

UNIVERSITY OF CALGARY

Force Production in Lengthened Myofibrils and Single Sarcomeres

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF KINESIOLOGY

CALGARY, ALBERTA

SEPTEMBER, 2010

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Abstract

It has been well documented that the steady-state isometric force generated by skeletal muscle is influenced by the contractile history of the muscle but there is very little agreement on the underlying mechanism. The most commonly held mechanism for the residual force enhancement following active muscle stretching involves sarcomere length re-distribution (and disruption). To gain insight into this history dependent behaviour of muscle contraction, specifically force-enhancement following active stretch, experiments were performed on rabbit psoas muscle myofibrils where all individual sarcomere lengths could be determined simultaneously with force during isometric and eccentric myofibrillar contractions. In single isometrically activated and then stretched myofibrils, force-enhancement following stretch occurred and while sarcomere length non-uniformities were detected, they exhibited stable behaviour and did not increase during stretch, therefore the development of sarcomere length non-uniformities were deemed to be an unlikely source of the enhanced force. Experiments were conducted on myofibrils reduced to a single sarcomere so as to preclude the possibility of non-uniform sarcomeres playing a role in the extra force following stretch. Enhanced force was observed in these single sarcomere preparations when compared to the isometric reference force uniquely demonstrating that force enhancement can occur in the absence of sarcomere overstretching. Finally, the role of titin in actively and passively lengthened myofibrils was investigated to determine whether the enhanced force following active stretch could be attributed to a molecular spring, as some have proposed. Single myofibrils were lengthened from the plateau region of the force-length relationship until mechanical failure (first observation of negative slope for the stress-time curve) was observed, with and without titin present, and it was determined that titin is crucial for force production in actively and passively lengthened myofibrils. While mechanical failure occurred at similar sarcomere length, failure force was four times higher in the actively compared to the passively stretched myofibrils. All myofibrils failed at sarcomere lengths beyond which actin-myosin based cross-bridge forces could be expected to contribute to the force at failure. These results suggest that the molecular spring titin plays a major role in sarcomere mechanics and that history dependent behaviour likely originates from a parallel elastic element and not from sarcomere length redistribution.

Preface

Each of the following four chapters is based on scientific manuscripts:

Chapter 3 is based on V. Joumaa, T.R. Leonard and W. Herzog. (2008). Residual force enhancement in myofibrils and sarcomeres. *Proceedings of the Royal Society B*. June 22; 275 (1641):1411-1419.

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Chapter 4 is based on T. Leonard, M. DuVall and W. Herzog. (2010). Force enhancement following stretch in a single sarcomere. *In Press. American Journal of Physiology-Cell Physiology* (September, 2010). Doi: 10.1152/ajpcell.00222.2010.

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Chapter 5 is based on T. Leonard and W. Herzog. (2010). Regulation of muscle force in the absence of actin-myosin-based cross-bridge interaction. *American Journal of Physiology-Cell Physiology*. 2010 July; 299(1):C14-20.

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Chapter 6 is based on T. Leonard, V. Joumaa and W. Herzog. (2010). An activatable molecular spring reduces muscle tearing during extreme stretching. *In Press. Journal of Biomechanics* (August, 2010). Doi: 10,1016/j.biomech. 2010.07.016.

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This dissertation is based on a collection of manuscripts, and therefore has some repetition in the introduction and methods sections of chapters three through six.

Acknowledgements

I would like to express my appreciation and sincere thanks to the following:

Dr. Walter Herzog for being my supervisor and providing me with the opportunity to pursue this degree and for his unwavering support and infectious enthusiasm.

Dr. Doug Syme and Dr. Darren Stefanyshyn for serving on my supervisory committee and providing guidance to me as a student.

Dr. Henk ter Keurs for serving on my dissertation committee.

Dr. Henk Granzier for traveling to Calgary to serve on my dissertation committee as the external examiner.

Dr. David Severson for serving as the neutral chair on my dissertation committee.

Dr. Venus Joumaa for reading my thesis and offering me excellent feedback.

Andrzej Stano, Hoa Nguyen and Azim Jinha for their help with the equipment needed to perform this work, and for being my friends.

My parents, Charles and Donna Leonard who have always encouraged me to work hard and improve myself.

My brothers Roger and Phil Leonard who listened patiently to me rant when things weren't going to plan.

Catherine and Gemma Leonard for their unstinting love and support.

Holly Hanna, Jessica Fordham, Glenda McNeil, Byron Tory and countless others who all make the lab a great place to work.

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Free employment of reason, in accordance with scientific method,
is the sole method of reaching truth.

Thomas H. Huxley, 1888.

Chapter One: Introduction

Animal movement and locomotion has fascinated humankind since the dawn of recorded history. The cave art drawings in Lascaux, France are more than just pictographs of prey species; they are 15,000 year old depictions of movement and strength. Our understanding of the mechanism underlying muscular movement has changed over time, from a mystical nature to one based on scientific principles and mechanics. Over 1800 years ago, Galen of Pergamum described how actions of muscles arose from the movement of the animal spirit (*pneuma pysicon*) traveling through hollow nerves to the muscle and resulting in the movement of the muscle. A more mechanical process was advocated by Rene Descartes (1596-1650) who proposed the “animal spirit” was actually a liquid substance rather than an invisible and weightless material, and which moved through the nerves to the muscle, but William Croone (1633-1684) disagreed. In his published work titled *De ratione motus musculorum* (1667) he argued that nerves did not contain the valves necessary to regulate the flow of a liquid nor were nerves large enough to facilitate the necessarily rapid movement of a fluid to inflate a muscle in a timely fashion. Rather, he proposed a process whereby small amounts of liquid moved through the nerve into the muscle which then mixed with arterial blood, resulting in a vigorous and expansive reaction or “fermentation” which then inflated the muscle resulting in contraction and subsequent force. Still later, Luigi Galvani (1737-1798) showed that electrical impulses could cause muscle to contract. He termed this “animal electricity” and proposed that this electricity was actually a liquid produced in the brain which traveled from the brain through nerves into the muscles where activation resulted. By the beginning of the twentieth century the lactic acid theory described the folding or shortening of proteins within muscle fibres upon activation which resulted in the production of contraction force.

By the 1950s however, it was shown that myosin filament length was actually constant in both activated and relaxed muscle (Huxley & Hanson, 1954; Huxley & Niedergerke, 1954) and it was proposed that the actin and myosin filaments would actually slide past each other, thereby allowing for shortening of the sarcomere without crumpling of the filaments (Huxley, 1957a; Huxley, 1969). At that time it was proposed by these two

groups, independently, (one headed by Andrew Huxley and one by Hugh Huxley) that linkages between actin and myosin might form upon activation but it was not until later that this idea was formulated more completely with cross-bridge connections arising from projections on the myosin binding actin and producing active contraction force.

As well as proposing this cross-bridge mechanism, a mathematical framework for predicting the observed force in experiments was provided (Huxley, 1957a). The key features of this important work were that each cross-bridge was an independent force generator and was neither influenced by any neighbouring cross-bridge nor was memory of previous activity retained. The filaments were required to be inextensible in length and thermal agitation (Brownian motion) was the driver of the required oscillations of the myosin binding pocket.

Work to test these predictions was done and published in 1966 (Gordon *et al.*, 1966b) and provided convincing evidence for the force generated by a muscle being proportional to the amount of filament overlap present and that each myosin based cross-bridge was an independent force producing unit with the binding sites being uniformly distributed along the filaments. Further work refined the sliding cross-bridge filament theory to incorporate rotation of the myosin head during the power stroke; (Huxley, 1969) and multiple binding states (Huxley & Simmons, 1971).

This sliding filament cross-bridge model of muscle has been near universally accepted by the scientific community, with refinements continuously produced. This is not to say that the current theory does not have weaknesses. The history dependent behaviour of muscle following length change has been a source of difficulty for the cross-bridge model (Walcott & Herzog, 2008) and early work showed convincing evidence that force after stretch exceeded the force observed for a muscle that was at the same final length but had not been stretched (Abbott & Aubert, 1952;Edman *et al.*, 1978). This enhanced force following stretch cannot be predicted using the force length relationship since the formulation of the cross-bridge theory requires that, for a given level of activation, the steady-state isometric force a muscle can produce is purely a function of its length. Several mechanisms have been proposed to explain this extra force following active stretch.

1.1 Mechanisms for force enhancement

1.1.1 *Altered cross-bridge kinetics.*

This mechanism involves some combination of either more cross-bridges being attached following stretch, or some or all of the cross-bridges can produce greater force. Both of these mechanisms contravene several of the key assumptions underlying the cross-bridge theory, namely that all cross-bridges produce the same force and that rate constants for attachment and detachment are based solely on the Huxley's "x-distance", that is the distance from a cross-bridge equilibrium position to its nearest actin attachment site. Evidence supporting altered cross-bridge kinetics as an explanation for force enhancement following stretch is inconclusive with some groups showing more attached cross-bridges following stretch (Linari *et al.*, 2000; Julian & Morgan, 1979b; Rassier & Herzog, 2005) and others no change (Sugi & Tsuchiya, 1988; Getz *et al.*, 1998).

1.1.2 *Engagement of a parallel elastic element.*

When the recruitment of a parallel elastic element within the sarcomere was first proposed as a mechanism for residual force enhancement, no structure had been identified at the time which could provide this extra force (Edman *et al.*, 1978). The recruitment of a passive element within the sarcomere (Edman & Tsuchiya, 1996) not only could explain the enhanced force following stretch but could also provide a satisfactory explanation of another behaviour observed when stretching an activated muscle, specifically, that steady state forces following stretch can be greater than that produced at the plateau of the force length relationship (Rassier *et al.*, 2003c; Edman *et al.*, 1978). This observed muscle behaviour cannot be directly accounted for by the current cross-bridge theory; however the recruitment of a parallel elastic element could offer a mechanism for producing force greater than that produced at a length where filament overlap is maximal by altering the spring constant of the elastic element. The possibility that titin (connectin) is involved as this parallel elastic element has already been proposed (Edman & Tsuchiya, 1996; Noble, 1992) but this was in frog single fibre experiments where other passive structures were present, which would include the

tendon, any collagen and basement proteins and other membranes such as the sarcolemma. In a reduced preparation such as a single myofibril, all passive force supporting structures external to the sarcomere are removed leaving titin as the sole source of passive tension (Horowitz *et al.*, 1989). A third filament (titin) that might change its spring constant or might interact with other structural or contractile proteins to change force based on contractile history, would be an evolution of the theory of muscular contraction.

1.1.3 *Sarcomere length non-uniformity.*

The mechanism that has gained the most support for explaining force enhancement following stretch is termed the “sarcomere length non-uniformity theory” (Julian & Morgan, 1979b; Morgan, 1994). The sarcomere is the smallest functional unit of striated muscle and with this structural rearrangement theory, individual sarcomeres within a myofibril undergo length changes with some sarcomeres becoming longer at the expense of others. Because of this variation in length, the ability of each sarcomere to generate force may be altered (due to the force-length relationship), and so the longer (weaker) sarcomeres are rapidly (Morgan, 1990) and forcibly lengthened further by the shorter (stronger) sarcomeres with the ultimate result being that the longer sarcomeres are pulled to extreme lengths where no actin-myosin filament overlap is present and these overstretched sarcomeres are then supported by passive means alone. Simultaneously, the shorter sarcomeres shorten further, placing them at a more advantageous length (when compared to the reference isometric contraction on the descending limb of the force-length relationship), thus accounting for the higher observed force following stretch and thereby providing a mechanism for residual force enhancement without contradicting the assumptions inherent to the cross-bridge model. This sarcomere length non-uniformity theory works best on the inherently unstable (Hill, 1953) descending limb of the force-length relationship. However, previous work to demonstrate the functioning of this mechanism has not been at a sufficiently low structural level where individual sarcomere lengths could be measured or where the issues surrounding the compliance of the tendon could be negated by removal of the tendon (Morgan *et al.*, 2000) or where

sarcomere length was recorded simultaneously with force (Rassier *et al.*, 2003a) or where the preparation was not given sufficient time for steady state force to redevelop after the stretch (Telley *et al.*, 2006b). Therefore a preparation with the ability to resolve the lengths of individual sarcomeres in a muscle and the force produced without other structures (i.e. tendons or membranes) being present would offer the best opportunity to determine whether sarcomere length non-uniformities do indeed develop following active lengthening of a muscle and whether these non uniformities can account for the enhanced force seen in the literature.

Force enhancement experiments where titin material properties could be altered while measuring individual sarcomere length and muscle force simultaneously could give insight into the mechanism involved in force enhancement following stretch and the possible role of titin in the production of this force.

1.2 Thesis objectives.

The general objectives of this thesis were to attempt to gain some understanding of the mechanism responsible for force enhancement following stretch in skeletal muscle and the possible role played by titin.

