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MECHANICAL BEHAVIOR AND LENGTH ADAPTATION OF RABBIT BLADDER SMOOTH MUSCLE

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MECHANICAL BEHAVIOR AND LENGTH ADAPTATION OF RABBIT BLADDER SMOOTH MUSCLE

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Acknowledgment

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List of Abbreviations

0Ca .......................................................... Nominally Ca^{2+} Free Solution
APS .......................................................... Adjustable Passive Stiffness
ASM .......................................................... Airway Smooth Muscle
ATP .......................................................... Adenosine Triphosphate
CCh .......................................................... Carbachol
DSM .......................................................... Detrusor Smooth Muscle
ICC .......................................................... Interstitial Cell of Cajal
KPSS .......................................................... 110 mM KCl
Lo ............................................................ Optimum Length
L-Ta .......................................................... Length-Active Tension
L-Tp .......................................................... Length-Passive Tension
L-Tt .......................................................... Length-Total Tension
MLCK ...................................................... Myosin Light Chain Kinase
MLCP ...................................................... Myosin Light Chain Phosphatase
NPSS ....................................................... Normal Physiological Salt Solution
OAB ........................................................ Overactive Bladder
PGE_2 ..................................................... Prostaglandin E_2
RC .......................................................... Rhythmic Contraction
S1 ..............................................................................................................................Series 1
S2 ..............................................................................................................................Series 2
S3 ............................................................................................................................. Series 3
SRC ................................................................. Spontaneous Rhythmic Contraction
Ta ................................................................. Active Tension
To ................................................................. Optimum Tension
Tp ................................................................. Passive Tension
Tt ................................................................. Total Tension
VLC ................................................................. Voiding-like Contraction
VSM ................................................................. Vascular Smooth Muscle
Dedication

To my father, Mohammad Almasri, and to my mother, Ghaya Alanani

who waited so long for this moment

Say: Truly, my prayer and my service of sacrifice, my life and my death, are (all)
for Allah, the Cherisher of the Worlds, No partner hath He: this am I
commanded, and I am the first of those who bow to His will.

(The Holly Quran, 6: 162-163)
Abstract

MECHANICAL BEHAVIOR AND LENGTH ADAPTATION OF RABBIT BLADDER SMOOTH MUSCLE

By Atheer Mohammad Almasri, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

Major Director: John E. Speich
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Overactive bladder (OAB), involuntary contractions during bladder filling, is a common condition affecting 17% of the adult population worldwide, and in the U.S. ranks ahead of diabetes in a list of the 10 most common chronic disorders (Mullins 2009). Mechanical mechanisms contributing to OAB are not completely understood and because of the unique function and broad volume range of the bladder, there may be mechanical characteristics that distinguish detrusor smooth muscle (DSM) in bladder from other smooth muscles.
Recent studies have shown that the length-passive tension curve in DSM exhibits adjustable passive stiffness (APS) characterized by a passive curve that can be shifted along the length axis as a function of strain history and activation history; however, the mechanical mechanisms responsible for APS remain to be determined. Also, whether DSM exhibits a dynamic length-active tension relationship, as has been identified in airway and vascular smooth muscles, has not been investigated.

This dissertation focused on both the passive and active length-tension relationships in DSM and the mechanical mechanisms responsible for these relationships. The first objective was to study the impact of APS on the length-total tension relationship and identify the mechanical mechanisms responsible for generating APS. The second objective was to determine whether the length-active tension relationship is adaptive and identify specific mechanical mechanisms contributing to any adaptive behavior.

The results showed that a shift in the length-passive tension curve due to APS corresponded with a shift in the length-total tension curve in DSM, and that APS was 27.0±8.4% of active tension at the optimum length for active tension generation. Most importantly, low-grade rhythmic contraction (RC), which can occur spontaneously in rabbit and human bladders, regenerated APS.

Results also showed that the length-active tension curve shifted due to stretch to and then activation at long lengths, as well as either multiple KCl-induced maximal contractions or RC. Thus, DSM exhibits length adaptation, and RC may contribute to both APS and length adaptation. Because increased RC has been correlated with OAB,
understanding RC, APS and length-adaptation in bladder may enable the identification of specific targets for new treatments for OAB.
CHAPTER 1 INTRODUCTION AND BACKGROUND

1.1. Motivation

Overactive Bladder (OAB) is one of the most common causes of bladder control problems. It is believed that OAB is due to uncontrolled spontaneous activity of the (bladder) detrusor smooth muscle (DSM) wall during the bladder filling phase (Rovner and Wein 2002), causing increased urgency (a strong desire to void); frequency of urination; and in the worst case, involuntary loss of urine (urge incontinence) (Milsom, Stewart et al. 2000). OAB affects over 17% of the worldwide population individuals (Mullins 2009), and has a significant impact on the quality of life, limitations on activities, and finances of those individuals who suffer from OAB symptoms. Therefore, it is worthy to investigate potential causes of OAB because this research could lead to improved treatment options.

Currently, the drugs used to treat overactive bladder are not specific to the smooth muscle in the bladder, and therefore can affect smooth muscles in other parts of the body. Therefore, the next step in targeting OAB is to identify aspects of contraction regulation unique to DSM. Among the three types of muscles; skeletal, cardiac, and smooth, this dissertation is focused on the mechanical function of detrusor smooth muscle (DSM) in the bladder wall.
The overall objective of this study is to investigate both passive and active length adaptation and the impact on the total tension. This study is focused the adaptive behavior of smooth muscle, particularly rabbit bladder smooth muscle, which is distinct from skeletal muscles which do not show adaptation.

The following introduction highlights some background information about muscle types, smooth muscle structures and contraction, with an emphasis on bladder smooth muscle. Furthermore, some important mechanical characteristics such as length-tension relationships; passive, active and total tension; preconditioning and length-adaptation are discussed.

1.2. Muscles Types

Muscles are the contractile tissues of the body. They are classified as skeletal, cardiac, and smooth muscles, and their function is to produce force and cause motion. Skeletal muscle tissue is named for its location, attached to bones. It is striated, which means the cells contain alternating light and dark bands that are perpendicular to the long axes of the fibers (Fung 1993; Guyton and Hall 2000). Skeletal muscle tissues are designed to contract or relax voluntarily. Cardiac muscle tissue forms the bulk of the wall of the heart. Like skeletal muscle tissues, cardiac muscle tissues are striated. Unlike skeletal muscle, cardiac muscle contraction is usually involuntary. Muscles in which striations cannot be seen are called smooth muscles. These muscles contract involuntary and this dissertation considers this type of muscles.

1.3. Smooth Muscles

Smooth muscles are located in the walls of hollow internal structures such as blood vessels, airways, the stomach, intestines, and urinary bladder (Fung 1993; Pollard
and Earnshaw 2002). Smooth muscle cells are generally long, spindle shaped cells, and therefore they are wide in the middle and narrow to almost a point at both ends (Garfield and Somlo 1985). Smooth muscle cells have a single centrally located nucleus. The cells range in size from 5 to 10 µm in diameter in the center of the cell and from 300 to 600 µm in length (Garfield and Somlo 1985). The size of smooth muscle cells varies between different tissues and species. Smooth muscle cells contain the same contractile proteins-actin and myosin- as skeletal and cardiac muscles; however, these proteins are laid out in a different pattern (Fung 1993). Smooth muscle in the bladder is called detrusor smooth muscle (DSM), and is the main focus in this dissertation.

1.4. The Bladder and its Function

The bladder is a hollow, muscular, and elastic organ. The main functions of the bladder are storage of urine and emptying the urine produced by the kidneys. Each kidney has its own ureter through which urine travels to the bladder (Andersson and Arner 2004). The size of the bladder varies over a large range between species (bladder capacity: mouse, ~0.15 ml; rat, ~1 ml; human, ~ 500 ml) (Andersson and Arner 2004). The empty human bladder is shaped somewhat like a pear. The bladder composed of two main parts as shown in Fig 1.1. The body, which is the major part of the bladder in which the urine collects, and the neck, which is the funnel-shaped extension of the body (Andersson and Arner 2004). The bladder neck is 2-3 cm long, and its wall composed of detrusor muscle interlaced with a large amount of elastic tissues. The muscle in this area is called the internal sphincter. Its natural tone normally maintains the bladder neck empty of urine and therefore, prevents emptying of the bladder until the pressure in the main part of the bladder rises above a critical threshold. Beyond the bladder neck, the
urethra passes through the urogenital diaphragm, which contains a layer of muscle called the external sphincter of the bladder. This muscle is a voluntary skeletal muscle, in contrast to the muscle of the bladder body and bladder neck, which contain only involuntary smooth muscle. The external of sphincter muscle is under voluntary control of the nervous system and can be used to consciously prevent urination even when involuntary controls are attempting to empty the bladder (Guyton and Hall 2000; Costanzo 2002). Once the stretch-receptors in the trigone, which is a triangular space between the two ureters and the bottom opening of the bladder, become stretched by a certain degree of bladder filling, the brain receives a signal that the bladder is full and should be emptied. The ring shaped muscle sphincter closing the bottom is relaxed, and the detrusor contracts to release the urine. The bladder flattens and changes shape as contraction occurs. If the signal to void is ignored, the bladder continues filling, but eventually the bladder will completely empty itself. Babies always pass urine this way, and it can take years for a child to master the control of passing urine.
1.5. Bladder Pressure and Wall Tension

When there is no urine in the bladder, the pressure is approximately zero centimeters of water but by the time 30 to 50 milliliters of urine has collected, the pressure rises to 5-10 centimeters of water. Additional urine from 200 to 300 milliliters can collect with only a small additional rise in pressure. This constant level of pressure is caused by intrinsic tone of the bladder wall itself. Beyond 300 to 400 milliliters, collection of more urine in the bladder causes the pressure to rise rapidly (Guyton and Hall 2000; Andersson and Arner 2004). Detrusor smooth muscle fibers in the bladder wall extend when the urine starts flowing into the bladder. These fibers that lay in the bladder wall mainly are extracellular matrix proteins such as elastin and collagen.
especially when the bladder is full. The main focus of this dissertation is the length-tension relationship of rabbit DSM.

1.6. Smooth Muscles Machinery Contractile Proteins

Actin is one of two main proteins responsible for contraction of muscle cells. Actin filaments are composed of many subunits of the globular protein G-actin. Actin occurs in muscle cells as a polymer, F-actin, which resembles two strings of beads twisted around each other into thin filaments (Fung 1993; Pollard and Earnshaw 2002). Each actin filament is about 1 µm long (Andersson and Arner 2004). The filaments occur in regular structures, alternated and interwoven with thick filaments that contain myosin, the other major muscle protein. There are four different actin isoforms in smooth muscles α-, β-, and two forms of γ-actin. The distribution of the actin isoforms has been reported to differ and it is possible that the different actin isoforms have different functions in the cytoskeleton. Actin is attached to dense bodies throughout the cell and also attached to the cell membrane in numerous areas. Smooth muscle tissues contain a much higher ratio of F-actin to myosin, which may play a role in maintaining structural integrity of smooth muscle cells (Tang and Anfinogenova 2008).

Myosins are a large family of proteins found in muscle cells (Fung 1993). Myosin is made up of 200 or more individual myosin molecules. Each myosin molecule consists of tail and two heads (Guyton and Hall 2000). The total length of each myosin filament is almost 1.6 µm (Andersson and Arner 2004).

1.7. Preconditioning

To obtain reliable length-tension curves tissues must generally be preconditioned (Fung 1993). Preconditioning is defined as reductions in stiffness to a steady state value
upon repeated stretches. Preconditioning is a well known term in rubber and elastomers mechanics, in which the lost stiffness due to damage is not recoverable within a reasonable time frame (Harwood, Mullins et al. 1967). Conversely, preconditioning in living tissues can be categorized as reversible or irreversible. Reversible preconditioning can be due to viscoelasticity of the connective tissues. One form of preconditioning is strain-softening, which is due to damage and breakage of the crosslinking proteins. In rubber, strain softening is essentially irreversible; however, in living tissues some strain softening can produce irreversible tissue damage, but some breakage of molecular structures is reversible as will be described in the APS section of this dissertation introduction. Generally, only data of preconditioned tissues are presented in length-tension curves (Fung 1993).

1.8. Passive Tension

In smooth muscles, tension has both passive and active components as shown in Fig 1.2. Passive force is the force exerted by the muscle as it is stretched when it is not stimulated (Speich, Quintero et al. 2006). This is due to the elastic elements in the muscle fiber and those surrounding fibers, which are primarily connective tissues. In an elastic object such as a rubber band, the passive force is very small below a certain length and as the muscle is stretched above this length, passive force rises. The rise becomes steeper as the muscle is stretched to greater and greater lengths (i.e., the same length change gives greater changes in passive force at longer muscle lengths). The length-passive tension curve, as seen in Fig 1.2, is an increasing exponential curve. In smooth muscles the passive tension is a significant fraction of the total tension at the optimum region, which makes smooth muscles different from skeletal muscles (Seow 2000). In smooth muscles,
there are many candidate structures that may contribute to passive force. Collagen, the basic structural protein in the living tissues, and elastin, an extracellular protein, are the most likely candidates especially at long lengths (Speich, Borgsmiller et al. 2005). There are other possible intracellular candidates that may play a role in passive tension. In skeletal muscles, the protein titin plays a role in passive force bearing, and there is a similar protein in smooth muscles called smitin, which may contribute to passive force (Kim and Keller 2002). Also, there are other intracellular structures may play a role in passive tension including any protein associated with force transmission through dense body (McGuffee, Mercure et al. 1991), as well as actin-myosin cross-bridges or auxiliary actin and myosin cross-linking proteins, may contribute to passive force in smooth muscle. For example, filamin is a cross-linking protein involved in forming F-actin networks, and desmin appears to play a role in transmission of both active and passive tensions (Wang and Singer 1977; Speich, Borgsmiller et al. 2005). Actin-myosin might become crosslinked and the level of crosslinking might be regulated. For example, caldesmon and calponin can bind both actin and myosin, and both proteins have been associated with formation of crosslinkins between actin and myosin (Sutherland and Walsh 1989; Marston, Pinter et al. 1992; Haeberle 1994). Actin-myosin crossbridges are discussed in the following section.
1.9. Active Force

Active force is the additional force (above the passive force) observed when the muscle is stimulated and solely in living tissues. The active force is due to the interaction between actin-myosin crossbridges. The “rowing” action (Fig 1.3) of the actin-myosin crossbridges causes relative sliding of actin and myosin filaments, which shortens the cells and develops force. The level of active force produced is dependent on the number of active crossbridges producing positive work, which is dependent on muscle length. Fig 1.2 also shows a length-active tension curve for smooth muscle. The active curve is somewhat parabolic with ascending and descending limbs and a plateau region in which the muscle can produce approximately the same amount of active force, not as skeletal muscles where the active curve has a single maximum active tension value (Seow 2000). It has been recognized that smooth muscle cells can function over a long range of length. Uvelius (Uvelius 1976) has shown that bladder smooth muscle cells can function over a 7-fold length range.
Smooth muscles are capable of generating at least as much as force as striated muscles. In skeletal muscles, the active force developed is in direct proportion to the degree of overlap between actin and myosin filaments; but in smooth muscles the active force is not strictly a function of the overlap between actin and myosin. The contractile relationship of myosin with actin appears to be less organized in smooth muscles (Battistella-Patterson, Wang et al. 1997; Wang, Pare et al. 2001), and the contractile unit has never been clearly understood. Mechanically speaking, the contractile unit which is considered a “unit cell” has never been identified in smooth muscles relative to striated muscles in which the “unit cell” is well organized and understood. However, smooth muscles contraction theories are based on mimicking the striated muscles contractile sarcomeres or “unit cells”.

1.10. Smooth Muscle Contraction

Contraction of the detrusor muscle is a major step for emptying the bladder. DSM cells fuse with one another so that low resistance electrical pathways exist from one muscle cell to the other. Therefore, an action potential can spread throughout the detrusor muscle, from one muscle cell to the next, to cause contraction of the entire bladder at once (Guyton and Hall 2000). It is widely known that the principal physiological events underlying the contraction of smooth muscle are similar to those of skeletal and cardiac muscles (Webb 2003). Contractions in smooth muscles are initiated by actions potentials that occur in the smooth muscle cell membrane. The depolarization of the action potential opens calcium channels in the membrane. Stimulation of smooth muscle results in an elevation in intracellular calcium, which binds to the protein calmodulin. The calcium-calmodulin complex then activates the enzyme myosin light chain kinase (MLCK), when
myosin light chain kinase is activated; it attaches a phosphate group to, or phosphorylates, myosin. When myosin is phosphorylated, it can cyclically bind to actin to form a crossbridge, move through a power stroke to develop force, release from actin, and return to its original orientation to begin the next cycle, Fig 1.3A-D.

