length of 0.622 \( L_o \). This steady state
length was, however, elevated from that of its corresponding $L_{cycle}$ of 0.462 $L_n$ at the identical loading conditions imposed at the beginning of the protocol. Strain amplitude also differed for the identical loading conditions with the second exposure resulting in larger strain amplitude compared to the first.

The $L_{cycle}$'s obtained at the end of each force amplitude were averaged across seven separate strips. All strips maintained greater than 85% of its initial at the end of the protocol with the mean final tension being 93.1% of $T_n$ ± 7.4%. The pooled results of the seven separate strips showing mean $L_{cycle}$ versus force amplitude are shown in Figure 5-3 and listed in Table 5-1. Like the time response of the representative strip, the pooled result show that $L_{cycle}$ is influenced by the amplitude of force fluctuation despite the maintenance of a constant cycle-averaged force imposed on the muscle. The mean, static equilibrium length that the strips contracted to under isometric conditions of 32% $T_n$ was 0.46 $L_n$. As force fluctuations of 4% and 8% were imposed, muscle length remained very near the static equilibrium length (0.46 $L_n$). However, when the force amplitude was increased to 16% or greater, the muscle lengthened, reaching a steady-state mean length of 0.52 $L_n$. The repeated-measures ANOVA with a Bonferroni correction, showed that the difference in length for $\Delta T =16\% T_n$ is statistically significant from the length determined under isotonic, steady-state conditions to a $p < 0.005$. For $\Delta T =24\%$ and $32\% T_n$, the differences in $L_{cycle}$ are statistically significant to $p < 2 \times 10^{-6}$ and $p < 5 \times 10^{-10}$, respectively. Finally, as the amplitude of fluctuation is reduced to 8% after the largest fluctuation of 32%, the muscle shortens to but yet remains longer than the length corresponding to the same loading conditions as exposed to at the early stages of the protocol.

5.5.1. The effect of tidal force fluctuations on muscle length: length-tension loops

Figure 5-4 shows the steady-state length-tension traces for each amplitude of force fluctuation. These length-tension loops correspond to the time point representing the final fluctuation in tidal force and the resulting length fluctuation at each amplitude in the protocol. These length-tension traces show that for force fluctuations of amplitude 8% $T_n$ or less, the loops remained near the length determined by its steady state, isotonic length-tension relation. However, as the amplitude of force fluctuation was increased to 16% $T_n$ or greater, the loop shift to the right (i.e. the muscle lengthened), the chord slope of
the loops decrease (i.e. the muscle became less stiff), and the width of the loops increased (i.e. the muscle became more hysteretic).

Table 5-1: The effects of force amplitude ($\delta T$) on mean cycle-averaged length ($\bar{L}_{cycle} / L_o$) ± SD, with p-values for $\bar{L}_{cycle} / L_o$ compared to the isotonic $\bar{L}_{isotonic} / L_o$. N=7.

<table>
<thead>
<tr>
<th>$\delta T$</th>
<th>$\bar{L}_{cycle} / L_o$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0.4621 ± 0.0870</td>
<td>N/A</td>
</tr>
<tr>
<td>4%</td>
<td>0.4529 ± 0.0815</td>
<td>p &gt;&gt; 0.05</td>
</tr>
<tr>
<td>8%</td>
<td>0.4609 ± 0.0760</td>
<td>p &gt;&gt; 0.05</td>
</tr>
<tr>
<td>16%</td>
<td>0.5172 ± 0.0699</td>
<td>p &lt; 0.005</td>
</tr>
<tr>
<td>24%</td>
<td>0.5936 ± 0.0696</td>
<td>p &lt; 2x10^{-6}</td>
</tr>
<tr>
<td>32%</td>
<td>0.7129 ± 0.0723</td>
<td>p &lt; 5x10^{-10}</td>
</tr>
<tr>
<td>2nd 8%</td>
<td>0.6225 ± 0.0848</td>
<td>p &lt; 2x10^{-67}</td>
</tr>
</tbody>
</table>

Figure 5-3: Steady-state $\bar{L}_{cycle}$ response to tidal force amplitude.
Figure 5-4: The steady-state length-tension loops during imposed force fluctuations on maximally activated ASM. Each loop is re-plotted on a magnified scale. Note that as the amplitude of force fluctuation is increased, the loops shift to the right, the chord slope decreases, and the loop area increases.

5.5.2. Time course of tissue states

The time-course of cycle averaged length ($\bar{L}_{cycle}$), normalized stiffness, hysteresivity, and the strain amplitude are shown in Figure 5-5. In the initial phase of isotonic shortening, no force oscillations were applied and thus there are no force-length loop-based indices of stiffness, strain, and hysteresivity. The imposition of the 4% $T_o$ force amplitude provides the initial window into the states of stiffness, strain, and hysteresivity. As the force amplitude is increased from 4% $T_o$ to higher amplitudes, stiffness decreases and hysteresivity increases, and strain amplitude increase virtually instantaneously in response to the new force amplitude. The transients generated during the initial transition to the subsequent amplitude are short-lived and dissipate within several cycles for stiffness, and over several minutes for hysteresivity. Length, stiffness, and hysteresivity at the end of each force amplitude stage are shown in
Figure 5-6. The results are tabulated in Table 5-2. These results similarly show that as the force amplitude is increased, stiffness decreases and hysteresivity increases.

Figure 5-5: Time course of tissue state response to imposed tidal force fluctuations Cycle averaged muscle length (\( \bar{L}_{\text{ave}} \)) is normalized by \( L_0 \). Stiffness is normalized by its value at \( \delta T = 4\% \) \( T_0 \) (\( K_1 \)). Hysteresivity is not normalized. Strain amplitude (\( \varepsilon \)) is shown as a percent of \( L_0 \). The values at the top represent the sequence of tidal force amplitudes (as a percent of \( T_0 \)) applied onto the muscle during the force fluctuation protocol. A time bar representing 120 minutes is shown at the bottom of the graph.
Table 5-2: Steady-state muscle states in response to tidal force fluctuation amplitude $\delta T$. Mean values with standard deviations listed in the parentheses. $N=7$

<table>
<thead>
<tr>
<th>$\delta T$ (% $T_o$)</th>
<th>$\bar{L}_{cycle}/L_n$</th>
<th>$K/K_4$</th>
<th>$\eta$</th>
<th>$\varepsilon$ (% $L_n$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0.462 (0.087)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>4%</td>
<td>0.453 (0.082)</td>
<td>1</td>
<td>0.172</td>
<td>0.105 (0.009)</td>
</tr>
<tr>
<td>8%</td>
<td>0.461 (0.076)</td>
<td>0.920</td>
<td>0.188</td>
<td>0.215 (0.024)</td>
</tr>
<tr>
<td>16%</td>
<td>0.517 (0.070)</td>
<td>0.755</td>
<td>0.220</td>
<td>0.516 (0.046)</td>
</tr>
<tr>
<td>24%</td>
<td>0.594 (0.070)</td>
<td>0.591</td>
<td>0.274</td>
<td>0.968 (0.037)</td>
</tr>
<tr>
<td>32%</td>
<td>0.713 (0.072)</td>
<td>0.411</td>
<td>0.318</td>
<td>1.816 (0.056)</td>
</tr>
<tr>
<td>2nd 8%</td>
<td>0.623 (0.085)</td>
<td>0.786</td>
<td>0.195</td>
<td>0.251 (0.063)</td>
</tr>
</tbody>
</table>

Figure 5-6: Steady-state tissue state response to imposed tidal force fluctuations

5.5.3. **Time controls**

The results of the time control experiment (Figure 5-7) show that muscle contracts to its static equilibrium length and remains near that length throughout the duration of the protocol. The results show a slight increase in muscle length that occurs in the final phase of the protocol. This increase was, however, limited to 4% of $L_n$. 

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Figure 5-7: Pooled time control results. The solid curve represents the ensemble average of the time control experiments (N=5). The open diamonds and error bars represent the average muscle length and its standard deviation at the time points corresponding to the end of each tidal force fluctuation amplitude. Note that throughout the entire duration of the time-control protocol, muscle length remained close to its static equilibrium length of 0.46 \( L_n \). The insert shows an expanded time scale of the initial 12 minutes of contraction.

5.6. RESULTS-THEORETICAL

The time course of the theoretical response of muscle length to tidal force fluctuations is shown in Figure 5-8. With activation, muscle length shortened to its static equilibrium length of 0.46 \( L_n \) over 1800 seconds (30 minutes). At \( t=30 \) minutes, a tidal force amplitude of \( \delta T = 4\% T_n \) produced small strain fluctuations in muscle length but cycle averaged muscle length (\( \bar{L}_{st} \)) remained near its static equilibrium length of 0.46 \( L_n \). As \( \delta T \) was increased to 8\% \( T_n \), \( \bar{L}_{st} \) again remained near its static equilibrium length of 0.46 \( L_n \). However, as the amplitude of force fluctuation was increased to 16\% \( T_n \), the muscle lengthened rapidly from its isotonic steady-state length (Figure 5-8B). An overshoot response in muscle length occurred. As \( \delta T \) was further increased to 24 and 32\% of \( T_n \), \( \bar{L}_{st} \) again increased with an overshoot transient and reached a new steady-state after roughly 500 seconds (9 minutes). Finally, as the force amplitude was decreased to 8\% of \( T_n \) at the end of the protocol, the muscle re-shortened to its initial length of 0.46 \( L_n \) (This re-shortening is not shown on Figure 5-8).
Figure 5-8: Representative muscle length response to tidal force fluctuation. The cycle-averaged tension was maintained at 32% of $T_n$ throughout the duration of the entire experiment. The numbers at the top of the graph represent the amplitude of tidal force fluctuations as a percent of $T_n$. Small fluctuations in length that occurred in response to the applied tidal force fluctuations were suppressed by averaging length over each cycle.

The time-course of length, stiffness, hysteresivity, and strain for the HHM theory are shown in Figure 5-9. In the initial phase of isotonic shortening, no force oscillations were applied and thus there are no force-length loop-based indices of stiffness, strain, or hysteresivity. With the imposition of $\delta T = 4\% T_n$, muscle length remained at its static equilibrium length. As the $\delta T$ was increased to $8\% T_n$, muscle length remained at its static equilibrium length, stiffness decreased slightly, hysteresivity increased slightly, and the resulting strain amplitude increased approximately two-fold over that which occurred for $\delta T = 4\% T_n$. As $\delta T$ was increased to $16\% T_n$ or greater, the muscle lengthened, became less stiff, and became more hysteretic. As occurred in the muscle length response, stiffness and hysteresivity responses also showed an overshoot-type transient that dissipated within 200 seconds.
5.7. DISCUSSION

The main finding of this chapter was that tidal force fluctuations drove muscle length to one longer than that predicted by the assumption of static equilibrium conditions. Additionally, force fluctuations decreased stiffness and increased hysteresivity when compared to its static equilibrium values. These changes in muscle states were characterized by graded stretch-effect relations where the greater the tidal force amplitude, the greater the departures from their values measured in conditions approximating the isometric steady state. The lengthening response to force fluctuations appeared to exhibit threshold-like behavior, in which an amplitude of 8% \( T_o \) or less did not alter cycle-averaged muscle, length but increasing force amplitude to 16% \( T_o \) or more resulted in marked lengthening of the muscle. Stiffness decreased and hysteresivity increased with increasing force amplitude. Using stiffness and hysteresivity as rough indices to the numbers of actin-myosin interactions, and the rate of turnover of
CHAPTER 5: FLUCTUATION-DRIVEN MUSCLE LENGTHENING

Those interaction\(^2\) [Fredberg'97], these results suggest that tidal force, like tidal strain, decreases the numbers of actin-myosin interactions and increases their rate of turnover.

Contrary to the theoretical results which showed no effect of load history on muscle state, the experimental results showed that as the force amplitude was decreased to 8\% \(T_n\) after exposure to the force amplitude of 32\% \(T_n\), the muscle did not re-shorten to its initial static equilibrium length. This increased length was also accompanied consistently by a decrease in muscle stiffness but no change in hysteresivity. Thus, for the same loading conditions, two muscle states emerged, with the difference being the history of exposure to force fluctuations. (In the future, the behavior that different steady-state muscle lengths emerge for the identical load is loosely termed "shortening plasticity.")

For the applied isotonic load, the steady-state length to which the muscle strips contracted compares well with existing length-tension data of isotonic contractions. In the isotonic phase of the experiment, contraction against the isometric load of 0.32 \(T_n\) resulted in a mean length of 0.462 \(L_n\) + 0.087 \(L_n\). This is comparable to the length-tension relation results of Stephens, which shows that for an isometric load of 0.32 \(T_n\), canine TSM is capable of contracting to roughly 45-50\% of \(L_n\) [Stephens'77]. A comparison between the time course of length change between the current study and that of Stephens are, however, quite disparate. For experiments as described in this thesis, the time course of the isotonic contraction against a load of 0.32 \(T_n\) required on the order of 60 minutes to reach its steady-state length. This is in sharp contrast to the results Stephens, who showed that under electric field stimulation, isotonically loaded canine TSM strips reached its maximal shortening within 12 seconds into stimulation [Stephens'77]. This difference may have, however, arisen from the mode of stimulation (electrical field stimulation for Stephens, acetylcholine for this study)\(^3\). Nevertheless, this difference suggests that the mode of stimulation plays an influential role in the rapidity of the contraction where electric field stimulation is likely the most rapid means of inducing contraction in \textit{in vitro} preparations.

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\(^2\) As discussed in Chapter Two.

\(^3\) Canine tracheal tissue received from the lab of N.L. Stephens was contracted isotonically with acetylcholine and required 30-60 minutes to reach steady-state. This is comparable to the results obtained with bovine tracheal tissue. Hence, the difference in species cannot account for the disparity in the time required.
5.7.1. Fluctuation driven lengthening - mechanism

The theoretical model provides some insights into the mechanism of the fluctuation-driven lengthening response. The parameters defining the muscle model used in this study was identical to that used in the theoretical investigation of Chapter Four. Moreover, the theoretical results of Chapter Four showed decreased muscle force and stiffness was a direct result of tidal strain amplitude reducing the attached fraction of crossbridges. Applied to the theoretical results of this chapter, this suggests that the lengthening response to force fluctuation occurs as a compensatory mechanism in order to restore the number of attached crossbridges through an increase in filament overlap.

The results show that tidal stretch, in the form of force fluctuations, are capable of driving airway smooth muscle states (length, stiffness, hysteresivity) into dynamically-determined states distinct from those associated with static equilibrium conditions. This behavior was shown both experimentally and theoretically. A comparison of the steady-state muscle states (Figure 5-10) shows that the HHM theory, which incorporates the equation of motion for the interaction between actin and myosin alone, is capable of closely mirroring the experimentally observed changes in muscle state. This result then supports the central hypothesis that the tidal force fluctuations act at the level of the acto-myosin interaction to drive airway smooth muscle state into one distinct from those associated with static equilibrium conditions. Like the observed suppressive effect of tidal length fluctuations on muscle tone, tidal force fluctuations lengthens contracted airway smooth muscle. Moreover, changes in associated muscle states of stiffness and hysteresivity are consistent with a perturbed equilibrium of actin-myosin binding dynamics.
Figure 5-10: Theoretical and experimental length response to tidal force fluctuations. The simulation results are shown with the lines. Experimental results are shown with the circles. The triangles represent the final state for the second application of $\delta F = 8\% F_c$.