1.2.1 Objective #1.

While force enhancement has been observed in a variety of preparations scaling from whole human muscle down to single fibres, it has never been investigated in a single myofibril. Given that the myofibril lacks extra-sarcomeric structures, force enhancement observed at this level would become a sarcomeric property. Therefore, specific aim #1 of this thesis was to test if force enhancement is observed in isolated myofibrils and whether it might be associated with sarcomere length non-uniformity and instability.

1.2.2 *Objective #2.*

The sarcomere length non-uniformity theory is the most accepted explanation at present for force enhancement following stretch and the ultimate test of that theory is the testing for force enhancement in an isolated single sarcomere. This has not been done previously for reasons of technical difficulty although a single sarcomere preparation has recently been used to investigate isometric contractions (Pavlov *et al.*, 2009b). This test then would clearly and uniquely answer the question of whether sarcomere length non-uniformity is required for force enhancement following stretch. The specific aim #2 of this thesis was to test if force enhancement is observed in a mechanically isolated single sarcomere.

1.2.3 *Objective #3.*

It has been suggested, and we have reported (Herzog & Leonard, 2002) that force enhancement might be associated with a passive structural element and not with actin-myosin based cross-bridge interactions. One way to test this hypothesis would be to slowly pull myofibrils beyond actin-myosin filament overlap and see what active and passive forces develop. If a passive structural element plays a role in force enhancement, we would expect differences in active and passive forces beyond overlap, while if it played no role, we would expect the active and passive forces to be the same beyond actin-myosin overlap. Specific aim #3 of this thesis was to test if force enhancement is observed in the absence of actin-myosin based cross-bridge forces.

1.3 Overview of dissertation

This thesis continues with a review of the relevant literature (Chapter 2) which provides a historical framework for the studies and the background information on force enhancement following stretch and the role of titin as a potential mechanism in force enhancement and failure. Specific aim #1 is addressed by chapter 3, specific aim #2 is

addressed by chapter 4 and specific aim #3 is addressed by chapters 5 and 6. Chapters 3 to 6 are written as separate manuscripts for publication. The thesis closes with a summary (Chapter 7) which summarizes the findings and proposes future research directions and questions.

Chapter Two: Literature Review

2.1 Force production in skeletal muscle

2.1.1 *Overview of muscle architecture*

The primary function of skeletal muscle is to change length and produce force across joints which results in movements such as running, swimming or flying. Skeletal muscle is a form of striated muscle which at the largest anatomical scale spans joints and is surrounded by a tough fascia layer as well as by a secondary layer called the epimysium which includes fat cells and collagen fibres. The first step down in structural size is to the muscle fascicle which is a collection of muscle fibres surrounded by a connective tissue sheath called the perimysium. A further reduction in anatomical scale results in a single muscle cell (fibre) which is a collection of myofibrils arranged in series and in parallel. A single fibre is enveloped by a connective tissue layer called the endomysium as well as a membrane associated with ion movement (the sarcolemma). Each fibre is associated with a single motoneuron which is responsible for the activation of that cell. Stepping down further is the structure known as the myofibril. A myofibril is a collection of serially arranged sarcomeres, with each sarcomere being the contractile unit of muscle (Au, 2004). The sarcomere is composed of a complex network of proteins such as the thick and thin filaments as well as the Z-disc and titin. The portion of the sarcomere which contains the Z-disc (composed primarily of α -actinin; (Blanchard *et al.*, 1989)) and the thin filament, is termed the I-band while the A-band region includes the entire thick filament plus the area where the thick filament and the thin filament interdigitate. The thin filament is composed primarily of actin but also includes troponin, tropomyosin (Ebashi & Ebashi, 1965) and nebulin (Wang & Williamson, 1980). The thick filament is composed primarily of myosin but also includes myosin binding protein C (Starr & Offer, 1971) as well as tightly bound titin (Furst D.O. *et al.*, 1988). The giant protein titin spans the half-sarcomere, connecting the Z-disc to the center of the thick filament (Houmeida *et al.*, 1995).

2.1.2 *Sliding filament cross-bridge model*

Thick filaments are composed of myosin molecules with a molecular weight of 420,000 Daltons and these molecules are formed into two chained helical rods with two heads which can interact with actin. Thin filaments are composed of G-actin subunits formed into F-actin filaments combined with troponin, tropomyosin and nebulin (Squire, 1997). The current widely accepted theory for the contraction of muscle is based on findings demonstrating that myosin and actin filaments act like rods with the myosin drawing the actin into the A-band (Huxley & Hanson, 1954; Huxley & Niedergerke, 1954) rather than the previously held view that the two filaments became bound together during activation and that shortening or crumpling of this assembly resulted in the shortening of muscle (Huxley, 1957a). The first description of the bands or striations in skeletal muscle being due to two overlapping sets of filaments (Hanson & Huxley, 1953) was important in that the overlap of the filaments set the stage for thinking that the filaments could slide with respect to each other. The precise process whereby force could be generated during this relative sliding movement was not described in 1954 but the authors of both papers alluded to “actin-myosin linkages” (Huxley & Hanson, 1954) and suggested that “if a relative force is generated between actin and myosin at each of a series of points in the region of overlap then the tension per filament should be proportional to the number of these points...” (Huxley & Niedergerke, 1954). This idea of “linkages” producing force in muscle was expanded to include a mathematical framework for predicting force based on filament overlap, assumed actin and myosin to be essentially inextensible (Suzuki & Sugi, 1983) or extensible only to a very small extent (Kojima *et al.*, 1994) and allowed for an actin binding pocket on a movable myosin “side piece” which oscillated back and forth due to thermal agitation, Figures 2.1 and 2.2 (Huxley, 1957a).

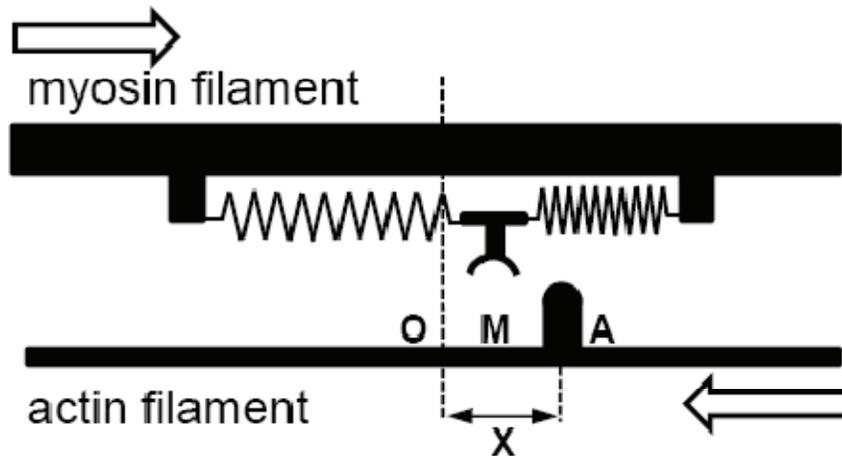


Figure 2.1: Proposed cross-bridge mechanism whereby myosin filament attaches to actin. Binding pocket M moves by Brownian motion and can attach to actin binding site A. Successful binding of M to A then results in the bound complex moving towards O (equilibrium position) producing the relative movement of actin with respect to myosin as shown by the arrows. Adapted from Huxley, 1957a.

This thermal ratchet incorporated a mechanism whereby action that pulled the actin towards the center of the myosin was favoured strongly over the reverse, thus generating stable shortening of the muscle. This theory incorporated attachment and detachment rates that were derived from experimental work by others (Hill, 1938) but did not accurately predict the heat produced in muscle during eccentric contractions.

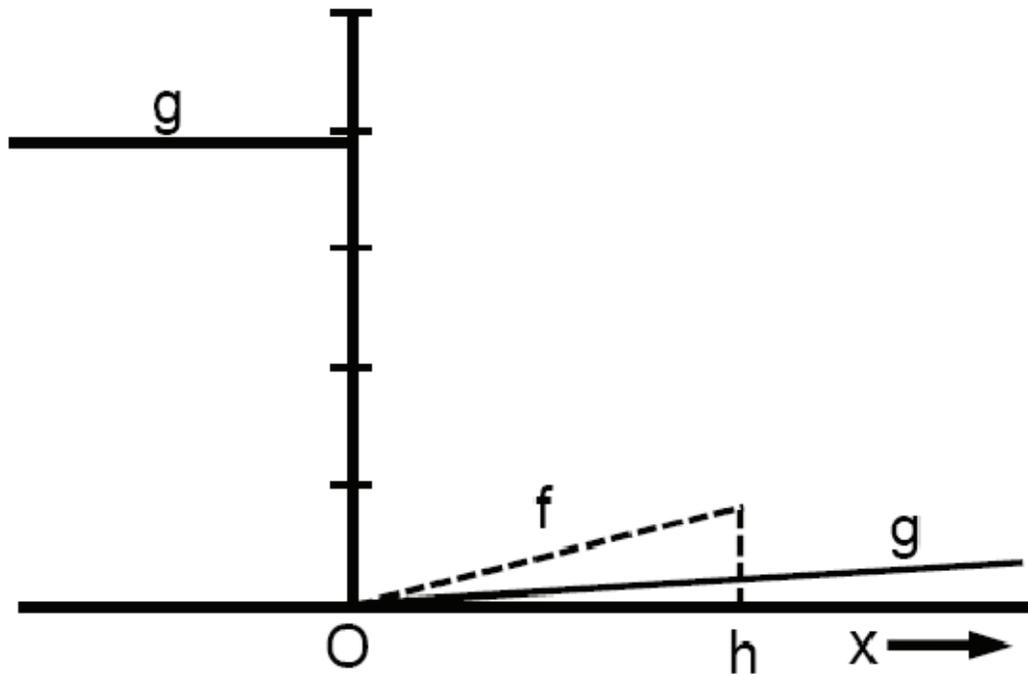


Figure 2.2: Attachment and detachment rates as a function of x distance from the original cross-bridge model. Attachment (f) and detachment (g) rates for M and A from Figure 2.1 for positive values of X result in active force production while the high detachment rate (g) to the left of equilibrium prevents force build-up which would inhibit shortening of the muscle. Adapted from Huxley, 1957a.

Refinements to the model were added to include a working stroke of 10 nm, 3 working states and an elastic region on the cross-bridge (Huxley & Simmons, 1971). Further work using crystallography described the structural basis of the cross-bridge model (Rayment *et al.*, 1993) with 5 states and that following the power stroke and release of ADP, ATP would re-bind to the myosin, resulting in the unbinding of rigor bound cross-bridges.

2.1.3 *Force length relationship*

Experimental evidence in frog semitendinosus single fibres had shown that for isometric contractions, force produced at resting length was maximal while activation at shorter or longer fibre length resulted in less force, with greater length increments away from

resting length resulting in larger decrease in force (Ramsey & Street, 1940). Concerns about sarcomere length striation irregularities within the muscle fibre meant that a specialized fibre preparation was needed, where a region of the fibre was “segment clamped” which would prevent these irregularities (at least for the portion of the fibre which was “clamped”) and allow for a cleaner determination of the force length relationship for fibres tested isometrically on the descending limb of the relationship. This clever experiment was performed by (Gordon *et al.*, 1966b) and the results, particularly the linear decrease of force resulting from each incremental increase in sarcomere length, provided strong support for the proposed cross-bridge model. Certain assumptions were made, specifically that myosin binding sites were distributed uniformly along the actin filament, that the actin and myosin filament must be inextensible and that the probability of an attachment for each cross-bridge is independent of all others and produce essentially the same force. The profile of the force-length relationship shows a plateau region containing a flat force-sarcomere length region as expected, and a linear descending limb. The experimental data fitted the predictions of the myofilament filament length data very well. Figure 2.3 illustrates the experimental data based force-length relationship for a single frog fibre with schematic representations showing key transition points in the relationship based on filament lengths reported in the literature.