**Fig 1.3.** A diagram showing the “rowing” action of the actin-myosin crossbridges.

The cycling of the actin myosin crossbridges in smooth muscles is much slower than in skeletal muscles, therefore skeletal muscles contract faster than smooth muscles. A typical smooth muscle tissues begins to contract 50 to 100 msec after it is excited, reaches full contraction about 0.5 second later and then declines in contractile force in another 1 to 2 sec, giving a total contraction time of 1 to 3 seconds (Guyton and Hall 2000). This is about 30 times as long as a single contraction of an average skeletal muscle. Because of the many types of smooth muscle, contraction of some types can be as short as 0.2 sec or as long as 30 sec. Thus, the speed of smooth muscle contraction is only a small fraction of that of skeletal muscle. However, although most skeletal muscles
contract and relax rapidly, smooth muscle contractions can be prolonged tonic contractions, sometimes lasting hours.

When the intracellular calcium concentration decreases, myosin is dephosphorylated by the enzyme myosin light chain phosphatase (MLCP). In this case, myosin can still interact with actin, but the attachments are called latchbridges. The latchbridges do not detach, or they detach slowly (Murphy 1988); therefore, they maintain a tonic level of tension in the smooth muscle with little consumption of adenosine triphosphate (ATP) which is the intracellular energy transfer in cell biology (the fuel of the cell). Ratz et al (Han, Speich et al. 2006) found in their study about saphenous arteries that the sustained isometric force was 40% less in saphenous artery than in femoral artery, and they attributed that to lack of latchbridge formation in saphenous artery. Whether bladder smooth muscle uses latchbridges remains to be determined. Speich et al (Speich, Borgsmiller et al. 2005) suggested a role of latchbridges as a potential site for generation of variable passive force, will be discussed later in the discussion section in this dissertation.

When the intracellular calcium concentration decreases below the level necessary to form a calcium-calmodulin complex, relaxation of the muscle occurs (Fung 1993; Guyton and Hall 2000; Costanzo 2002; Webb 2003; Andersson and Arner 2004).

1.11. Spontaneous Rhythmic Contraction (SRC)

Spontaneous rhythmic contraction (SRC) is a phenomenon common to many muscular organs (Fung 1993). Detrusor smooth muscle is not totally passive during filling phase (Gillespie 2004). Instead, detrusor smooth muscle of many mammalian species such as rabbits (Shenfeld, McCammon et al. 1999), rats (Drake, Gillespie et al.
2006; Kanai, Roppolo et al. 2007), cats (Gillespie 2004), guinea pig (Drake, Harvey et al. 2003), mice (Herrera, Etherton et al. 2005; Lagou, Drake et al. 2006; Brown, Bentcheva-Petkova et al. 2008; Ekman, Andersson et al. 2009) and human (Biers, Reynard et al. 2006; Brading 2006), display SRC during the filling phase. The mechanism generating this rhythm activity is still unknown, but it is believed that in order to produce rhythmic activity interstitial cells of Cajal (ICC) act as pacemaker cells (Gillespie 2004). The ICCs may generate the agonist prostaglandin E2 (PGE2) to cause rhythmic contraction (Collins, Klausner et al. 2009).

Thus, there are two distinct contractile phenomena in the bladder: spontaneous rhythmic contraction (SRC) during the filling phase and voiding contraction during the voiding phase. The physiological role of the SRC remains to be determined. This study reveals a potential role for SRC which will be discussed in chapters 3 and 4 of this dissertation.

1.12. Length Range of Smooth Muscles

Cardiac muscle cells can function within 20%-25% length range (Allen, Jewell et al. 1974), and skeletal muscle cells can function within a 3-fold total length range (Gordon, Huxley et al. 1966). In the contrary, it has been recognized for a long time that smooth muscles can function over a long length range because of the large volume changes required by some hollow organs. Uvelius (Uvelius 1976) has shown that bladder smooth muscle cells can function over a 7-fold length range. For example, detrusor smooth muscles tissues, which are the main focus in this dissertation, are subjected to large changes in volume during filling and voiding compared to other kinds of muscles. The smooth muscle of detrusor is to continuously changing mechanical conditions during
normal physiological cycles. As bladder volume increases and decreases with each cycle, the detrusor smooth muscle is stretched and retracted. The urinary bladder can decrease its volume several hundred-fold within 1 min (Ford, Seow et al. 1994), and it has been shown that bladder muscle cells develop active force over a 7-fold length range (Uvelius 1976). This is much greater than the 3-fold total range of filament sliding over which force is generated in skeletal muscle (Gordon, Huxley et al. 1966). Using a systematic approach Gordon et al. (Gordon, Huxley et al. 1966) demonstrated the full length-tension curve in frog skeletal muscle fibers. They reported that tension generation was nearly constant at sarcomere lengths from approximately 2.0µ to 2.25µ. Tension fell sharply at lengths both shorter and longer than those identified as the plateau, such that tension generation was only possible between lengths of 1.27µ and 3.65µ.

Therefore, it seems highly unlikely that a fixed filament could accommodate the large functional length range of some smooth muscles. Thus, a likely explanation of the smooth muscles behavior is that plastic (dynamic) changes in the filament vary the number of the contractile units, so as to accommodate large length changes. The dynamic behavior of both active and passive tension will be studied in the dissertation, along with an explanation of the mechanical mechanisms that might be responsible for this behavior.

1.13. Dynamic Length-Tension Curves for Smooth Muscle

Until the 1990s the L-T relationships in smooth muscle were generally considered to be static (Stephens, Kroeger et al. 1969; Mulvany and Warshaw 1979), with a single passive tension value and a single maximum active tension value for each muscle length, and the fixed sliding filament mechanism explains this behavior. Although a few studies from the 1920s (Brocklehurst 1926), 1960s (Speden 1960) and 1970s (Herlihy and
Murphy 1973) showed that Tp and/or Ta values at a particular muscle length could be different if the measurements were made during muscle loading versus unloading, these differences were attributed to incomplete inactivation, irreversible stretch, viscoelasticity, or re-orientation of muscle fibers during strip preparation.

Stephens et al in 1969 (Stephens, Kroeger et al. 1969) studied trachealis mongrel dogs airway smooth muscle and they found that the length-tension curve resembles the length tension curve of skeletal muscles. The active tension curve demonstrates the same type of length dependency as skeletal muscles; which can be explained by the sliding filament theory (Gordon, Huxley et al. 1966). The passive tension is low the maximum length which is one of the distinct properties of skeletal muscles.

In 1971 Gordon et al studied (Gordon and Siegman 1971) the length-tension relationship of rabbit taenia coli. The results from their study indicated that rabbit taenia coli has “static properties similar to skeletal and other smooth muscles.” Also, because the active tension depends on the muscle length and because of the existence of the optimum active tension that means the sliding filament theory (Gordon, Huxley et al. 1966) applies on rabbit taenia coli smooth muscle.

One of the first studies was completed by Herlihy and Murphy (Herlihy and Murphy 1973), in which they set out to more fully characterize the passive and active tension of hog carotid artery smooth muscle over a series of lengths. They noted that different in the length-passive tension curves could be obtained dependent on the protocol used to obtain passive tension. They suggested that a series of stretches to a maximal length followed by release to shorter length provide the best estimate of passive tension. Thus, the length-history of VSM affects passive tension generated at a particular length.
Mulvany and Warshaw (Mulvany and Warshaw 1979) later estimated that the working range of VSM was 0.38-fold Lo to 1.82-fold Lo, where Lo is the length at which the tissue is able to generate maximal tension. When relaxed, isolated smooth muscle cells stretched to lengths greater than resting produced similar forces to that achieved at resting length. The large length range over which smooth muscle has been shown to generate tension has called into question a static sarcomeric structure or the fixed sliding filament model that is known to exist in skeletal muscle. Thus, all of the old studies assumed that smooth muscles are static, which contradicts with the most recent studies and with what will be shown in this dissertation.

More recent studies (Pratusevich, Seow et al. 1995; Wingard, Browne et al. 1995; Xu, Gillis et al. 1997; King, Paré et al. 1999; Seow 2000; Fust and Stephens 2005; Speich, Borgsmiller et al. 2005; Bosse, Sobieszek et al. 2008; Syyong, Cheung et al. 2008; Speich, Almasri et al. 2009) studies showed that the length-tension relationships are dynamic, and some smooth muscles have the ability to accommodate large length changes without compromising the ability to generate tension (Fig 1.4, active force).

In 1995, Seow et al (Pratusevich, Seow et al. 1995) showed that canine tracheal muscle is able to maintain constant active force over a 3-fold length change. It was found that altering the passive length of the muscle preparation results in a reduction in active force elicited immediately after the length alteration. If the muscle is then allowed to adapt to the new length, the active force recovers fully to the level before the length change. The adaptation process consists of a brief stimulation of the muscle electrically every 5 min, and it takes 4-6 activations for the muscle to recover fully. This dynamic
behavior was explained by the dynamic nature of the subcellular structures in airway smooth muscle. Therefore, the airway smooth muscle length-tension curve is dynamic.

Wingard et al. (Wingard, Browne et al. 1995) later expanded on Murphy’s work (Herlihy and Murphy 1973) using a similar protocol to obtain passive tension and also found the presence of two passive tension curves. The passive tension generated with gradual tissue lengthening exceeded that generated with gradual shortening and the authors attributed the difference in curves to an irreversible increase in the compliance of the tissue in contact with the steel mounting posts. Thus, following maximal stretch, the length of viable tissue capable of contributing to the passive tension, as well as active tension, is reduced. Others have suggested a possible role for Ca\(^{2+}\)-independent crossbridges or passive structures as responsible for resistance to stretch (Rembold 1992).

Seow (Seow 2000) showed that the length-passive tension of vascular smooth muscle is time dependent and therefore not static. In his study the carotid artery length of adult New Zealand white rabbit was changed from Lref to 0.67, 0.83, 1.17 or 1.33Lref in random order. Once the muscle was set to a new length, a 2 min period was allowed for the passive viscoelastic response of the muscle to settle, an electrical stimulus was then applied to elicit an isometric contraction. The muscle was then allowed to adapt at that length for ~30 min while it was stimulated isometrically once every 5 min. Seow obtained two different passive and active-length curves at 2 minutes and at 27 minutes after a length change (Fig 1.4, passive force). Thus, passive and active tension in vascular smooth muscle is also dynamic and can be adapted to different lengths as a function of activation.
In 2005 Fust et al (Fust and Stephens 2005) found that airway smooth muscle can adapt to different length between 0.7Lo to 1.3Lo and the muscle can produce the same amount of active tension. The authors explained this behavior as the cell undergoes a length perturbation, myosin filaments become dephosphorylated and depolymerized. While the cell adapts to the new length, myosin filaments will repolymerize until there are enough contractile units for the cell to generate the same amount of force during a contraction as before the perturbation.

Seow et al in a recent study (Syyong, Cheung et al. 2008) determined that pulmonary arterial sheep lung smooth muscle is capable of adapting to length changes (especially shortening) and regain its contractile force. In their study the pulmonary arterial smooth muscle was changed from Lref to either 0.8Lref or 0.6Lref. Once the muscle was fully adapted at reference length (Lref), the resting (nonstimulated) muscle was then quickly (within 1s) released to either 0.8Lref or 0.6Lref and allowed to adapt at the shortened lengths through regular electrical field stimulation (once every 5 min) until active force reached a plateau. Then, the muscle was fully adapted at either 0.8 Lref or 0.6Lref before it was quickly stretched (at resting state) to Lref again. It was then allowed to recover through regular stimulation at Lref until it was fully adapted to the stretched length, which was not different from the active force found at Lref before quick release. Their major conclusion of their study was that pulmonary arterial smooth muscle is able to adapt to changes in cell length with significant active force changes only observed in the large release (from Lref to 0.6 Lref) or stretch (from 0.6 Lref to Lref). They attributed that due to rearrangement of the contractile filaments and optimization of the overlap between the thin (actin) and thick (myosin) filaments may be responsible for the active
force recovery. The changes in passive tension after the length change was less than the changes in active force and the passive force recovery may reflect passive stress relaxation in the tissue, but active muscle tone could also make up a portion of it.

In a recent study in 2008 by Seow et al (Bosse, Sobieszek et al. 2008) explained adaptation by two phases. The first phase partial disassembly of the contractile apparatus and cytoskeletal structures occurs, followed by a second phase in which reassembly of the structures occurs at the new cell length. The authors observed a substantial increase in myosin filament content and they proposed that this increase is due to additional contractile units incorporated into the contractile apparatus during the process of length adaptation. Whether DSM exhibits a dynamic length-tension relationship remains to be determined and is one of the main objectives of this dissertation.


A previous study by Speich et al. (Speich, Borgsmiller et al. 2005) in 2005 showed that passive detrusor smooth muscle exhibits viscoelastic softening that is reversible within a short period of time (~ 10 min) and irreversible strain softening which are revealed by a shift in length-passive tension curve. The strain softening is irreversible unless the tissue is contracted either by using KCl or Carbachol (CCh). It was also found that a 3 min contraction was sufficient to regain the stiffness. Therefore, DSM has a passive stiffness can be regained by contraction, and maintained unless the tissue is stretched. Thus, passive tension is dynamic and can be adapted to different lengths as a function of activation and stretch history, as shown in Fig 1.4. Because the passive stiffness can be adjusted, this characteristic was labeled as adjustable passive stiffness (APS) (Speich, Dosier et al. 2007). Therefore, there is a reversible strain softening in
DSM can be regenerated by a contraction at short length. The present study will confirm that DSM exhibits APS and will examine how APS influences the total tension in DSM. Furthermore, factors that may influence APS formation will be examined.

![Diagram of shifting active and passive length-tension curves](image)

**Fig 1.4.** Illustration of a shifting active and passive length-tension curves (Bosse, Sobieszek et al. 2008).

### 1.15. Potential Adaptation Mechanisms

A recent study on length adaptation of airway smooth muscle by Bosse et al (Bosse, Sobieszek et al. 2008) defined length adaptation as “a relatively rapid process in which smooth muscle regains contractility after experiencing a force decrease induced by length fluctuation.” Length adaptation in smooth muscles can be made by a single contraction (Seow, Pratusevich et al. 2000; Gunst and Wu 2001), a series of brief activations (Pratusevich, Seow et al. 1995; Silberstein and Hai 2002), or a continuous submaximal activation over a period of tens of minutes (McParland, Tait et al. 2005), or adaptation can also occur in a relaxed muscle set at a fixed length over a period of hours (Wang, Pare et al. 2001; Martinez-Lemus, Hill et al. 2004) or days (Arner, Malmqvist et al. 1984; Zeidan, Nordstrom et al. 2000; Naghshin, Wang et al. 2003).
There are different mechanisms that may explain the molecular structure changes during adaptation: changes in filament length, numbers, arrangement, and regulation. Sloway and colleagues have suggested that changes in actin filament length may play a key regulatory role (Dulin, Fernandes et al. 2003; Solway, Bellam et al. 2003) whereas Seow and colleagues have suggested that the structural design of the myosin filaments may change (Pratusevich, Seow et al. 1995; King, Paré et al. 1999; Seow, Pratusevich et al. 2000; Mitchell, Seow et al. 2001; Herrera, Kuo et al. 2002; Qi, Mitchell et al. 2002; Seow 2005; Bosse, Sobieszek et al. 2008). Gunst and colleagues have suggested that there is alteration of the connection of the actin filament to the focal adhesion at the cell boundary and accompanying cytoskeleton reorganization that are influenced by the histories of activation and mechanical stretching (Gunst, Wu et al. 1993; Gunst, Meiss et al. 1995; Mehta and Gunst 1999; Tang, Mehta et al. 1999; Gunst and Wu 2001; Zhang and Gunst 2008). Whether these proposed adaptation mechanisms occur in detrusor smooth muscle remains to be determined and some of these mechanisms be studied in this dissertation.

1.16. Dissertation Objectives

This dissertation focused on both the passive and active length-tension relationships in DSM and the mechanical mechanisms contributing to these relationships. The first objective for this dissertation was to identify mechanical mechanisms responsible for the generation of APS and the impact of APS on the length-total tension relationship. More specifically, the following hypotheses concerning APS were tested:

Hypothesis (1): A shift in the length-passive tension curve in DSM due to APS produces a shift in the length-total tension curve.
Hypothesis (2): APS formation in DSM depends on the number of contractions, magnitude of contraction, and length of contraction.

Hypothesis (3): A “voiding-like” contraction plus RC or RC alone at a short muscle length can regenerate APS in DSM.

Hypothesis (4): Actin and myosin crossbridges participate in APS regeneration.

The second objective of this dissertation was to determine whether the length-active tension relationship is adaptive and to identify specific mechanical mechanisms that contribute to any adaptive behavior. More specifically, the following hypotheses were tested:

Hypothesis (1): The length-active tension curve in DSM is not fixed (static), but instead preconditioning shifts the length-active tension curve.

Hypothesis (2): The length-total tension curve in DSM shifts as a result of preconditioning, APS and length adaptation of the length-active tension curve.

Hypothesis (3): The change in active tension in DSM at short lengths due to preconditioning identified in hypotheses (1) and (2) was due to adaptation of the length-active tension curve and that adaptation can be reversed by multiple contractions.

Hypothesis (4): Isometric rhythmic contractions will produce length adaptation of length active tension curve.