5.7.2. Alternative mechanisms of fluctuation driven lengthening

The muscle strips studied maintained more than 90% of their isometric force generating capacity, thus ruling out mechanisms based on damage or fatigue of the muscle preparation over the duration of the experiment. Time control studies show that in the absence of large amplitude force fluctuations, the muscle contracts to, and remains fixed at, its static equilibrium length throughout the duration of the protocol, ruling out mechanisms based on decreased activation through degradation of ACh, receptor downregulation/desensitization, or similar mechanisms. The effect of neural reflexes or prostanoid release, as considered in Chapter Four, were not directly addressed in these set of experiments. It is,
however, unlikely that these mechanisms account for the lengthening response observed as the results of Chapter Four showed that inhibition of these mechanisms did not alter the tension or stiffness response to length oscillations. In support are pilot studies using the identical force fluctuation protocol with muscle strips treated with indomethacin ($10^{-6}$ M) which showed lengthening responses nearly identical to non-treated strips.

The HHM theory, which considers the binding dynamics of actin-myosin alone, was capable of capturing the effect of fluctuation-driven lengthening. The qualitative similarities between the theoretical and experimental results support the conclusion that both are linked by a common mechanism at the level of the actin-myosin bond. Moreover, the control experiments have ruled out many alternative hypotheses. Taken together, these results support the hypothesis that the principle mechanism underlying fluctuation driven lengthening is the disruption of actomyosin binding that drives the binding dynamics of actin and myosin into a perturbed equilibrium.

There remain, however, several features of the experimental response that the HHM theory, and thus a perturbed equilibrium of crossbridge dynamics, cannot account for. The first discrepancy is the difference between the rate of the lengthening response to imposed force fluctuations. A comparison between the experimental and theoretical time courses of lengthening (Figure 5-11) reveals that the lengthening response obtained experimentally occurs at a rate roughly ten-fold slower than that predicted by the theoretical results. Moreover, the theoretical time response exhibits an overshoot not observed experimentally. Modifying the HHM theory model parameters to reflect a process that evolves at a rate tenfold slower is not a simple remedy likely to resolve this discrepancy as the parameters chosen by Hai & Murphy reflect both mechanical and biochemical response indices such as ATP consumption and myosin phosphorylation. Alteration of the model parameters would likely lead to changes in other states such as the steady-state length response, phosphorylated myosin fraction, and/or ATP consumption, each requiring yet a new set or parameters. The disparity in time scales then suggests an additional slow process acting as a mechanical impediment to lengthening. Many additional proteins are known to influence actin-myosin binding and also the overall mechanical behavior of a cell. It is plausible that one or many of these proteins act to slow the process of lengthening by interacting with actin or myosin or both simultaneously. In speculation, caldesmon is one such molecule which may influence the
mechanical interaction between acting and myosin filaments where caldesmon functions as a bridge to mechanically link actin and myosin filaments. Through its proposed function as a mechanical bridge, caldesmon may also act as a mechanical impediment to lengthening by impeding relative motion between filaments.

![Experimental](image1)

![Theoretical](image2)

Figure 5-11: A comparison between the experimental and theoretical muscle length time responses to tidal force fluctuation. Note the difference in time scale of the two plots where the experimental evolution of muscle length occurred over 10.5 hours. This is in comparison to the theoretical results that show a rapid equilibration of each stage and a total time of 5000 seconds (1.3 hours). Also note the shape of the length transients where the experimentally observed lengthening occurs asymptotically. This is in contrast to the overshoot response obtained by the theory.

A second characteristic that the HHM theory cannot account for is the effect of load history. As shown in the experimental results, exposure to the sequence of increasing force fluctuations resulted in a partial loss of the muscle strips to re-contract to its isometric equilibrium length even as force amplitude was decreased to $8\% T_0$ or completely ceased. In contrast, the theoretical response showed a lengthening

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response that was independent of load history, lengthening for large amplitudes and re-contracting back to its initial isotonic equilibrium length as the amplitude was decreased to $8\% T_\text{c}$ or less. This suggests that a perturbed equilibrium of crossbridge dynamics cannot account for the effect of load history.

Nevertheless, the results, when taken in total, point to a perturbed equilibrium of actin and myosin dynamics as a key mechanism in the fluctuation-driven lengthening response of ASM. Despite the clear discrepancies in the rate of the lengthening response, the lack of a load history effect, and the apparent differences between the length and force fluctuation effect, the HHM theory, which incorporated only crossbridge mechanisms, was capable of representing several important features of the lengthening response observed experimentally. Namely, the HHM theory exhibited a threshold at a force amplitude of $\delta f = 8\% T_\text{c}$; the same amplitude which the length threshold occurred experimentally. Theoretical steady-state lengths in response to the imposed force fluctuations were also strikingly similar to that obtained experimentally. Lastly, the qualitative agreement between the experimental and theoretical results for stiffness, hysteresivity, and amplitude of cyclic strain, support the hypothesis that force fluctuations act to disrupt actomyosin binding and drive the binding dynamics of actin and myosin into a perturbed equilibrium and in part, may account for a significant fraction of the lengthening effect observed.

5.8. SUMMARY

The results of this chapter establishes the central hypothesis, showing that tidal force fluctuations were capable of driving airway smooth muscle into a dynamically-determined state markedly different from that associated with static equilibrium conditions. Of these tissue states, mean muscle length – the state that determines airway caliber – increased in a threshold-like manner to the amplitude of the applied force. This dependence of muscle length on force amplitude, not predicted under the prevailing assumption that muscle length is determined by an equilibrium of static forces, can profoundly alter the minimal caliber at which the airway can constrict to. At one extreme of the loading conditions imposed, a force amplitude of $32\% T_\text{c}$ about a mean tension of $0.32 T_\text{c}$ drove cycle-averaged muscle length from $45\% L_\text{c}$ (the length determined under steady-state isotonic conditions) to one nearly two-fold greater. Using the airway model of Chapter Three showed that this two-fold increase in muscle length is not
small. If the effect of mucosal thickness is considered, the airway model showed that this increase in muscle length corresponded to a 700-fold decrease in resistance of that airway.

The theoretical investigation using the HHM theory showed that tidal force fluctuations were capable of producing a lengthening effect that closely mirrored the experimental results. In the lengthening effect, the theoretical model also exhibited a threshold at $8\% T_n$: a threshold identical to the experimental results. Moreover, the associated muscle states of stiffness and hysteresivity followed qualitatively the experimentally observed results. The ability of the theoretical results to closely mirror all of the experimentally observed tissue states then suggests that tidal force fluctuation act to modulate airway smooth muscle length at the level of the actin-myosin interaction and that lengthening occurs as a result of a perturbed equilibrium of acto-myosin binding dynamics.

Interestingly, the experimental results also showed that load history influenced the length to which the muscle contracted. Specifically, airway smooth muscle exposed to a tidal force amplitude of $8\% T_n$ and lengthened with increasing force amplitudes was unable to re-contract to its initial length once tidal force fluctuations stopped. In contrast, the HHM showed no dependence on load history. This then suggests that both the lengthening effect of force fluctuations and load history effect are mediated not only by a perturbed equilibrium of acto-myosin dynamics by also by additional processes. This then led to the consideration of the alternate mechanisms of stretch-induced deactivation, contractile element and cytoskeletal remodeling, sarcomere popping, and force-fluctuation induced changes in tissue volume, that may account for the force-fluctuation modulation of muscle length and the bistable state. These mechanisms are addressed in more detail in the following chapter.
Chapter Six

THE EFFECT OF TIDAL LOAD HISTORY: SHORTENING PLASTICITY

6.1. OVERVIEW

Chapter Six investigates the influence of tidal load history on muscle contractile state and in particular, on muscle length. The results of Chapter Five showed that within a single sustained activation, ASM lengthened when tidal force fluctuations were applied. However, once lengthened, muscle length did not return to its initial static equilibrium length once the tidal force amplitudes were ceased. These muscle strips retained on average, 93% of their isometric tension, thus ruling out torn or damaged muscle as an explanation. This behavior is termed "shortening plasticity." This chapter investigates further shortening plasticity and plausible mechanisms to account for it. Of the five alternative mechanisms considered (sarcomere inhomogeneity, tissue volume change effects, cytoskeletal remodeling, contractile element reorganization, and stretch-induced deactivation), the two mechanisms of tissue volume changes and sarcomere inhomogeneity (i.e. sarcomere popping) are addressed. The experimental evidence will show that shortening plasticity is unlikely a result of tissue volume changes or sarcomere inhomogeneity effects (i.e. sarcomere popping). The remaining mechanisms, while not addressed through direct experiments, are addressed using a combination of the results of this and previous chapters, associated experimental evidence, and/or simple testable predictions.
6.2. EXPERIMENTAL MATERIALS & METHODS

The experimental investigation described below was designed to address directly the alternative mechanisms of sarcomere inhomogeneity/sarcomere popping and tissue volume changes. As described in the previous chapters, bovine tracheal smooth muscle strips (Research 87) were used in these experiments. The preparation methods, solutions, bath conditions, and experimental apparatus are identical to those described in Chapters Four and Five. In all experiments, the tissue strips were allowed one hour of equilibration. Optimal length \( L_o \), and peak tension \( T_o \) with \( 10^{-4} \) M acetylcholine, were determined in an identical manner to that described in Chapter Five.

Data analysis

Cycle-averaged tension, stiffness, and hysteresivity were calculated from the measured length and force signals using the equations defined in Chapter Two. The reader is referred to Fredberg, et al., for details of the calculation [Fredberg'89]. Except where noted, cycle-averaged muscle states are presented as the mean \( \pm \) standard deviation. To account for the variability in stiffness between strips, the normalization factor \( T_o / L_o \) was used to account for differences in muscle cross-sectional area and length. Hysteresivity was not normalized, as it is a non-dimensional index. In each case, "N" represents the number of muscle strips used for each study. The Student’s t-test was used to compare the results between groups.

6.3. SARCOMERE POPPING AS A MECHANISM FOR SHORTENING PLASTICITY?

How sarcomere popping might result in shortening plasticity can be illustrated with a hypothetical length-tension characteristic of a single sarcomere (Figure 6-1). In this length-tension characteristic optimal length is \( S_o \). For tensions less than optimal, there is a range of lengths in which two different lengths are capable of developing the same tension. That is, for a sarcomere length \( S_1 \) where \( S_1 < S_o \), there is a second stable sarcomere length \( S_2 \) (where \( S_2 > S_o \)) that generates the same tension. While hypothetical, this form of the sarcomere length-tension characteristic predicts that a single tension can result in one of two different lengths – the key feature of shortening plasticity. A plausible scenario can then be constructed where a sarcomere operating at \( S_1 \) is exposed to a transient force that
exceeds the optimal tension such that the sarcomere pops to \( S_1 \). In this scenario, the important feature is that while the initial and final tensions are identical, the lengths are not the same due to differences in the load history. This general scheme is used to generate testable predictions of sarcomere popping, if it occurs, on shortening plasticity.

Figure 6-1: Hypothetical sarcomere length-tension relation. For a tension less than the maximal (threshold tension), the sarcomere operates in the normal range of its length-tension relation (S1). If, however, tension exceeds the threshold for a sufficient duration, the sarcomere lengthens beyond So and “pops.” Lengthening stops where the applied tension matches the sarcomere length tension relation in the popped range (S2). If tension is reduced, note that muscle length does not return to its normal operating length (S1) unless tension falls drastically below the trough tension shown.

This scheme, extended to muscle tissue behavior is developed as follows. It is first assumed that ASM tissue may be modeled with many non-identical sarcomeres (non-uniform in optimal tension and length) linked in series. As described above, a sarcomere pops when exposed to a load that exceeds the optimal tension for that sarcomere. It then follows that in a population of non-uniform of sarcomeres, weaker sarcomeres pop before stronger ones as the applied tension is increased. In terms of the population, higher tensions “pop” a larger proportion of sarcomeres. This argument is essential in formulating the following prediction of sarcomere popping on shortening plasticity in ASM tissue. The
prediction is that if shortening plasticity is a result of sarcomere popping then a given peak tidal load is linked to a unique proportion of popped sarcomeres and thus a unique amount of shortening plasticity in length.

In the context of the shortening plasticity that occurs after fluctuation-driven lengthening, it then follows that for a given mean load and tidal force amplitude, a unique proportion of sarcomeres are popped (and that this proportion results in shortening plasticity). If so, a second exposure to the identical mean load and tidal force amplitude should not change the proportion of popped sarcomeres and hence, the degree of shortening plasticity should remain unchanged. This is illustrated in Figure 6-2.

![Diagram]

Figure 6-2: A hypothetical loading pattern and steady-state muscle length response predicted by sarcomere popping. If the tidal loading 1 and 2 are identical, sarcomere popping predicts that the subsequent steady state isotonic lengths will be equal to each other, but greater than the static equilibrium length ($L_{eq}$). The degree of shortening hysteresis is shown by $\Delta L$, the difference between the initial isotonic static equilibrium lengths and subsequent steady-state isotonic lengths after being lengthened by tidal force fluctuations. Note that $\Delta L$ for the second and third isotonic loads are equal, as predicted by sarcomere popping.

6.3.1. The repeated tidal-isotonic loading protocol

This protocol tests the hypothesis that sarcomere inhomogeneity (i.e. sarcomere popping) accounts for shortening plasticity. First, optimal length $L_{o}$, and optimal force, $T_{o}$, was determined as described in Chapter Five. The loading protocol proceeded as follows. The muscle was contracted isometrically with $10^{-4}$M acetylcholine (Sigma) until a tension of $0.32T_{o}$ (plus a slight offset to compensate for passive tension) was reached. When muscle tension reached the target tension, the contraction mode was shifted from isometric directly to isotonic in which the isotonic load was
maintained at the target throughout the contraction. The muscle was allowed to contract for 120 minutes until muscle length reached a steady-state length. At \( t = 120 \) minutes, a sinusoidal force fluctuation (0.2Hz) of either 16, 24, or 32\% \( T_n \) in amplitude was superimposed on the steady component of the load of 0.32 \( T_n \). Force fluctuations were maintained for 120 minutes while muscle length reached a new equilibrium length. At \( t = 240 \) minutes, the fluctuations were stopped and the muscle was again allowed to re-contract against the identical isotonic load (0.32 \( T_n \)) for 180 minutes. At \( t = 420 \) minutes, a sinusoidal force fluctuation of identical amplitude to the first was again superimposed on the threshold load and maintained for an additional 120 minutes. At \( t = 540 \) minutes, the fluctuations were again ceased and the muscle was allowed, for the third time, to contract isotonically against the identical load of 0.32 \( T_n \) for 180 minutes. Throughout the protocol, mean tension, as averaged over each cycle, was maintained constant at 0.32 \( T_n \). The sequence of loads for this protocol is illustrated in Figure 6-3. It is noted that a tidal force amplitude of 8\% \( T_n \) was not used as the results of Chapter Five showed that force amplitudes less than or equal to 8\% \( T_n \) did not significantly increase muscle length.