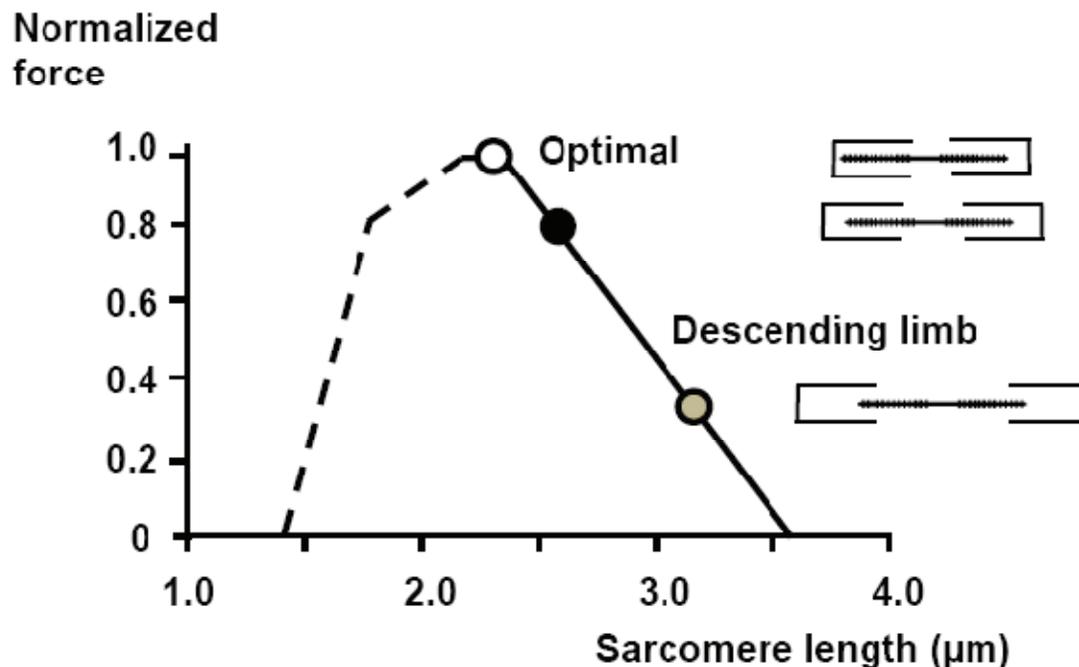


Figure 2.3: Representative force-length relationship. Optimal sarcomere length (SL) (white circle) provides optimal actin-myosin filament overlap on the plateau region of the relationship and maximal force for a given activation condition. As SL increases on the descending limb of the relationship (black solid line), filament overlap decreases and so active force produced also decreases (black and grey circles respectively). Adapted from Gordon et al., 1966b.

The length of vertebrate actin and myosin filaments has been reported extensively (Herzog *et al.*, 1992; Walker & Schrodt, 1974; Page & Huxley, 1963; Trombitas *et al.*, 1993; Granzier *et al.*, 1991; Burkholder & Lieber, 2001; Kruger *et al.*, 1991; Littlefield & Fowler, 2002) with the myosin being reported as relatively invariable at 1.6 µm while actin filaments vary with the species investigated; rat at 1.04 µm to 1.09 µm, cat at 1.12 µm, rabbit at 1.07 µm to 1.09 µm, frog at 0.925 µm to 0.975 µm, monkey at 1.16 µm and human at 1.27 µm. One set of experiments (Sosa *et al.*, 1994) involved tracking filament changes during contraction and then quick freezing the specimens to determine filament length; no filament changes were observed and lengths were essentially the same as those published by other investigators. Changes in actin filament length will not fundamentally alter the shape of the force length relationship but they will alter some key parameters of the relationship. Specifically, the length at which the plateau starts and the

sarcomere length where myofilament overlap is lost, will shift rightwards along the relationship with longer actin filaments. The length of the plateau is determined by the bare zone at the center of the myosin and is reported as typically being 0.17 μm (Walker & Schrodt, 1974). Regardless of the variation found in the length of the thin filament across species, it is well accepted that sarcomere length is the principle determinate of the force produced during an isometric contraction.

2.2 History Dependent Behaviour of Muscle

2.2.1 *General description of muscle force with active stretch*

Active stretching of muscle (eccentric contractions) produces a rise in force during the stretch but once the stretch has stopped and the muscle has reached a steady length (isometric contraction) the force decays and reaches a steady state, see Figure 2.4, (blue trace). The speed and the magnitude of the stretch both influence the force produced during the stretch, while the final length of the muscle following the stretch determines the steady state force after the stretch.

2.2.2 *Increased force with stretch*

It is important to distinguish between the two types of force increase observed with activated muscle lengthening. The first type of force increase is called “force enhancement during stretch” and is observed in activated muscle subjected to a ramp lengthening. It manifests itself as an increase in force that contains an initial rapid rise in tension, followed by a slower increase in force that rises to a peak with the end of the ramp stretch (Mutungi & Ranatunga, 2001) and then decays with time (Noble, 1992). While not universally understood, these forces are thought to result from the forced detachment of weakly bound cross-bridges and then, the increase of forced detachment and rapid reattachment of strongly bound cross-bridges. The lower ATP consumption observed during stretch when compared to isometric contractions has been attributed to these weakly bound cross-bridges, which are able to resist the force of stretch without large energy requirements (Linari *et al.*, 2003). This rise in force during active stretch

was described previously (Hill, 1953) and is sensitive to the speed of lengthening; greater speed of lengthening resulting in greater force during the stretch. This is part of the force-velocity relationship and was accounted for in the cross-bridge model by A. F. Huxley in 1957, although the predictions of force during stretch were over estimated by the model.

2.2.3 *Increased force following stretch*

The second type of force increase relating to lengthening of active muscle is insensitive to the speed of stretch but is influenced by the magnitude of the stretch imposed. This force is characterized by a long lasting increase in the steady state force following a stretch when compared to an isometric contraction at the same final specimen length (Abbott & Aubert, 1952;Edman *et al.*, 1982;Edman *et al.*, 1978;Cavagna & Citterio, 1974) and is termed the “residual force enhancement following stretch”. This extra force is long lasting (up to 20 seconds in whole cat muscle (Herzog & Rassier, 2002), is relatively insensitive to the speed of the stretch (Edman *et al.*, 1978) but it is dependent on the amplitude of the stretch (Edman *et al.*, 1978;Edman *et al.*, 1982;Abbott & Aubert, 1952;Sugi & Tsuchiya, 1988). It has been observed on the ascending limb of the force-length relationship by some (Hisey *et al.*, 2009;Herzog & Leonard, 2002;Peterson *et al.*, 2004) but not by others (Edman *et al.*, 1978;Edman *et al.*, 1982;Julian & Morgan, 1979b), the plateau region (Peterson *et al.*, 2004) and consistently on the descending limb (Julian & Morgan, 1979b;Abbott & Aubert, 1952;Morgan *et al.*, 2000). This extra force has been observed at different structural levels in muscle; whole human muscle in vivo (Lee & Herzog, 2002;De Ruyter *et al.*, 2000;Oskouei & Herzog, 2005;Pinniger & Cresswell, 2007), whole muscle in situ (Pinniger *et al.*, 2006;Morgan *et al.*, 2000;Schachar *et al.*, 2002;Abbott & Aubert, 1952;Deleze, 1961), single fibres (Edman *et al.*, 1978;Bagni *et al.*, 2002;Sugi & Tsuchiya, 1988;Sugi, 1972;Peterson *et al.*, 2004;Hill, 1977) but has never been studied at the myofibrillar level.

Figure 2.4 shows two traces, one where a cat soleus muscle was contracted isometrically (red trace) and a second trace (blue) with the same stimulation parameters but the muscle was initially at a shorter length at the onset of stimulation and then actively stretched to a

longer length which corresponded to the same length as the isometric contraction. The enhanced force is observed to be long lasting and remains above the isometric force for the duration of the test.

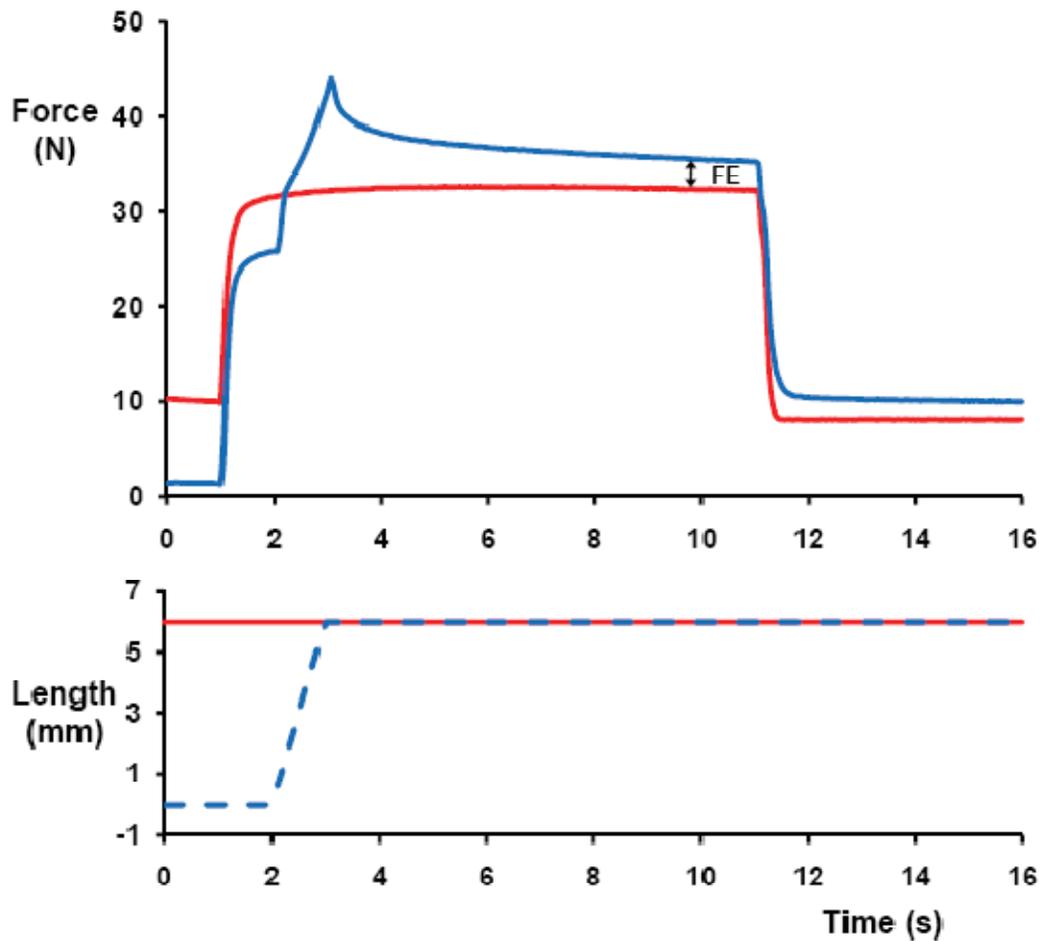


Figure 2.4: Force enhancement data for intact cat soleus muscle. Enhanced force following active stretch (top panel blue trace) is seen at steady state (10 s) when compared to the isometric force (top panel, red trace) at the same final muscle length. Bottom panel shows the muscle length for the red isometric reference with blue representing the activated muscle, initially at a shorter length [and stronger when compared to the red trace, once passive forces are removed from the red trace] and then actively stretched to the same final length. Adapted from Herzog and Leonard, 2002.

2.2.4 *Possible mechanisms*

Within the framework provided by the force length relationship (Gordon *et al.*, 1966b), residual force enhancement following stretch cannot be explained since the lengthened activated muscle and the isometrically activated muscle are at the same final test length. They must therefore have the same amount of filament overlap and produce the same force. This observation poses obvious difficulties for the cross-bridge model (Noble, 1992) but mechanisms have been proposed to address the models short comings.

2.2.4.1 Altered cross-bridge kinetics

Cross-bridge kinetics have been suggested as a possible mechanism. The extra force following active stretch may originate within the cross-bridges themselves with extra force per cross-bridge being produced and/or an increase in the number of cross-bridges bound to the actin. This would be testable by a stiffness test (a rapid and small stretch) which could show the proportion of attached cross-bridges between the two experimental conditions. Results of such stiffness tests have been rather ambiguous, with some groups observing increased stiffness (Herzog & Leonard, 2000;Linari *et al.*, 2000;Rassier & Herzog, 2005) while others have found no increase in stiffness following active stretch (Sugi & Tsuchiya, 1988). The difficulty with this cross-bridge kinetics model is that the extra force/more bound cross-bridges must persist over many attachment/detachment cycles following the length change and last potentially for many seconds. If force enhancement following stretch is based in cross-bridges, and if stiffness is not increased (implying more attached cross-bridges), then more force generated for each cross-bridge would produce the enhanced force observed. No general agreement exists on whether altered cross-bridge kinetics is the mechanism underlying force enhancement with some researchers arguing they are (Rassier *et al.*, 2005) while others conclude this is not the case (Edman *et al.*, 1982;Bagni *et al.*, 2002;Julian & Morgan, 1979b).

2.2.4.2 Sarcomere length non-uniformity

A second possible mechanism responsible for force enhancement following stretch has been attributed to the development of sarcomere length non-uniformities on the descending limb of the force-length relationship (Morgan *et al.*, 2000;Edman & Tsuchiya, 1996;Morgan, 1990;Julian & Morgan, 1979b). This proposed mechanism is underpinned by the presence of shorter (stronger) and longer (weaker) sarcomeres in a fibre, with the shorter sarcomeres being located nearer the ends of the fibre, and the inherent instability of the descending limb of the force-length relationship (Hill, 1953;Zahalak, 1997). With these two preconditions met and the fact that force production is dependent on the amount of filament overlap present as described by the force-length relationship (Gordon *et al.*, 1966b), then eccentrically contracting muscle will have some sarcomeres that are initially less capable of supporting the stretch force and will be lengthened and hence become even weaker. This progression of ever longer sarcomere length and resulting increased weakness inevitably results in the sarcomere being rapidly pulled to a length where no filament overlap is possible and all the force is sustained by purely passive structures (Morgan, 1994;Morgan, 1990). In experiments comparing isometric contractions to eccentric contractions on the descending limb where residual force enhancement is observed, the overstretching of some sarcomeres during stretch will allow other sarcomeres to lengthen less than they otherwise would have (resulting in these sarcomeres being at shorter lengths than those in the purely isometric reference contraction) and therefore being stronger, thus accounting for the enhanced force compared to the isometric test. See Figure 2.5.