1.17. Contributions of the Dissertation

Studies have shown that the length-tension relationships in airway and vascular smooth muscles are dynamic and can adapt to length changes over a period of time. Previous studies from our lab have shown that the passive length-tension relationship in rabbit detrusor smooth muscle is dynamic and the muscle exhibits APS characterized by
a length-passive tension curve that can shift along the length axis as a function of strain history and activation history. To the best of my knowledge, this is the first study to show that the active length-tension relationship in detrusor smooth muscle is dynamic and can adapt over a broad length range for bladder function.

The bladder of all known mammalian species, including humans, displays SRC activity during the filling phase. This rhythmic activity has largely been ignored by those that believe it occurs and the physiological function of rhythm has yet to be identified. Based on the literature and preliminary data from our lab I expect to find that SRC is responsible for regenerating APS and for adapting the muscle. If this hypothesis is correct, this will be the first study to identify the function of SRC in bladder DSM.

In summary, this dissertation is the first to investigate the dynamic length-active tension behavior in detrusor smooth muscle and mechanical mechanisms that may participate in this behavior. Ultimately, these results may be helpful in the identification of treatment for overactive bladder (OAB). Since low-grade rhythmic contractions occur in human bladder, we could potentially identify a physiological role of these rhythmic contractions. Moreover, since elevated levels of RC have been shown in patients with OAB disorder, a defect in the generation of APS may be associated with this disorder. Thus, understanding APS and length adaptation may be essential to understanding a potential mechanism for OAB.

1.18. Dissertation Organization

This study of the mechanical behavior of bladder smooth muscle consists of five chapters. This chapter has provided background information from the relevant literature and listed the main objectives and expected contributions of this dissertation. Chapter 2
discusses the experimental methods used in this study. Chapter 3 presents the results of an APS regeneration study; while chapter 4 focuses on adaptation of active-length tension curve in rabbit DSM. Chapter 5 provides a discussion of the results, whereas chapter 6 provides conclusions and recommendations for future research.
CHAPTER 2 MATERIALS AND METHODS

2.1. Choice of Animal Model

Rats, rabbits, mice and pigs have been the main focus of numerous studies in various biological scientific research disciplines. Although there are marked differences in bladder size, capacity, compliance, physiology, and pharmacology among these species, they still have many common characteristics with humans.

The detrusor muscle, the muscle of the urinary bladder wall, consists of three layers of smooth (involuntary) muscle fibers. The cells of the outer and the inner layers are oriented longitudinally, and those of the middle layer are mostly arranged in a circular configuration. In the human detrusor, bundles of the muscle cells of varying size, usually millimeters in diameter, are surrounded by connective tissue rich in collagen (Andersson and Arner 2004). Within the main bundles, the smooth muscles cells may exist in groups of small functional units. The orientation and interaction between the smooth muscle cells in the bladder are important, since this will determine how the bladder wall behaves and what effect activity in the cells will have on its shape and pressure. For small animal like rabbits, the muscle bundles are less complex and the patterns of the arrangement simpler than in the human bladder (Andersson and Arner 2004), but in both rabbits and humans the muscle consists of three layers of smooth
muscle fibers and most of the fibers are arranged longitudinally (Guyton and Hall 2000; Andersson and Arner 2004).

In this study New Zealand White Rabbits were used for a number of reasons. First, the detrusor consists of three layers of smooth muscle fibers and most of the bundles are arranged longitudinally, as in humans. Second, it is relatively easy to cut strips from the rabbit bladder because the bundles are clearly demarcated. Third, unlike mice, the size of the rabbit bladder is large enough to provide the number of tissue strips necessary for some experiments. Fourth, the cost is reasonable when compared to larger animals such as pigs. Fifth, there are extensive research results published on rabbit bladder, so using this species will allow the results from this study to be compared to the literature. Finally, this dissertation builds upon prior studies from our laboratory which used New Zealand White Rabbits.

2.2. Tissue Preparation

All experiments involving animals were conducted within the appropriate animal welfare regulations and guidelines and were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (IACUC). Whole bladders were obtained from adult female New Zealand White rabbits (2-4 kg) which were pre-anesthetized prior to death through sternotomy, because the heart was used for other investigations. Bladders were washed, cleaned of adhering tissues, including fat and serosa, and stored in cold (0–4°C) normal physiologic salt solution (NPSS). Thin strips (~0.2 mm thick) of longitudinal upper DSM, free of underlying urothelium and overlying serosa, were cut from the bladder wall above the trigone and close to the dome by following the natural bundling clearly demarcated when bladders were in cold buffer as
seen in Fig 2.1. Also, as clearly seen in Fig 2.1 the rabbit bladder bundles are aligned in a longitudinal direction therefore it is easy to cut strips without sacrificing the integrity of the muscle cells.

**Fig 2.1** An image showing a posterior surface of a female New Zealand White rabbit bladder.

### 2.3. Solutions

Normal physiological salt solution (NPSS) is composed of (in mM) 140 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.6 CaCl₂, 1.2 Na₂HPO₄, 2.0 morpholinopropanesulfonic acid (adjusted to pH 7.4 at either 0 or 37°C, as appropriate), 0.02 Na₂EDTA, and 5.6 dextrose. Nominally calcium free solution (0Ca⁺²) is composed of (in mM) 140 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 Na₂HPO₄, 2.0 morpholinopropanesulfonic acid (adjusted to pH 7.4 at either 0 or 37°C, as appropriate), 0.02 Na₂EDTA, and 5.6 dextrose. Muscle contractions were induced with KPSS which is composed of 110 mM KCl (Speich, Borgsmiller et al. 2005; Speich, Quintero et al. 2006; Speich, Dosier et al. 2007; Speich, Almasri et al. 2009).
2.4. Muscle Strip Set-up

Each tissue was secured by small clips to a micrometer for manual length adjustments and a computer-controlled force sensor to record force (Fig 2.2). Voltage signals were digitized (PCI-6024E, National Instruments), visualized on a computer screen as force in grams (g), and stored electronically for analyses.

![Schematic of the experimental set-up.](image)

**Fig 2.2.** Schematic of the experimental set-up.

2.5. Length-Tension Measurement Protocol and Reference Length Determination

Following equilibration, DSM strips were subjected to one of the L–T curve or adaptation protocols that are described in the next chapters along with the results. Each of these protocols involved passive and/or total tension measurements (with some exceptions), and Fig 2.3A-B shows the typical measurement protocol that was performed.
at each muscle length. DSM strips were incubated for 2 min in 0Ca to eliminate active
tone and SRC (Jezior, Brady et al. 2001; Ratz and Miner 2003), stretched or released to
the muscle length of interest if necessary, incubated for an additional 2 min in 0Ca,
incubated in NPSS for 3 min, and then incubated in KPSS for 1 min to induce a
contraction. Additional time in the protocol, not shown in Fig 2.3A, was used to change
solutions in the 4-bath apparatus and to stretch the muscle strips.

In some of the APS regeneration protocols in chapter 3 in this dissertation were
performed based on a reference length (L_{ref}) determination for each tissue. To determine
L_{ref}, tension measurements were made at 3 mm increments (Fig 2.3C) until a peak value
on the L-Ta curve was identified, and this length was designated as L_{ref}. Due to tissue
variability, L_{ref} was 9, 12, or 15 mm (Fig 2.3D).

It has previously shown that stress relaxation in a DSM strip can take an hour or
more to approach equilibrium (Speich, Quintero et al. 2006). The primary objective in
many experiments was not to determine a fully stress relaxed T_p value, but rather to
obtain a reliable measure of T_p at the time of activation, regardless of whether the tissue
had completely stress relaxed to a minimum T_p value. Therefore, T_p was measured
immediately before stimulation with KPSS. The time for each segment of the protocol in
Fig 2.3A was selected, to provide the tissues with time to stress relax toward (but not
always completely to) a steady-state value and to enable the complete experiment to be
performed in a reasonable amount of time.
Fig 2.3. Tension measurement protocol diagram.
Tension data for a pair of DSM strips are provided in Fig 2.3B to illustrate the protocol. Strip 1 (black line) exhibited less tension in NPSS than in 0Ca, indicating additional stress relaxation while in NPSS. In contrast, Strip 2 (gray line) exhibited SRC and greater tension in NPSS than in 0Ca, indicating that active tone was developed while in NPSS which exceeded any additional stress relaxation while in NPSS. To account for both of these possibilities, “passive” tension was taken to be the lower value of either the final tension in NPSS or the final tension in 0Ca as shown in Fig 2.3A. Ta was calculated by subtracting Tp from Tt, the peak total KCl-induced tension. The typical sample data in Fig 2.3B demonstrate that Tp should be measured during incubation in 0Ca to eliminate active tone and that 2 min in 0Ca and 3 min in NPSS appear sufficient to allow stress relaxation to achieve a pseudo steady-state. In some cases, when the values of the NPSS were higher than the 0Ca values, a curve fitting was introduced. Two phase decay curve fitting for the data was either to predict the passive tension values after 5 min or after 7 min.

Preconditioning was performed to remove any “kinks” in the tissue (Ford and Gilbert 2007); to establish any permanent damage that will occur due to stretching, especially local damage at the clamps; and most importantly to remove the amount of passive tension due to APS that will be lost due to strain-softening the tissue to the maximum length in the protocol (Speich, Borgsmiller et al. 2005; Speich, Quintero et al. 2006; Speich, Dosier et al. 2007).

To determine whether tissues contracted more forcefully when exposed to NPSS before stimulation with KPSS, compared to exposure to 0Ca, Tp and Ta were measured for three isometric contractions performed at 9 mm using the timing from Fig 2.3A-B,
however NPSS was replaced with 0Ca before the 1st and 3rd contractions. Therefore, the DSM strips were incubated in 0Ca for 7 min total before the 1st and 3rd contractions and in 0Ca for 4 min and then NPSS for 3 min before the 2nd contraction. As shown in Fig 2.4, Tp in NPSS was not significantly different from Tp in 0Ca; however, Ta induced by KPSS following incubation in NPSS was significantly greater than following incubation in 0Ca. Therefore, in order to achieve maximal KCl-induced contractions in the protocols, 0Ca solution was replaced with NPSS before each contraction (Speich, Almasri et al. 2009).

**Fig 2.4.** Rationale for 3 min NPSS treatment following incubation of tissues in 0Ca and preceding stimulation in NPSS.

### 2.6. Statistics and Curve Fitting

Statistical analysis was performed using Prism (5.0, GraphPad Software, Inc.). The D'Agostino and Pearson omnibus normality test available in Prism was used to determine if selected data were normal. When comparing two groups, a *t*-test was used to determine significant differences, and when comparing more than one group, a one-way ANOVA with the post-hoc Student-Newman-Keuls test was used where appropriate.
The null hypothesis was rejected at p<0.05. The sample size (n value) refers to the number of animals and not the number of tissues.
Passive tension during bladder filling is critical, because it directly affects the signals responsible for urgency and positions the crossbridges in preparation for bladder emptying. Therefore, dysfunction during the storage/filling phase could lead to OAB symptoms, such as increased urgency and increased voiding frequency. Furthermore, dysfunction during the voiding phase could lead to symptoms such as hesitancy, weak stream, and incomplete bladder emptying.

Urinary bladders of many mammalian species such as rabbits (Shenfeld, McCammon et al. 1999) and human (Biers, Reynard et al. 2006; Brading 2006), display SRC during the filling phase. SRC during filling displays weaker contractile amplitude than the “full” contraction responsible for voiding (Collins, Klausner et al. 2009). Thus, there are two distinct contractile phenomena in the bladder: SRC during the filling phase and voiding contraction during the voiding phase. Until recently (Drake, Mills et al. 2001; Biers, Reynard et al. 2006), SRC activity has largely been ignored and its physiological function has yet to be identified.

The objective of the experiment in this chapter of this dissertation was to determine, 1) if APS is responsible for a significant percentage of the total tension in DSM, 2) the effect of the number and duration of KCl-induced contractions on APS
formation, and 3) if physiological phenomena of micturition and filling, specifically voiding contraction and SRC, are involved in APS formation.

3.1. The Importance of APS in DSM

The L-T curve protocol illustrated in Fig 3.1A was designed to confirm that a shift in the length-passive tension curve due to APS produces a shift in the length-total tension curve. Tissues first underwent two maximal KCl-induced contractions at the 3 mm length, “wake up,” to ensure tissue viability. Tissues were then preconditioned seven times in 0Ca to the maximum length in the protocol, 15 mm (Fig 3.1A, white column), because it has previously shown at our laboratory that peak Tp decreases with each stretch until it reaches equilibrium in 6-7 stretches (Speich, Borgsmiller et al. 2005). The maximum length in the protocol, 15 mm, was chosen as it is 5-fold initial length of 3 mm and incorporates much of the 7-fold length range over which DSM can function (Uvelius 1976). Following the seven preconditioning stretches to 15 mm, L-T measurements were made at increasing lengths from 3.75 to 15 mm (Fig 3.1A, “Loading” protocol) and then decreasing lengths from 15 to 3.75 mm (“Unloading” protocol). According to our previous studies at the lab, it is expected to see greater Tp during the “loading” compared to the “unloading” protocol because we previously showed that contractions at shorter muscle lengths establish APS (in this case the contraction at 3.75 mm) (Speich, Borgsmiller et al. 2005), and it is expected to see less Tp during the “unloading” protocol because of a reduction in APS due to the preceding preconditioning (strain-softening, stretch) to the longer length (15 mm). Furthermore, based on previous studies from our lab (Speich, Dosier et al. 2007) of the effect of APS on Ta at a single muscle length, it is expected the L-Tt curve to be modulated by L-Tp with little change in the L-Ta curve.
DSM strips subjected to the protocol illustrated in Fig 3.1A produced the L-T curves in Figs 3.1B-F. Tp for the unloading protocol was significantly less than for the loading protocol at lengths greater than 3.75 mm (Fig 3.1D). Based on previous studies from our lab (Speich, Borgsmiller et al. 2005; Speich, Quintero et al. 2006; Speich, Dosier et al. 2007), we attribute the additional Tp identified by the loading protocol to APS established by the contractions at the shorter lengths, especially 3.75 mm, and then lost following the stretch to 15 mm (Speich, Borgsmiller et al. 2005; Speich, Quintero et al. 2006; Speich, Dosier et al. 2007). Tt was also significantly less for the unloading protocol compared to the loading protocol at lengths greater than 3.75 mm (Fig 3.1B). In contrast, Ta values for both protocols were not different between 12 and 14.25 mm, and Ta for the unloading protocol was greater than for the loading protocol at both 14.7 and 15 mm (Fig 3.1C). Thus, the downward shift in the L-Tt curve for the unloading protocol compared to the loading protocol was primarily due to the shift in the L-Tp curve at lengths greater than 7.5 mm, and the L-Ta and L-Tp curves shifted in opposite directions (loading versus unloading) at 14.7 and 15 mm.

The L-Ta curves for the loading and unloading protocols both have ascending and descending limbs with a peak at 14.7 mm (Fig 3.1C). Furthermore, the T_{APS} curve, calculated as the difference in the loading and unloading Tp curves attributed to APS, also has ascending and descending limbs with a peak value at 14.7 mm (Fig 3.1C). T_{APS} was a substantial 27.0±8.4% of active tension at this length. For the loading protocol, passive tension was greater than 10% of total tension at lengths of 7.5 mm and above (Fig 3.1F). For the unloading protocol, passive tension was a smaller fraction of total tension, contributing less than 10% at lengths of 13.25 mm and less. At 14.7 mm, where peak Ta
was identified, Tp was a sizeable 32.7±7.4% of Tt on the loading curve and 20.5±5.4% of Tt on the unloading curve (Fig 3.1F). The reason Tp/Tt appears greater for the “unloading” curve than the “loading” curve at 3.75 mm is likely because of increased APS due to the contraction at 7.75 mm before the Tp measurement at 3.75 mm.
Fig 3.1. A: L-T curve protocol for preconditioned tissues. Following seven preconditioning stretches to 15 mm, L-T measurements were made at increasing lengths from 3.75 to 15 mm (“Loading” protocol) and then decreasing lengths (“Unloading” protocol). Black circles indicate KCl-induced contractions. B–E: Tension values (mean±SE, Loading: solid line, Unloading: dashed-line) were normalized to the maximum Ta value for each tissue, Ta_ref. B: L-Tt curves. C: L-Ta curves and Tp due to APS, TAPS (dotted line). D: L-Ta curves. E: L-T curves from panels B–D shown together. F: Tp as a fraction of Tt, (Tp/Tt). B–D and F: * indicates that the loading and unloading values are significantly different (paired-t, p<0.05, n=7).