![Diagram](image)

Figure 6-3: The repeated isotonic-tidal loading protocol. The amplitude used in this figure represents \( \delta T = 24\% T_n \). The frequency of force fluctuations is not shown to scale.

### 6.3.2. The repeated tidal-isotonic loading protocol results

The results of this loading protocol are described sequentially, by loading phase with comparisons across the different loading amplitude. (Recall that the loading pattern consisted of repeated isotonic and tidal loading patterns.) Representative time histories of the airway smooth muscle length response for fluctuation amplitude of 16, 24, & 32\% \( T_n \), are shown in Figure 6-4.
The addition of $10^{-4}$ M acetylcholine caused a rapid rise in muscle tension to the threshold of $0.32 T_o$. Per protocol, the mode of contraction was switched from isometric to isotonic and muscle shortening occurred. While the loading in this initial isotonic phase is identical across the different groups, slight differences in steady-state length occurred. The following sections describe chronologically the results of the remaining phases of the protocol.

Figure 6-4: The muscle length response to the three amplitudes of the repeated isotonic-tidal protocol. For $\delta T = 16\% T_o$, the initial exposure to tidal loads does not result in subsequent shortening plasticity. This is in contrast to $\delta T = 24\% T_o$ and $\delta T = 32\% T_o$, which show that muscle length does not return to its initial isotonic static equilibrium length once the force fluctuations are stopped (a.k.a. shortening plasticity). The second exposure, however, results in a shortening plasticity in all three loading amplitudes considered. Moreover, note that the magnitude of fluctuation driven lengthening increases on the second exposure to tidal force fluctuations when compared to the first.
CHAPTER 6: SHORTENING PLASTICITY

Force fluctuations drive the lengthening of airway smooth muscle

At $t = 120$ minutes, the loading condition was switched from isotonic ($T_{iso} = 32\% T_n$) to a fluctuation tidal load of amplitude 16, 24, or 32$\% T_n$. In all cases, the mean (cycle-averaged) load was maintained constant at 32$\% T_n$. Figure 6-4 shows the cycle-averaged lengthening response for the three $\delta T$'s. Muscle lengthening occurred for all amplitudes of tidal load. Moreover, lengthening followed a graded effect to $\delta T$ where the greater $\delta T$'s resulted in greater lengthening. Similarly, hysteresis was greater for larger $\delta T$'s while muscle stiffness $K/(T_n/L_n)$ was less for larger $\delta T$. Steady-state length, stiffness, and hysteresis are listed in Table 6-1.

Table 6-1: The effect of tidal force amplitude on steady-state muscle states for the single amplitude loading protocol. Values are shown as mean ± standard deviations.

<table>
<thead>
<tr>
<th>Muscle states</th>
<th>$\delta T = 16% T_n$</th>
<th>$\delta T = 24% T_n$</th>
<th>$\delta T = 32% T_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=4</td>
<td>N=4</td>
<td>N=3</td>
</tr>
<tr>
<td>Initial isotonic muscle length (in $L_n$'s)</td>
<td>0.497 ± 0.125</td>
<td>0.453 ± 0.078</td>
<td>0.493 ± 0.129</td>
</tr>
<tr>
<td>Fluctuation-driven length (in $L_n$'s)</td>
<td>0.552 ± 0.112</td>
<td>0.605 ± 0.078</td>
<td>0.789 ± 0.210</td>
</tr>
<tr>
<td>Stiffness, $K$ (normalized by $T_n/L_n$)</td>
<td>20.177 ± 4.354</td>
<td>18.105 ± 1.963</td>
<td>12.077 ± 3.721</td>
</tr>
<tr>
<td>$\eta$</td>
<td>0.211 ± 0.086</td>
<td>0.207 ± 0.051</td>
<td>0.259 ± 0.036</td>
</tr>
<tr>
<td>$\varepsilon$ (% $L_n$)</td>
<td>0.72 ± 0.178</td>
<td>1.10 ± 0.27</td>
<td>2.39 ± 0.74</td>
</tr>
<tr>
<td>$t_{50}$ (min)</td>
<td>1.17</td>
<td>2.92</td>
<td>4.17</td>
</tr>
</tbody>
</table>

Shortening plasticity is dependent on the amplitude of the preceding tidal load.

At $t = 240$ minutes the load was switched from the sinusoidal tidal pattern to isotonic ($T_{iso} = 0.32 T_n$). All muscle strips then re-shortened against the isotonic load and progressed to a steady-state length after approximately 120 minutes. For $\delta T = 16\% T_n$ (Figure 6-4a), cessation of tidal force fluctuations resulted in complete re-contraction to its previous steady-state isotonic length. In this case,
there was no effect of tidal load on subsequent muscle length (i.e. no shortening plasticity). In contrast, for $\delta T = 24\% T_n$ (Figure 6-4b), the cessation of tidal force fluctuations did not result in the complete re-shortening and muscle length remained longer than its initial isotonic length. Similarly, this occurred for $\delta T = 32\% T_n$ (Figure 6-4c). For the later two $\delta T$'s, subsequent shortening was affected by the prior load. For tidal amplitude of 16% $T_n$, there was no shortening plasticity. However, for tidal amplitude of 24% $T_n$ or greater, shortening plasticity occurred.

The magnitude of shortening plasticity $\Delta L_{iso}$, is measured by the difference in steady-state isotonic lengths before and after exposure to the tidal force. The results show that shortening plasticity, as represented by $\Delta L_{iso}$, increased with increasing force amplitude (Table 6-2). (Note: There were no stiffness, hysteresivity, and strain results as no cyclic oscillations in force or length was applied.)

<table>
<thead>
<tr>
<th>$\delta T$</th>
<th>Initial isotonic muscle length (in $L_n$'s)</th>
<th>Isotonic muscle length after $\delta T$ (in $L_n$'s)</th>
<th>$\Delta L_{iso}$ (in $L_n$'s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16% $T_n$ N=4</td>
<td>0.497 ± 0.125</td>
<td>0.490 ± 0.108</td>
<td>-0.007</td>
</tr>
<tr>
<td>24% $T_n$ N=4</td>
<td>0.453 ± 0.078</td>
<td>0.545 ± 0.039</td>
<td>0.092</td>
</tr>
<tr>
<td>32% $T_n$ N=3</td>
<td>0.493 ± 0.129</td>
<td>0.666 ± 0.223</td>
<td>0.173</td>
</tr>
</tbody>
</table>

A second exposure to an identical tidal force fluctuation results in greater muscle lengthening than that of the initial exposure

At $t = 480$ minutes, the loading was again switched from isotonic ($T_{iso} = 32\% T_n$) to tidal force loading. For each strip, a tidal amplitude identical to that used at $t = 120$ minutes, was applied. Figure 6-4 shows the lengthening response for the three cases of load amplitude. In each case, the re-imposition of the tidal force fluctuation resulted in lengthening. Parallel to the response at $t = 120$ minutes, the results show a graded effect of $\delta T$ on lengthening. Different from the response at $t = 120$ minutes is that
the final lengths were, however, longer on the second exposure when compared to the first. This increased lengthening for the same amplitude of occurred in for all three \( \delta T \)'s. Additionally, the second exposure to \( \delta T \) resulted in a greater decrease in stiffness when compared to the first (Table 6-3). The change in hysteresivity are less consistent with a slight increase in the second exposure when compared to the first for \( \delta T = 16\% T_n \) & 32 \( T_n \), and a slight decrease for \( \delta T = 24\% T_n \). Steady-state \( \bar{L}_{\text{rest}} \), stiffness, hysteresivity, and strain, for the three different amplitudes investigated at both first and second exposures are listed in Table 6-3.

Table 6-3: Comparison of muscle states at first and second exposures to tidal force fluctuations. Steady-state muscle length, stiffness, hysteresivity, and tidal strain amplitude are shown as mean ± standard deviations (standard deviations are shown in parentheses). Values in the “1\(^{\text{st}}\)” column represent steady state values when exposure to the first tidal force fluctuation period in the protocol. Likewise, values in the “2\(^{\text{nd}}\)” column represent steady-state values on the second exposure to tidal force fluctuations in the loading protocol. Note: the first exposure values for \( \delta T = 24\% T_n \) are different from the prior values due to the deletion of one experiment that did not run successfully during the second exposure.

<table>
<thead>
<tr>
<th>Muscle states</th>
<th>( \delta T = 16% T_n )</th>
<th>( \delta T = 24% T_n )</th>
<th>( \delta T = 32% T_n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=4</td>
<td>0.497±0.125</td>
<td>0.453 ± 0.078</td>
<td>0.493 ± 0.129</td>
</tr>
<tr>
<td>Initial isotonic muscle length, ( L_{\text{iso}} ) (in ( L_n )')s</td>
<td>1(^{\text{st}})</td>
<td>2(^{\text{nd}})</td>
<td>1(^{\text{st}})</td>
</tr>
<tr>
<td>Tidal force exposure</td>
<td>Fluctuation-driven length (normalized by ( L_{\text{iso}} ))</td>
<td>(1.19) (0.078)</td>
<td>(1.268) (0.277)</td>
</tr>
<tr>
<td></td>
<td>Stiffness, ( K ) (normalized by ( T_n / L_n ))</td>
<td>(20.18) (4.35)</td>
<td>(20.05) (2.46)</td>
</tr>
<tr>
<td></td>
<td>( \eta )</td>
<td>(0.211) (0.086)</td>
<td>(0.207) (0.051)</td>
</tr>
<tr>
<td></td>
<td>( \varepsilon ) (% ( L_n ))</td>
<td>(0.72) (0.18)</td>
<td>(0.99) (0.22)</td>
</tr>
</tbody>
</table>
Isotonic steady-state muscle length is not unique

In the final stage of this protocol ($t = 600$ minutes), the loading was again switched from tidal force fluctuations to isotonic ($T_{iso} = 0.32 T_n$). Muscle shortening occurred in response. However, for all cases, the final muscle length remained longer than that at the previous isotonic stages at $t = 240$ and $480$ minutes (Figure 6-4.), despite identical isotonic loads for each stage. Steady-state lengths are summarized in Table 6-4 & Figure 6-5. The results show a cumulative effect of tidal loads on shortening plasticity where each exposure to tidal force fluctuations leads to a subsequent loss of shortening ability. Interestingly, while full re-contraction was observed for a single exposure to $\delta T = 16\% T_n$, a second exposure to $\delta T = 16\% T_n$ resulted in incomplete re-contraction.

Table 6-4: Summary of steady-state muscle length responses for the repeated isotonic-tidal loading protocol. For $\delta T = 16$, 24, & 32% $T_n$. Muscle lengths are normalized to $L_i$ (The initial steady-state isotonic length. $L_{iso}$). (Muscle length is re-normalized to its initial isotonic length rather than $L_i$ to show more clearly the changes in isotonic steady-state length.) Values are shown as mean ± standard deviations. These results are plotted in Figure 6-5 below.

<table>
<thead>
<tr>
<th>Loading</th>
<th>$\delta T = 16% T_n$</th>
<th>$\delta T = 24% T_n$</th>
<th>$\delta T = 32% T_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=4</td>
<td>N=4</td>
<td>N=3</td>
</tr>
<tr>
<td>Isotonic I</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>Tidal I</td>
<td>1.12 ± 0.08</td>
<td>1.37 ± 0.30</td>
<td>1.60 ± 0.14</td>
</tr>
<tr>
<td>Isotonic II</td>
<td>0.99 ± 0.05</td>
<td>1.23 ± 0.26</td>
<td>1.34 ± 0.19</td>
</tr>
<tr>
<td>Tidal II</td>
<td>1.24 ± 0.17</td>
<td>1.73 ± 0.88</td>
<td>2.08 ± 0.49</td>
</tr>
<tr>
<td>Isotonic III</td>
<td>1.16 ±0.17</td>
<td>1.62 ± 0.81</td>
<td>1.81 ± 0.47</td>
</tr>
</tbody>
</table>
6.4. TISSUE VOLUME CHANGES AS A MECHANISM FOR SHORTENING PLASTICITY?

Tissue/cell volume and changes in volume have been identified as factors that influence the length to which ASM can contract. While the mechanism(s) of how tissue volume changes alters ASM length is not yet clear, experimentally induced changes in cell volume show that increased cell volume, as induced by hypotonic bathing solutions, decreases the ability of ASM to contract to short lengths [Meiss’92] [Sato’94]. This then leads to the possibility that shortening hysteresis arises from tidal force fluctuation-induced changes in tissue/cell volume. To address this possibility, the following section describes a protocol in which changes in tissue volume are measured at the end of several loading patterns.

Figure 6-5: Steady-state lengths at each state of the repeated isotonic-tidal loading protocol. Note that muscle length is normalized to $L_{\text{iso}}$ (the static equilibrium isotonic length for a isotonic load of 32% $T_0$). The values are listed above in Table 6-4.
6.4.1. Tissue weight and shortening hysteresis protocol

This protocol was designed to assess the effect of tissue volume on muscle shortening. Wet and desiccated tissue weights were used to determine whether tidal loading results in tissue volume changes and whether tissue volume changes, in turn, affect muscle length or shortening. Optimal length $L_o$, and optimal force $T_o$, was determined in the usual manner. The protocol proceeded as follows. The muscle was contracted isometrically with $10^{-4}$ M acetylcholine until a tension of $0.32T_o$ (plus a slight offset to compensate for passive tension) was reached. As muscle tension reached the target tension, the contraction mode was shifted immediately from isometric to tension-control mode where a sinusoidal force fluctuation of amplitude at $32\%T_o$ at 0.2Hz, superimposed on the target load of $0.32T_o$, was applied to the muscle. The large amplitude tidal load was continued for 60 minutes. At $t = 60$ minutes, force amplitude was reduced to $8\%T_o$. (Analogous to the loading protocol used in Chapter Five.) This reduced amplitude was maintained for an additional 60 minutes. Cycle-averaged tension was maintained constant at $32\%T_o$ throughout the duration of the protocol. This tidal load pattern is illustrated in Figure 6-6. At the end of the protocol, the tissue was cut from the attached clips and excess solution was blotted off with task wipers (Kimwipes EX-L). The tissue strips were dehydrated in acetone for a minimum of 96 hours and then transferred to desiccant for 36 hours. Dry muscle weights were then measured.

The identical procedure was applied to muscle strips from the same trachea that were loaded isotonically ($T_{isot} = 32\%T_o$) or zero load. An identical weighing and desiccating procedure was used to determine the wet and dry weights for these control strips.

![Figure 6-6: the tissue weight and shortening hysteresis protocol. Note that this protocol begins with a large tidal force fluctuation. This is in contrast to the previous protocols, which have begun with a long phase of isotonic contraction. The mean tension for this load is maintained at 32\%T_o through the protocol.](image-url)
6.4.2. Tissue volume change protocol results

The results of this loading protocol are described sequentially. The time response of mean (cycle-averaged) muscle length is shown in Figure 6-7.