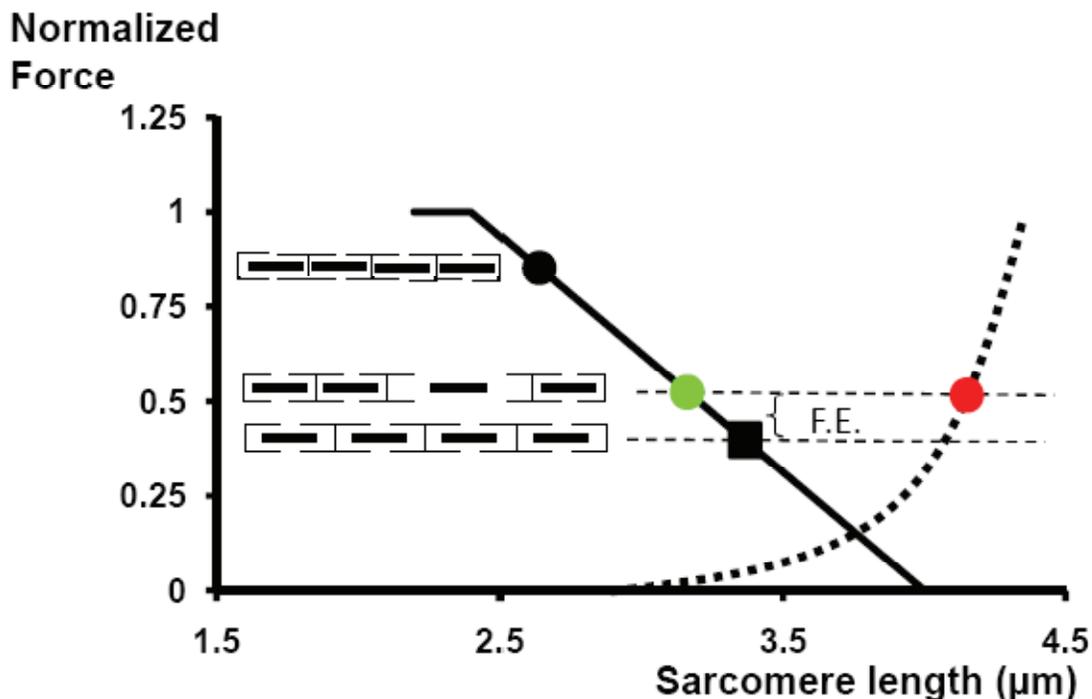


Figure 2.5: Sarcomere length non-uniformity mechanism for force enhancement. Isometric contractions at short sarcomere length (SL) and long SL, black circle and black square respectively produce force based on degree of filament overlap. Eccentric contraction from short SL (2.6 μm) to 3.4 μm results in a muscle that is the same length as the isometric 3.4 μm (black square) but the development of SL non-uniformities results in some overstretched sarcomeres placed beyond actin-myosin filament overlap where they are supported purely by passive force structures (heavy dashed line, red circle) with other sarcomeres now shorter (and stronger, green circle) than the isometric reference (black square) resulting in the enhanced force (FE).

Evidence for overstretched sarcomeres has come indirectly from either rapid freezing or chemical fixation of the eccentrically contracted muscle followed by histological preparation and imaging (Brown & Hill, 1991; Lieber *et al.*, 1996; Wood *et al.*, 1993; Talbot & Morgan, 1996). Experiments investigating sarcomere overstretching or sarcomere dispersion with direct observation have concluded that overstretching (or disruption) was not observed (Telley *et al.*, 2006b), that yield was not observed at full activation (Shimamoto *et al.*, 2009) or that non-uniformities were not able to account for the observed residual force enhancement following stretch (ter Keurs *et al.*,

1978;Pinniger & Cresswell, 2007;Rassier *et al.*, 2003a). The presence of sarcomere length non-uniformities has been described on eccentrically contracted muscle using techniques such as laser diffraction on single fibres (Edman *et al.*, 1978;Edman *et al.*, 1982) and direct length measurements of individual sarcomeres in myofibrils (Rassier *et al.*, 2003a). Since sarcomere overstretching was not observed in either of these experiments, the question must be posed: “ how do sarcomeres at steady state length and with different sarcomere lengths (and presumably different amounts of filament overlap) support the same force?” In whole muscle or even single fibres it is possible that forces are supported through extra-sarcomeric structures such as collagen (Purslow, 1989;Purslow & Trotter, 1994), the sarcolemma (Dulhunty & Franzini-Armstrong C., 1975), basement proteins like desmin (Shah *et al.*, 2004) or lateral force transmission (Jaspers *et al.*, 1999;Street, 1983;Huijing, 1999) but in a single myofibril, none of these structures are present and so the force supported by each sarcomere (in series) must be identical.

2.2.4.3 Engagement of a parallel elastic element

Force enhancement may arise from cross-bridges or sarcomere length non-uniformities but has also been proposed to arise from the recruitment of a passive element (Edman *et al.*, 1978) intrinsic to the sarcomere (Edman & Tsuchiya, 1996). Force enhancement following stretch has been suggested to be comprised of two components, an active and a passive component (Kosterina *et al.*, 2009;Herzog & Leonard, 2002;Lee & Herzog, 2002;Lee *et al.*, 2007;Rassier & Herzog, 2004;Joumaa *et al.*, 2007;Rassier *et al.*, 2005) with the giant protein titin thought likely to be involved in some way. Figure 2.4 shows the total force enhancement (at approximately 10 seconds) and the passive force enhancement is observed once the muscle has been deactivated (12 seconds and beyond). If there were no passive force enhancement present following muscle deactivation, the force traces should overlap. Passive force enhancement is a significant fraction of the total force enhancement and can contribute up to 84% of the total force enhancement in cat soleus muscle (Herzog & Leonard, 2002) and up to 59% in myofibrils (Joumaa *et al.*, 2007). The passive force enhancement can only arise within a parallel elastic element

since the enhanced force persists following deactivation (relaxing) of the muscle; this behaviour precludes a contribution by cross-bridges or by sarcomere overstretching. The most likely candidate for this parallel elastic element is titin (Herzog & Leonard, 2002; Tatsumi *et al.*, 2001; Edman & Tsuchiya, 1996; Joumaa *et al.*, 2007). Titin is a 1 μm long protein with a molecular weight of up to 3.7 megaDaltons which spans the half-sarcomere, (Furst D.O. *et al.*, 1988; Tskhovrebova & Trinick, 2002). Figure 2.6 shows a schematic representation of several titin isoforms and the location of the titin molecule within the sarcomere.

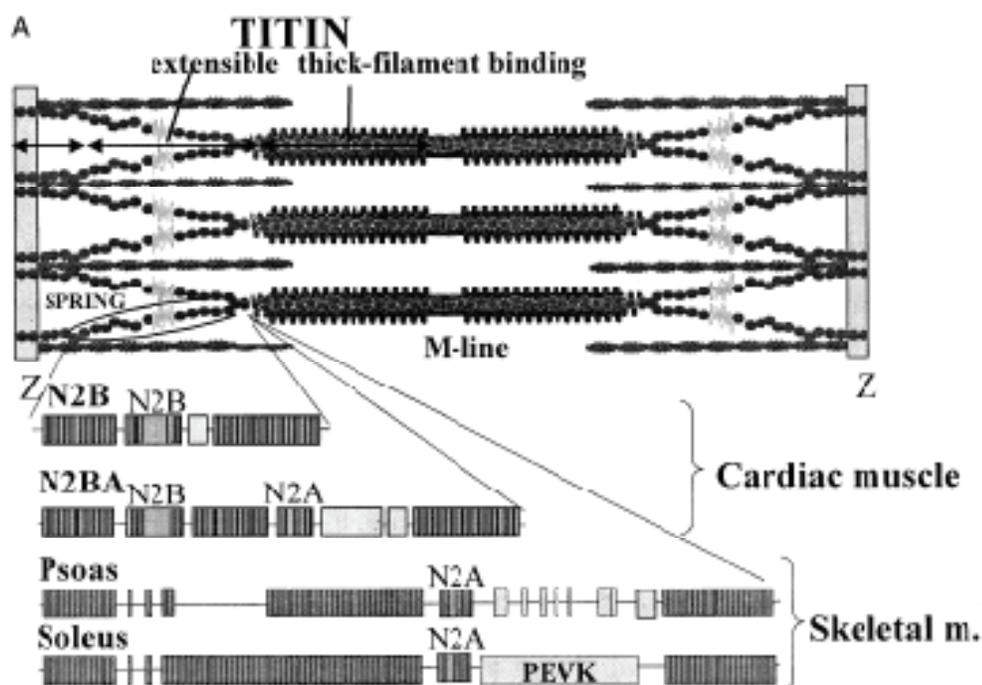


Figure 2.6: Layout of titin in sarcomere. Titin spans the Z-disc to the M-line region of the sarcomere. Titin in and near the Z-disc is inextensible as is the titin in the A-band region of the sarcomere (here titin binds to the thick filament). The remaining I-band region is elastic and functions as a molecular spring. The molecular spring region varies in sequence due to differential splicing. The sequences of four different isoforms are shown at the bottom, made up of the Ig domains, the PEVK domain and the unique sequences.

From Granzier and Labeit, (2006). *Used with permission.*

Titin is thought to provide A-band centering (Horowitz *et al.*, 1986; Granzier & Labeit, 2007) and help return the sarcomere to its resting length after stretch (Trombitas & Pollack, 1993; Maruyama *et al.*, 1977). Furthermore, titin is thought responsible for the production of the majority of passive force observed in muscle fibres and myofibrils, Figure 2.5; heavy dashed line (Horowitz, 1992; Linke *et al.*, 1994; Wang *et al.*, 1991; Granzier *et al.*, 2000; Freiburg *et al.*, 2000; Linke *et al.*, 1996; Trombitas *et al.*, 1998; Maruyama, 1997; Prado *et al.*, 2005).

Since force enhancement following stretch is observed to be long lasting (up to 25 seconds after the end of the ramp stretch) and is insensitive to the speed of stretch but increases with the magnitude of the stretch imposed (Herzog & Leonard, 2002), a structural protein could nicely explain the passive component of residual force enhancement. The exact mechanism whereby titin might be recruited to contribute force to the residual force enhancement following active stretch is unknown. It has been suggested that the binding of calcium to regions of the titin molecule (specifically the PEVK region) affects the intrinsic stiffness of titin (Tatsumi *et al.*, 2001; Labeit *et al.*, 2003b; Bagni *et al.*, 2002; Campbell & Moss, 2002; Herzog & Leonard, 2002; Granzier *et al.*, 2002). For example, it has been suggested that the PEVK region of titin has a greater affinity for calcium and so becomes stiffer during eccentric compared to isometric contractions (Bagni *et al.*, 2002; Herzog & Leonard, 2002; Campbell & Moss, 2002), but recent results suggest that calcium binding to titin only accounts for a very small part (maximally 25%) of the observed passive force enhancement following stretch.

Titin can also become stiffer if the resting length of the molecule is reduced. Titin-actin interactions have been well documented in cardiac and skeletal muscle (Granzier *et al.*, 2002; Granzier *et al.*, 1997; Li *et al.*, 1995; Linke *et al.*, 2002; Kulke *et al.*, 2001; Yamasaki *et al.*, 2001) and titin is known to bind the actin at the Z-disc, making this region of titin functionally stiff (Linke *et al.*, 1997; Kulke *et al.*, 2001; Linke *et al.*, 2002; Trombitas & Granzier, 1997).

2.3 Summary

There is overwhelming evidence that residual force enhancement is a property of skeletal muscle, but this history dependent behaviour is not accounted for by the cross-bridge model of muscle contraction. Force enhancement following active lengthening appears to have two contributory elements. One is an active component that may result from cross-bridge actions or sarcomere length non-uniformities (or a combination of both), or may involve the recruitment of a parallel elastic element such as titin in some force-regulated manner. The second is a passive force enhancement component which appears to originate in a parallel elastic element exclusively (since it is observed in relaxed muscle following eccentric contraction) but whether this passive force enhancement is due solely to calcium induced stiffness changes or to changes in the native length of titin, or a combination of the two, remains to be resolved. There is not enough evidence to preclude any one of these three mechanisms as the underlying source of the force observed in the total residual force enhancement and therefore all three may contribute to this history dependent behaviour. Myofibrils offer the best hope of understanding the nature of force enhancement since the preparation offers individual sarcomeres in series and the ability to determine length for each, a lack of passive force structures other than titin, the ability to manipulate cross-bridge kinetics through the use of chemical agents such as BDM or various concentrations of calcium, as well as being able to selectively treat filaments by means of chemical agents such as KCl, gelsolin and trypsin.