3.2. Regeneration of APS in DSM

3.2.1. Effect of Number or Length of KCl-induced Contractions on APS Regeneration

The APS regeneration protocols in this dissertation were performed based on a reference length (Lref) for each tissue. To determine Lref, tension measurements were made at 3 mm increments (Fig 2.2C) until a peak value on the L-Ta curve was identified, and this length was designated as Lref. Due to tissue variability, Lref was 9, 12, or 15 mm (Fig 2.2D). The protocol in Fig 3.2A was designed to test the hypothesis that APS regeneration, which it has previously shown in previous studies form our lab, can be generated by contractions at short muscle lengths (Speich, Borgsmiller et al. 2005; Speich, Quintero et al. 2006; Speich, Dosier et al. 2007), but what remains to be determined is the effect of the number of contractions and on the duration of each
contraction, which is one of the main objectives of this chapter. Following the
determination of Lref (Fig 2.2B), six pair of Tp measurements were made at Lref (Fig
3.2A, points 3 & 6, 8 & 12, 14 & 16, 18 & 20, 22 & 24, 26 & 29) while in 0Ca. Before
each pair of Tp measurements, tissues were preconditioned seven times in 0Ca to reduce
Tp due to APS at shorter muscle lengths by stretching to 133% Lref and then releasing to
until they were approximately slack (Fig 3.2A, points 2, 7, 13, 17, 21, and 25).

Following preconditioning and release to Lref, the first pseudo-steady-state Tp
measurement was made after 1 min of isometric tension redevelopment. Between the
first and second Tp measurements in each set, tissues were released to 50% Lref and
subjected to two, three, or one contraction(s) (Fig 3.2A, points 4-5, 9-11, 15,
respectively) to potentially restore APS lost as a result of the immediately preceding
preconditioning. The timing of most contraction cycles was identical to the tension
measurement cycle in Fig 2.2A, which consisted of 4 min in 0Ca, then 3 min in NPSS,
and finally 1 min in KPSS to induce each contraction. The exceptions were a longer, 24
min contraction following 2 min in 0Ca (Fig 3.2A, point 19; Fig 3.2C, sample data) and
incubation in 0Ca for 4 min without a contraction (Fig 3.2A, point 23). Following the
contraction(s) or incubation in 0Ca, tissues were stretched to Lref in 0Ca, and the second
pseudo-steady-state Tp measurement was made after 10 min of isometric stress
relaxation. The second measurement was made after 10 min at Lref, compared to 1 min
for the first measurement; because the tissues took longer to stress relax than to redevelop
to a pseudo-steady-state Tp value (Fig 3.2D). The difference between the relaxed and
redeveloped Tp values at Lref represented the amount of APS restored by the
contraction(s) at 50% Lref (Fig 3.2D). Based on previous studies from our lab with APS
it is expected to find that the amount of APS generated increased with the number of contractions and with the length of contraction.

DSM strips subjected to the protocol in Fig 3.2A showed an increase in Tp of 0.33±0.09 g at Lref (point 6 minus 3, mean±SE) following two 1 min contractions at 50% Lref, which represented 28.4±8.7% of Ta produced by the first contraction. The normalized increases in Tp following three 1 min contractions and following one long 24 min contraction (Fig 3.2C) at 50% Lref were not different than the increase due to two contractions (Fig 3.2B), although the average normalized increases (1.05±0.07 and 1.23±0.15, respectively) were greater (Fig 3.2B). The increase in Tp following a single 1 min contraction was significantly less than the increase due to two contractions (Fig 3.2B, 0.72±0.04, *) and significantly greater than after incubation in 0Ca with no contraction (Fig 3.2B, 0.55±0.05, Ω). Finally, two subsequent contractions produced an increase in Tp (0.94±0.12) that was not different from the increase produced by the original pair of contractions (Fig 3.2B). Together, these results show that the amount of APS restored is dependent on both the number of contractions and on the duration of contraction, with two or three short 1 min contractions or one long 24 min contraction at shorter length restoring the most APS.

To verify that the timing of the Tp measurements was appropriate, two-phase exponential curves were fit to the average 1 min Tp redevelopment data for measurement points 3 and 22 (Fig 3.2D, black and gray lines, respectively, fitted lines are broken) and for the average 10 min Tp relaxation data for points 6 and 24. These curves were extrapolated to 20 min, and the predicted Tp increase of 0.35 g following two
contractions (point 6 minus 3) corresponded well with the 0.33±0.09 g calculated using the 1-min redeveloped and 10 min relaxed Tp values. Likewise, the predicted steady-state increase of 0.18 g following incubation in 0Ca at 50% Lref corresponded well with the 0.17±0.03 g calculated using the 1 min redeveloped and 10 min relaxed Tp values. This timing for Tp measurements was used in the other protocols in this study.

A. Percentage of L\text{ref}

Data Point in Stretch Sequence

B. Passive Tension Increase

C. Tension (T/T\text{max})

Number or Length of Contraction

Time (min)
Fig 3.2: A: Protocol to determine the effect of the number of contractions and the duration of a contraction on APS reformation. Following the determination of Lref (Fig 2.2C), six pairs of Tp measurements were made at Lref (gray bars) while in 0Ca. Before each pair of Tp measurements, tissues were strain softened seven times in 0Ca by stretching to 133% Lref and then releasing to until they were approximately slack (white bars). Then, Tp was measured after 1 min of isometric tension redevelopment at Lref (points 3, 8, 14,18,22 and 26), one or more contractions were performed at 50% Lref (hatched bars), and then Tp was measured at following 10 min of stress relaxation at Lref (points 6, 12, 16, 20, 24, and 29). Between each pair of Tp measurements two contractions, three contractions, one contraction, a long 24 min contraction, 4 min incubation in 0Ca with no contraction, or two contractions, respectively, were performed (points 4-5, 9-11, 15, 19, 23, and 27-28, respectively). Small black circles at the top of the hatched bars indicate a contraction during that tension measurement cycle. B: Tp increase at Lref due to each set of contractions normalized to the increase due to two contractions (e.g., Tp for points (6 minus 3)/(6 minus 3)). Values are mean±SE, * indicates a significant difference compared to 1.0 (paired-t, p<0.05, n=4), and Ω indicates that incubation in 0Ca without contraction produced a significantly smaller increase in Tp compared to one contraction, (paired-t, p<0.025, n=4). C: Data from one experiment showing a 24 min KCl-induced contraction and the wash out (w) of the KPSS by 0Ca. D: 1 min Tp redevelopment for data points 3 and 22 (inset) and 10 min Tp relaxation for data point 6 following two contractions and data point 24 following incubation in 0Ca (mean values, black and gray lines, respectively, n=4). Two-phase exponential curves fit to the redevelopment data (dotted lines) and relaxation data (dashed lines), extrapolated to 20 min (R²≥0.993 for all fits).
3.2.2. Effect of “Voiding-Like” Contraction ± Induced RC on APS

The protocol in Fig 3.3A was designed to test the hypothesis that a “voiding-like” contraction (VLC) followed by 15 min of induced RC causes regeneration of APS, but that a VLC followed by 15 min of incubation in NPSS without RC does not permit APS regeneration at 50% Lref. Following the determination of Lref (Fig 2.2C) in four DSM strips from the different rabbits, tissues were preconditioned seven times to 133% Lref and Tp was measured at Lref before and after two contractions at 50% Lref, as in the previous protocol (Fig 2.2A, data points 1-6). Next, tissues were strain softened to 133% Lref, released to Lref where Tp was measured after 1 min of isometric tension redevelopment (Fig 3.3A, points 7-8). Then, tissues were subjected to a VLC in which they were contracted with KPSS at Lref until Tt reached its maximum value and then manually released (~20-30 sec) to 50% Lref while remaining in KPSS to mimic a voiding contraction (Fig 3.3A, point 8). Following the VLC, each of the four strips was first incubated in NPSS+SC560 (10⁻⁹ M to 3x10⁻⁷ M), a cyclooxygenase-1 (COX-1) inhibitor, to abolish or prevent any natural SRC (Collins, Klausner et al. 2009) (Fig 3.3A, point 9). Then, PGE₂ (10⁻⁹ M to 3x10⁻⁷ M) was added to NPSS in two tissue baths and CCh (Klausner, Rourke et al. 2009) (0.1 or 0.3 µM) was added to NPSS in one bath until RC was established in the three tissues. Finally, 1.0 µM nifedipine-calcium ion channel blocker- was added to one of the baths containing PGE₂ to abolish the RC (Shenfeld, McCammon et al. 1999). Each RC agonist or antagonist was added in half-log increments beginning at 10⁻⁹ M and ending with at most 3x10⁻⁷ M, until RC was either established by PGE₂ or CCh or abolished by SC560 or nifedipine. Then, Tp was measured following 10 min of stress relaxation at Lref to determine the effect of the VLC plus the 15 min with or
without RC on the restoration of APS (Fig 3.3A, point 10). Finally, tissues were
preconditioned again to 133% Lref, and Tp was measured at Lref before and after two
contractions at 50% Lref, as in the previous protocol (Fig 3.3A, points 25-25, Fig 3.3A,
11-15). Based on previous work from our lab showing APS is restored by contractions at
a short muscle length (Speich, Borgsmiller et al. 2005; Speich, Quintero et al. 2006;
Speich, Dosier et al. 2007), it is expected to find that either a VLC alone or a VLC
followed by RC would also restore APS, thus identifying a potential physiological
mechanism for generating APS.

DSM strips subjected to the protocol in Fig 3.3A showed an increase in Tp at Lref
following two 1 min contractions at 50% Lref (Fig 3.3B, 2 Cont.), as in the previous
protocol. The normalized increases in Tp following a VLC plus 15 min of PGE₂ or CCh-
induced RC (0.86±0.11 and 0.81±0.07, respectively, n=4) were not significantly different
from the increase due to the two control contractions (Fig 3.3B-C). In contrast, the
increase in Tp of (0.71±0.05, n=4) following a VLC plus 15 min in NPSS with RC
induced with PGE₂ and then quickly abolished with the calcium channel blocker
nifedipine (Fig 3.3B-C) was reduced compared to the increase due to the two control
contractions. Furthermore, the increase in Tp was also reduced to (0.54±0.07, n=3)
following a VLC plus 15 min in NPSS without induced RC and with natural RC inhibited
with SC560 (Fig 3.3B-C). In all tissues, except those exposed to nifedipine, which
apparently did not wash out well, two subsequent contractions produced increases in Tp
that were not different from the increases produced by the original pair of contractions
(Fig 3.3B).
Finally, in an additional experiment, one VLC+90 min of PGE₂-induced RC restored 0.86±0.10 Tp which was not statistically different from two control contractions (p<0.05, n=5) and was the same average amount restored by one VLC+15 min of PGE₂-induced RC (i.e. both 15 min and 90 min of RC restored 0.86 Tp).
**Fig 3.3. A:** Protocol to determine whether a “voiding-like” contraction (VLC) followed by 15 min of (RC) causes regeneration of APS. Following the determination of Lref (Fig 5C), three pairs of Tp measurements were made at Lref (gray bars) while in 0Ca as in the previous protocol (Fig 8A). The first and third pairs measured the amount of Tp restored by two 1 min KCl-induced contractions at 50% Lref (points 4-5 and 13-14, hatched bars) following seven strain softening stretches to 133% Lref (white bars). The second pair of Tp measurements (points 8 &10) measured the amount of Tp restored by a VLC (point 8, arrow) plus 15 min in NPSS with or without induced RC at 50% Lref (hatched bar, point 9).

**B:** Tp increase at Lref due to the following: two contractions (2 Cont), one VLC plus 15 min RC induced with PGE2 (SC560+PGE2), one VLC plus 15 min RC induced with CCh (SC560+CCh), one VLC plus 15 min in NPSS with RC induced with PGE2 and then quickly abolished with nifedipine (SC560+PGE2+nifidipine), and one VLC plus 15 min in NPSS with RC abolished by SC560 (SC560). Data were normalized to the increase due to two contractions (e.g., Tp for points (6 minus 3)/(6 minus 3)) Values are mean±SE, and * indicates a significant difference compared to 1.0 (paired-t, p<0.05, n=3-4).

**C:** Selected data from one experiment showing induced RC due to either PGE2 or CCh, and absence of RC due to either SC560 or nifedipine.

3.2.3. **Effect of VLC or 15 min RC on APS**

The protocol in Fig 3.4A was designed to determine whether the APS restored following a VLC plus RC in the previous protocol (Figs 3.3) was restored as a result of the VLC or the RC, i.e., whether APS was restored during voiding or filling, or both. Following the determination of the Lref (Fig 2.2C), tissues were preconditioned seven times to 133% Lref, and Tp was measured at Lref before and after two contractions at 50% Lref, as in the previous protocols (Figs 3.2A, 3.3A, points 1-6). Next, tissues were preconditioned to 133% Lref, released to Lref where Tp was measured after 1 min of isometric tension redevelopment (Fig 3.4A, points 7-8). Then, tissues were subjected to a VLC to 50% Lref, incubated for 4 min in 0Ca at that length, and then returned to Lref where Tp was measured as before (Fig 3.4A, points 8-10). Next, tissues were again preconditioned to 133% Lref, released to 100% Lref where Tp was measured as before, released without contraction to 50% Lref where RC was established with PGE2 as before. Then, following 15 min of rhythm, tissues were returned to Lref in 0Ca where Tp was
measured as before. Based on the results from the protocol in Fig 3.3, it is expected to find that the VLC did not restore more APS than incubation in 0Ca, but that RC alone did restore more APS than incubation in 0Ca or a VLC.

DSM strips subjected to the protocol in Fig 3.4A showed an increase in Tp at Lref following two 1 min contractions at 50% Lref (Fig 3.4B, 2 Cont.), as in the previous two protocols. The normalized increase in Tp of 0.63±0.06 following a VLC was less than the increase due to the control contractions (Fig 3.4B, VLC), while the increase in Tp of 0.88±0.07 due to 15 min of PGE₂ induced RC without a VLC was not significantly different from the increase due to the two control contractions (Fig 3.4B, RC, \( p<0.05 \), \( n=4 \)). Thus, a VLC alone appears to restore less APS than RC alone.
**3.2.4. Effects of Staurosporine and Distilled Water on APS**

The protocol in Fig 3.5A was designed to determine whether the APS restored following incubation in 0Ca in the protocol in Fig 3.2 was restored due to an active, regulated process or due to passive viscoelasticity. Tp was measured at Lref before and after 15 min of incubation in 0Ca at 50% Lref, and again following 15 min of incubation in 1µM staurosporine in 0Ca, an agent known to inhibit many of the protein kinases responsible for increasing the degree of myosin light chain phosphorylation (Speich, Quintero et al. 2006), at that length to determine whether calcium-independent crossbridge attachment or slow cycling might be responsible for Tp development.

In a final experiment added to the end of the protocol in Fig 3.3A, Tp was measured at Lref after preconditioning (strain softening) to 133% Lref and again after 40 min of incubation in distilled water at 50% Lref to kill the tissues. After the first 20 min of this incubation, tissues were exposed to KPSS for 1 min to determine if they could contract. After the second Tp measurement at Lref, tissues were (preconditioned) strain softened a final time to 133% Lref and Tp was measured at Lref. It is expected to find that if the fraction of Tp restored during incubation in 0Ca was due to passive
viscoelasticity, then restoration of Tp would not be inhibited by staurosporine and might be exhibited by non-viable tissues.

**Fig 3.5.** **A:** Protocol to determine whether APS restored following incubation in 0Ca is inhibited by 1µM staurosporine. Following the determination of Lref (Fig 2.2B), four pairs of Tp measurements were made at Lref (gray bars) while in 0Ca as in the previous protocols (Fig 3.2A and 3.3A). The first and fourth pairs measured the amount of Tp restored by two 1 min KCl-induced contractions at 50% Lref (points 4-5 and 17-18, hatched bars) following seven preconditioning stretches to 133% Lref (white bars). The second pair of Tp measurements (points 8 &10) measured the amount of Tp restored by 15 min in 0Ca at 50% Lref (white bar, point 9), the third pair of Tp measurements (points 12 & 14) measured the amount of Tp restored by 15 min in 0Ca+1µM staurosporine at 50% Lref (arrow, point 13), and the fourth pair of Tp measurements (points 16 & 19) measured the amount of Tp restored by two 1 min KCl-induced contraction at 50%Lref (points 17 & 18).

**B:** Tp increase at Lref due to the following: two contractions, 15 min in 0Ca, and 15 min in 0Ca+1 µM staurosporine, and two contractions. From a similar protocol the data of 40 min in distilled H2O are added to the results in Panel B. Data were normalized to the increase due to two contractions (e.g., Tp for points (6 minus 3)/(6
Values are mean±SE, * indicates a significant difference compared to 1.0 (paired-t, $p<0.05$, $n=4$), and Ω indicates that the increase in Tp was significantly less following incubation in 0Ca with staurosporine compared to without staurosporine, (paired-t, $p<0.025$, $n=4$).