![Graph showing muscle length over time with annotations for 32% \(T_o\) and 8% \(T_o\).]

Figure 6-7: The time-response of muscle length to the tissue volume loading protocol. The isotonic control case is included. Note that the imposition of \(\delta T = 32\% T_o\) from the onset of shortening reduced the amount of shortening compared with the isotonic control. At \(t = 60\) min, when the tidal amplitude is reduced to \(\delta T = 8\% T_o\), muscle shortening again ensues. However, the length to which the muscle shortens remains above that for the isotonic control.

**Time course of responses**

The addition of \(10^{-4}\) M acetylcholine caused a rapid rise in muscle tension to the isometric threshold of 0.32 \(T_o\) (not shown). Per protocol, the mode of contraction was switched immediately from isometric mode to tidal tension mode (\(T_{iso} = 0.32 T_o\), \(\delta T = 32\% T_o\), \(f = 0.2\) Hz). Shortening ensued but to a limited degree where muscle length contracted to only \(0.74 \pm 0.09 L_o\) after 60 minutes. As a note, roughly 80% of the total shortening was complete within the initial 4 minutes.

At \(t = 60\) minutes, \(\delta T\) was reduced to \(8\% T_o\) and the shortening re-ensued from \(0.74 L_o\) to a shorter length of \(0.61 \pm 0.11 L_o\). The pooled data for the cycle-averaged muscle length \(L_{ave}\), stiffness
$K (T_u / L_u)$, hysteresivity $\eta$, strain $\varepsilon$ at $t = 60$ ($\delta T = 32\% T_u$) and at $t = 120$ ($\delta T = 8\% T_u$) minutes into the protocol are listed in Table 6-5.

Table 6-5: Steady-state length, stiffness, hysteresivity, and strain results of the tissue volume change protocol. Values are shown as mean ± standard deviations.

<table>
<thead>
<tr>
<th>Muscle states</th>
<th>$\delta T = 32% T_u$ (t = 60 min.)</th>
<th>$\delta T = 8% T_u$ (t = 120 min.)</th>
<th>p (paired t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluctuation-driven length (in $L_u$'s)</td>
<td>0.741 ± 0.094</td>
<td>0.614 ± 0.108</td>
<td>0.001</td>
</tr>
<tr>
<td>Stiffness (normalized by $T_u / L_u$)</td>
<td>10.83 ± 1.48</td>
<td>22.71 ± 3.19</td>
<td>0.003</td>
</tr>
<tr>
<td>$\eta$</td>
<td>0.302 ± 0.063</td>
<td>0.155 ± 0.020</td>
<td>0.011</td>
</tr>
<tr>
<td>$\varepsilon$ (% $L_u$)</td>
<td>2.46 ± 0.34%</td>
<td>0.32 ± 0.47%</td>
<td>0.001</td>
</tr>
<tr>
<td>$t_{so}$ (min)</td>
<td>0.50</td>
<td>13.75</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Control case shortening results

Isotonic control experiments ($T_{iso} = 32\% T_u$) showed the typical isotonic response of asymptotic shortening to its final length of $0.457 \pm 0.089 L_u$ at $t = 120$ minutes. The length response of the zero-load control was not recorded. Length was not measured in the zero load control case. Comparison of muscle length between isotonic control and force fluctuation at $t = 60$ and $t = 120$ minutes reveals a statistically significant difference in muscle length with a $p < 0.003$ for the muscle lengths at $t = 60$ minutes, and $p < 2 \times 10^{-05}$ for muscle length at $t = 120$ minutes. The comparison of normalized length and the associated statistics are summarized in Table 6-6.
CHAPTER 6: SHORTENING PLASTICITY

Table 6-6: Comparison of tidal fluctuation cycle-averaged length ($\bar{L}_{\text{cycle}} / L_{n}$, N=4) and isotonic length ($L_{\text{iso}} / L_{n}$, N=33, time & treatment control) at identical time points after $10^{-4}$M ACh contraction. Tidal treatment amplitudes at $t = 60$ & 120 minutes are $\delta T = 32\% T_{n}$ & $8\% T_{n}$, respectively. Data shown as $\bar{L}_{\text{cycle}} / L_{n} \pm$ SD. p was calculated using the Student's t-test.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>$\bar{L}<em>{\text{cycle}} / L</em>{n}$</th>
<th>$L_{\text{iso}} / L_{n}$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tidal treatment (N=4)</td>
<td>isotonic control (N=33)</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.741 ± 0.094</td>
<td>0.499 ± 0.091</td>
<td>p &lt; 0.003</td>
</tr>
<tr>
<td>120</td>
<td>0.614 ± 0.108</td>
<td>0.457 ± 0.089</td>
<td>p &lt; 2x10^{-5}</td>
</tr>
</tbody>
</table>

**Tissue weight results**

Dry/Wet tissue weigh ratio data are shown below in Table 6-7. For the different loading conditions applied, the results show the zero-load control case with the lowest Dry/Wet ratio (and thus highest wet weight tissue gain). Dry/Wet tissue weight between the cases of isotonic control or force fluctuations did not differ much, with a roughly 3% difference between the average values (0.186 ± 0.013 versus 0.181 ± 0.002). Baseline wet/dry muscle weight ratio (obtained from muscle strips, which were given 60 minutes in the bath at 37°C) is not listed in Table 6-7, as the loss of one data value results in an unpaired comparison across loading condition. It is however noted that additional data, in combination with the available data from this study, shows that the muscle strips undergo a roughly 20% increase in wet weight over the 3 hours in the muscle bath when compared to baseline.

Table 6-7: The tissue weight results of the tissue volume protocol. Values are shown as mean ± standard deviations.

<table>
<thead>
<tr>
<th>Strip treatment or control type</th>
<th>Dry/Wet weight ratio</th>
<th>Wet/Dry weight ratio</th>
<th>Muscle Length $L/L_{n}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-load control (N=3)</td>
<td>0.174 ± 0.006</td>
<td>5.76 ± 0.19</td>
<td>N/A</td>
</tr>
<tr>
<td>Isotonic control (N=3)</td>
<td>0.186 ± 0.013</td>
<td>5.39 ± 0.36</td>
<td>0.457 ± 0.101</td>
</tr>
<tr>
<td>Treatment (force fluctuation, N=3)</td>
<td>0.181 ± 0.002</td>
<td>5.54 ± 0.08</td>
<td>0.597 ± 0.112</td>
</tr>
</tbody>
</table>
6.5. DISCUSSION

The principle findings of this chapter are as follows. Shortening plasticity - the ability of airway smooth muscle to assume more than one static equilibrium length for a given load - emerged in both a tidal amplitude and load-history dependent manner. For $\delta T = 16\% T_n$, muscle lengthening occurred and when the fluctuations were ceased, the muscle re-contracted to its initial static-equilibrium length. For a single exposure to a $\delta T = 16\% T_n$, there was no shortening plasticity. Similarly, for $\delta T = 24\% T_n$ or greater, muscle lengthening occurred while subjected to tidal force fluctuations. However, with cessation of tidal force fluctuations, the muscle did not re-shorten to its static equilibrium length but remained at a longer length. In each case, the isotonic stages imposed the identical isotonic load ($T_{iso} = 0.32 T_n$). However, for all but the case where $\delta T = 16\% T_n$, post-exposure muscle length remained longer than its initial static equilibrium length.

Load history was also a factor in the emergence of shortening plasticity with a cumulative effect as the muscle was subjected to repeated isotonic-tidal loading cycles. In the case of $\delta T = 16\% T_n$, the initial exposure did not initially result in any shortening plasticity. However, a second exposure to the identical tidal load ($\delta T = 16\% T_n$) resulted in both greater fluctuation-driven lengthening and a subsequent impairment in re-shortening when the fluctuations were ceased. Similarly, for tidal loads of $\delta T = 24\% T_n$ or greater, the second exposure to tidal loads resulted in greater fluctuation driven lengthening and a greater amount of shortening plasticity where each exposure to tidal loads resulted in an increase in the static equilibrium isotonic length.

In the investigation of tissue volume change effects, measurements of tissue weights showed that wet tissue weight increased roughly 20% over the duration of the protocol for both isotonic control and treatment strips. However, these increases in tissue weights were not correlated with any loading conditions or shortening plasticity effects. Wet/dry weight ratio for isotonic control strips were slightly larger (~3%) than the isotonic control strips, but the greatest wet/dry tissue ratio (and thus the largest wet weight increase) occurred in the zero-load control strips (4% greater than tidal load strips, 7% greater than isotonic control strips). While there appeared to be no consistent trend between loading pattern and increases in wet weight, strips exposed to tidal loads remained at steady-state lengths much longer than that of the isotonic controls (of $0.60 \pm 0.11 L_n$ versus $0.46 \pm 0.10 L_n$). While the slightly greater wet
weight of tidally-loaded muscle (3% over isotonic controls) suggests a possible relation between increased tissue volume and shortening hysteresis, existing data shows that this in not the case. The results of Meiss show that a 50% increase in cell volume increases the minimum shortening length by only 1%—an effect much weaker than the 3% increase in cell volume accounting for a 30% change in muscle length observed in these experiments. This then argues that shortening plasticity cannot be explained for by changes in cell volume.

Lastly, an auxiliary but significant finding was that tidal force fluctuations, when applied at the onset of contraction, are capable of inhibiting muscle shortening. Moreover, this inhibition in shortening resulted in a final length equivalent to that which occurred during fluctuation-driven lengthening. The equivalent effect of force fluctuations on length in both fluctuation-driven lengthening and fluctuation-inhibited shortening suggests a common mechanism for both processes.

These findings, then, suggest that the two alternate mechanism considered—sarcomere popping and tissue edema effects—are unlikely to account for shortening plasticity. In the case of sarcomere popping, this conclusion is drawn from the finding that the shortening plasticity behavior in this study was inconsistent with the predictions from the sarcomere model thought experiment discussed above. Namely, the prediction was that if sarcomere inhomogeneity, through the phenomenon of sarcomere popping, was a key mechanism in shortening plasticity, the magnitude of the shortening plasticity effect would be determined solely by the peak loads imposed on the muscle. The experimental results showed that this was not the case and in fact, the effect of shortening plasticity (and fluctuation-driven lengthening) increased as tidal force fluctuations were applied, ceased, and re-applied. This inconsistency argues that sarcomere popping, within the framework of a series sarcomere arrangement, is unable to account for shortening plasticity. It is however noted that this finding does not disprove the existence of sarcomere popping, which may occur in a manner different from that of simple series sarcomere popping or in combination with a separate mechanism. Similarly, the results of the tissue volume investigation did not reveal any consistent evidence suggesting that changes in tissue weight are responsible for shortening hysteresis of the magnitude observed.
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This then leads to the remaining alternate mechanism of stretch deactivation, cytoskeletal remodeling, and or contractile element reorganization as plausible mechanism for shortening plasticity. These alternative mechanisms are discussed below.

6.6. ALTERNATIVE MECHANISMS OF SHORTENING PLASTICITY

This section speculates on how the remaining mechanism considered might influence muscle length and possibly result in shortening plasticity.

Alternate mechanism 1: The stretch-induced deactivation of airway smooth muscle

As discussed in Chapter Four, there is evidence showing that airway smooth muscle length is capable of modulating activation level [Yoo’94]. If this mechanism also applies to tidal length changes, a plausible scenario arises where through the action of tidal length stretch, activation level is reduced and the muscle lengthens in response. Recent findings from our laboratory (The findings by Chak Wong in our laboratory, yet unpublished) have shown that myosin fractional phosphorylation is insensitive to tidal length changes of 0.25%, 2%, & 4% of $L_n$. If phosphorylation fraction is assumed as an index of activation level, this result suggests that small tidal length fluctuations do not measurably alter activation level within ASM. It is acknowledged, however, that ASM contractile behavior is not determined solely by activation level and myosin phosphorylation alone. In fact, there is evidence of phosphorylation independent mechanism in ASM contraction [Gerthoffer‘91] [Hai‘93]. Nevertheless, this result points to the simple conclusion that stretch-induced deactivation is not a likely mechanism for the effects of shortening plasticity observed.

Alternate mechanism 2: Series-parallel remodeling of the contractile apparatus.

Series or parallel-type reorganization of contractile elements, as proposed and demonstrated by Pratusevich & colleagues [Pratusevich’95], may contribute to the observed effect of lengthening in response to tidal force fluctuation. If, as considered in Chapter Five, this form of remodeling occurs
during tidal stretch, a parallel to series reorganization could result in increased muscle length for the identical load. A prediction that arises from this mechanism is that if muscle lengthening occurs as a result of additional serial contractile elements, stiffness should decrease with increasing length. The results in this and the previous chapter indeed show a decrease in stiffness congruent with the appearance of shortening plasticity.

Alternate mechanism 3: Contractile filament reorganization and cytoskeletal remodeling

As discussed in Chapter Five, Gunst & colleagues propose that the association between actin filaments and dense plaques are not fixed but plastic. That is, the binding of actin filaments to the membrane-associated plaques is not fixed but free to associate and dissociate throughout a contractile event [Gunst'95]. If during tidal loading the actin filament-dense plaque connections are broken and reformed, it is plausible that this rearrangement can alter the cell’s length-tension relation. If so, the different length-tension relation may account for different lengths for the identical load. In the case of the above result, it is notable that shortening plasticity is more pronounced as tidal loads are re-applied later in the contraction which may indicate a slow process in which structural changes in ASM occur over several minutes to hours.

6.7. CONCLUSIONS

The history of tidal stretch exposure is capable of influencing profoundly the steady-state static equilibrium length that the muscle can contract to. The effect of prior tidal load exposure of sufficiently large amplitude is characterized by a decreased capacity to shorten once the tidal force fluctuations have ceased. This investigation showed that this decreased capacity to shorten (shortening plasticity) was conditional on both the amplitude of the tidal fluctuations and its history to prior tidal load exposure. While this effect cannot be accounted for by crossbridge mechanisms alone, the emergence of shortening plasticity was conditional upon whether tidal force fluctuations first perturb ASM length away from it static equilibrium length. Therefore, while shortening plasticity per se cannot be attributed to crossbridge
mechanisms, it is appears to result as a consequence of upon whether tidal force fluctuations are capable of first perturbing ASM length away from static equilibrium.

The effects of tidal load history, which cannot be accounted for by crossbridge mechanisms, indicate that the length response to tidal loading is likely due to several factors. Of the five considered, these results of this chapter showed that sarcomere inhomogeneity effects and tissue volume change effects were unlikely to account for shortening plasticity. The mechanism for shortening plasticity remains to be discovered. However, of the alternative mechanism proposed, contractile element reorganization and cytoskeletal remodeling remain as likely candidates.
Chapter Seven

A TEST OF THE PERTURBED EQUILIBRIUM HYPOTHESIS - THE EFFECT OF STRETCH FREQUENCY

7.1.  OVERVIEW

This chapter investigates the effect of tidal stretch frequency on ASM contractile state. In doing so, this chapter serves as an additional test of the central hypothesis. That is, whether the effect of frequency is consistent with predictions of the perturbed equilibrium hypothesis. The effects of frequency are investigated using experiments and the HHM theory. The results show that indeed, the effects of tidal stretch frequency are consistent with predictions of the perturbed equilibrium hypothesis. Namely, that as the rate of tidal stretch is increased, muscle length, tension, stiffness, and hysteresivity depart further away from their static equilibrium values.