Chapter Three: Residual Force Enhancement in Myofibrils and Sarcomeres

3.1 Introduction

It has been known for a long time that when a muscle is stretched its force increases, and although the precise nature of this increase remains a matter of debate (e.g., Pinniger *et al.*, 2006), it was conceptually incorporated into the general behaviour of muscle contraction through the force-velocity relationship (e.g., Hill, 1938), and is accounted for in the cross-bridge theory (Huxley, 1957a). Similarly, when a muscle is actively stretched, and then held at the stretched length long enough for all transient force response to disappear, the isometric steady-state force at the stretched length is greater than the steady-state isometric force at that same length for a purely isometric contraction (Figure 3.1). This observation has first been described systematically by Abbott and Aubert (1952), and has been referred to as residual force enhancement (Edman *et al.*, 1982). In contrast to the force increase during stretch, the residual force enhancement following stretch has not been accounted for in the cross-bridge theory (Huxley, 1957a; Huxley, 1969; Huxley & Simmons, 1971; Rayment *et al.*, 1993). In fact, it can be shown that cross-bridge models are unable to predict residual force enhancement, except if one allows for muscle stretch to change the kinetics of the cross-bridge cycle permanently (Walcott & Herzog, 2006).

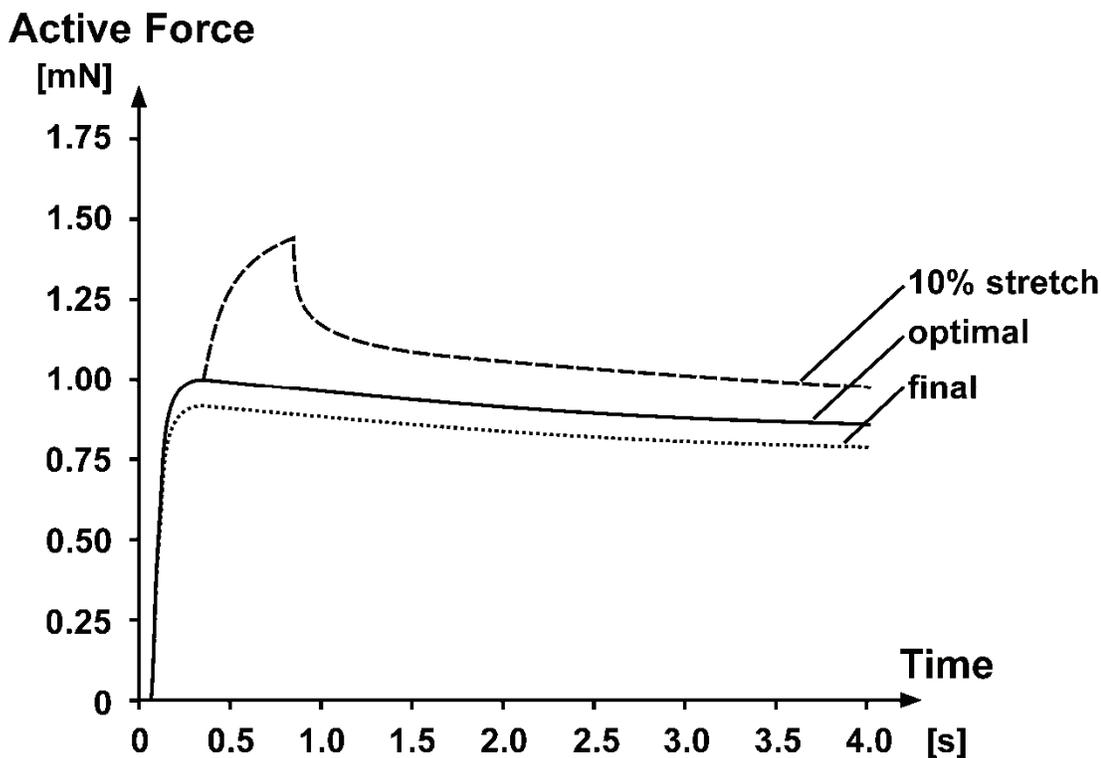


Figure 3.1: Force-time histories obtained from a single fibre of frog tibialis anterior for two isometric contractions at optimal (optimal) and final (final) lengths, and for a contraction in which the preparation was stretched from an initial to a final length. The stretch magnitude corresponded to 10% of the fibre length, and was performed at a speed of approximately 20% fibre length/s. Following the stretch, the fibre was held long enough for all force transients to disappear, so that the steady-state forces could be compared. The increase in isometric force following the stretch compared to the isometric force at the final length is defined as the residual force enhancement. The increase in isometric force following the stretch compared to the isometric force obtained at the optimal sarcomere length indicates the increase in force above the plateau of the force-length relationship. Adapted from (Rassier *et al.*, 2003c).

Residual force enhancement is known to increase with the magnitude of stretch (Abbott & Aubert, 1952;Edman *et al.*, 1978;Herzog & Leonard, 2002;Herzog & Leonard, 2005), at least to a certain threshold value (Bullimore *et al.*, 2007), appears to be independent of the speed of stretch (Edman *et al.*, 1982), but is sensitive to the initial length of the muscle (Edman *et al.*, 1982;Edman *et al.*, 1978). Residual force enhancement is long

lasting (>20s in cat soleus - Herzog & Rassier, 2002), but can be abolished by deactivating the muscle long enough for force to drop to zero (Abbott & Aubert, 1952;Morgan *et al.*, 2000). It occurs in human muscles activated electrically (De Ruyter *et al.*, 2000) and voluntarily (Lee & Herzog, 2002), in isolated muscle preparations (e.g., Abbott & Aubert, 1952;Herzog & Leonard, 2002), and in single fibre or fibre bundle preparations (Sugi & Tsuchiya, 1988;Edman *et al.*, 1978;Edman *et al.*, 1982;Bagni *et al.*, 2004;Bagni *et al.*, 2002). Despite an abundance of experimental observations, the mechanisms underlying residual force enhancement remain a matter of debate (Herzog *et al.*, 2006;Herzog & Leonard, 2006;Morgan & Proske, 2006).

It has been suggested that residual force enhancement has a passive and an active component (Edman *et al.*, 1978;De Ruyter *et al.*, 2000;Herzog & Leonard, 2002;Herzog *et al.*, 2006). The passive component has been associated with the molecular spring titin and its interaction with calcium upon activation (Labeit *et al.*, 2003b;Bagni *et al.*, 2004;Bagni *et al.*, 2002;Joumaa *et al.*, 2007). The active component is associated with the so-called sarcomere length non-uniformity theory (Morgan *et al.*, 2000;Morgan, 1990;Morgan, 1994). This theory is based on the idea that sarcomeres on the descending limb of the force-length relationship are unstable as suggested by Hill (1953), and when stretched, this instability will cause differential elongation of sarcomeres: some might hardly be stretched at all, while others are stretched beyond actin-myosin filament overlap and are only held in force equilibrium with the “active” sarcomeres by their passive forces (e.g., Morgan *et al.*, 2000;Morgan & Proske, 2006). However, recently we demonstrated that sarcomeres can be perfectly stable on the descending limb of the force-length relationship in a single myofibril preparation (Rassier *et al.*, 2003a), but we were unable to measure force and sarcomere length simultaneously. Therefore, force enhancement could not be determined. Others have measured force and sarcomere length simultaneously in myofibril preparations following stretch (Telley *et al.*, 2006b), but they did not wait long enough (1s) for force to reach steady-state values and sarcomeres to finish the transient length changes associated with myofibril stretching, thus no insight into force enhancement was obtained.

The determination of force enhancement in single myofibrils is crucial for many reasons. First, if there was force enhancement in myofibrils, it would be possible to eliminate extra-sarcomeric structures as a cause; second, if there was force enhancement in the presence of steady sarcomere lengths, we could eliminate sarcomere length instability as a mechanism; third, since sarcomeres are mechanically arranged in series within a myofibril, measuring the force at the end of a myofibril gives the instantaneous force in each sarcomere; and finally, by measuring individual sarcomere lengths, it would be possible to calculate force enhancement for individual sarcomeres, thus providing novel insight into how force enhancement may affect the basic contractile unit of muscle.

3.2 Methods

3.2.1 Extraction of single myofibrils and experimental set-up

Strips of rabbit psoas were dissected and tied to small wooden sticks. These samples were stored in a rigor solution at -20°C . On the day of the experiments, the muscle strips were cut into pieces of about 2mm length using a #15 scalpel blade, and subsequently blended using previously described protocols (Rassier *et al.*, 2003a). The blended muscle was then put into a chamber whose bottom was a glass cover slip placed on top of an inverted microscope (Zeiss, Axiovert 200M, Germany). After a sufficient time for stabilization (5-10 minutes), the rigor solution was replaced with a relaxing solution, and myofibrils in suspension were washed away leaving those attached to the bottom of the experimental chamber. A myofibril with a good striation pattern was then selected and attached to a glass needle and motor at one end, and to a pair of nano-levers (Bartoo *et al.*, 1993; Fauver *et al.*, 1998) which allowed for myofibril force measurements, at the other end. A more complete treatment of the methods can be found in Appendix A of this thesis.

The image of the attached myofibril was projected onto a high-density linear photodiode array (Schafter and Kirschhoff Model SK10680DJR, Hamburg, Germany, theoretical

resolution of 6 nm) to give tracings of the myofibrillar striation pattern for identification of the A- and I-bands and the Z-lines. Sarcomere lengths were calculated from Z-line to Z-line, or when these could not be identified reliably, from the centroids of adjacent A-bands. Half-sarcomere lengths were calculated from the Z-line to the centroid of the corresponding A-bands.

3.2.2 *Protocol*

Once a myofibril was ready for mechanical testing, and a clear striation pattern could be observed, a ten-minute rest was given and then the relaxing solution was replaced by the activating solution causing contraction of the myofibril. Six myofibrils were tested isometrically at an average sarcomere length of 2.4 μm and 3.4 μm and the active and passive forces were determined. A further twelve myofibrils were activated isometrically at a short length, stretched while activated, and then held isometrically for another 30s until force transients had disappeared. Six of these myofibrils were stretched from a nominal average sarcomere length of 2.4 μm to 3.4 μm , while the remaining six myofibrils underwent a series of stretches, starting at different average sarcomere lengths and being subjected to variable stretch magnitudes (between 12% and 38% of the initial sarcomere length). For all myofibrils, the mid-diameter was measured using a calibrated eyepiece, and myofibril cross-sectional areas were calculated assuming a cylindrical myofibril shape. Forces were normalized relative to the cross-sectional areas to provide myofibril stress, which allowed for comparison of different sized preparations.

3.2.3 *Solutions*

The rigor, relaxing and activating solutions were identical to those described previously in our studies (e.g., Rassier *et al.*, 2003a). Briefly these are as follows:

Rigor solution (storage and homogenizing): 50 mM 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris), 100 mM sodium chloride, 2 mM potassium chloride, 2 mM magnesium

chloride, 10 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) all adjusted to pH=7.0.

Relaxing solution (passive tests): 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 64.4 mM potassium propionate, 9.45 mM sodium sulphate, 5.23 mM magnesium propionate, 10 mM EGTA, 0.188 mM calcium chloride, 7 mM adenosine triphosphate (ATP) at pCa = 8.0 and adjusted to pH = 7.0.

Activating solution (active contraction tests): 10 mM MOPS, 45.1 mM potassium propionate, 5.21 mM magnesium propionate, 9.27 mM sodium sulphate, 10 mM EGTA, 9.91 mM calcium chloride, 7.18 mM ATP, 10 mM creatine phosphate at 3.5 pCa and adjusted to pH = 7.0.

3.2.4 *Definition of force enhancement*

Force enhancement for the myofibril preparation was defined as the difference in the steady-state isometric force following the stretch (test) contraction, and the purely isometric (reference) contraction at the corresponding length (i.e. length at the end of the stretch), or as the difference in steady-state isometric force obtained prior to stretch and the corresponding force after stretch corrected for the average sarcomere lengths in the myofibril prior to and following stretch and accounting for the loss of myofilament overlap during stretch in accordance with Gordon et al. (1966b) but scaled to the actin-myosin lengths of rabbit skeletal muscle (Page & Huxley, 1963; Herzog *et al.*, 1992).