DSM strips subjected to the protocol in Fig 3.5A showed 0.63±0.10 in Tp at Lref following 15 min of incubation in 0Ca at 50% Lref and a 43% smaller increase of 0.36±0.05 in Tp following 15 min of incubation in 1µM staurosporine (Fig 3.5B). These results indicate that the Tp restored at Lref by 15 min in 0Ca at 50% Lref was actively restored by a staurosporine sensitive regulation system and not by passive viscoelastic effects. Tissues incubated in distilled water for 20 min did not contract when exposed to KPSS, and after an additional 20 min in distilled water at 50%Lref, tissues showed no increase or decrease in Tp (0.00±0.02, $n=4$) at Lref.
Bladder smooth muscle cells can undergo a 7-fold length change during filling (Uvelius 1976) and must be prepared to contract efficiently and void throughout this range. How the bladder prepares and positions its actin-myosin crossbridges to contract efficiently over this broad length range during the filling phase remains to be determined.

The passive and active L–T relationships for skeletal muscles are considered to be static, with a single passive tension value and a single maximum active tension value for each muscle length (Gordon, Huxley et al. 1966). Until the 1990s, the L-Tp and L-Ta relationships in smooth muscle were thought to be static, although a few earlier studies showed that Ta and/or Tp at a particular muscle length could differ if tension measurements were made during muscle unloading versus loading (Speden 1960; Herlihy and Murphy 1973). Surprisingly, adaptation of the active L-Ta curve has not been explored in bladder; however, because the length range over which DSM operates is considerably greater than airway and vascular smooth muscles, it is reasonable to expect that DSM would also undergo length adaptation.

The objective of the experiments described in this chapter was to examine the L-Ta relationship in DSM and to test the hypothesis that DSM exhibits length adaptation similar to vascular and airway smooth muscles. More specifically, the main objectives of
the present study are 1) to test the hypothesis that the L-Ta relationship in rabbit DSM exhibits length adaptation, 2) to test the hypothesis that preconditioning, and length adaptation each impact the maximum total tension produced at a particular DSM length, 3) to test the hypothesis that length adaptation at a particular length on the descending limb of the L-Ta curve shifts the complete curve to the right along the length axis, 4) to compare length adaptation at lengths on the ascending limb of the L-Ta curve with adaptation on the descending limb, and 5) to examine the effect of length adaptation on the passive-to-active tension ratio in rabbit DSM.

4.1. Effect of Preconditioning on the L-Ta Curve

The protocol in Fig 4.1A was designed to determine if the L-Ta curve displays stronger Ta values at short lengths before preconditioning as compared to after preconditioning and therefore shifts the L-Ta curve to the right. The L-Ta and L-Tp curves for three series (S1–S3) of five L-T measurements at increasing lengths from 3 to 15 mm are shown in Figs 4.1B-E. Data were grouped according to the optimal length for contraction, Lo1, corresponding to the optimal (maximum) Ta value, To1, for series S1. Data for tissues with Lo1 values at 6, 9, 12, and 15 mm are shown separately in panels B-E, respectively. The length axis was intentionally not normalized to Lo to demonstrate variability and the challenge of identifying a reference length for a particular muscle strip, especially if the tissue was not preconditioned. These data indicate that although great care was taken in hanging the tissue strips to ensure uniformity in muscle strip size and initial length, there was significant variation in Lo. This variability increased the challenge of designing and performing experiments; however, in this case the variability enabled us to quantify the effect of preconditioning on strips stretched well beyond the
initial Lo (Fig 4.1B-C), just beyond Lo (Fig 4.1D), and not quite to Lo (Fig 4.1E) using a single protocol. When data from all 12 experiments were grouped together, the resulting curves still display a shift in the L-Ta and L-Tp curves (Fig 4.1F).
**Fig 4.1.**

**A:** L-T curve protocol for tissues that were not preconditioned. Protocol consisted of three series (S1–S3) of five measurements at increasing lengths from 3 to 15 mm. **B–F:** L-Ta curves (mean±SE) for S1 (hollow symbols), S2 (solid symbols), and S3 (gray symbols, panel C only) and corresponding L-Tp curves (S1: dotted line; S2: dashed-dotted line; S3: dashed line, panel C only). Data from 12 experiments were grouped according to L01 and normalized to T01 (B–D) or T0_max (E). For S1–S3, peak (optimal) Ta values are labeled T01–T03 and correspond to optimal lengths L01 (6 mm, **B**; 9 mm, **C**; 12 mm, **D**; and ≥15 mm, **E**), L02 (12 mm, **B–C**; ≥15 mm, **D–E**), and L03 (12 mm, **C**). **B:** L-Ta and L-Tp curves for S1–S2 for tissues with L01 at 6 mm (n=3). **C:** L-Ta and L-Tp curves for S1–S3 for tissues with L01 at 9 mm (n=3). **D:** L-Ta and L-Tp curves for S1–S2 for tissues with L01 at 12 mm (n=4). **E:** L-Ta and L-Tp curves for S1–S2 for tissues with L01≥15 mm (n=2). **F:** L-Ta and L-Tp curves for S1–S2 for all 12 tissues (n=12). **B–F:** * indicates that Ta for S2 was significantly less than Ta for S1 at that particular length (paired-t, p<0.05). **C:** Ψ indicates that Ta for S3 was significantly less than Ta for S2 at that particular length (paired-t, p<0.05).
Comparing series S2 to S1 in Fig 4.1B for tissues with Lo1 at 6 mm, Ta was significantly less at shorter lengths, and To2 was less than To1 and was shifted to the right from 6 mm to 12 mm (Lo2). Similarly, comparing S2 to S1 in Fig 4.1C for tissues with Lo1 at 9 mm, Ta was significantly less at shorter lengths, and To2 was less than To1 and is shifted to the right from 9 mm to 12 mm (Lo2). For tissues with Lo1 at 12 mm in Fig 4.1D, Ta for S2 was significantly less than Ta for S1 at shorter lengths, and To2 was shifted to the right from 12 mm to ~15 mm or beyond. However, in contrast to the tissues with Lo1 at 6 or 9 mm (Figs 4.1B-C), Ta at 15 mm for S2 was not significantly different from To1 at 12 mm (Fig 4.1D, n=4, p>0.05). Furthermore, although Ta was significantly reduced in S2 compared to S1 at all shorter lengths, Ta values produced at the maximum length in the protocol, 15 mm, were not different for S1 and S2, regardless of the Lo1 value (Figs 4.1B-D). Together the data in Figs 4.1B-E reveal that preconditioning collapsed the ascending limb of the L-Ta curve when tissues were stretched beyond Lo1 and that the farther the tissue was stretched and contracted beyond Lo, the greater the increase (rightward shift) in Lo2 and the greater the decrease in To2. Furthermore, the reduction in Ta due to preconditioning was greater at shorter lengths compared to longer lengths. Most importantly, these data demonstrate that Lo can shift significantly upon preconditioning, and therefore preconditioning should be carefully considered during experiment design.

To determine whether a subsequent series of stretches and contractions would reveal additional changes in the L-Ta curves, S3 was examined (Fig 4.1C) for tissues with Lo1 at 9 mm. Comparing S2 with S3 in Fig 4.1C, Lo was not shifted to a longer
length, and compared to the decrease in Ta from S1 to S2 at 3, 6, and 9 mm, the decrease from S2 to S3 was considerably less.

The L-Tp curves for series S2 in Figs 4.1B-D exhibited a shift to the right compared to S1 that corresponded to the shift in the L-Ta curves. It is worthy noting that before preconditioning beyond Lo1 in S1, the passive to active ratio at Lo1, \((\text{Tp at To1})/\text{To1}\), was 18.7±4.0% \((n=10\), data from Figs 4.1B-D). After preconditioning in S2, Tp at Lo1 was reduced (Figs 4.1B-D); however because of the shift to the right from Lo1 to Lo2 and the reduction in Ta from To1 to To2, the passive to active ratio at Lo2, \((\text{Tp at To2})/\text{To2}\), increased to 39.5±5.0% \((n=6\), data from Figs 4.1B-C). Thus, while Tp at a given length was reduced by preconditioning, the combined shifting of the L-Ta and L-Tp curves due to preconditioning produced a higher Tp/Ta ratio at Lo2.

4.2. Effect of Preconditioning on the L-Tt Curve

The L-T curve protocol illustrated in Fig 4.2A was designed to determine whether the L-Tt curve shifts due to preconditioning and whether any shift was a result of a change in Tp, Ta or both. The protocol consisted of two series (S1–S2) of measurements at lengths between 3 and 11 mm as shown in Fig 4.2A. Smaller length steps were taken at longer muscle lengths to better identify any peak in the L-Ta curve in this region. Based on previous studies from our laboratory (Speich, Borgsmiller et al. 2005; Speich, Quintero et al. 2006; Speich, Dosier et al. 2007), it is expected to find that changes in both Tp and Ta shifted the L-Tt curve.

The L-T curve protocol illustrated in Fig 4.2A produced the two L-Tt curves in Fig 4.2B. Tt for series S2 was significantly lower at each length, revealing that preconditioning from S1 shifted the L-Tt curve for S2 downward and to the right. Ta was
reduced more at shorter than longer lengths, while Tp was reduced more at longer than shorter lengths (Fig 4.2C). Thus the change in Tt between S1 and S2 was due to the change in Ta at shorter lengths (Fig 4.2E, on the ascending limb of the S1 L-Ta curve in Fig 4.2C) and due to the change in Tp at longer lengths (Fig 4.2E, on the descending limb of the S1 L-Ta curve in Fig 4.2C).
Fig 4.2. A: L-T curve protocol consisting of two series measurements (S1 and S2) to determine if the L-Tt curve shifts due to preconditioning. B: L-Tt curves for S1 (solid line, hollow symbols) and S2 (dashed line, solid symbols). * indicates that Tt for S2 was significantly less than Tt for S1 at that particular length ($p<0.05$, $n=4$). C–D: Full (C) and zoomed (D, box from C) L-Ta curves. Localized peak Ta values for S1 and S2 are labeled To1a (C), To1b (D), and To1c (D) and To2a (C, D), To2b (D) and To2c (D). C: L-Tp curves for S1 (solid line) and S2 (dotted line). C: * indicates that Ta for S2 was significantly less than Ta for S2 at a particular length less than 9 mm (paired-t, $p<0.05$, $n=4$). D: At 10.75 and 11 mm, Ta values for S1 were not different from the corresponding values for S2 (paired-t, $p>0.05$, $n=4$). The second Ta values measured at 10.5 mm were significantly greater than the first for both S1 and S2 ($\psi$, paired-t, $p<0.05$, $n=4$), forming counter-clockwise loops suggesting adaptation to that length region. E: Changes in Tt (solid line), Ta (dashed line) and Tp (dashed-dotted line) between S1 and S2 for lengths between 3 and 10.5 mm. The change in Tt between S1 and S2 was due to the change in Ta at shorter lengths and due to the change in Tp at longer lengths.

4.3. Adaptation of the L-Ta Curve

4.3.1. Adaptation of the L-Ta Curve at Lengths Greater than $L_0$

Fig 4.2D shows zoomed L-Ta curves from the boxed region of Fig 4.2C. In this region, the Ta values at 10.75 and 11 mm for S1 were not different from the corresponding values for S2, indicating that preconditioning did not significantly alter Ta near the maximum length in the protocol, 11 mm (Fig 4.2D, $n=4$, $p>0.05$). However, the second Ta values measured at 10.5 mm (during unloading) were significantly greater than...
the first (during loading) for both S1 and S2 (Fig 4.2D), forming counter-clockwise loops suggesting adaptation of Ta to that length region. Furthermore, the To1c and To2c values at 10.5 mm were not significantly different from the To1a value at 6 mm (Figs 4.2C-D), indicating that for both S1 and S2, the tissues were able to adapt back to their initial To1 values while at 175% of Lo1.

4.3.2. L-Ta Curves with Multiple Ascending and Descending Limbs

In Fig 4.2C, the stretches and contractions in S1 produced an L-Ta curve with an ascending limb that reached To1a at 6 mm and a descending limb between 6 and 10.5 mm. A second ascending limb was produced between 10.5 and 11 mm, with peak To1b at 11 mm, and a second descending limb was produced during unloading between 11 and 10.5 mm with peak value To1c at 10.5 mm (Fig 4.2D). Thus the L-Ta curve for S1 has two ascending limbs and two descending limbs. The L-Ta curve for S2 follows a similar pattern with multiple ascending and descending limbs and three peak values, To2a-To2c. The presence of multiple ascending and descending limbs indicates that there is not a single, static L-Ta curve for DSM.

4.3.3. Adaptation of the L-Ta Curve at Lengths Less than Lo

The L-T protocol shown in Fig 4.3A was designed to test the hypothesis that the change in Ta at short muscle lengths due to preconditioning identified with the previous protocols in Figs 4.1 and 4.2 was due to adaptation of the Ta curve and that the adaptation could be reversed by multiple contractions at a short length. This protocol consisted of two series of stretches and tension measurements between 3 and 9 mm (S1 and S2) followed by a series of six tension measurements at 3 mm and for some tissues measurements at 6 and 9 mm (S3). Adaptation at short lengths has been chosen to
minimize any changes in Tt due to APS which represents a greater fraction of Tp are present at longer muscle lengths. This protocol will determine whether any reduction in Ta at a short length due to preconditioning to a longer length could be restored by multiple contractions at the short length and could therefore be attributed to length adaptation and not to tissue damage.

Data from tissues subjected to the L-T protocol in Fig 4.3A were categorized according to the shape of the L-Ta curve produced by S1. Data for tissues producing at least 10% greater Ta at 9 mm than at 6 mm during S1 are shown in Fig 4.3B and were categorized as tissues with a “more steep ascent 6-9 mm.” Data for tissues producing less than 10% greater Ta at 9 mm than at 6 mm during S1 are shown in Fig 4.3C and were categorized as tissues with a “less steep ascent 6-9 mm.” Data for tissues producing greater Ta at 6 mm than at 9 mm during S1 are shown in Fig 4.3D and were categorized as tissues with a “descent 6-9 mm.” Finally, data from a protocol in which 0Ca was replaced with NPSS are shown in Fig 4.3E and categorized as tissues with an “ascent 6-9 mm, no 0Ca.”

For each of the four categories, Ta for contraction 8 was significantly less than Ta for contraction 2 at 3 mm (Fig 4.3F, compare data points 2 and 8 for each category), following the stretches to and contractions at 6 and 9 mm (Fig 4.3A, points 3-7). For tissues not stretched to the descending limb of the L-Ta curve, Ta at 3 mm was reduced by 55.8±4.9% for the “more steep ascent” group and by 62.9±7.2% for the “less steep ascent” group following the stretches to and contractions at 6 and 9 mm (Fig 4.3F, compare data points 2 and 8). For the two “ascent” groups combined, Ta at 3 mm was reduced by 58.6±4.1% (n=15). For tissues stretched to the descending limb of the L-Ta
curve, Ta was reduced by 84.5±2.8% at 3 mm, following the stretches to and contractions at 6 and 9 mm (Fig 4.3F, compare data points 2 and 8).

For each of the four categories, the multiple contractions at 3 mm in S3 produced increasingly greater Ta (Fig 4.3F, data points 8-13). For the “more steep ascent” group, Ta produced by the sixth consecutive contraction at 3 mm reached 103.3±12.3% of, and was not significantly different from, the second pre-stretch contraction at that length (Fig 4.3F, compare data points 2 and 13). For the “less steep ascent” group, Ta produced by the sixth consecutive contraction at 3 mm reached 73.9±10.8% of the second pre-stretch contraction at that length (Fig 4.3F, compare data points 2 and 13). For the two “ascent” groups combined, the sixth contraction in S3 produced 91.5±9.1% of the Ta produced by the second pre-stretch contraction at 3mm in S1. Together, these data show that multiple contractions at 3 mm enabled Ta to adapt to a greater value and in some cases back to its pre-stretch value at that length.

For tissues stretched to the descending limb of the L-Ta curve, multiple contractions at 3 mm enabled Ta to adapt, but Ta only returned to 47.8±7.2% of the pre-stretch Ta value at that length by the sixth contraction (Fig 4.3F, “descent 6-9 mm”, compare data points 2 and 13). However, the level of adaptation of Ta for series S3, T_adapt ((Ta of first minus Ta of last contraction in S3)/Ta_max), was not significantly different for the four categories (“13-8”, Fig 4.3F). These results indicate that a significant portion of the change in Ta at short lengths due to preconditioning was due to adaptation and not irreversible tissue damage. Furthermore, the adaptation could be reversed by KCl-induced contractions at a short length. Finally, replacing 0Ca with NPSS
in the protocol did not change the trends in the data (Fig 4.3E-F, “Ascent 6-9 mm, no 0Ca”), indicating that adaptation was not an artifact of the 0Ca solution.
Fig 4.3. **A:** Protocol to identify adaptation of the L-Ta curve at 3 mm consisting of two series of stretches and tension measurements between 3 and 9 mm (S1 and S2) followed by a series of six tension measurements at 3 mm and for some tissues measurements at 6 and 9 mm (S3). **B-D:** L-Ta (solid lines) and L-Tp (dotted lines) curves for S1 (diamonds), S2 (triangles) and S3 (squares) (mean±SE at 6 and 9 mm). Data were categorized according to the shape of the L-Ta curve produced by S1 and normalized to Ta_max. **B:** Data for tissues producing at least 10% greater Ta at 9 mm than at 6 mm during S1 were categorized as tissues with a “more steep ascent 6-9 mm” (n=9 for data points 1-13 and n=8 for data points 14-15). **C:** Data for tissues producing less than 10% greater Ta at 9 mm than at 6 mm during S1 were categorized as tissues with a “less steep ascent 6-9 mm” (n=6, data points 1-13 only). **D:** Data for tissues producing greater Ta at 6 mm than at 9 mm during S1 were categorized as tissues with a “descent 6-9 mm” (n=5 for data points 1-13 and n=3 for data points 14-15). **D:** Data for tissues incubated in NPSS for 7 min during each tension measurement cycle, instead of 4 min in 0Ca and 3 min in NPSS (see Fig 2.2A) were categorized as tissues with an “ascent 6-9 mm, no 0Ca” (n=4). **F:** Ta values (±SE) for contractions at 3 mm, corresponding to the data in panels B-E, for S1 (dark gray), S2 (white) and S3 (light gray), along with the increase in Ta from contractions 8 to 13 (T_adapt, “13-8”, calculated as Ta for 13 minus Ta for 8). **F:** *
or NS indicates whether Ta for contraction 8 or 13 at 3 mm was (*) or was not (NS) significantly different from the Ta value for contraction 2 at 3 mm for that category and ψ indicates T_adapt (“13–8”) was not different from T_adapt for the other categories.