7.2.  THE PERTURBED EQUILIBRIUM HYPOTHESIS AND ITS PREDICTIONS

The central hypothesis of this thesis is that tidal stretch is capable of perturbing airway smooth muscle length, tension, stiffness, and hysteresivity away from its static equilibrium steady-state through the direct and disruptive action of stretch on the actin-myosin crossbridge. If the static equilibrium steady-state is conditional upon whether airway smooth muscle is given sufficient time to accommodate to imposed length or tension loads, then increasing the magnitude of length or tension perturbations or decreasing the accommodation time should result in a departure of airway smooth muscle from its static equilibrium steady-state. Chapters Four and Five showed that as the amplitude of length fluctuations or force fluctuations were increased, airway smooth muscle responded with falling tension or increasing length, respectively. These changes in tension or length were characterized by a graded stretch-effect
relation where the greater the amplitude, the greater were the departures from their values measured in conditions approximating the isometric or isotonic steady state.

If the departures from isometric or isotonic steady state are attributable to a perturbed equilibrium rendered by tidal stretch, the hypothesis predicts three factors capable of influencing the contractile state of muscle. The first is that of tidal stretch amplitude, which was addressed in Chapters Four and Five.

The second is that the frequency of tidal stretch. Like tidal stretch amplitude, the frequency of tidal stretch should influence the degree to which these departures occur. Moreover, the direction of change is predicted to occur such that an increase in frequency (and thus a decrease in the accommodation time) results in a greater departure from the static equilibrium steady-state. It then follows that for the case tidal length fluctuations, increased stretch frequency should produce a decrease in muscle tension (i.e. a greater difference between isotonic steady-state tension and that which is developed under tidal stretch). Likewise, increased tidal force frequency should produce increased lengthening as measured from the static equilibrium steady-state isotonic length. While heuristic in nature, these predictions are supported by the results of the HHM theory, which are described below.

A final factor, complementary to that of frequency is a modification in the intrinsic "speed" of muscle. Namely, that for a given frequency and amplitude of tidal stretch, faster muscle is able to accommodate to its static equilibrium state much more rapidly that normal muscle and thus, remain closer to static equilibrium. This facet of the investigation is addressed in Appendix A.

Of the three, this chapter focuses on the effects of frequency on muscle state.

7.3. THE PREDICTIONS OF THE HHM THEORY

As argued above, increased frequency should result in greater departures from static equilibrium steady-state. The HHM theory, when evaluated with several different tidal length fluctuation or force fluctuation frequencies, supports this argument. The theoretical steady-state response to tidal length fluctuations at frequencies of 0.1, 0.2, and 0.4 Hz are shown in Figure 7-1. Likewise, the theoretical length response to tidal force fluctuations at frequencies of 0.1, 0.2, and 0.4 Hz are shown in Figure 7-2.
These theoretical results show that as predicted using the heuristic argument above, increased frequency results in a greater decrease in tension for the case of tidal length fluctuations and increased lengthening effect for tidal force fluctuations. These theoretical results are used as predictions of the central hypothesis that tidal stretch, through the direct and disruptive action of stretch on the actin-myosin crossbridge, is capable of perturbing airway smooth muscle length, tension, stiffness, and hysteresivity away from its static equilibrium steady-state. These predictions are compared to the experiment results as described below.

![Graph showing the effect of tidal length fluctuations on muscle tension](image)

**Figure 7-1:** HHM theory results of the effect of tidal length fluctuations on muscle tension (cycle-averaged). For higher frequencies of strain, the HHM theory shows a steeper decline in muscle tension. Cycle averaged muscle tension is normalized to $T_n$.

### 7.4. EXPERIMENTAL MATERIALS & METHODS

The experiments in this chapter used bovine TSM strips and identical preparation methods and apparati described in Chapters 4, 5, & 6. The reader is referred to these chapters for details.
7.4.1. **The tidal length fluctuation protocol at 0.1, 0.2, or 0.4Hz**

Activated muscle strips (10^-4 M ACh) were exposed to tidal length fluctuations at a frequency of either \( f = 0.1, 0.2, \text{ or } 0.4 \text{Hz} \). The protocol followed a sequence of tidal length amplitude (0.25, 0.5, 1, 2, 4, & 8% of \( L_n \)), with each amplitude maintained for the identical time duration (600 seconds for \( \delta L = 0.25\% L_n \), 300 seconds each for \( \delta L = 1, 2, 4, \text{ & } 8\% L_n \) identical to that described in Chapter Four.

![Graph](image)

Figs. 7-2: HHM theory results of the effect of tidal force fluctuations on muscle length. For higher frequencies of tidal force, the HHM theory shows more lengthening for a given force amplitude.

7.4.2. **The tidal force fluctuation protocol at 0.1, 0.2, or 0.4Hz**

With the exception of using different frequencies of tidal force fluctuation, the protocol used in these experimental mirrored that described in Chapter Five. For this protocol, activated muscle strips (10^-4 M ACh) that were isotonically contracted \( (T_{est} = 0.32 T_n) \) to its static equilibrium steady-state length. The muscle was then subjected to a tidal force fluctuations at a frequency of either \( f = 0.1, 0.2, \text{ or } 0.4 \text{Hz} \). The protocol followed the identical sequence of tidal force amplitude (4, 8, 16, 24, & 32% of \( T_n \)), with each amplitude maintained for the identical time duration (60 minutes for \( \delta T = 4, 8, \text{ and } 16\% T_n \), 90 minutes for \( \delta T = 24\% T_n \), and 120 minutes for \( \delta T = 32\% T_n \)).
7.4.3. Data analysis

Cycle-averaged tension, length, stiffness, and hysteresivity were calculated from the measured length and force signals using the equations defined in Chapter Two. The reader is referred to Fredberg, et al., for details of the calculation [Fredberg'89]. Except where noted, cycle-averaged muscle states are presented as the mean ± standard deviation. To account for the variability in stiffness between strips, a normalization factor of $T_n/L_n$ was used to account for differences in muscle cross-sectional area and length. In each case, "N" represents the number of muscle strips used for each study. The Student's t-test was used to compare the results between groups.

7.5. RESULTS

The results are presented for steady-state values of length, tension, stiffness, and hysteresivity. The time evolution of these state changes, are qualitatively similar to that described in Chapters Four and Five and are not presented. The results of both protocols are described below.

7.5.1. The effects of tidal length fluctuations (0.1, 0.2, or 0.4Hz) on steady-state tension, stiffness, and hysteresivity

The effect of tidal length fluctuations at $f = 0.1$, 0.2, or 0.4Hz on tension, stiffness, and hysteresivity are shown in Figure 7-3. The results show that increasing amplitudes of $\delta L$ resulted in progressive decreases in tension for the three frequencies tested. That is for length amplitudes of 1% $L_n$ or less, there was little change from isometric steady-state tensions. However, as the amplitude of $\delta L$ was increased beyond 1% $L_n$, tension decreased. Frequency modulated this effect by increasing the slope of the response of $\delta L$ on tension. That is, higher frequency resulted in a greater decrease in tension for any $\delta L > 1% L_n$ (Figure 7-3a)

Similarly, this trend was also observed in the effect of frequency on normalized stiffness. That is, for length amplitudes of 0.5% $L_n$ or less, there was little change from baseline stiffness (stiffness at $\delta L = 0.25% L_n$) for the frequencies tested but for $\delta L = 1% L_n$ or greater, stiffness decreased where higher
frequencies caused a faster fall from baseline stiffness (Figure 7-3c). Interestingly, at low values of \(\delta L\), higher frequencies result in higher values of stiffness. The increased rate of decline associated with higher frequencies, however, results in a convergence of all tension curves for as \(\delta L\) approaches 8% \(L_o\).

Figure 7-3: The effect of tidal length fluctuation frequency on muscle state. A) Steady-state, cycle averaged tension versus strain amplitude. Increasing amplitude results in a decrease in tension. Moreover, increasing the frequency increases the slope of the tension-amplitude curves. B) Hysteresivity versus strain amplitude. Note that baseline values of hysteresivity (values at a strain amplitude of 0.25%) increase with decreasing frequency. The results show a vertical shift in all the curves as frequency is varied. C) Stiffness versus strain amplitude. The results suggest that stiffness increases as the frequency of tidal strain is increased. A faster rolloff, however, results in equivalent values of stiffness at high strain amplitudes. Note: A normalization factor of \(T_o/L_o\) is used where \(T_o/L_o\) reflects the stiffness contribution due to strip dimension. Normalization with \(T_o/L_o\) allows a dimension-normalized (thickness and length normalized) comparison across different strips and across different frequencies.
Lastly, hysteresivity, while difficult to interpret due to the dependence of baseline values on frequency, shows little change for \( \delta L \leq 1\% L_n \) and a considerable increase for \( \delta L > 1\% L_n \). The effect of increased frequency may be roughly described as a downward shift in the response of hysteresivity but no change in the threshold or slope of the response as frequency is varied (Figure 7-3b).

Interestingly, tidal length amplitude (\( \delta L \)), in contrast to tidal frequency, appears to have a more pronounced effect in perturbing muscle states away from those associated with static equilibrium conditions. This may be seen with a rough comparison where a four-fold increase in \( \delta L \) from 1 to 4\% \( L_n \) or 2 to 8\% \( L_n \) resulted in a roughly 40\% decrease in \( T_n \) whereas a four-fold increase in frequency from 0.1 to 0.4Hz resulted in a maximal decrease of 9\% \( T_n \).

7.5.2. The effects of tidal force fluctuations (0.1, 0.2, or 0.4Hz) on steady-state length, stiffness, and hysteresivity

Parallel to the above, the effect of tidal force fluctuations at \( f = 0.1, 0.2, \) or 0.4Hz on muscle length, stiffness, and hysteresivity are shown in Figure 7-4. The results show that for force amplitudes of 8\% \( T_n \) or less, there was little change from static equilibrium isotonic length for all three frequencies tested. However, as the amplitude of \( \delta T \) was increased beyond of 8\% \( T_n \), length increased in a threshold like manner. While the large difference in baseline (static equilibrium length) at \( f = 0.2\)Hz from the other two makes it difficult to compare the magnitude of lengthening across different frequencies, a comparison between the cases \( f = 0.1\)Hz and \( f = 0.4\)Hz shows that the lengthening effect for a given \( \delta T \) was greater for higher \( f \) (Figure 7-4a).

The effect of tidal frequency on stiffness is shown using the stiffness versus \( \delta T \) curves for each frequency (Figure 7-4c). For all frequencies, stiffness decreased in a linear fashion as \( \delta T \) was increased. Again, this decrease was accelerated for \( \delta T \) at higher frequencies with the slope for \( f = 0.4\)Hz slightly steeper than the other two curves. Additionally, higher frequencies resulted in a higher baseline stiffness (baseline = the stiffness at \( \delta T = 4\% T_n \)). In this case, stiffness was greatest for \( f = 0.2\)Hz, followed by the stiffness for \( f = 0.4\)Hz, then \( f = 0.1\)Hz.
Figure 7-4: The effect of tidal force fluctuation frequency on muscle state. A) Steady-state, cycle averaged tension versus strain amplitude. Increasing amplitude results in a decrease in tension. Moreover, increasing the frequency increases the slope of the tension-amplitude curves. B) Hysteresivity versus strain amplitude. Note that baseline values of hysteresivity (values at a strain amplitude of 0.25%) increase with decreasing frequency. The results show a vertical shift in all the curves as frequency is varied. C) Stiffness versus strain amplitude. Note that stiffness is normalized by To/Lo so that comparisons between different strips and across different frequencies can be made without a complete normalization of the effects of frequency. The results suggest that stiffness increases as the frequency of tidal strain is increased. A faster rolloff, however, results in equivalent values of stiffness at high strain amplitudes.

Hysteresivity, showed a near linear increase as \( \delta T \) was increased for all frequencies. The effect of frequency in this case followed a distinct trend where the lowest frequency \( (f = 0.1\text{Hz}) \) resulted in the
CHAPTER 7: A TEST OF THE PERTURBED EQUILIBRIUM HYPOTHESIS - THE EFFECT OF STRETCH FREQUENCY

highest values of hysteresivity across the range of $\delta T$'s, followed by that for $f = 0.2\text{Hz}$, and finally $f = 0.4\text{Hz}$ (Figure 7-4b).

Lastly, the resulting strain amplitudes are shown in Figure 7-4d. For all three frequencies, the amplitude of strain increased as $\delta T$ was increased. This increase appeared parabolic in nature with a near linear increase for $\delta T = 16\% T_n$ or less, and an disproportionate increase for $\delta T > 16\% T_n$. The curves also show that for $\delta T = 16\% T_n$ or less, the effect of frequency is negligible but at tidal force amplitudes of $\delta T > 16\% T_n$, the curves diverge with the highest strain amplitude occurring for $f = 0.1\text{Hz}$ followed by $f = 0.4\text{Hz}$, and finally $f = 0.2\text{Hz}$.

7.6. DISCUSSION

The principle findings of this chapter are as follows. For imposed tidal length fluctuations, mean tension responded in a threshold-like fashion to tidal length changes where for $\delta L = 1\% L_n$ or less, mean tension remained near its static equilibrium tension but for $\delta L$ greater than $1\% L_n$, tension decreased significantly below its isometric steady-state values. The $\delta L$ at which the threshold occurred was $\delta L = 1\% L_n$ for all frequencies tested. For $\delta L$ greater than $1\% L_n$, higher frequencies steepened the falling response of tension to tidal length fluctuations. That is, for a given $\delta L$, the greatest decrease in tension occurred for the highest frequencies. The effect of $\delta L$ frequency on stiffness mirrored those described for tension. That is, increasing $\delta L$ resulted in a threshold-like decrease in stiffness and increasing the frequency of $\delta L$ hastened this response. Hysteresivity, however, responded to an increase in $\delta L$ frequency with a vertical shift in value but no change to the threshold point or to the slope of the hysteresivity versus $\delta L$ response.

For all frequencies tested tidal force fluctuations produced a threshold-like lengthening response where for $\delta T = 8\% T_n$ or less, the muscle remained near its static equilibrium length but for $\delta T > 8\% T_n$, the muscle lengthened. The effect of frequency on fluctuations-driven lengthening was to increase the amount of lengthening as frequency was increased, as predicted by the hypothesis. Stiffness, at all frequencies, decreased as $\delta T$ was increased. The effect of frequency appeared shift the response of stiffness to $\delta T$ vertically with higher frequency resulting in higher stiffness. Hysteresivity followed a similar trend where increasing $\delta T$ resulted in increasing hysteresivity for all but the highest $\delta T$. 

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Hysteresivity appeared to respond to increasing frequency with a downward shift in the curves rather than a change in slope or change in threshold amplitude.