Force enhancement for individual sarcomeres was obtained in the same way as defined for the whole myofibril with the exception that the individual sarcomere lengths and the corresponding filament overlap (Herzog *et al.*, 1992; Page & Huxley, 1963) and expected force for the reference and test contractions were accounted for individually. This had to be done as sarcomere length in the reference and test contractions could not be assumed to be the same, despite the same myofibril length, and since sarcomere lengths increased for all sarcomeres in each myofibril during stretching.

3.3 Results

Myofilament lengths in rabbit skeletal muscles are 1.65 μm for myosin and between 1.07 μm and 1.09 μm for actin (Page & Huxley, 1963; Herzog *et al.*, 1992). Therefore, assuming an actin length of 1.08 μm , the plateau of the sarcomere force-length relationship occurs between 2.26 μm (twice the actin filament length plus the width of the Z-line – 0.1 μm) and 2.43 μm (2.26 μm plus the width of the bare zone in the middle of myosin – 0.17 μm ; (Herzog *et al.*, 1992). Similarly, the end of the descending limb of the force-length relationship occurs at 3.91 μm (twice the actin length, plus the length of myosin, plus the width of the Z-line). Isometric reference measurements at mean sarcomere lengths of 2.4 μm and 3.4 μm gave average steady-state stresses of 194 $\text{nN}/\mu\text{m}^2$ and 43 $\text{nN}/\mu\text{m}^2$, respectively (Figure 3.2), thereby confirming expected maximal values at optimal length reported for mammalian skeletal muscles, when due account is taken of the temperature (21°C) at which the experiments were performed (Ranatunga & Wylie, 1983), and approximating the expected linear decrease in force with loss of myofilament overlap at increasing sarcomere lengths (Gordon *et al.*, 1966b).

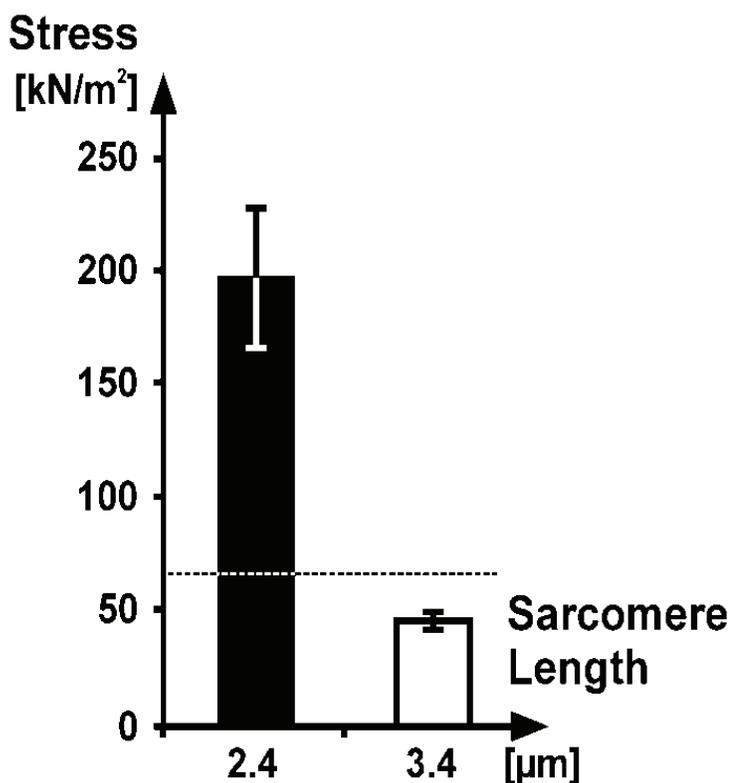


Figure 3.2: Stresses (total force/cross-sectional area) of single myofibrils (n=6) at an average sarcomere length of 2.4 μm and 3.4 μm . Note that the stress was much greater at 2.4 μm than 3.4 μm , thereby indicating that all stretch experiments were performed on the descending part of the force-sarcomere length relationship. Single myofibrils were obtained from rabbit psoas and all experiments were performed at a temperature of 21°C. Dashed horizontal line represents predicted stress for 3.4 μm based on the mean stress observed at 2.4 μm .

All myofibrils showed residual force enhancement following stretch (Figure 3.3) averaging (means \pm SD) $328 \pm 250 \text{ nN}/\mu\text{m}^2$ or $386 \pm 251\%$ of the isometric reference stresses. Similarly, all individual sarcomeres of all myofibrils showed force enhancement (Figure 3.4), but sarcomeric force enhancement within the same myofibril differed substantially because of the different lengths of sarcomeres prior to and following stretch (Figure 3.3). For 11 out of the 12 myofibrils, stretching resulted in residual force enhancement whose forces were greater than the isometric reference forces at optimal sarcomere length. The average force enhancement above plateau for the 12 myofibrils was $106 \pm 85\%$, or essentially a doubling of the maximal isometric forces obtained for

the purely isometric reference contractions, for an average stretch of $35 \pm 15\%$ of the initial sarcomere length.

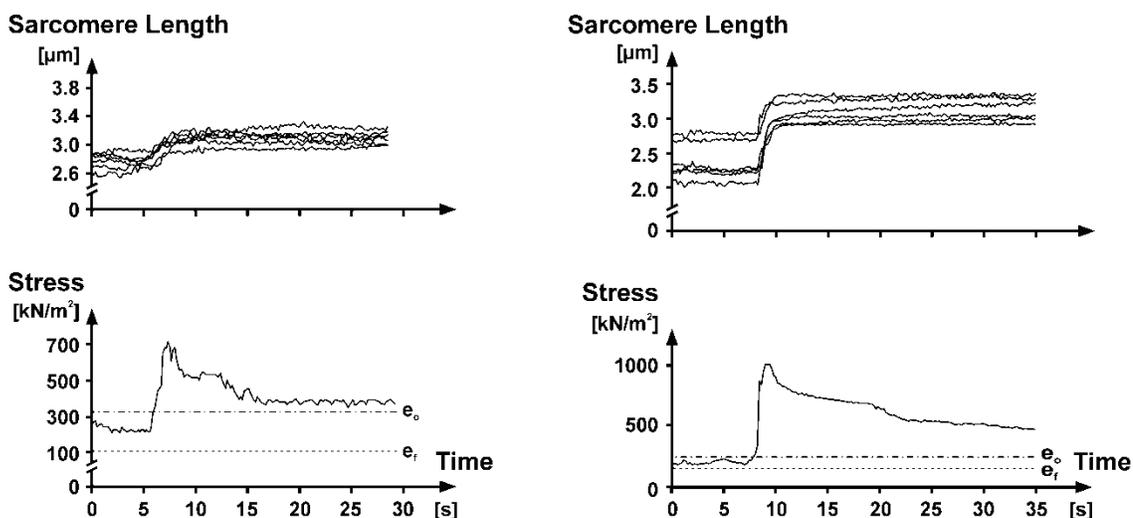


Figure 3.3: Sarcomere length and stress (force/cross-sectional area) as a function of time for two exemplar myofibrils stretched from an initial sarcomere length on the descending limb (left panel) or near the plateau (right panel) of the force-length relationship. Sarcomere lengths are non-uniform for the isometric contraction prior to stretch and remain non-uniform following stretch. Stresses increase during stretch dramatically and remain greater after stretch (for at least 25s) compared to the stresses prior to stretch, and greater than the expected stresses (based on myofilament overlap for the average sarcomere length) at the optimal length (e_0) and the final length achieved after the stretch (e_f).

Sarcomere lengths prior to and following stretch were non-uniform, and for the stretch magnitudes used here, all sarcomeres elongated during myofibril stretch, albeit not necessarily by the same amount (Figure 3.3). Statistical analysis of the slopes of the sarcomere lengths vs. time graphs for the past 10s of “steady-state” revealed that 55 sarcomeres had a zero slope, 18 sarcomeres had a small positive slope, and 6 had a small negative slope.

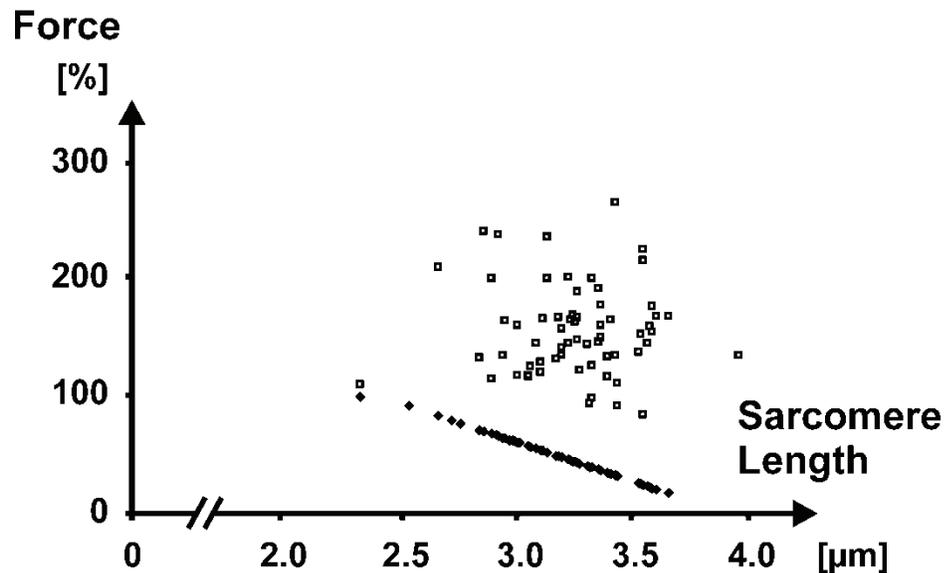


Figure 3.4: Predicted forces for single sarcomeres based on the myofilament overlap theory and the actin and myosin filament lengths for rabbit skeletal muscle (filled diamonds), and the corresponding observed sarcomere forces achieved following activated myofibril stretching (open squares).

However, all positive slopes were so small that it would have taken minutes to pull these sarcomeres beyond myofilament overlap ($3.91 \mu\text{m}$). In order to investigate if the observed sarcomere length changes were small, random fluctuations or systematic instabilities in the sense of Hill (1953) and Morgan *et al.* (2000), the shortest and longest sarcomere in each myofibril were considered further. The instability theory states that long sarcomeres are weak and short sarcomeres are strong on the descending limb of the force-length relationship (Hill, 1953), and predicts the short (strong) sarcomeres to shorten and the long (weak) sarcomeres to be stretched and pulled beyond overlap (Morgan *et al.*, 2000; Morgan & Proske, 2006). However, this expected pattern was never observed in any of the twelve myofibrils, while the opposite, the shortest sarcomere being stretched and the longest shortening, was observed in one myofibril (Figure 3.5). Therefore, any small remnant shortening or stretch of individual sarcomeres seemed to be random and not associated with sarcomere length instabilities as predicted by Hill (1953).

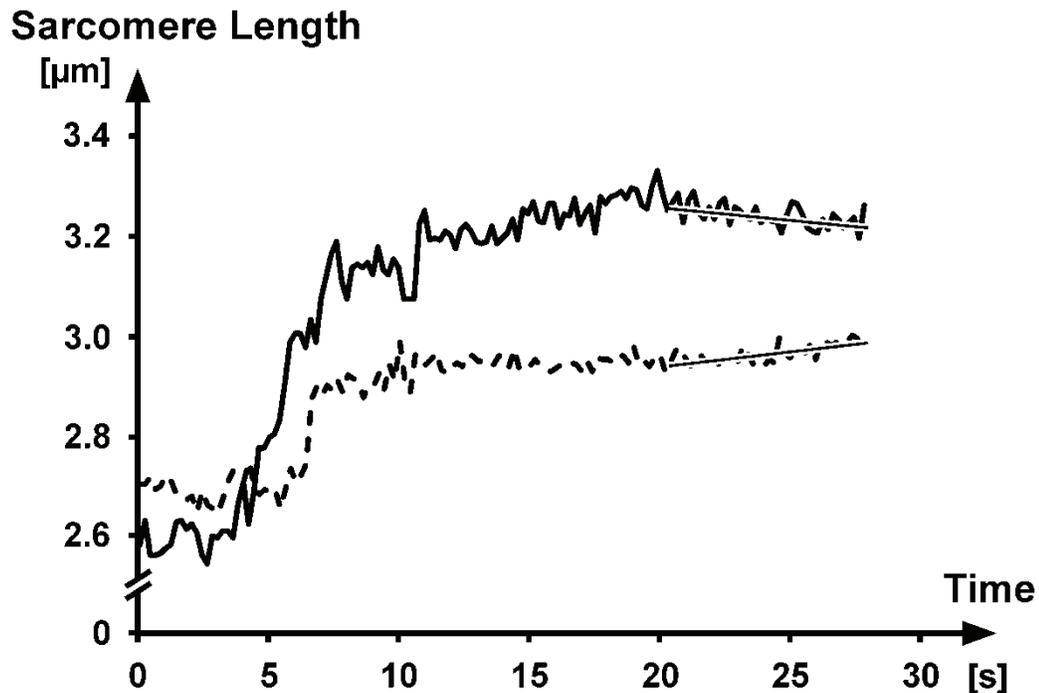


Figure 3.5: Sarcomere lengths as a function of time for two sarcomeres from the same myofibril. The two sarcomeres are those that end up being the longest and shortest following active myofibril stretching. Note that the stretch magnitude is substantially different for the two sarcomeres and that the shorter sarcomere prior to stretch becomes the longer sarcomere following stretch. Note further, that the long sarcomere after stretch is not pulled beyond myofilament overlap by the short sarcomere. Rather, the long sarcomere is slightly shortening towards the end of the contraction at the expense of the short sarcomere, which is slowly elongating.