4.4. The Dynamic Active/Passive Tension Ratio

4.4.1. Adaptation on the Original Descending L-Ta Limb and then on the Descending Limb

The protocol shown in Fig 4.4A was designed to examine the effect of length adaptation on the ascending and descending limbs of the L-Ta curve, on the L-Tp curve, and on the passive-to-active tension ratio. This protocol consisted of 20 of the tension measurements illustrated in Fig 2.2C. The contraction 1 was considered a “wake up” contraction to confirm that the tissue was viable (Fig 4.4A, data point 1), and contractions 2-6 were performed 3 mm length steps from 3 to 15 mm (Fig 4.4A, points 2-6). The purpose of these contractions was to identify the muscle length corresponding to the maximum Ta value, which was labeled the initial optimal length, Lo1, for tissues in which a descending limb was identified.

Following identification of the initial L-Ta curve between 3-15 mm, contractions 7-8 examined the effect on the L-Ta and L-Tp curves of releasing to an intermediate length, 9 mm; isometrically contracting; and then returning to the maximum length in the protocol, 15 mm (Fig 4.4A, points 7-8). Based on our previous findings, it is expected to find that Ta was reduced at the intermediate length due to a stretch and contraction beyond Lo1, but that Ta was not reduced at the maximum length (Speich, Almasri et al. 2009). It is also expected based on previous studies from our laboratory to find that Tp was reduced at both lengths due to a loss of APS (Speich, Borgsmiller et al. 2005; Speich, Quintero et al. 2006; Speich, Dosier et al. 2007; Speich, Almasri et al. 2009).
Contractions 9-19 examined the effect on the L-T curves of multiple contractions at the maximum length in the protocol, 15 mm (Fig 4.4A, points 9-13) and then multiple contractions at an intermediate length, 9 mm (Fig 4.4A, points 14-19). Based on our prior studies (Speich, Almasri et al. 2009), it is expected to find that multiple contractions at each length increased Ta at that length.

Finally, contraction 20 examined the effect of adaptation to an intermediate length, 9 mm, on the subsequent Tp and Ta values at the maximum length, 15 mm (Fig 4.4A, point 20). Based on our previous studies from our laboratory, it is expected to find that the multiple contractions at 9 mm increased Tp at 15 mm due to the restoration of APS that had been lost due to the previous stretches to 15 mm (Speich, Borgsmiller et al. 2005; Speich, Dosier et al. 2007). It is also expected to find that adaptation to 9 mm reduced the subsequent Ta value at 15 mm and therefore adapted the tissue away from 15 mm.

The results from the protocol in Fig 4.4A for tissues with Lo1 at 12 mm are presented in Fig 4.4B-G. Following release from 15 mm, both Ta and Tp at 9 mm were reduced relative to their previous values (Fig 4.4B, point 7 vs. point 4), indicating that the stretch to and contraction at 15 mm on the descending limb adapted the tissues away from their initial values at 9 mm on the original ascending limb. However, following a return to 15 mm, both Ta and Tp were not different from their previous values at that length (Fig 4.4B, point 8 vs. 6), indicating that the release to and single contraction at 9 mm on the original ascending limb did not adapt the tissues.

Five subsequent contractions at 15 mm (Figs 4.4C and 4.4F, points 9-13) significantly increased Ta, and conversely, significantly decreased Tp. Lo shifted from
12 to 15 mm, and the adapted To value at 15 mm was not different from To1 at 12 mm (Fig 4.4C, point 13 vs. 5). Similarly, the adapted Tp value at 15 was not different from the original Tp value at Lo1 (Fig 4.4C, point 13 vs. 5). Together these findings indicate that the multiple contractions at 15 mm on the original descending limb adapted the tissues to that length. Furthermore, following subsequent release to 9 mm, Ta was reduced compared to previous values (Fig 4.4C, point 14 vs. 7 and 4), indicating that the six contractions at 15 mm produced additional adaptation away from 9 mm.

Five subsequent contractions at 9 mm (Figs 4.4D-E, points 15-19) significantly increased both Ta and Tp, but not to their original values (Fig 4.4D, point 19 vs. 4), indicating some adaptation at that length. Furthermore, upon return to 15 mm, Ta was reduced compared to the most recent value at that length (Fig 4.4D, point 20 vs. 13), but not compared to the original value (Fig 4.4D, point 20 vs. 6). Conversely, Tp was increased at 15 mm compared to the most recent value (Fig 4.4D, points 20 vs. 13) and returned to a value not different from its original value (Fig 4.4D, point 20 vs. 6). Together these findings indicate that the six contractions at 9 mm on the original ascending limb adapted to 9 mm and away from 15 mm.

Most importantly, the six contractions at 15 mm adapted the passive-to-active tension ratio, Tp/Ta, from 0.540±0.084 to 0.223±0.033 at that length on the original descending limb (Fig 4.4G, point 8 vs. 13), such that it was not different from the initial Tp/Ta ratio of 0.208±0.033 at Lo1 (Fig 4.4G, point 13 vs. 5).
Fig 4.4. A: L-T curve protocol consisting of 20 contraction cycles (see Fig 2.2). B–F: L-Ta and L-Tp curves for tissues that produced a L-Ta curve with an ascending limb between 3 and 12 mm (data points 1-5); a peak (optimal) Ta value, To1, at 12 mm, Lo1;
and a descending limb between 12 and 15 mm (points 5-6). Values were normalized to To1, and * indicates tension values were significantly different (PAIRED-T, P<0.05, n=7 at points 1-19 and n=4 at point 20). B-D: Data points are labeled 1-20. B: Following release from 15 mm, Ta and Tp at 9 mm were reduced relative to their previous values (point 7 vs. point 4), and returned to values not different from their previous values at 15 mm (point 8 vs. 6). C and F: Six contractions at 15 mm (points 8-13) significantly increased (adapted) Ta, and conversely, significantly decreased Tp. C: Lo shifted from 12 to 15 mm, and the adapted To value at 15 mm was not different from the original To value at 12 mm (point 13 vs. 5). Similarly, the adapted Tp value at 15 was not different from the original Tp value at Lo1 (point 13 vs. 5). Following release to 9 mm, Ta was reduced compared to previous values (point 14 vs. 7 and 4). D-E: Six contractions at 9 mm (points 14-19) significantly increased (adapted) both Ta and Tp, but not to their original values (point 19 vs. 4). D: Upon return to 15 mm, Ta was reduced compared to the most recent (adapted) value (point 20 vs. 13), but not compared to the original value (point 20 vs. 6). Conversely, Tp was increased at 15 mm compared to the most recent value (point 20 vs. 13) and returned to a value not different from its original value (point 20 vs. 6). G: Before adaptation, the Tp/Ta ratio at 15 mm on the descending limb was significantly greater than at Lo1 (point 8 vs. 5, Ω); however, following adaptation at 15 mm, the Tp/Ta ratio was reduced such that the value at the new Lo was not different than at Lo1 (point 13 vs. 5). E-G: Mean values±SE.

4.4.2. Adaptation on the Descending L-Ta Limb and then at Lo1

The results from the protocol in Fig 4.4A for tissues with Lo1 at 9 mm are presented in Fig 4.5. Following release from 15 mm, both Ta and Tp at 9 mm were reduced relative to their previous values (Figs 4.5A and 164.5, point 7 vs. point 4), indicating that the stretch to and contraction at 15 mm on the descending limb adapted the tissues away from their initial values at Lo1, 9 mm. When tissues were returned to 15 mm, Ta returned to a value not different from its previous value at that length (Fig 4.5A, point 8 vs. 6), indicating that the release to and contraction at Lo1 did not adapt Ta. Tp at 15 mm did not return to its previous value at that length (Fig 4.5A, point 8 vs. 6), indicating a reduction in APS.

Five subsequent contractions at 15 mm (Figs 4.5B, 4.5E and 4.5H; points 9-13) significantly increased Ta, and conversely, significantly decreased Tp, suggesting adaptation to that length. Furthermore, subsequent release to and contraction at 9 mm
revealed a new ascending limb between 9 and 15 mm (Fig 4.5B, arrow, points 14 and 13), indicating that Lo had shifted from 9 mm to 15 mm. In addition, Ta at 9 mm was reduced compared to previous values (Fig 4.5B, point 14 vs. 7 and 4), indicating that the six contractions at 15 mm produced additional adaptation away from Lo1. Together these findings indicate that the multiple contractions at 15 mm on the original descending limb adapted the tissues to that length; however, the peak, adapted Ta value at 15 mm remained less than To1.

Five subsequent contractions at 9 mm (Figs 4.5C and 4.5F-G; points 15-19) significantly increased both Ta and Tp, but not to their original values (Figs 4.5C and 4.5F, point 19 vs. 4), indicating some adaptation back to that length. Furthermore, upon return to 15 mm, Ta was reduced compared to the most recent value at that length (Fig 4.5C, point 20 vs. 13), but not compared to the original value (Fig 4.5C, point 20 vs. 6). Conversely, Tp was increased at 15 mm compared to the most recent value (Fig 4.5F, point 20 vs. 13) and returned to a value not different from its value before adaptation (Fig 4.5F, point 20 vs. 8), but less than its original value (Fig 4.5F, point 20 vs. 6). Together these findings indicate that the six contractions at Lo1 adapted tissues back to that length and away from 15 mm; however, the adapted Ta value at Lo1 remained less than To1.

Adaptation at 15 mm reduced Tp/Ta at 15 mm (point 13 vs. 8, Ω); however, the Tp/Ta ratio at 15 mm on the descending limb was 5-to-10-fold greater than at Lo1 (Fig 4.5I-J, points 8 and 13 vs. 4). Furthermore, adaptation at 9 mm did not increase the Tp/Ta ratio to its original value at 9 mm (14 and 19 vs. 4, ψ).
Fig 4.5. A–C: L-Ta curves for tissues subjected to the protocol in Fig 4.4A that produced an ascending limb between 3 and 9 mm (data points 1-4); a peak (optimal) Ta value, To1, at 9 mm, Lo1; and a descending limb between 9 and 15 mm (points 4-6). Values were normalized to To1, and * indicates tension values were significantly different (paired-t,
p>0.05, n=9). **D–F:** L-Tp curves corresponding to the L-Ta curves in panels A–C. **A–F:** Data points are labeled 1-20. **A and D:** Following release from 15 mm, Ta and Tp at 9 mm were reduced relative to their previous values (point 7 vs. point 4). When returned to 15 mm, Ta, but not Tp, returned to a value not different from its previous value at 15 mm (point 8 vs. 6). **B, E and H:** Six contractions at 15 mm (points 8-13) significantly increased (adapted) Ta, and conversely, significantly decreased Tp. **B:** Following release to 9 mm, Ta was reduced compared to previous values (point 14 vs. 7 and 4). The ascending limb between points 14 and 13 reveals that Lo shifted from 9 to 15 mm; however, the peak (adapted) Ta value at 15 mm remained less than To1. **C and F-G:** Six contractions at 9 mm (points 14-19) significantly increased (adapted) both Ta and Tp, but not to their original values (point 19 vs. 4). **C:** Upon return to 15 mm, Ta was reduced compared to the most recent (adapted) value (point 20 vs. 13), but not compared to the original value (point 20 vs. 6). **F:** Conversely, Tp was increased at 15 mm compared to the most recent value (point 20 vs. 13) and returned to a value not different from its value before adaptation (point 20 vs. 8), but less than its original value (point 20 vs. 6). **I–J:** The Tp/Ta ratio at 15 mm on the descending limb was 5-to-10-fold greater than at Lo1 (points 8 and 13 vs. 4). Adaptation at 15 mm reduced Tp/Ta at 15 mm (point 13 vs. 8, Ω). Adaptation at 9 mm did not increase the Tp/Ta ratio to its original value at 9 mm (14 and 19 vs. 4, ψ). **G-J:** Mean values±SE.

### 4.4.3. Adaptation at Longer and then Shorter Lengths on the Ascending L-Ta Limb

The results from the protocol in Fig 4.4A for tissues that produced a maximum Ta value at 15 mm are presented in Fig 4.6. Following release from 15 mm, Tp, but not Ta, at 9 mm was reduced relative to its previous value (Fig 4.6A, point 7 vs. point 4), indicating that the stretch to and contraction at a longer length on the ascending limb caused a reduction in APS, but did not significantly adapt Ta from its original value at 9 mm. Furthermore, following a return to 15 mm, both Ta and Tp returned to values not different from their previous values at that length (Fig 4.6A, point 8 vs. 6), indicating that the release to and contraction at 9 mm on the original ascending limb did not adapt the tissues.

Five subsequent contractions at 15 mm (Figs 4.6B and 4.6E, points 9-13) significantly increased Ta, and conversely, significantly decreased Tp, indicating that the multiple contractions at 15 mm on the ascending limb adapted the tissues to that length.
Furthermore, following subsequent release to 9 mm, Ta and Tp were reduced compared to their original values (Fig 4.6B, point 14 vs. 4), indicating that the six contractions at 15 mm produced adaptation away from 9 mm.

Five subsequent contractions at 9 mm (Figs 4.6C-D, points 15-19) significantly increased both Ta and Tp, such that they were not different from their original values at 9 mm (Fig 4.6C, point 19 vs. 4), indicating that the previous adaptation away from that length had been reversed. Furthermore, upon return to 15 mm, Ta was reduced compared to the most recent value at that length (Fig 4.6C, point 20 vs. 13), but not compared to the original value (Fig 14.6C, point 20 vs. 6). Conversely, Tp was increased at 15 mm such that it returned to a value not different from its original value (Fig 4.6C, point 20 vs. 6). Together these findings indicate that the six contractions at 9 mm adapted the tissue back toward 9 mm and away from 15 mm.

Finally, the Tp/Ta ratio decreased from 0.229±0.042 to 0.117±0.020 following multiple contractions at 15 mm (Fig 4.6F, point 13 vs. 8, \(\Omega\)). These ratios are comparable to the ratio of 0.208±0.033 identified at Lo1 in Fig 4.6G, indicating that although Lo was not identified in Fig 4.6A, the initial Lo would have likely been near 15 mm.
Fig 4.6. A–E: L-Ta and L-Tp curves for tissues subjected to the protocol in Fig 4.4A that produced a L-Ta curve with an ascending limb between 3 and 15 mm (data points 1-6) with a maximum Ta value, Ta_max1, at 15 mm. Values were normalized to Ta_max1, and * indicates tension values were significantly different (paired-t, p<0.05, n=7 at points 1-19 and n=5 at point 20). A-C: Data points are labeled 1-20. A: Following release from 15 mm, Tp, but not Ta, at 9 mm was reduced relative to its previous value (point 7 vs. point 4), and upon return to 15 mm, both Tp and Ta returned to values not different from their previous values at 15 mm (point 8 vs. 6). B and E: Six contractions at 15 mm (points 8-13) significantly increased (adapted) Ta, and conversely, significantly decreased Tp. B: Following release to 9 mm, Ta and Tp were reduced compared to their original values at 9 mm (point 14 vs. 4). C-D: Six contractions at 9 mm (points 14-19) significantly increased (adapted) both Ta and Tp, such that they were not different from their original values at 9 mm (point 19 vs. 4). C: Upon return to 15 mm, Ta was reduced compared to the most recent (adapted) value (point 20 vs. 13), but not compared to the original value (point 20 vs. 6). Conversely, Tp was increased at 15 mm such that it returned to its original value (point 20 vs. 6). F: The Tp/Ta ratio decreased following multiple contractions at 15 mm (point 13 vs. 8, Ω). D-F: Mean values±SE.
4.4.4. Effect of Adaptation on the Ascending and Descending Limbs

This protocol, illustrated in Fig 4.7A, was designed to test the hypothesis that length adaptation at a particular muscle length on the descending limb of the L-Ta curve shifts the complete curve to the right along the length axis. For this protocol, two adjacent strips of DSM were cut from each bladder and examined simultaneously. Contraction (see Fig 2.2C) were performed at 3 mm steps (Fig 4.7A, points 1-5) until a peak (optimal) Ta value, To1, was identified at length Lo1, which was at either 12 or 15 mm. Tissues were then subjected to five additional contractions at Lo1+3 mm (Fig 4.7A, points 6-10), and it is expected based on the previous Figs in this dissertation (For example, Figs 4.2C, 4.3, 4.4, 4.5, 4.6) to see increased Ta at Lo1+3 mm, indicating adaptation to that length. Next, one DSM strip was contracted at Lo1+6 mm (point 11, hatched bar) while the corresponding strip cut from the same bladder was contracted at decreasing steps from Lo1 to Lo1 minus 6 mm (points 11-13, shaded bars). It is also expected that if the L-Ta curve exhibited length adaptation, as in ASM and VSM, then Lo1+3 mm would become a new Lo and that steps to shorter and longer lengths would reveal ascending and descending limbs, respectively, that were shifted to the right relative to the original limbs.