The results, when taken together, show that the effects of frequency are largely consistent with the predictions of the perturbed equilibrium hypothesis. That is, as predicted, higher frequencies of tidal stretch resulted in greater departures of muscle length, tension, and stiffness from static equilibrium values. This is reflected by the results where increasing $f$ resulted in a greater decrease in tension, or a greater lengthening effect, at each amplitude of length or force fluctuation, respectively. Interestingly, higher frequencies (of $\delta L$ or $\delta T$) did not appear to cause a leftward-shift the threshold amplitudes, as might be predicted by the hypothesis.

*The influence of stretch amplitude versus stretch frequency*

For both the data and HHM theory, tidal length amplitude ($\delta L$ or $\delta T$), in contrast to tidal frequency, appeared to have a greater effect in perturbing muscle states away from those associated with static equilibrium conditions. A rough comparison reveals that a four-fold increase in $\delta L$ from 1 to 4% $L_n$ or 2 to 8% $L_n$, resulted in roughly a 40% decrease in $T_n$, whereas a four-fold increase in frequency from 0.1 to 0.4Hz resulted in a maximal decrease of 9% $T_n$. This pattern is also mirrored in the effects of tidal force fluctuations where a four-fold increase in amplitude from 8 to 32% $T_n$, produced, on average, lengthening of roughly 30% $L_n$, whereas a four-fold increase in frequency from 0.1 to 0.4Hz resulted in an increased lengthening of only 9% $L_n$. While the comparisons are made with the steepest portions of the tension versus $\delta L$ or the length versus $\delta T$ curves, this comparison is justified by the result that for the tidal stretch frequencies investigated, there was no consistent evidence that a frequency threshold existed between 0.1 and 0.4Hz.

A speculation for the greater effect of stretch amplitude over frequency may be attributable to the nature of the actin-myosin bond and its sensitivity to stretch. Recall from the sliding filament theory that while myosin is in a continuous binding and detachment cycle with actin, its detachment rates are sensitive to the strain that each myosin head is exposed to. If as assumed in the sliding filament theory, excess detachment occurs only when the myosin head is strained beyond its nominal range, imposing a small amplitude of tidal strain induces a excess detachment of the sub-population of myosins that are
near the boundaries of the nominal range. However, increasing the rate at which this strain amplitude occurs does not change fraction the sub-population of myosin near the boundaries (Figure 7-5). This argues that while the amplitude of stretch has the potential to affect all myosin-actin bonds, tidal frequency is capable of only affecting the sub-population of myosin-actin bonds that are already influenced by strain.

![Image of Figure 7-5](image_url)

Figure 7-5: The effect of amplitude versus frequency on the crossbridge distribution. A) A hypothetical spatial distribution of myosin attached to actin at static equilibrium, steady-state. B) The imposition of small tidal strain (ε) drives the myosin near the boundaries into regions of excess detachment and results in a decreased number of attached myosin near the boundaries. C) Increasing the amplitude by four-fold (4ε) results in a four-fold increase in the myosin sub-population that is pushed into the regions of excess detachment. D) Maintaining the same amplitude of strain but increasing the frequency by four-fold decreases the number of attached myosins near the boundaries. Note however that the net effect of increasing frequency is small compared to the effect of increasing the amplitude of strain by fourfold. This net effect is represented by the increase in white area within the dotted boundary.

Functionally, this results in a preferred effect of tidal stretch through amplitude rather than frequency. A brief extrapolation of this effect in the context of breathing patterns and airway caliber is that fast and rapid shallow breathing is less capable at relaxing the airways than a slow deep inspiration.
from FRC to TLC and back. The effect of frequency however, has its own implications in the context of the perturbed equilibrium hypothesis in that if frequency modulates the effect of tidal stretch, the dual argument is that ASM, itself, through changes in the speed at which it can accommodate to these length changes, is also capable of modulate the effect tidal stretch. This dual mechanism is considered in the following chapter in greater detail.

7.7. CONCLUSION

The results of this investigation support, to a large extent, the central hypothesis that tidal stretch is capable of perturbing airway smooth muscle length, tension, stiffness, and hysteresivity away from its static equilibrium steady-state through the direct and disruptive action of stretch on the actin-myosin crossbridge. This is supported by the predictions of the HHM theory which showed that a stretch perturbed binding equilibrium of myosin and actin led to the tissue level changes in muscle length, tension, stiffness, and hysteresivity and that the experimentally observed changes were consistent with the predictions generated by the HHM theory. While crossbridge mechanism alone cannot account for all of the observe effects tidal stretch, (example in point: shortening plasticity), these results provide additional evidence that a tidal stretch-perturbed myosin–actin binding cycle may be invoked to explain the observed effects of muscle lengthening and tension suppression.
8.1. OVERVIEW

This chapter addresses the functional implications for each of the tidal stretch-modified behaviors observed in Chapters Five through Seven. In particular, the ability of tidal tension fluctuations to drive ASM from its static equilibrium length to longer lengths determined by a perturbed equilibria, and how this alters the classical model of airway narrowing, is addressed. In extrapolating the functional implications of the perturbed equilibrium ASM behavior, the findings of the previous chapters are address in the following themes of 1) fluctuation driven-lengthening (Chapter Five), 2) shortening plasticity (Chapter Six), 3) the time constants of fluctuation driven lengthening and tension fluctuation-inhibited shortening (Chapter Six), and 4) frequency, $\nu_{\text{max}}$, and MLCK effects (Chapters Seven and Appendix A). Finally, a framework for the dynamic regulation of airway lumen caliber (versus the static equilibrium regulation) is proposed in which tidal lung inflation plays a fundamental role in setting airway smooth muscle length.

8.2. THE PERTURBED EQUILIBRIUM HYPOTHESIS AND ASM LENGTH REGULATION

This thesis has shown that in the setting of tidal stretch, airway smooth muscle length is not set by a balance of static forces but is regulated by a dynamic process sensitive to the amplitude of tidal
stretches imposed. For sufficiently large stretch, the muscle lengthens to a new length longer than that set under static equilibrium conditions. The central hypothesis of this thesis asserts that this lengthening and the associated changes in stiffness and hysteresivity are mechanistically rooted in the ability of tidal stretch to perturb airway smooth muscle from its static equilibrium state through the direct and disrupted action of tidal stretch on the actin-myosin crossbridge. Indeed, the experimental evidence and the theoretical findings based on the HHM theory argue that the perturbed equilibrium hypothesis plays an important role in regulating airway smooth muscle length under the setting of tidal forces.

The classical view assumes that maximally activated smooth muscle length is set by a mechanical balance between the distending load and muscle tension. In addition to the distending load and muscle tension, the perturbed equilibrium hypothesis yields three additional determinants, each unanticipated in the classical view and each capable of profoundly influencing the final length that airway smooth muscle. The determinants, as borne from the perturbed equilibrium hypothesis, were 1) the amplitude of tidal stretch (Chapter Five, in the form of tidal force amplitude) 2) the frequency of tidal stretch (Chapter Seven), and 3) the intrinsic speed of ASM (Appendix A). Reinterpreted in the context of airway hyperreactivity, the perturbed equilibrium hypothesis proposes that a principle factor leading to AHR may be that tidal lung inflations are unable to drive ASM away from static equilibrium conditions and into a perturbed equilibrium of myosin-actin binding. The perturbed equilibrium hypothesis then argues that AHR may arise from tidal lung inflations that are insufficient in amplitude or frequency to drive ASM away from static equilibrium and/or ASM that is refractory to the lengthening effects of tidal force fluctuations (secondary to tidal lung inflation.). These ideas are developed below.

8.2.1. Fluctuation driven lengthening and its functional implications

If it is assumed that FRC corresponds to a muscle load of $0.32\, T^*_n$, normal tidal breathing corresponds to $\delta T = 11 - 13\%\, T^*_n$, and deep inspirations correspond to $\delta T = 30\%\, T^*_n$, the experimental results predicts a steady-state static equilibrium ASM length of $0.45\, L^*_n$ at FRC, a perturbed equilibrium length of $0.48-0.49\, L^*_n$ for normal tidal breathing, and a perturbed equilibrium length of $0.72\, L^*_n$ for small deep inspirations respectively (Figure 8-1). Functionally, the differences between static equilibrium,
normal tidal breathing, and DI determined lengths are not small. If mucosal thickness as used in [Macklem '95] is accounted for, an increase in muscle length from 0.45 $L_n$ to 0.72 $L_n$ corresponds to an increase in the lumen radius from less than 0.1 mm to 0.51 mm. If airway resistance is assumed to scale to the inverse fourth power to airway diameter$^1$, this five-fold increase in lumen radius then translates into a minimum 700-fold reduction in airway resistance. This scenario emphasizes the functional consequences of airway smooth muscle length remaining at its static equilibrium length versus the ability of tidal tension fluctuations to drive airway smooth muscle length into a perturbed equilibrium. Namely, static equilibrium and its associated muscle length lead to a constricted airway with a small lumenal area and a high airflow resistance. Likewise, a perturbed equilibrium that might occur with deep inspirations results in a substantially larger lumenal area, and a marked decrease in local airway resistance. While the above estimates suggest that normal tidal breathing alone is unable to dilate the airways, it is interesting to note that tidal breathing lies perched just beyond the edge of static equilibrium where an occasional DI may help to maintain the patency of the airway lumen.

![diagram](image)

Figure 8-1: Fluctuation-driven lengthening muscle length and ASM radius as a function of tidal tension fluctuation amplitude. For normal tidal breathing about FRC, the resulting tidal tension fluctuations are estimated at 11-13% $T_e$, which drive muscle length slightly above its static equilibrium length. For deep inspiration, the tidal tension fluctuations are estimated at 32% $T_e$ or greater, which then drives muscle length to 0.72% $L_n$ or greater. The resulting change in airway smooth muscle radius is illustrated with the airway cartoons. Note that the inner mucosal layer is not accounted for in the estimate of the airway lumenal area.

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$^1$ Based on the assumption of Poiseuille flow through the airways.
8.2.2. Is AHR a consequence of a collapse to static equilibrium conditions?

It is established that excessive airway narrowing is limited in non-asthmatics who have initiated deep inspirations before or at the onset of bronchoconstriction challenge [Nadel'61] [Fish'81] [Woolcock'84]. In contrast, asthmatics challenged in the same manner display a reduced or absent bronchodilatory effect of deep inspiration [Orehk'80] [Fiss'81] [Lim'87]. The perturbed equilibrium hypothesis applied to these observations suggests that deep inspirations in the non-asthmatic are capable of driving airway smooth muscle far from its static equilibrium length to one more closely associated with the bronchodilated state. Likewise, it also suggests that deep inspirations in the asthmatic are unable to perturb ASM from its static equilibrium and narrowing progresses to a bronchoconstricted state. This mechanism then leads to two possible explanations for why DI fails to reverse bronchoconstriction in the asthmatic. The first is that the tidal load fluctuations that arise from DI's in the asthmatic are insufficient in amplitude\(^2\) to drive ASM into a perturbed equilibrium. While there is no direct evidence showing that the tidal loads in the asthmatic airway are different from that of a non-asthmatic, it is plausible that the tissue and/or morphological changes observed in the asthmatic airway (adventitial remodeling, increased connective tissue, peribronchial edema, and increased airway wall thickness) contribute to a shielding of ASM from the tidal fluctuations of deep inspiration in the asthmatic lung. Indeed, increased thickness of the adventitial tissue surrounding the airways, when incorporated into the elastic recoil relations, is implicated as a factor that reduces the elastic recoil loads transmitted to ASM [Wiggs'92] [Lambert'93]. While the effects of these morphological changes on the tidal loads transmitted to the airways have yet to be established, the perturbed equilibrium hypothesis provides a clear prediction that these morphological changes, if they act to decrease the tidal loads transmitted to the airway, predispose the airway to excessive narrowing.

A second explanation for the failure of a DI to dilate the airways may lie in ASM itself where the tidal loads on the airways of an asthmatic and non-asthmatic are equivalent but that the ASM of an asthmatic is refractory to the effects of tidal stretch. As put forth in Chapter Seven and Appendix A, the perturbed equilibrium hypothesis predicts that for the same tidal loading pattern, ASM with faster

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\(^2\) While the results in Chapter Seven showed that frequency plays a role in the perturbed equilibrium hypothesis, in the physiological range of amplitude and frequency, amplitude exerted a much greater lengthening effect.
intrinsic cycling rates is less likely to escape from static equilibrium than normal muscle would. The theoretical investigation in Appendix A revealed that two modifications of the governing rate constants (of three modifications considered) were capable of such an effect. Namely, the global increase in rate constants and an increase in the rate constants that regulate MLCK activity resulted in ASM behavior that was refractory to the effects of tidal stretch where lengthening to tidal tension loads was reduced. While a global increase in rate constants has not been established in sensitized or asthmatic ASM, a two-fold increase in MLCK activity has been identified in allergen-sensitized ASM [Stephens’98]. This finding, coupled to the predictions of the HHM theory, then provides a plausible mechanism linking allergen sensitization, increased airway responsiveness, increased velocity of shortening, and increased MLCK activity. In this mechanism, MLCK activity is increased through the allergen sensitization process. This increase in MLCK activity then results in the biophysical changes of increased ATPase activity and an increase in the unloaded velocity of shortening [Stephens’98]. This increase in MLCK activity acts by shifting the myosin binding cycle away from the slowly cycling latch-state toward the rapid cycling state and thereby forestalls the perturbing effect of tidal stretch on the myosin binding equilibrium. As interpreted through the perturbed equilibrium hypothesis, these changes act to stabilize the static equilibrium state and thus renders the muscle resistant to the lengthening effect of tidal stretch.

8.3. A UNIFIED VIEW OF AIRWAY HYPERREACTIVITY

As described in the two scenarios above, the perturbed equilibrium hypothesis reveals a possible mechanism to explain why DI fails to reverse bronchoconstriction in the asthmatic airway and how allergen sensitization, through an increased speed of muscle, leads to AHR. Moreover, the perturbed equilibrium hypothesis provides a means of linking seemingly unrelated observation in asthma and AHR where any factor that decreases the tidal loads transmitted to ASM or decreases the ability of the applied loads to drive ASM away from static equilibrium is a factor that predisposes the airway to hyperresponsiveness. The perturbed equilibrium hypothesis may provide an effective framework for linking inflammation-associated tissue changes of the asthmatic airways to AHR.
8.3.1. Asthma, inflammation, airway hyperreactivity, and the perturbed equilibrium hypothesis

While airway inflammation, AHR, and airway obstruction are three hallmark features of the asthmatic lung, the mechanism relating the three remain rather mysterious [Fredberg’99]. Of the three, airway inflammation is generally believed to be the initial event that then leads to AHR and obstruction, but how this occurs remains fragmentary. As mentioned in Chapter Three, the static equilibrium view of airway narrowing predicts that AHR should arise through either a increase in the magnitude of muscle tension acting on the airway, or through a reduction in the elastic load against which the muscle acts. Yet, the available data remains inconclusive in demonstrating increased ASM tension or a decrease in the elastic load in the asthmatic lung. What has been reported in asthmatic airways are morphological changes airway tissues (adventitia, airway wall, mucosa, submucosa, etc.), and biophysical changes in ASM – increased unloaded shortening velocity, increased shortening capacity, an increased compliance of the internal resistor, and possible changes in relaxation [Stephens’98] [Stephens’93]. Yet through the classical models of airway narrowing, these changes are either irrelevant in determining the static equilibrium length-tension point that defines airway diameter, or exert only a marginal influence on the static equilibrium airway diameter such that the effect of each of these changes is alone unable to account for the excessive airway narrowing that occurs in asthma.