Force enhancement has been shown to increase with increasing stretch magnitudes and increasing final sarcomere lengths (Edman *et al.*, 1982; Abbott & Aubert, 1952). In this study, sarcomeric force enhancement was positively correlated to the initial sarcomere lengths (Figure 3.6a), but not the stretch amplitude (Figure 3.6b) as one might have expected, except for single sarcomeres of myofibrils for which the stretch magnitude was small (e.g. Figure 3.6c; four sarcomeres from a single myofibril that was stretched by 12% of its initial length).

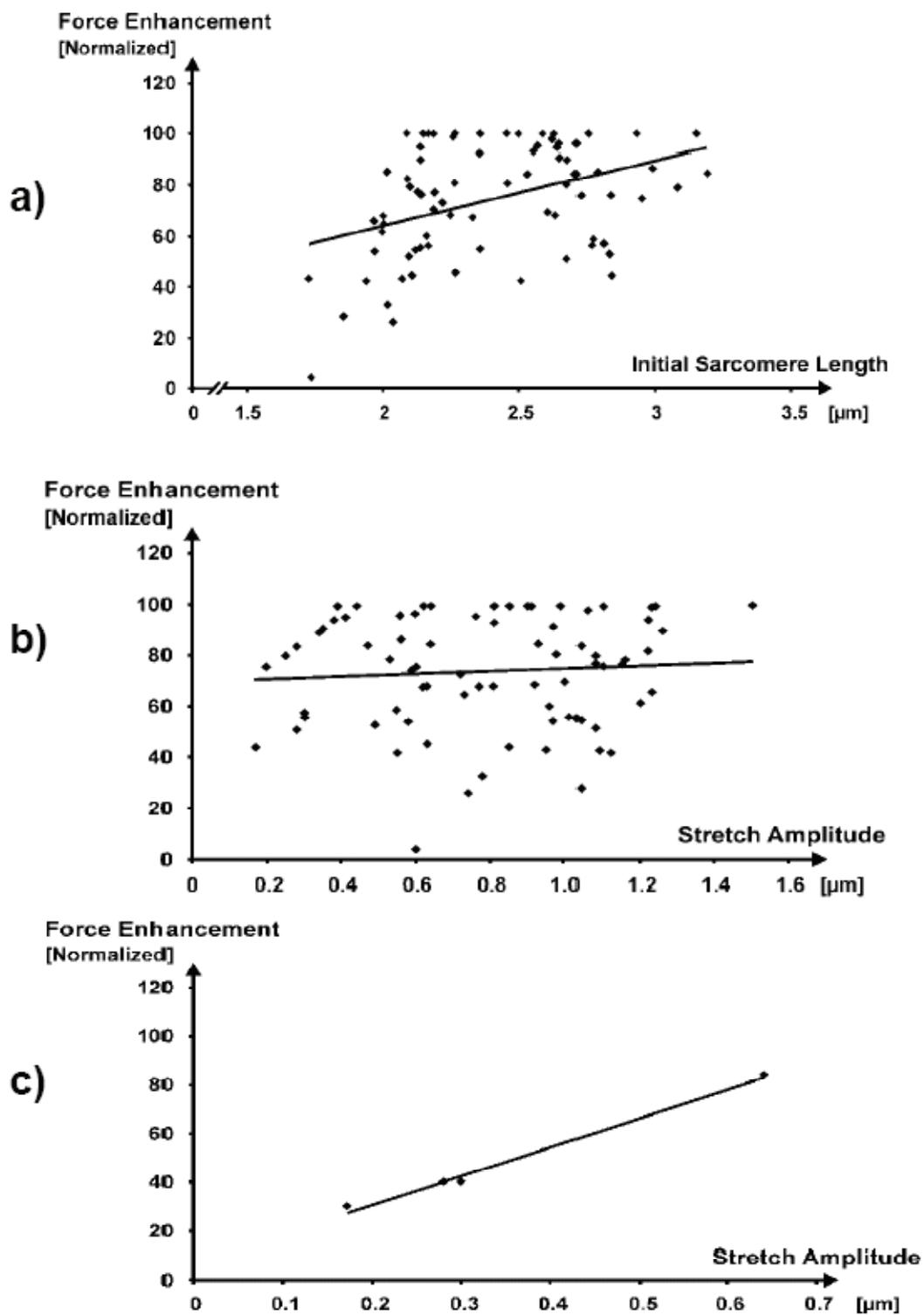


Figure 3.6: Normalized force enhancement as a function of initial sarcomere

length (a) (the average sarcomere length of the myofibril prior to stretching) and stretch amplitude (b and c).

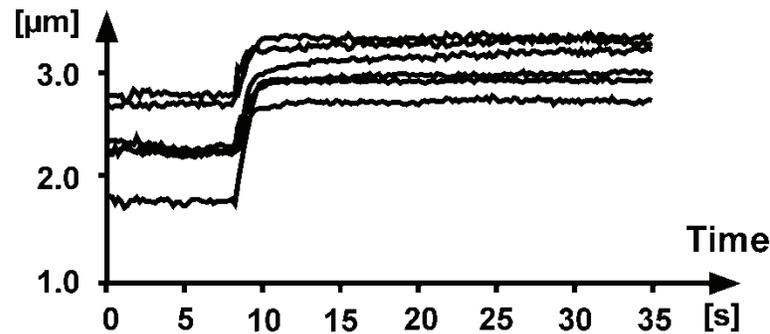
Figure 3.6 (a): Force enhancement was normalized relative to the maximal force enhancement observed in one sarcomere for each of the twelve myofibrils tested. Data for all twelve myofibrils is pooled in these figures. There is a small but statistically significant relationship ($R^2=0.20$, $p<0.05$) between force enhancement and initial sarcomere length.

Figure 3.6 (b): Normalized force enhancement as a function of sarcomere stretch magnitude for all sarcomeres from all twelve myofibril preparations. There was no statistically significant relationship between these two variables for the conditions of this test.

Figure 3.6 (c): Force enhancement as a function of sarcomere stretch amplitude in a single myofibril, where the total stretch magnitude was small (12% of the initial sarcomere length). For this scenario, there is a positive correlation between sarcomere stretch and force enhancement ($R^2=0.98$).

Since sarcomere length stability was observed in all myofibril preparations, but sarcomere lengths prior to and following stretch were not uniform, sarcomeres at different lengths on the descending limb of the force-length relationship, and thus presumably different actin-myosin overlap, supported the same amount of force. One might argue that this is caused by differences in the contractile materials within neighbouring sarcomeres. However, if that was the case, sarcomere lengths should remain at a constant ratio on the descending limb of the force-length relationship, but that is not necessarily the case (Figures 3.8a and 3.8b). Furthermore, corresponding half-sarcomeres, which share the central myosin filament and thus would be expected to have identical contractile material on each side of the half-sarcomere, also do not necessarily support the same amount of force on the descending limb of the force-length relationship (Figure 3.9).

a) Sarcomere Length



b)

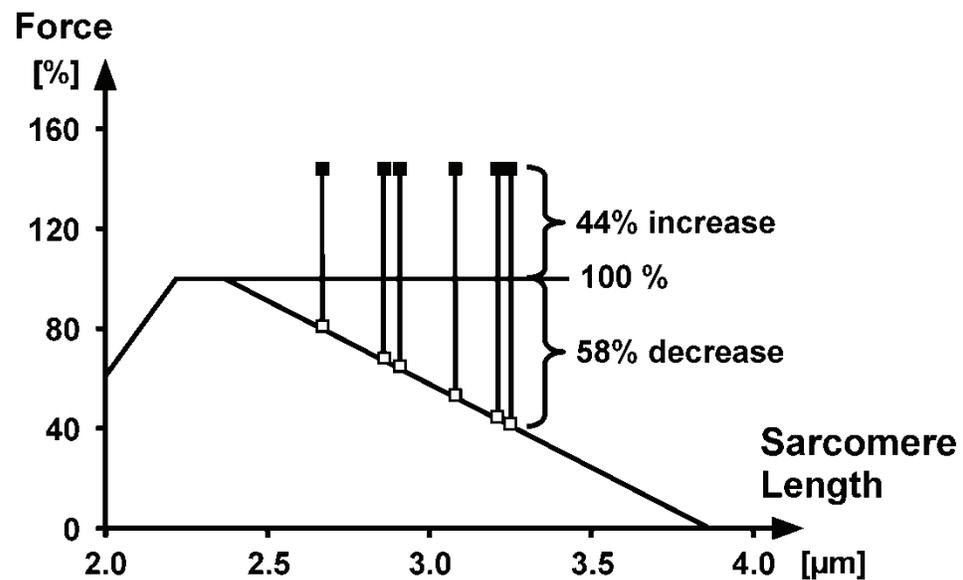
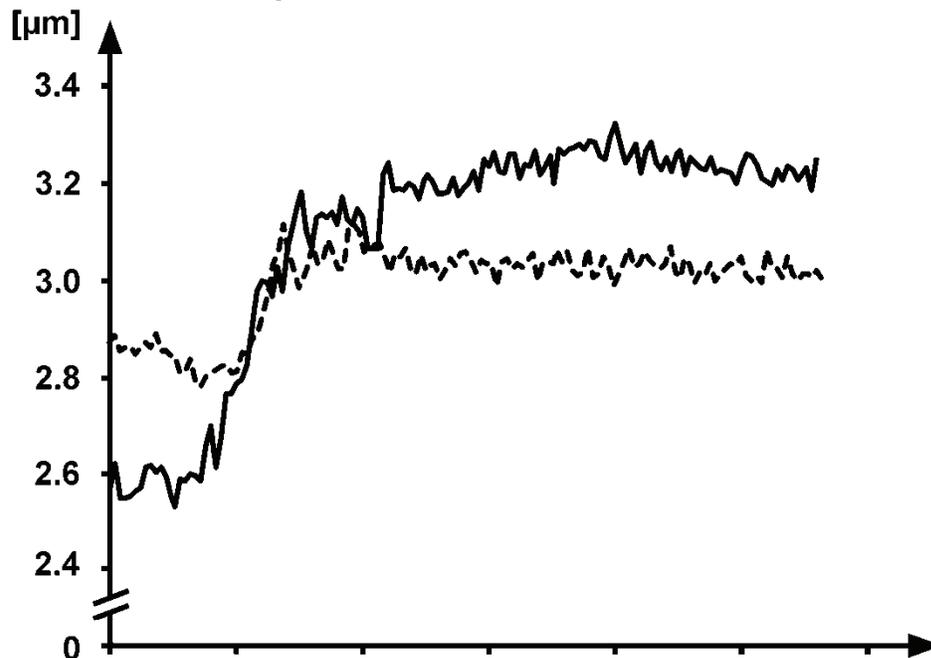


Figure 3.7: Sarcomere lengths vs. time for all six sarcomeres of a single myofibril (a), and the corresponding force-length relationship for single sarcomeres based on the myofilament overlap theory (b) and the actin and myosin filament lengths for rabbit skeletal muscle (open squares connected by solid lines), and the corresponding sarcomere forces achieved following myofibril stretching (filled squares). For the longest sarcomere following stretch (about $3.25 \mu\text{m}$), the expected force is 58% lower than the maximal isometric force at the plateau of the force-length relationship, while the actual force was 44% above the maximal force. The vertical line connecting the open and filled squares represents the force enhancement for each single sarcomere. Note that the shortest sarcomere (following stretch) should be about twice as strong as the longest sarcomere based on myofilament overlap.

a) Sarcomere Length



b) Sarcomere Length

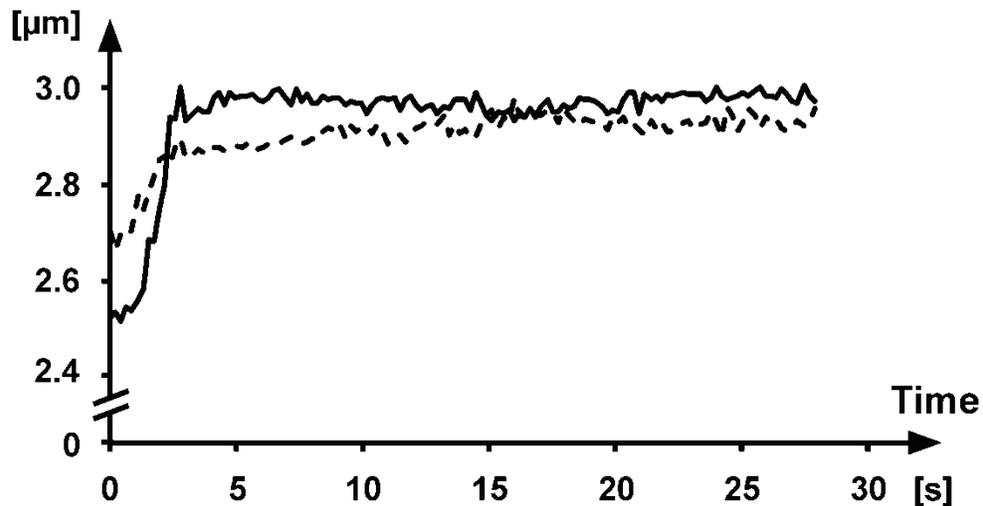


Figure 3.8: Sarcomere length as a function of time for two pairs of sarcomeres from two myofibrils stretched on the descending limb of the force-length relationship. Note that in both cases, the short sarcomere prior to stretch becomes the long sarcomere after stretch. In (a), the stretch magnitudes are different by a factor of three, and in (b), the difference is approximately a factor of two.