Figs 4.7B-C show L-Ta and L-Tp curves before (solid lines) and after (dashed lines) adaptation at Lo1+3 mm (Figs 4.7B-C, points 5-10, arrows, intermediate points not shown). The six contractions at Lo1+3 mm significantly increased (adapted) Ta at that length (Fig 4.7B, point 10 vs. 5, arrow) such that it was not different from To1 (Fig 4.7B, point 10 vs. 4) and both the ascending and descending limbs of the L-Ta curve shifted to the right along the length axis. The six contractions at Lo1+3 mm significantly decreased Tp at that length (Fig 4.7C, point 10 vs. 5, arrow) and the L-Tp curve also shifted to the
right. Furthermore, before adaptation, the Tp/Ta ratio at Lo1+3 mm on the descending limb was significantly greater than at Lo1 (Fig 4.7D, point 5 vs. 4, Ω); however, following adaptation the Tp/Ta ratio at Lo1+3 mm was reduced such that it was not different from the original value at Lo1 (Fig 4.7D, point 10 vs. 4).
**Fig 4.7. A:** L-T curve protocol in which contractions (see Fig 2.2) were performed at 3 mm steps (points 1-5) until a peak (optimal) Ta value, To1, was identified at length Lo1. Tissues were then subjected to 5 additional contractions at Lo1+3 mm (points 6-10). Then one DSM strip was contracted at Lo1+6 mm (point 11, hatched bar) while a corresponding strip from the same rabbit was contracted at decreasing steps from Lo1 to Lo1 minus 6 mm (points 11-13, shaded bars). **B-C:** L-Ta and L-Tp curves before (solid lines) and after (dashed lines) adaptation at Lo1+3 mm (points 5-10, arrows, intermediate points not shown). Mean values (±SE) were normalized to To1, and * indicates a tension value was significantly different from the original value at that length (paired-t, p<0.05, n=4). **B:** The six contractions at Lo1+3 mm significantly increased (adapted) Ta at that length (point 10 vs. 5, arrow) such that it was not different from To1 (point 10 vs. 4) and the ascending and descending limbs of the L-Ta curve shifted to the right. **C:** The six contractions at Lo1+3 mm significantly decreased Tp at that length (point 10 vs. 5, arrow) and the L-Tp curve shifted to the right. **D:** Before adaptation, the Tp/Ta ratio at Lo1+3 mm on the descending limb was significantly greater than at Lo1 (point 5 vs. 4, ω); however, following adaptation the Tp/Ta ratio at Lo1+3 mm was reduced such that it was not different from the original value at Lo1 (point 10 vs. 4).

### 4.5. Effect of Multiple Contractions versus Time in NPSS or NPSS±Rhythmic Contraction on Isometric Ta

An additional protocol was designed to determine if the increase in Ta (previous protocols Figs 4.3B, 4.4, 4.5, 4.6) during successive isometric contractions on the ascending limb of the L-Ta curve identified could be achieved by simply with waiting in NPSS, waiting in NPSS with RC induced with PGE2 or waiting in NPSS with RC blocked by SC560. Following an initial contraction at 3 mm, tissues were stretched in 0Ca to 12 mm and tension measurements were made to determine the Tp/Ta ratio. If necessary, tissue lengths were adjusted and the Tp/Ta ratio was measured again until it was 18±4%, corresponding to the Tp/Ta ratio at the initial Lo identified using the protocol in Fig 4.1A. Using this length as a reference length, Lref, tissues were subjected to the protocol in Fig 4.8A. To adapt tissues back and forth between Lref and 0.8Lref, control tissues were subjected to three sets of three tension measurement cycles (see Fig 2.2B&C) at Lref alternated with three sets of four contraction cycles at 0.8Lref (Fig
To determine if tissues would adapt as well during incubation in NPSS, PGE$_2$, or SC560 as during multiple KCl-induced contractions, the first three 8-min tension measurement cycles in the second set of measurements at 0.8L$_{ref}$ (Fig 4.8A, points 12-14, Test) were replaced with an equivalent duration of 24 min of incubation in NPSS, PGE$_2$, or SC560. Similarly, the second and third measurement cycles of the final set of measurements at 0.8L$_{ref}$ (Fig 4.8A, points 20-21, Test) were replaced with 16 min of incubation in NPSS, NPSS+PGE$_2$, or NPSS+SC560.

Following the determination of a reference length (11.66±0.10 mm, mean±SE, n=9) at which the Tp/Ta ratio (18.4±0.7%, range 15-22%) corresponded to the Tp/Ta ratio at Lo1 for the data in Fig 4.1 (18.7±4%), tissues were subjected to the protocol in Fig 4.4A to determine if Ta adapted as much during incubation in NPSS, PGE$_2$, or SC560 as during multiple KCl-induced contractions. Ta increased during initial control contractions in both Test and Control groups (8 vs. 5, *). However, Ta did not increase following incubation neither in NPSS nor SC560 for 24 min (Fig 4.8B, 15 vs. 5, NPSS Test and SC560 Test), but did increase following incubation in PGE$_2$ for 24 min (Fig 4.8B, 15 vs. 5, PGE$_2$ Test) and three isometric contractions (Fig 4.8B, 15 vs. 12 and 5, KPSS Control).

The degree of adaptation for control tissues was 17.55±2.58%, and 15.13±1.69% for the first and third sets of contractions at 0.8L$_{ref}$ (Fig 4.8C, 8 minus 5, and 22 minus 19) and the degree of adaptation for test tissues with PGE$_2$ was 12.24±2.06% and 16.79±3.31% for the first and third sets of PGE$_2$ test at 0.8L$_{ref}$ (Fig 4.8C, 8 minus 5 and 22 minus 19). Although the average value of Ta increased following 16 min in NPSS, this apparent increase was not statistically significant (Fig 4.8B, 22 vs. 5). Moreover, the
degree of adaptation following 16 min in NPSS was not greater than zero (Fig 4.8C, 22 minus 19, NPSS Test). In addition, the average value of Ta increase following 24 or 16 min in the presence of SC560 was not statistically significant (Fig 4.8B, 22 vs. 5) and the degree of adaptation either after 24 min or 16 min was not greater than zero too (Fig 4.8C, 8 minus 5 or 22 minus 19, SC560 Test)

These results show that 16-24 min of incubation in NPSS or SC560 did not, whereas three KCl-induced contractions or 16-24 min in PGE2 did, permit tissues to adapt at 0.8Lref. Therefore, the most important result is that time alone will not cause adaptation but RC will cause adaptation.
Fig 4.8. A: Protocol to determine if Ta increased as much following incubation in NPSS, PGE$_2$, SC560 as following multiple KCl-induced contractions. Control tissues were subjected to three sets of three 8-min tension measurement cycles at Lref alternated with three sets of four cycles at 0.8Lref. For “Test” tissues, cycles 12-14 and 20-12 at 0.8Lref were replaced with 24 and 16 min incubations in NPSS, PGE$_2$, SC560, respectively. B: Ta values for the first and fourth cycles of each series of measurements at 0.8Lo were normalized to Ta for the first contraction at 0.8 Lo, Ta$_5$ (data point 5, ±SE). Ta increased during initial control contractions in both groups (8 vs. 5, * p<0.05 compared to 1). Ta did not increase following incubation in NPSS or SC560 for 24 min (15 vs. 5, Test, n=5), but did increase following incubation in PGE$_2$ for 24 min (15 vs. 5, Test, n=5) and following three isometric contractions (15 vs. 12 and 5, Control, n=4). C: Ta adapted (increased) following initial control contractions in both groups (8 minus 5, Ω p<0.05 compared to zero). However, Ta did not significantly adapt following 16 min in NPSS or SC560, but did adapt following incubation in PGE$_2$ KCl-induced contractions (22 minus 19, Test vs. Control, Ω p<0.05 compared to zero).
CHAPTER 5 DISCUSSION

5.1. Generation of APS in DSM

APS in detrusor is responsible for a substantial fraction of Tp and Tt at longer muscle lengths (Fig 3.1F) and is reduced upon stretch to and release from longer muscle lengths (Speich, Borgsmiller et al. 2005). Fig 5.1 summarizes the increases in pseudo-steady-state Tp measured at Lref following one or more muscle activations and/or drug treatments at 50%Lref (described in chapter 3, Figs 3.2-3.5). A single, maximal, 24 min KCl-induced contraction at 50%Lref or two or three 1 min KCl-induced contractions at 50%Lref led to the greatest increases in Tp at Lref (Figs 3.2-3.5). Although, these stimulations did not mimic the physiological behavior of the bladder, they revealed that the duration and/or number of stimulations influenced the increase in Tp and motivated the investigation of the effect of two physiological phenomena, voiding contraction and RC, on the restoration of APS.

SRC in rabbit DSM produces ~5-12% of peak Ta (Ratz and Miner 2003), and because SRC occurs in human bladders (Gillespie 2004; Brading 2006), the most important finding of this study may be that at least 15 min of RC induced with PGE$_2$ or CCh permitted the regeneration of APS lost to preconditioning (strain softening) and led to increases in Tp that were not different from increases produced by two maximal KCl
contractions (Figs 3.3 and 5.1). In contrast, a single maximal KCl-induced contraction or a voiding-like contraction without subsequent RC yielded smaller increases in Tp (Figs 3.2-3.4 and 5.1). Together, these findings suggest that the duration of contraction may influence the restoration of APS to a greater degree than the magnitude of the contraction.

**Fig 5.1.** Summary of the normalized increases in Tp at Lref following selected stimuli at 50%Lref for the protocols in chapter 3, values are normalized to the increase in Tp following two maximal 1 min KCl-induced contractions.

Ta produced by a long, 24-min contraction had two phases, a fast (phasic) phase that lasted ~1 min and a long tonic phase with weaker amplitude that lasted ~23 min (Fig 3.2C). This tonic phase permitted a single long KCl-induced contraction to generate significantly more APS than a short contraction with little or no tonic phase (Figs 3.2 and 5.1). Similarly, a VLC without subsequent RC restored less APS than RC alone (Figs 3.4 and 5.1). Furthermore, a VLC without subsequent RC did not lead to a greater restoration
of APS than incubation in 0Ca (Figs 3.4, 3.5, and 51), indicating that the VLC did not contribute to the restoration of APS. Thus, a clear trend identified in Fig 5.1 was that single, maximal, short, phasic contractions restored less APS than tonic contractions and restored no more APS than incubation in 0Ca (Fig 5.1, compare “1 min KCl Contraction,” “VLC+4 min 0Ca” and “VLC+15 min SC560” with “15 min PGE2 RC” and the three tests involving both VLC and RC, and with “15 min 0Ca” and “4 min 0Ca”). Prior studies from our lab support the hypothesis that APS is caused by slowly detaching crossbridges (Speich, Borgsmiller et al. 2005; Speich and Ratz Submitted September 2009).

The data in Fig 5.1 indicate that approximately half of the APS restored by two maximal KCl-induced contractions was also restored in a nominally Ca\(^{2+}\)-free environment (Fig 5.1, “15 min 0Ca,” “VLC+4 min 0Ca” and “4 min 0Ca”). However, the general kinase inhibitor staurosporine, or prolonged exposure to the COX-1 inhibitor SC560, inhibited the restoration of APS in 0Ca by approximately half (Fig 5.1, compare “15 min Staurosporine” and “VLC+90 min SC560”, “15 min 0Ca” and “4 min 0Ca”), which indicates that APS generation may be regulated in part by a calcium-independent staurosporine sensitive pathway. A Ca\(^{2+}\)-free solution abolishes basal (spontaneous) RC, and completely prevents KCl from causing contraction (Jezior, Brady et al. 2001; Ratz and Miner 2003). However, a Ca\(^{2+}\)-free solution reduces but does not abolish basal MLC phosphorylation when tissues are stretched beyond their slack length. More importantly, a Ca\(^{2+}\)-free solution does not reduce the high basal MLC phosphorylation level (~20%) when tissues are at slack length (Ratz and Miner 2003). Moreover, release from a longer muscle length to the slack length actually increases the level of basal MLC
phosphorylation, (i.e., Lref to 50%Lref) (Ratz and Miner 2003). These data together are consistent with a model suggesting that restoration of APS when DSM was released to slack length (i.e., 50% Lref) in a Ca$^{2+}$-free solution was due to an increase in MLC phosphorylation resulting in activation of slowly detaching crossbridges. The fraction of APS that was abolished only by distilled water could be due purely to mechanical alterations in nonlinear viscoelastic structures rather than to biochemical regulated mechanisms. Alternatively, fluid redistribution alone involving passive or active processes could have produced greater pseudo-steady state Tp at Lref when tissues were released to that length compared to when they were stretched to that length.

5.2. Shifting of the L-Ta and L-Tp Curves due to Preconditioning, and Length Adaptation

One important contribution provided by this study is strong evidence that the L-Ta relationship in DSM can best be described by a series of curves over a broad range of muscle lengths rather than by a unique parabolic curve (For example, Figs 4.1, Fig 4.2). To the best of our knowledge, this is the first study to identify length adaptation of the L-Ta curve in DSM (Speich, Almasri et al. 2009). The data in Figs 4.1B–D and 4.2C show that the L-Ta and L-Tp curves shifted down and to the right following a series of contractions at progressively longer lengths indicating that preconditioning caused the L-T curves to shift to longer lengths. These data also indicate that the further a tissue strip was stretched beyond Lo, the greater the shift in the L-Ta curve (compare Figs 4.1, B with D, and 4.3, B with D), but if a tissue strip was not stretched beyond Lo, there was no significant change in Ta at intermediate lengths (Fig 4.4B, Ta at 6 mm, and trend in Fig 4.1E).
The results in Fig 4.2E demonstrate a decrease in Tt at short lengths that was due to a decrease in Ta upon preconditioning, and Fig 4.3, B–F, shows that at least some of this decrease in Ta could be reversed by adaptation resulting from one or more additional contractions at that short length. Neither the increase in Ta at 3 mm (Fig 4.3, B–F) nor the decrease in Ta at 3 mm following preconditioning (Figs 4.1, B and C, 4.2C, and 4.3, B–E) resulted from a decrease in Tp due to stress relaxation across the repeated contraction cycles because Tp was essentially zero at this length and Tp was measured before each contraction. In Fig 4.3B, the reduction in Ta at the short length following preconditioning was completely restored, indicating that the reduction was due to adaptation and not irreversible preconditioning. However, for tissues preconditioned beyond the initial Lo, the data in Fig 4.3D indicate that approximately half of the reduction in Ta upon preconditioning was reversible and attributable to adaptation, while the other half appeared irreversible.

Thus preconditioning can induce both a reversible and an irreversible shift in the L-Ta curve. The reversible portion of the rightward shift in the L-Ta curve following preconditioning is due to length adaptation. Moreover, Fig 4.2, C and D, reveals that in addition to the adaptation due to preconditioning in S1, multiple contractions at longer muscle lengths in S2 adapted the optimal length from Lo2a to Lo2c. In summary, length adaptation of the L-Ta relationship in DSM has been identified, and it is characterized by a decrease in Ta due to subsequent stretches to and contractions at progressively longer lengths and by an increase in Ta as a result of one or more contractions at or near a particular muscle length. Another important contribution of this study is evidence that the L-Tt curve is shifted by changes in the L-Tp curve in addition to length adaptation of the
L-Ta curve. Fig 4.2E demonstrates a decrease in Tt at longer lengths that is due to a decrease in Tp resulting from preconditioning. Together, the data in Figs 4.1, 4.2, and 4.3 demonstrate that the L-Ta and L-Tp relationships in rabbit DSM are dynamic functions of both strain and activation history (Speich, Almasri et al. 2009).