As argued above, the classical model of airway narrowing in which static equilibrium is presumed to prevail in the airways is untenable in light of the findings that ASM state does not remain near its static equilibrium under the influence of tidal stretch. With this, the dynamic view of AHR provides a possible framework with which to explain observations that appear unrelated to the excessive airway narrowing and obstruction of asthma. In this framework, a plausible case can be constructed by which the morphological changes that occur in asthma, possibly a result of the inflammatory process, act to decrease the tidal loads transmitted to the airways. These changes, which have been identified as an increase in adventitial thickness [Wiggs’92] [Lambert’93], increases in airway wall thickness [Ebina’90A] [Wiggs’92] [Carroll’93] peribronchial and lumenal edema [Yager’89], and increases in the submucosal and mucosal layer of asthmatic lungs [Wiggs’92] may act to decrease the elastic loads in both mean and tidal magnitude exerted on the ASM.
Biophysical and biochemical changes identified in asthmatic and allergen sensitized ASM may influence the degree to which tidal lung inflations are capable of perturbing ASM state away from static equilibrium. Indeed, the theoretical results of Appendix A identified the an increase in MLCK activity, is capable of maintaining ASM near static equilibrium conditions by favoring a rapidly cycling state of myosin over the slow cycling latch state. Lastly, as identified in Chapter Seven, the influence of tidal stretch frequency on perturbed equilibrium muscle length, while small, may also influence the lengthening effect of tidal force fluctuations.

Reiterating the statement of Drazen & Haley, AHR, as it occurs in asthma, may indeed be the final phenotypic expression of a multitude of distinct pathobiological processes [Drazen’98].

8.3.2. Can the perturbed equilibrium hypothesis link the universe of AHR phenotypes?

As discussed above, the perturbed equilibrium hypothesis suggests that failure to escape static equilibrium due to insufficient tidal force amplitude or frequency and/or muscle that is refractory to the effects of tidal force fluctuations are potential factors in AHR. Reductions in tidal force fluctuations may arise through a shielding effect of tissue remodeling associated with the airway inflammation of asthma, through a decrease in tidal volume, or a decrease in the elastic recoil of the lung. In addition to asthma, these instances bring to mind emphysema, normative aging, spinal cord injury, restrictive disorders of the chest wall, and obesity, each which are known to be associated with airway hyperresponsiveness or asthma [Sparrow’94] [Singas’96] [Boyer’96] [Camargo’98]. Similarly, nocturnal asthma may be linked to the above phenotypes where sleep is associated with decreases in both tidal volume and breathing frequency. Finally, the perturbed equilibrium hypothesis might also help to explain why AHR associated with exercise-induced asthma begins only after exercise has ceased and when tidal lung volumes have declined to resting levels [Fredberg’98B].

Alternatively, as discussed above, AHR may result from airway smooth muscle that is refractory to the lengthening effects of tidal force fluctuations. Increased cycling rates (velocity of shortening), as observed in asthma, differences between mouse species, allergen sensitization, and immaturity are also
associated with airway hyperresponsiveness [Stephens'98B] [Fan'97] [Antoinissen'79] [Tepper'95] [Ikeda'92]. While speculative, it is interesting that the perturbed equilibrium hypothesis may link seemingly unrelated phenotypes of AHR.

8.4. THE IMPAIRED BRONchodilATOR RESPONSE IN NON-ASTHMATICS: A CASE AGAINST THE PERTUBED EQUILIBRIUM HYPOTHESIS?

The perturbed equilibrium hypothesis provides a plausible mechanism for the impaired bronchodilator response in the asthmatic lung, through either a reduction in the magnitude/amplitude of the tidal loads or a reduced effectiveness of these loads to perturb the static equilibrium of ASM (i.e. ASM that is refractory to the influence of tidal stretch.). However, the experimental findings coupled to \textit{in vivo} estimates of tidal force amplitude argue that AHR should never occur in the presence of deep inspirations. While this prediction holds true in the case where deep inspirations are initiated before or at the onset of bronchoconstriction challenge non-asthmatics, it fails in predicting that the same individuals, if challenged without deep inspiration at the onset of challenge, are unable to dilate their airways with the return of deep inspiration [Skloot'95]. This result then suggests that ASM in non-asthmatics become refractory to the effects of tidal stretch. The relevance of this finding is that superficially, it is inconsistent with the experimental findings that show tidal tension fluctuations equally capable in preventing shortening to its static equilibrium length or lengthening muscle that has first reached its static equilibrium length (Figure 8-2)
Figure 8-2: The experimental results of tension fluctuation-driven lengthening and tension fluctuation-inhibited shortening. The imposition of a large tidal tension amplitude (\( \delta T = 32\% T_n \)) at the onset of contraction inhibits shortening and maintains muscle at a length of \( 0.741 \pm 0.094 \ L_n \). If shortening first allowed to progress to static equilibrium isotonic length and then tidal tension fluctuations are imposed, the muscle lengthens to a length of \( 0.789 \pm 0.210 \ L_n \). While the lengths are different, these results of Chapter Six shows that tidal tension fluctuations imposed before of after static equilibrium conditions are reached results in equivalent perturbs lengths.

While there are several plausible mechanisms for this observation, a closer examination of the experimental results reveals that the findings of Skloot et al. may be explained for by the rate at which tension fluctuation lengthening occurs. Figure 8-3 illustrates the time response of lengthening using the experimental results of Chapter Six, for the case where \( \delta T = 32\% T_n \). Experimentally, the imposition of a tidal tension fluctuation drove ASM length from a static equilibrium length of \( 0.493 \pm 0.129 \ L_n \) to a perturbed equilibrium length of \( 0.789 \pm 0.210 \ L_n \), a length change that is consistent with a with a potent bronchodilator effect. One feature of the response, however, is that this evolution of this length change occurred over a time span of 120 minutes with a time constant that may be roughly estimated to be on the order of several minutes (4.2 minutes). Plotted on an expanded time scale, the time course of lengthening shows that large tidal fluctuations of \( \delta T = 32\% T_n \) increased muscle length by only \( 0.1 \ L_n \) over a period of two minutes (24 cycles). If for the moment, each tidal tension fluctuations in this response is assumed to represent a single DI, the response suggests that each DI is capable of dilating the airway no more than \( 0.5\% \ L_n \) per deep inspiration. Functionally then, this slow progression of lengthening is equivalent to a refractory state of muscle in which the effects of tidal tension fluctuations evolve over a time scale
manifold greater than the duration of a DI itself. This extrapolation is consistent with the findings of Skloot & colleagues, who showed that three successive deep inspirations initiated after constriction caused a small but progressive improvement in pulmonary function [Skloot'95]. These results then suggest that if muscle is allowed to first contract to the “latch” state, the ability of deep inspiration to dilate the airway in the steady-state is not impaired in but that each inspiration cause only a small incremental dilation of the airway. In the explanation of this behavior, it may be useful to think of muscle that has reached its isotonic steady-state as equivalent to a frozen or slush state. That is if muscle is first allowed to contract to is static equilibrium length, thereby converting its rapidly cycling myosins into the slowly cycling latch state (frozen or slush state), the subsequent imposition of tidal stretch is then governed by the rate constants of latch (slush) and lengthening evolves at a commensurate rate a well.

Figure 8-3: The evolution of fluctuation driven muscle lengthening shown in two time scales. A) Shows the progression of cycle averaged muscle length from the onset of tidal tension fluctuations until 60 minutes into the response. While the lengthening effect progresses such that muscle length is increased from $0.5 \, l_n$ to $0.74 \, l_n$, this increase evolves over a duration of 120 minutes. The time required for the response to reach 50% of its steady state value is estimated at 4.2 minutes. B) The first two minutes of the lengthening response when tidal tension fluctuations are applied. If each cycle is assumed to represent a single deep inspiration, this response shows that the lengthening effect occurs on a time scale nearly two orders of magnitude greater than the duration of a DI itself (250 seconds versus 5 seconds). In this figure, the response shows that 24 cycles are capable of increasing muscle length an average of no more than 0.5% $l_n$ per cycle.
8.5. SHORTERING PLASTICITY: THE BONUS OF A PERTURBED EQUILIBRIUM STATE

The functional implications of tension-fluctuation driven lengthening on AHR were discussed above and many of the same functional implications are relevant to the behavior of shortening plasticity. Namely, that the ability to drive airway smooth muscle length away from its static equilibrium length and maintain this elevated muscle length provides a potent protective mechanism against excessive airway narrowing. In the shortening plasticity response, two features which may have particular significance in protecting the airways against AHR are 1) the maintenance of a muscle length longer that the static equilibrium length even when the tidal fluctuations in load are ceased and 2) the potentiating effect of shortening plasticity on subsequent fluctuation driven lengthening. As discussed above, the ability to perturb muscle length away from its static equilibrium length through fluctuations driven lengthening, or in this case through shortening plasticity, is important as it increases the luminal area of the airway and substantially decreases airway resistance. This is reflected in the first feature of shortening plasticity in which ASM length could be maintained at a length longer that the static equilibrium length even when tidal fluctuations were ceased. The second also contributes to increase muscle length through an amplification of lengthening response to tidal tension fluctuations and a cumulative increase in muscle length even when the tidal tension fluctuations are ceased (Figure 8-4).

The phenomena of shortening plasticity is profound as it provides a means of protecting the airway through a lasting, inhibitory effect on subsequent shortening effect and a means of amplifying both the this effect and that of fluctuation driven lengthening on subsequent applications of tidal loads. Yet, the emergence of this effect was conditional upon the whether fluctuation driven lengthening occurred. That is, shortening plasticity required that tidal stretch first perturb airway smooth muscle away from its static equilibrium length and into a perturbed equilibrium before any of the above behaviors could occur. While the mechanism of shortening plasticity itself could not be attributed to a perturbed binding equilibrium of myosin binding, these results suggest that a perturbed equilibrium of myosin binding may be a precursor or a catalyst, for the subsequent changes in the cell contractile machinery that is hypothesized to underlie the effect of shortening plasticity.
Figure 8-4: The shortening plasticity results of Chapter Six and its implications on airway narrowing. Muscle length is first allowed to contract isotonically to its static equilibrium isotonic length. This is used to represent a closed airway. The imposition of tidal tension fluctuations lengthens the muscle and thus result in a dilation of the airway lumen. The cessation of the tidal loads result in a slight re-shortening of the muscle, but remains elevated above its initial static equilibrium length and thus maintains a roughly equivalent airway luminal area. The re-imposition of tidal tension fluctuations, however results in a lengthening response amplified above the previous fluctuation driven lengthening response, and the airway lumen dilates to an even greater extent. The final state where the tidal loads are ceased results in a re-contraction, but again this re-contraction is slight. Moreover, this final muscle length is nearly equivalent to the initial fluctuation driven length, but it does not require the large tidal amplitude for the maintenance of this length.

A perturbed equilibrium as a self-reinforcing mechanism

The functional implications of this conditional emergence is profound as it suggest that airways that are unable to escape from static equilibrium forgo two important phenomena that protects the airways from excessive narrowing. The first is the behavior that tidal stretch, if sufficient to perturb the
static equilibrium of ASM, is capable of exerting a lengthening effect on ASM and prevent excessive airway narrowing from ever occurring. The second effect arises from the cascade that that airways that are unable to escape from static equilibrium also are unable to benefit from the lasting and amplifying effects associated with shortening plasticity. Using the socioeconomic analogy, airway smooth muscle that is capable of escaping from the chains of static equilibrium are then exposed to new opportunities of shortening plasticity and subsequent tension-fluctuation lengthening amplification that stabilize against a return to static equilibrium while ASM stuck in static equilibrium must toil at is current level. It is a classic case of the rich get richer and the poor remain poor.

While interesting, this analogy also leads to the suggestion that the effect of tidal stretch on ASM and the ability to drive ASM away from static equilibrium with tidal stretches results in a self-reinforcing system by which lengthening leads to more lengthening through a cascade of events that ultimately prevents excessive airway narrowing from occurring. Similarly, an inability to escape from static equilibrium leads to excessive airway narrowing and a state of ASM that is without the benefit of these bronchoprotective mechanisms. Although the pervious section proposed that the rate of lengthening as n explanation for bronchoconstriction refractory to DI in normals, this self-reinforcing mechanism may also play a role in this refractory bronchoconstriction. It is plausible that a temporary loss of the bronchoprotective effect of deep inspiration in the non-asthmatic results in the subsequent loss of the of bronchoprotective cascade of events that were observed in the shortening plasticity investigation.

8.6. CONCLUSIONS

This thesis establishes that in the setting of tidal force fluctuations, such as those that occur during tidal breathing, airway smooth muscle state (length, stiffness, hysteresivity) is not set by a static equilibrium but by a dynamic process conditional upon the amplitude of the tidal forces applied. The central hypothesis proposed in which these changes in muscle state from static equilibrium were mechanistically based on the ability of tidal stretch to directly disrupt to binding equilibrium of myosin-actin crossbridges was supported by the experimental evidence and the theoretical investigations of the HHM theory.
Importantly, these results argue against the classical models of airway narrowing, which assume that airway smooth muscle length, and thus airway caliber, is set by a balance of static forces. This thesis has shown that in an environment of tidal force fluctuations, airway smooth muscle length is not set by a static equilibrium but that muscle lengthening occurs when the muscle is subjected to tidal force fluctuations of sufficiently large amplitude. While the classical models of airway narrowing are useful as they define the caliber to which the airway tends to if given enough time, these models are limited in explaining airway behavior under tidal breathing and in particular, deep inspiratory maneuvers.

Fluctuation-driven muscle lengthening, as a phenomenon, is important as it may help to explain some of the current mysteries in AHR such as why deep inspiration (DI) fails to dilate the airways in spontaneous asthmatic bronchoconstriction. In the context of fluctuation driven lengthening, the reduced ability to dilate the airways of the asthmatic with deep inspiration may lie in the simple explanation that the tidal forces generated are insufficient in magnitude to drive the process of fluctuations-driven lengthening or that the process occurs on a time scale so slow as to resemble a state that is refractory to the dilatory effects of DI.

Fluctuation-driven lengthening and its hypothesized mechanism of a perturbed binding equilibrium of myosin reveals a simple mechanism for the regulation of ASM length in the setting of tidal force fluctuations from which several predictions can be made. In particular, the perturbed equilibrium hypothesis reveals the unanticipated determinants of tidal force amplitude, tidal force frequency, and intrinsic speed of the muscle itself, as factors capable of regulating ASM length. These predictions are significant as it provides a simple framework from which the links between asthma, airway inflammation, and airway hyperreactivity may be explained. Additionally, the hypothesis appears capable of linking seemingly unrelated phenotypes of AHR to an inability of tidal stretch to perturb the myosin-actin binding equilibrium away from static equilibrium conditions. Quite simply, the perturbed equilibrium hypothesis, in combination with the classical models of airway narrowing, proposes that airway smooth muscle length is regulated by the mean, amplitude, and frequency of the load in combination with the speed and strength of the airway smooth muscle against which it acts. This view provides a simple prism from which many of the puzzles of AHR can be addressed.
While a perturbed equilibrium of myosin binding is a powerful tool from which seemingly unrelated phenotypes of AHR may be unified, it clearly is not the entire picture in defining AHR. As shown in Chapter Six (the investigation of shortening plasticity), airway smooth muscle length is not determined by crossbridge mechanisms alone. Indeed current smooth muscle research is revealing a rich and complex regulatory scheme of airway smooth muscle contraction that is far from being absolutely defined. The perturbed equilibrium hypothesis is clearly not the end, but the beginning of a new way of thinking about airway smooth muscle length regulation and hopefully factors that lead to AHR and asthma.
Chapter Nine

BIBLIOGRAPHY


Appendix A

A PRELIMINARY INVESTIGATION INTO THE EFFECT OF MAXIMAL VELOCITY OF SHORTENING ($V_{\text{max}}$) AS A MODULATOR OF TENSION FLUCTUATION-DRIVEN LENGTHENING

A.1. OVERVIEW

This section is presented to the reader as the report of preliminary experiments with sensitized canine airway tissue and a brief investigation with the HHM theory. Specifically, this investigation addresses a prediction of the perturbed equilibrium hypothesis in that influence of muscle speed and velocity of shortening as a modulation factor of force-fluctuation driven lengthening. While the theoretical results as obtained through the HHM theory are consistent with the hypothesis, the experimental results are inconclusive.

A.2. THE PERTURBED EQUILIBRIUM HYPOTHESIS AND ITS PREDICTIONS

Recall from Chapter Seven that the perturbed equilibrium hypothesis generated three factors that could influence the amount of force fluctuation lengthening. The first was the amplitude of the tidal force (Chapter Five). The second was the frequency of the tidal force (Chapter Seven). The Final factor was the intrinsic "speed" of the muscle.

Within the framework of the perturbed equilibrium hypothesis, it is argued that an increased frequency of tidal stretch, by decreasing the time available for equilibration to the new load, results in a muscle state further away from static equilibrium steady-state conditions. Similarly, this argument can be
A.3. THE HHM THEORY, $v_{\text{max}}$, AND ITS PREDICTIONS ON TENSION FLUCTUATION-DRIVEN LENGTHENING

The maximal velocity of shortening $v_{\text{max}}$, in most cases, is thought to reflect the intrinsic rates of bridge cycling in smooth muscle (and skeletal muscle as well). Therefore, an increase in $v_{\text{max}}$ reflects an increase in these intrinsic cycling rates over the nominal rates. As argued above, and increase in the intrinsic cycling rates (and thus an increase in $v_{\text{max}}$) is predicted to influence muscle length where increased $v_{\text{max}}$ results in lesser departures from static equilibrium steady-state. Modulating $v_{\text{max}}$ in the HHM theory can occur through several modes. In this thesis, three modes of parameter modulation capable of increasing $v_{\text{max}}$ are considered. The three modes are

1) A global increase or decrease in all rate constants $k_1$ to $k_5$.

2) An increase in the attachment and detachment rates of myosin from actin ($k_1$, $k_4$ & $k_7$ or equivalently, “$f(x)$” and “$g(x)$” in the sliding filament theory.)

3) A increase or decrease in the constants regulated by MLCK and MLCP ($k_1, k_2, k_5$, & $k_6$.)

While the first two parameter variations are hypothetical changes that might occur in muscle with higher $v_{\text{max}}$, the third variation of changes in MLCK and/or MLCP activity is based on actual changes within airway smooth muscle as reported by Stephens et al. [Stephens’98]. In their investigation, Stephens & colleagues showed that ragweed pollen sensitization causes a two-fold increase in MLCK activity through an increase in MLCK content and a measurable increase in $v_{\text{max}}$.

The predictions for these three cases in the case of tension fluctuation-driven lengthening are shown in Figure A-1. The predictions show that for the first and third cases, an increase in the rate constants results in decreased lengthening and vice versa. However, in the second case where the attachment and detachment rates alone are increased, the HHM theory predicts counter-intuitively, an increase in lengthening. These preliminary predictions show that while each increase in the rate constants are associated with an increase in $v_{\text{max}}$, increased $v_{\text{max}}$ does not necessarily predict an outcome of decreased lengthening. Similarly, a comparison of the two remaining cases of a global increase in rate constant and a local increase in the MLCK-MLCP rate constants reveals that a change in MLCK rate constants only is more effective at inhibiting the lengthening effect of tidal tension fluctuation. Taken
together, these predictions show that the regulatory scheme of the HHM theory is sufficiently complicated that predictions, which may seem intuitively obvious can fail. While specific changes that are capable of increasing $u_{MAX}$ in smooth muscle are possible, these results show that specific patterns in this increase can either amplify or suppress the lengthening effect of tension fluctuations. This suggest that $u_{MAX}$, while sufficiently accurate as an index of cycling rates, may not accurately predict whether tidal stretch is capable of perturbing the static equilibrium of ASM. The above predictions, combined with the experimental results, are discussed in greater detail below.

Figure A-1: The HHM theory predictions for the proposed parameter changes. In all figures: nominal parameters (solid lines) increase in rate constants (dashed lines), decrease in rate constants (dotted lines). A) The effect of global changes in all rate constants increased or decreased by 50% - Note that a global increase in rate constants result in less lengthening. B) The effect of changes in attachment and detachment rates $f_p$, $g_p$, & $g$ - Counter to the heuristic prediction that increasing cycling rates should result in less lengthening, the HHM theory predictions show the opposite. That is increasing the attachment ad detachment rates results in a increased lengthening effect. C) The effect of changes in MLCK-MLCP - Again the constants regulating MLCK & MLCP ($k_i$, $k_3$ & $k_4$ & $k_5$) are increased or decreased by two-fold. Similarly, an increase in the rate constant result in a decreased lengthening response.
A.4. EXPERIMENTAL MATERIALS & METHODS

Ragweed-pollen sensitized and control canine ASM from the laboratory of N. L. Stephens was used in this study. The expected behavior of these two tissues is that sensitized tissue has a $v_{\text{MAX}}$ roughly 30 to 50% higher than controls [Antonissen '79] [Stephens '86]. The experiments were conducted blind. The tissue strips were prepared in an identical manner to the methods described in Chapter Four and same experimental apparatus was used. The reader is referred to these chapters for details. Likewise, in all experiments, optimal length $L_o$, and peak tension $T_o$ with $10^{-4}$M acetylcholine, were determined in an identical manner.

A.4.1. The tidal tension fluctuation protocol

The protocol used in this study is identical to that described in Chapter Five. As a review, the protocol proceeded as follows. Activated muscle strips ($10^{-4}$M ACh) that were isotonically contracted ($T_o = 0.32 T_u$) to its static equilibrium steady-state length. The muscle was then subjected to tidal tension fluctuations ($f = 0.2\text{Hz}.$) through a sequence of tidal tension amplitudes (4, 8, 16, 24, 32, &8% of $T_u$). Each amplitude was maintained according to the following: 60 minutes for $\delta T = 4, 8$, and $16\% T_u$, 90 minutes for $\delta T = 24\% T_u$, 120 minutes for $\delta T = 32\% T_u$, and 120 minutes for $\delta T = 8\% T_u$.

A.4.2. The quick-release protocol for $v_{\text{MAX}}$

The maximal velocity of shortening $v_{\text{MAX}}$ was measured at different time points after the onset of stimulus using the quick-release technique as described in [Stephens '77]. In this protocol, muscle strips were taken from tissue adjacent to that used in the tidal tension fluctuation protocol. The protocol proceeded with isometric contraction via electric field stimulation. At a predetermined time point into the contraction, the load on the muscle was reduced (quick-released) to approximately 100 mg of tension using a servo controller. The 100 mg afterload was typically less than 2% of $T_u$. The resulting muscle shortening was recorded and the velocity of shortening $100 \text{ms}$ after the quick-release was used to represent $v_{\text{MAX}}$. This stimulation-release protocol was repeated at five-minute intervals using different
predetermined release times into the contraction. The release times used in this protocol were $t = 2, 4, 8, 15, 30$, and 60 seconds after the onset of electric field stimulation.

A.4.3. **Data analysis**

Cycle-averaged length, stiffness, and hysteresivity were calculated from the measured length and force signals using the equations defined in Chapter Two. The reader is referred to Fredberg, et al., for details of the calculation. Except where noted, cycle-averaged muscle states are presented as the mean ± standard deviation. The data was grouped according to sensitization or control (unsensitized).

A.5. **RESULTS**

The results are presented with the steady-state values of length, stiffness, and hysteresivity. The time evolution of these state changes, are qualitatively similar to that described in Chapters Five and are not presented. The results are described below.

A.5.1. **The response of sensitized ASM to tidal force fluctuations**

The results of tidal tension fluctuations on sensitized canine ASM are shown in Figure A-2. Similar to the previous experiments, the results show that the muscle lengthened as the amplitude of tidal tension were increased from zero to 32% $T_n$. A noteworthy difference between this and the previous results using bovine ASM is a decrease in the threshold amplitude at which lengthening occurs. In the bovine ASM experiments, recall that the threshold occurred at $\delta T = 8\% T_n$. A second difference is seen in the magnitude of lengthening where for bovine ASM, the muscle lengthened roughly 1.7 fold over its static equilibrium isotonic length. For the sensitized canine ASM, the maximal lengthening, which occurred for a $\delta T = 32\% T_n$, was roughly two-fold greater than its static-equilibrium isotonic length. Lastly, even in the case of sensitized canine ASM, a reduction in $\delta T$ after lengthening occurred did not result in a re-contraction of length and the muscle remained lengthened above its length for the initial exposure to $\delta T = 8\% T_n$. 

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Similarly, Figure A-2c shows the effect of tidal tension fluctuation on stiffness for sensitized canine ASM. Again, these changes mirrored that of the previous bovine ASM experiments in Chapter Five where an increase in $\delta T$ resulted in a decrease in muscle stiffness. Again, a reduction in tension amplitude to $\delta T = 8\% T_o$ after lengthening occurred resulted in an increase in stiffness, but that this stiffness remained below that for its initial exposure to $\delta T = 8\% T_o$. Hysteresivity, likewise, showed an increase as $\delta T$ was increased (Figure A-2b). Lastly, the resulting strain amplitudes (Figure A-2d) shows disproportionate increase in strain amplitude as $\delta T$ was increased.

Figure A-2: Tension fluctuation result for both sensitized (solid lines) and control (dotted lines) ASM. A) The lengthening response to tension fluctuations. B) The hysteresivity response to $\delta T$. C) Normalized stiffness. D) The resulting strain amplitude response. All values represent the steady-state length, hysteresivity, tension, and strain amplitude for the applied tension amplitude.
A.5.2. The response of control ASM to tidal tension fluctuation

The results of force tension fluctuations on the control canine ASM are shown in Figure A-2 superimposed with the result of the sensitized canine ASM. Similar to the sensitized ASM response, the control ASM lengthened as the amplitude of tidal tension was increased from zero to 32% $T_o$. Moreover, while not statistically significant, the controls responded in less lengthening when compared to the sensitized strips. This pattern of decreased response to tidal tension fluctuations is also observed in Figures A-2b and A-2d, where the decrease in stiffness and the increases in strain amplitude were less for the control strips compared to the sensitized strips. In the case of hysteresivity, no pattern is apparent with the response of sensitized ASM starting with a higher baseline value but then crossing over as the amplitude of $\delta T$ is increased to 32% $T_o$. As a note, the responses between the sensitized and control ASM are similar to the extent that no statistical difference was detected for the number of samples used. (N=11 for the sensitized ASM, N=4 for the controls).

A.5.3. $u_{\text{max}}$ of sensitized and control canine ASM

Figure A-3 shows the resulting $u_{\text{max}}$ for both sensitized and control groups. The responses show a $u_{\text{max}}$ of 0.5-0.6 $L_o / s$ at $t = 2$ seconds, that decreased asymptotically to values of 0.32-0.38 $L_o / s$ at $t = 30$ seconds or more. A comparison between the sensitized and control $u_{\text{max}}$ reveals an unexpected result where the control muscle strips resulted in higher values of $u_{\text{max}}$ than the sensitized. This difference, however, not statistically significant for the number of samples used.
Figure A-3: The maximal velocity of shortening as measured in sensitized and control canine ASM. Each time point represents the time into the contraction at which $V_{MAX}$ was measured. $N_{sensitized} = 11, N_{control} = 4$.

A.6. DISCUSSION

The principal finding of this investigation is that when exposed to tidal tension fluctuations, sensitized ASM appeared to respond with greater length increases than non-sensitized controls. While the differences are not statistically significant, this finding is opposite to the expected response that sensitized ASM should respond with less lengthening due to its intrinsically faster cycling rates. A second unexpected finding was that an increase in $V_{MAX}$ for the sensitized tissue was not established.

In both cases, the low number of non-sensitized control samples ($N=4$), the time from when the tissue is excised and delivered to our laboratory ($> 30$ hours), and perhaps a failure on the quick-release technique may have resulted in these results which were contrary to expected
A.7. CONCLUSION

The HHM theory provided predictions that were important in understanding the implications of the perturbed equilibrium hypothesis and its speculation that increased intrinsic cycling rates of the actin-myosin binding cycle could delay and possibly prevent departures from static equilibrium conditions brought on by tidal stretch. While this was shown to be true in most cases, one exception was found in the case where an increase in attachment and detachment rates resulted in more lengthening. A comparison of the two remaining cases showed that a increase in all rate constants resulted in muscle that was less resistant to the lengthening effect of tidal force fluctuations compared to a specific increase in the rate constants that regulate MLCK & MLCP only.

In regard to the effect of specific increases in rate constants, the HHM theory showed that the experimentally established increase in MLCK activity for sensitized ASM, when applied to the rate constants regulating the MLCK-MLCP reaction, results in behavior that is strikingly similar to reduced bronchodilator response in the asthmatic lung. While global increases in all rate constants, or equivalently, a decrease in tidal stretch frequency, was shown to decrease the lengthening response to tidal tension fluctuations, this local increase in MLCK-MLCP rate constants appeared to confer the largest effect in preventing tidal stretch induced lengthening. Interestingly, this parameter variation points to a possible mechanism based on the biochemical regulation of ASM where this mechanism may underlie the inability of tidal lung inflation to modulate airway caliber.

Experimentally, this investigation was unable to reproduce the established behavior of a 50% increase in $v_{max}$ for ragweed pollen-sensitized canine ASM versus non-sensitized controls. This may have been a result of the technique used or even a loss of sensitization effect. While the results are inconclusive, the HHM theory provides a interesting prediction in that if the experimental techniques are sufficiently refined, that the increase in MLCK activity, as occurs during sensitization, should also result in an increase in $v_{max}$ and a decreased susceptibility to the lengthening effect of tidal force fluctuations.