The irreversible portion of the rightward shift in the L-Ta curve following preconditioning may be due breakage of some intermediate connective proteins. Arner et al (Sjuve, Arner et al. 1998) found that mice urinary bladders lacking desmin-an intermediate filament- produce less active tension at Lo compared to bladders with desmin. Therefore, desmin may participate in active tension development. Tang et al (Wang, Li et al. 2006) studied the role of vimentin-another intermediate filament- in trachealis dogs smooth muscle and they found that lacking vimentin inhibits active tension development, but it did not inhibit the myosin light chain phosphorylation. Fig 4.2D (S2 to S3) shows at a long length the muscle produces the same optimum active tension value (To1c) at a different length (i.e. length-adaptation). Thus, unless there is damage at least to desmin or vimentin or any other intermediate filament, smooth muscle type can adapt to the ambient muscle length, resulting in shifts along the length axis in the optimum muscle length for production of maximum active tension and a dynamic active length tension curve. Also, adaptation to maximum active tension may be limited to a certain length range. Beyond this limit, the muscle will show partial adaptation (Fig 4.5, adaptation at 15 mm) or possibly no adaptation.

5.3. L-T Curves from other Studies

In Fig 5.2, length-active tension (L-Ta) and length-passive tension (L-Tp) curves from Figs 4.1C-D, 4.4, and 4.5 in chapters 3 and 4 are compared with curves
approximated from studies by Uvelius (Uvelius 1976) and Longhurst, et al. (Longhurst, Kang et al. 1990) and normalized to the To and Lo. In Fig 5.2, active and passive data from series S1 and S2 in Figs 4.1C and 4.4 (diamonds, circles, respectively) appear to be consistent with those of Uvelius and Longhurst, et al., when normalized to To1 and Lo1 (Fig 5.2 A-D). When data from S2 in Figs 4.1C or 4.7B are normalized to To1 and Lo1 (solid circles), they are clearly different from the other data in Fig 5.2E; however, if they are normalized to To2 and Lo2 (solid triangles), then they are consistent with the published data, except that the ascending Ta limb is shifted somewhat down and to the right. If the data in S1 were not recorded, as might be the case if tissues are preconditioned before the L-Ta curve is produced, then only the data from S2 would be observed, and the significant shift from the S1 curve to the S2 curve (Fig 5.2, compare solid circles to triangles) would not be observed; however, if normalized to both Lo and To, the S2 curve would appear consistent with tissues that were not preconditioned (compare diamonds to triangles). Three key observations from this comparison are that (1) DSM strips clearly produce a somewhat parabolic L-Ta curve, (2) a single DSM strip can produce two very different L-Ta curves (Fig 5.2, compare diamonds with solid circles), and (3) normalization of the L-Ta curve to both Lo and To may hide important characteristics, like length adaptation, by making two very different curves appear similar (Fig 5.2, compare diamonds with triangles).
Fig 5.2. Normalized L-Ta and L-Tp curves. A: active tension data approximated from (Uvelius 1976) normalized to To and Lo (square), active tension data approximated from (Longhurst, Kang et al. 1990) normalized to To and Lo (circle), active tension data from...
S1 in Fig 4.1C normalized to To1 and Lo1 (Ta/To1, diamond), and active tension data from S1 in Fig 4.1D normalized to To1 and Lo1 (circle). B: active tension data approximated from (Uvelius 1976) normalized to To and Lo (square), active tension data approximated from (Longhurst, Kang et al. 1990) normalized to To and Lo (circle), active tension data from S1 in Fig 4.4 normalized to To1 and Lo1 (Ta/To1, diamond), and active tension data from S1 in Fig 4.5 (circle). C: Passive tension data approximated from (Uvelius 1976) normalized to To and Lo (square), passive tension data approximated from (Longhurst, Kang et al. 1990) normalized to To and Lo (circle), passive tension data from S1 in Fig 4.1C normalized to To1 and Lo1 (Ta/To1, diamond), and passive tension data from S1 in Fig 4.1D (circle). D: Passive tension data approximated from (Uvelius 1976) normalized to To and Lo (square), passive tension data approximated from (Longhurst, Kang et al. 1990) normalized to To and Lo (circle), passive tension data from S1 in Fig 4.4 normalized to To1 and Lo1 (Ta/To1, circle), and passive tension data from S1 in Fig 4.5 (circle). E: Data from S1 in Fig 4.1C normalized to To1 and Lo1 (Ta/To1, diamond) and data from S2 in Fig 4.1C normalized to To1 and Lo1 (Ta/To1, circle) and normalized to To2 and Lo2 (Ta/To2, triangle). F: data from S1 in Fig 4.7B normalized to To1 and Lo1 (Ta/To1, diamond) and data from S2 in Fig 4.7B normalized to To1 and Lo1 (Ta/To1, circle) and normalized to To2 and Lo2 (Ta/To2, triangle).

5.4. Implications on Future Studies

Given evidence that both the L-Tp and L-Ta curves in DSM are dynamic and can shift as a function of strain and activation history, the choice of a suitable reference length becomes quite challenging. Initially, all tissues in the protocols in this study were essentially slack with \( Tp \approx 0 \) at 3 mm; however, the optimum tension occurred at 6 mm in some tissues and at 15 mm or beyond in others (Figs 3.2, 3.3, 3.4, 3.5 (Lref determination protocols are not shown, 4.1, 4.3, 4.4, 4.5, and 4.6). Also, even with prior preconditioning, a stretch to 15 mm shifted slack length from 3.5 mm to 7 mm in some tissues (Fig 3.1). Therefore, passive slack length may not be a suitable reference length. Furthermore, the Lo shifted from 6 mm to 11 mm and then to 10.5 mm in some tissues (Fig 4.2B-C) and the Lo shifted also from 12 mm to 15 mm (Fig 4.7C), and a shift in Figs (4.1, 4.7); therefore, Lo may also not be a suitable reference length. Thus, for studies involving DSM strips, the dynamic nature of the L-Ta and L-Tp curves should be
considered when choosing a reference length. In many of the protocols in this study, tissues were stretched from an initial length of 3 mm to a maximum length and then grouped based on the trends in the L-Ta curve.

Length-adaptation identified in this study can cause an increase in isometric Ta over time. Therefore, future pharmacological studies that involve measurements of Ta should consider length adaptation.

5.5. Potential Length-Adaptation Model

Several mechanisms for length adaptation of smooth muscle have been proposed and investigated primarily in ASM. There are three different mechanisms that may explain the molecular structure changes during adaptation. Sloway and colleagues have suggested that changes in actin filament length may play a key regulatory role (Dulin, Fernandes et al. 2003; Solway, Bellam et al. 2003) whereas Seow and colleagues have suggested that the structural design of the myosin filaments themselves may change (Pratusevich, Seow et al. 1995; King, Paré et al. 1999; Seow, Pratusevich et al. 2000; Mitchell, Seow et al. 2001; Herrera, Kuo et al. 2002; Qi, Mitchell et al. 2002; Seow 2005; Bosse, Sobieszek et al. 2008). Gunst and colleagues attributed adaptation to shifting of the sites where actin filaments connect to dense bodies and to actin polymerization (Gunst, Wu et al. 1993; Gunst, Meiss et al. 1995; Mehta and Gunst 1999; Tang, Mehta et al. 1999; Gunst and Wu 2001; Zhang and Gunst 2008). Understanding myosin structural design changes (myosin polymerization) and actin polymerization during Ta adaptation were not considered in this study.

Latchbridge state is a special state in which crossbridges in smooth muscles following muscle activation, become dephosphorylated yet remain attached to create
latchbridges that are capable of maintaining tension in which smooth muscle maintains force with minimal consumption of ATP (Dillon, Aksoy et al. 1981; Murphy, Ratz et al. 1987; Hai and Murphy 1988; Murphy, Rembold et al. 1990; Speich, Borgsmiller et al. 2005). Previous studies have proposed that APS could be due to latchbridges (Speich, Borgsmiller et al. 2005). Our data in Figs 4.2, along with the data in Figs 4.4, 4.5, and 4.6, demonstrate that adaptation of Tp, (i.e. changes in APS) can occur while a DSM strip is isometric. Thus, the isometric increase in Tp following a contraction on the ascending limb of the L-Ta curve (Figs 4.4, 4.5, and 4.6, points 14-19) could be due to latchbridges that are formed during internal shortening due to the contraction and revealed as the crosslinks are loaded upon internal lengthening during relaxation. Furthermore, the isometric decrease in Tp following a contraction at Lo or on the descending limb of the L-Ta curve (Figs 4.4, 4.5, and 4.6, points 8-13) could be due to the breakage or regulated release of these crosslinks. If APS is due to crosslinked crossbridges or latchbridges, then the decrease in Tp corresponding to the increase in Ta during adaptation on the descending limb (Fig 4.4, points 8-13) suggests that the increase in Ta may be due to the conversion of crossbridges responsible for Tp into active crossbridges. The increase in both Ta and Tp during adaptation on the descending limb (Fig 4.4, points 14-19) could also support this hypothesis if a fraction of the actively cycling crossbridges were crosslinked or latched to increase Tp, which subsequently maintained a more efficient arrangement of thick and thin filaments and increased the number of active crossbridges to produce greater Ta.

Additional studies are necessary to determine whether adaptation of Ta in DSM is caused by changes in the arrangement, length, number and/or regulation of contractile
filaments or other mechanisms. We speculate that length adaptation plays a significant role in bladder physiology and that alterations in this system could potentially play a role in bladder disorders; therefore, understanding the mechanisms responsible for length adaptation may provide information essential to the development of therapies targeting overactive bladder and other bladder contractile disorders. We believe that one of the physiological functions of length adaptation is to broaden the muscle’s functional length range so the bladder is able to void well over this length range. Understanding, the molecular mechanisms may enable us to target specific proteins to modify DSM behavior.

5.6. Smart Materials Behavior in Smooth Muscles

Scientists define smart materials as materials that have one or more properties that can be significantly changed in a controlled fashion by external stimuli, such as stress, temperature, moisture, pH, electric or magnetic fields (Harrison and Ounaies 2002). Therefore, in this broad definition of smart materials, one can fit any materials that can change any of their properties as an effect of an external stimulus. Thus, biomaterials and in particular smooth muscles can be included in this definition. If a bladder smooth muscle strip is stretched, its properties change and it becomes limp and the strip does not have stiffness as it originally had (For instance Figs 3.1, 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6), but if a bladder smooth muscle strip exposed to a contraction, multiple contractions, or rhythmic contractions (similar to an electrical stimulus) at a certain length, the muscle becomes stiffer (For instance Figs 3.2, 4.8). Thus, in a certain way the bladder smooth muscle acts as a smart material in which the stress-strain curve shifts to the right as a result of stretch and to the left as a result of contraction. Therefore, a smart material
model with multiple inputs and outputs may be suitable for modeling this complex behavior of DSM.

5.7. Potential Clinical Significance

Overactive Bladder (OAB) is one of the most common causes of bladder control problems. It is believed that OAB is due to uncontrolled spontaneous activity of the detrusor smooth muscle wall during the bladder filling phase (Rovner and Wein 2002), causing increased urgency (a strong desire to void); frequency of urination; and in the worst case, involuntary loss of urine (urge incontinence) (Milsom, Stewart et al. 2000). The studies show that OAB affects over 17% of the worldwide population individuals (Mullins 2009). Therefore, OAB has a significant impact on the quality of life and limitations on activities to those individuals. Although, OAB occurrence increases with age (Milsom, Stewart et al. 2000), the occurrence is not normal at any age.

RC occurs in all mammalian bladders studied thus far, including human bladders (Biers, Reynard et al. 2006; Brading 2006). Human bladders show that the occurrence of spontaneous rhythmic contractions is greater in detrusor strips taken from clinically unstable bladders than in strips taken from patients with normal bladders (Kinder and Mundy 1987). Thus, we speculate that there is a threshold level of SRC in the bladder wall. Above this level, the bladder may show OAB symptoms, because some patients with OAB show increased spontaneous rhythmic activity (Brading 1997). Therefore, further investigation of stable and unstable bladders is necessary to determine if there is an appropriate level of RC. Some level of RC appears to be necessary for APS and length-adaptation, however, level of RC should not be high to a point where RC may contribute to OAB symptoms.
APS is important and may play a crucial role especially during the bladder filling phase. The likely roles of APS in bladder function might be to maintain bladder shape while allowing the bladder to accommodate urine at relatively low pressures and to maintain efficient spacing between actin and myosin filaments to enable efficient active tension generation for voiding throughout the broad range of muscle lengths over which detrusor operates.

Localized stretching of the bladder wall affects the signaling system that leads to urgency (Coolsaet, Van Duyl et al. 1993), and that could lead to increase sensory nerve activation during the filling phase and consequently contribute to OAB symptoms. Insufficient stiffness (no or very low level of rhythmic contraction) could allow increased deformation of the bladder and therefore increased bladder wall strain during changes in abdominal loading, which could lead to premature urgency. Elevated stiffness (high level of rhythmic contraction) could lead to increased pressure and bladder wall stress, which could also lead to premature urgency. Thus, any dysfunction in passive tension during the filling phase could contribute to OAB. Additional studies are necessary to determine whether the elevated levels of RC in patients with OAB may contribute to any dysfunction involving bladder wall stiffness or may be an attempt to compensate for a dysfunction involving bladder wall stiffness.

We also speculate that length adaptation plays a significant role in bladder physiology and that pathologic alterations in this system could potentially play a role in bladder disorders. The results in this study (Fig 4.8) reveal that RC adapts the muscle at a short length. Thus, understanding the molecular mechanisms responsible for length
adaptation in DSM may provide information essential for the timely discovery of therapies to specifically target OAB and other bladder contractile disorders.

Overall, the data from this study show that RC can regenerate APS (Figs 3.2-3.5) and can cause adaptation (Fig 4.8) in rabbit detrusor. Therefore, this study has potentially identified two physiological roles of RC in bladder. Also, the data from this study are consistent with a bladder model that either includes a single crossbridge system that can be regulated to generate both Ta and APS or includes two contractile systems, one responsible for voiding, which produces the phasic portion of a sustained isometric contraction, and one responsible for RC and generation of APS, which produces the tonic portion of a sustained isometric contraction (Fig 3.2C). Both models are consistent with Gillespie’s conclusion that micturition (voiding) and non-micturition activity (rhythmic contraction) in the bladder are controlled by distinct systems (Gillespie 2004).
6.1. Conclusions

This dissertation focused on both the passive and active length-tension relationships in DSM and the mechanical mechanisms contributing to these relationships. The first objective was to identify mechanical mechanisms responsible for the generation of adjustable passive stiffness (APS) and the impact of APS on the length-total tension relationship. The results show that:

1. A shift in the length-passive tension curve in DSM due to APS produced a shift in the length-total tension curve.

2. APS formation in DSM depended on the number of contractions, magnitude of contraction, and duration of contraction, and as the number, magnitude, and duration of contractions increased more APS was generated.

3. Rhythmic contractions at a short muscle length regenerated more APS than a “voiding-like”, indicating that APS regeneration may occur in the filling phase rather than the voiding phase.

4. APS regeneration may be due to an active regulated process.
The second objective of this dissertation was to determine whether the length-active tension relationship is adaptive and to identify specific mechanical mechanisms that contribute to any adaptive behavior. The results show that:

1. The length-active tension curve in DSM was not fixed (static) as in skeletal muscle, but instead was dynamic shifted due to preconditioning.

2. The length-total tension curve in DSM shifted as a result of preconditioning, APS and length adaptation of the length-active tension curve.

3. A decrease in active tension at short muscle lengths following preconditioning was due to adaptation and that adaptation was at least partially reversed by multiple KCl-induced contractions at that muscle length.

4. Isometric rhythmic contractions produced length adaptation of the length-active tension curve.

Overall, DSM showed a dynamic length-tension relationship as previous identified in airway and vascular smooth muscles. To the best of my knowledge, this is the first study to show that the active length-tension relationship in DSM is dynamic and can adapt over a broad length range.

This study confirmed that rabbit bladder strips display spontaneous rhythmic contractile activity and the results suggest that physiological functions of SRC are to regenerate APS and to adapt the length-active tension relationship. This study is the first to identify these potential functions of SRC in bladder DSM. Ultimately, these results may be helpful in the identification of treatments for OAB. Since low-grade rhythmic contractions occur in human bladder and since elevated levels of RC have been shown in
patients with OAB disorder, understanding the role of RC in both APS and length adaptation may be essential to understanding a potential mechanism for OAB.

6.2. Future Studies

In addition to the future studies recommended in the preceding discussion, I recommend that the next steps in the study of APS include identifying the molecular structures responsible for APS. Actin polymerization inhibitors such as latrunculin B and cytochalasin D could be used to test the effect of actin polymerization on APS formation. The effect of APS and length adaptation in an intact bladder should also be studied.

Future studies related to length-adaptation of the active length tension curve in DSM could focus on specific molecular mechanism responsible for this behavior. I suggest that the role of Rho kinase be investigated using selective inhibitors such as H-1152 and Y-27632. In addition, the role of actin polymerization could be investigated using actin polymerization inhibitors, and the role myosin phosphorylation could be investigated using Western blot histology analysis to study the effect of these contractile proteins on length-adaptation behavior. Finally, APS and length adaptation could be investigated in other types of smooth muscle.
List of References


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