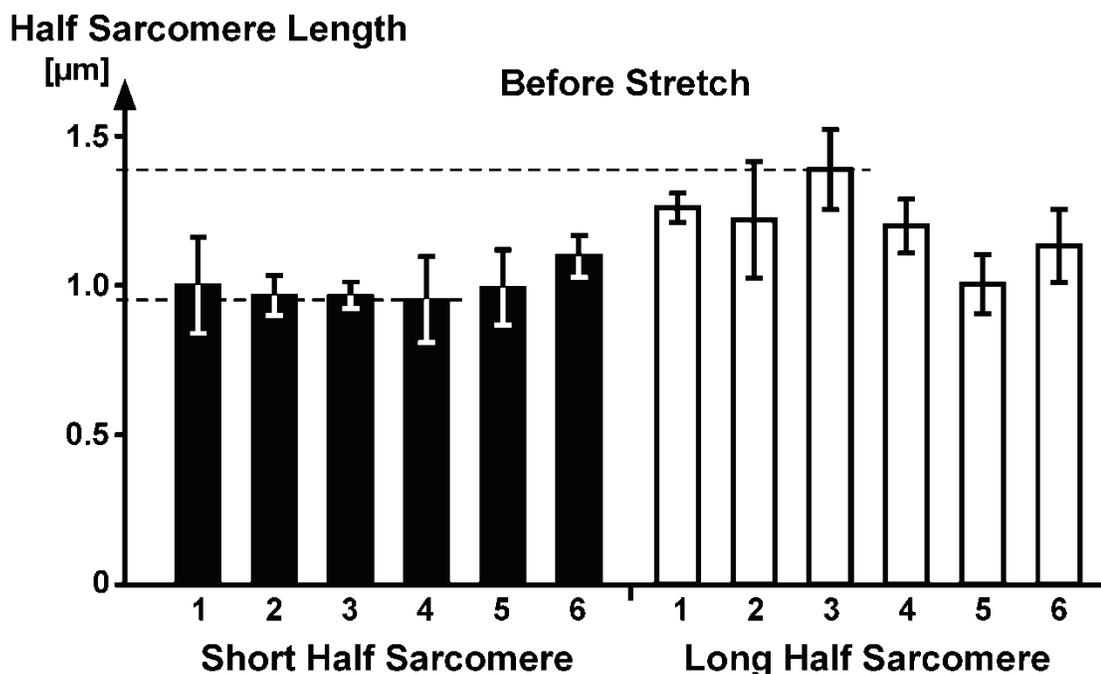


Figure 3.9: Short and long half-sarcomere length from an exemplar myofibril tested in this study. The shortest and longest half-sarcomeres are about 0.95 μm and 1.4 μm , while some half-sarcomeres are at optimal length for maximal force production based on the myofilament overlap theory (1.13 μm -1.22 μm). Despite these differences, which correspond to an expected force difference from the strongest to the weakest half-sarcomere of about 30%, all half-sarcomeres were perfectly stable while supporting identical forces.

3.4 Discussion

Here, we provide the first direct evidence of residual force enhancement in single myofibrils and individual sarcomeres, and further show that the isometric forces obtained at average sarcomere lengths corresponding to optimal actin-myosin overlap can be more than doubled if the isometric steady-state conditions are preceded by an appropriate stretch ($35 \pm 15\%$ of the initial sarcomere length). These findings are qualitatively consistent with earlier observations for *in situ* muscles (e.g., De Ruiter *et al.*, 2000; Lee & Herzog, 2002), isolated muscles (e.g., Abbott & Aubert, 1952; Herzog & Leonard, 2002), and single fibres or fibre bundles (Edman *et al.*, 1978; Edman *et al.*, 1982; Sugi &

Tsuchiya, 1988), although the magnitudes achieved are somewhat greater in single myofibrils than in any previously investigated preparation.

Recently, Telley et al. (2006b) measured simultaneous force and sarcomere lengths in single myofibril preparations with the aim to assess sarcomere and half-sarcomere dynamics during stretch. They observed myofibrils for just one second following stretch when force was still in its transient decay and sarcomere lengths were not constant, therefore, residual force enhancement could not be determined. However, their data for the stretch and initial transient phase are similar to ours, and there is reason to believe that they would have found residual force enhancement in their preparations as we did here. Their measurements were typically made at shorter sarcomere lengths than ours thereby suggesting that our findings on the descending limb would likely also hold for the plateau region of the sarcomere force-length relationship.

The residual force enhancement, calculated for individual sarcomeres according to the force length relationship, varied within a given myofibril. This is explained by the fact that sarcomere lengths prior to and following myofibril stretch were non-uniform and that the stretch magnitudes for the individual sarcomeres were not necessarily the same (e.g., Figures 3.3, 3.5 and 3.8). Sarcomere stresses in the enhanced state often exceeded the stresses obtained at optimal sarcomere lengths. It has been found that the magnitude of stretch and the length of a muscle or fibre prior to stretch are related to the magnitude of the force enhancement (Abbott & Aubert, 1952;Edman *et al.*, 1978;Edman *et al.*, 1982;Herzog & Leonard, 2002). However, in this study only the initial sarcomere lengths were loosely but statistically significantly correlated with the amount of force enhancement (Figure 3.6a) while the magnitude of sarcomere stretch was not (Figure 3.6b). The lack of correlation between stretch magnitude and sarcomeric force enhancement was surprising and is inconsistent with most previously published observations. However, Bullimore et al. (2007) showed that although force enhancement and stretch magnitude were well correlated for stretches of approximately 25% of the optimal fibre length, once stretch magnitudes were beyond 25%, force enhancement

remained approximately constant or even decreased slightly. The stretch magnitudes used in our preparations for 8 of the 12 myofibrils exceeded 25%, therefore, stretch magnitude and force enhancement might not have been correlated in this study because of the great stretch magnitudes used in two-thirds of the tests. This hypothesis is strengthened by the correlation obtained between force enhancement and the amplitude of the stretch within a myofibril where the average stretch magnitude was only 12% (Figure 3.6c).

Residual force enhancement in muscles and single fibres typically does not exceed 50%. However, here we measured enhanced forces for single myofibrils and sarcomeres that often were more than twice those measured on the plateau. Comparable force enhancement magnitudes have been observed in isolated fibre preparation treated with great amounts of 2,3-butanedione monoxime (BDM) (Lee *et al.*, 2007;Bagni *et al.*, 2004;Bagni *et al.*, 2002), or for fibres stretched to great final sarcomere lengths (Edman *et al.*, 1982). BDM limits cross-bridge attachment in the strongly bound state by inhibiting phosphate release (Herrmann *et al.*, 1992), thereby biasing the ratio of weakly to strongly bound cross-bridges towards the weakly bound state. This bias is associated with a great decrease in force, but only a small decrease in stiffness, as weakly bound cross-bridges are not expected to contribute (much) to force, but they contribute to the stiffness of fibres (Herrmann *et al.*, 1992;Regnier *et al.*, 1995). The great magnitudes of force enhancement in BDM treated preparations might be explained if we assume that there is a stretch-induced change of the ratio of weakly to strongly bound cross-bridges.

However, the single myofibrils used here were not treated with BDM. At this point, it is not clear why force enhancement in the myofibrils and sarcomeres is so much greater than in single fibre or muscle preparations. However, several possibilities exist. For example, all experiments were performed at $< 22^{\circ}\text{C}$, which is cold for mammalian muscle such as the rabbit psoas used in this study. Low temperatures have been associated with a bias of the weakly to strongly bound cross-bridges towards the weakly bound state (Decostre *et al.*, 2005;Linari *et al.*, 2005), similar to what has been observed for BDM

treated preparations. Therefore, the results observed here might be associated with temperature.

Another possibility for the great force enhancement could be the lengths at which final force enhancement measurements were made for many of the sarcomeres ($>3.4 \mu\text{m}$). For example, Edman et al. (Edman *et al.*, 1982) obtained force enhancement in excess of 60% in single fibre preparations at sarcomere lengths of about $2.8 \mu\text{m}$ (estimated from their Figure 3A). They further demonstrated a consistent increase in force enhancement with increasing stretch magnitudes and final sarcomere lengths, thereby suggesting that had they increased stretch magnitude or made measurements at sarcomere lengths greater than $2.8 \mu\text{m}$, force enhancement could easily have reached values in excess of 100%. In our case, many of the final sarcomere lengths were $>3.4 \mu\text{m}$, which produces reference forces of about 34% of the maximal isometric force at the plateau of the force-length relationship. For the case of Edman et al. (1982), a 60% force enhancement at $2.8 \mu\text{m}$ would have resulted in a 244% force enhancement at $3.4 \mu\text{m}$ in their preparation, assuming that the absolute force enhancement remained about the same, a conservative estimate, as they showed increasing absolute force enhancements with increasing final sarcomere lengths. Thus, a likely explanation for the vast force enhancements observed in this study is the fact that final sarcomere lengths were often very long and the associated predicted isometric reference forces were small. However, comparison with the literature is difficult, as experiments investigating force enhancement following stretch at sarcomere lengths of $3.4 \mu\text{m}$ or greater have not yet been performed.

Myofibrils are ideal preparations to determine the mechanical properties of individual sarcomeres, as sarcomeres are arranged strictly in series. That means, at any given time, the force transmitted by one sarcomere has to be the same as that of any other sarcomere, which has to be the same as the force measured at the end of the myofibril. Therefore, and in accordance with the sliding filament and cross-bridge theory, one would expect all sarcomeres to be of the same length on the descending limb of the force-length relationship so that myofilament overlap, and therefore steady-state force, would be the same for all sarcomeres. However, this was not the case. For example, in Figure 3.7, the

shortest sarcomere after stretch (about 2.7 μm) would be expected to produce about 80% of its maximal isometric force, while the longest sarcomere (about 3.3 μm) would be expected to produce about 40% of its maximal isometric force, a difference of 100% in force production ability between these two sarcomeres. Both sarcomeres were isometric when the measurements were made (Figure 3.7a), and this difference is not consistent with the myofilament overlap theory (Gordon *et al.*, 1966b).

One might explain the difference in sarcomere lengths (for the same isometric force) with a difference in the number of contractile proteins in one sarcomere compared to the other. For example, if one sarcomere had 20% more contractile proteins, it should be able to produce the same amount of force on the descending limb of the force-length relationship as another sarcomere even if its length was 20% greater than that of the other sarcomere. However, if this was the case, then length increases during myofibril stretching should be proportional, so that this explanation holds for the entire range of the descending limb of the force-length relationship. However, there are numerous examples where this is not the case, and one such example is shown in Figure 3.5, and another two are depicted in Figure 3.8. In each case shown (Figures 3.5 and 3.8), the short sarcomere prior to stretch becomes the long sarcomere after stretch. Since all sarcomeres are always on the descending limb of the force-length relationship (i.e., starting sarcomere lengths are greater than 2.43 μm), the results observed here cannot be explained by differences in contractile proteins between sarcomeres. This conclusion is further supported by pairs of sarcomeres starting at the same length prior to stretch and ending up at different lengths after stretch, or sarcomeres of different lengths prior to stretch ending up at the same length after stretch, observations that were made in this study (not shown) and observations made by us previously (Rassier *et al.*, 2003a; their Figures 3a and 4a, respectively).

Another possibility could be that differences in sarcomeric passive forces might account for the observed results. However, if this were the case, one would not expect the length reversal during stretch of the pairs of sarcomeres shown in Figures 3.5 and 3.8.

Furthermore, the average passive forces at sarcomere lengths of 3.0 μm to 3.2 μm have been reported to be about 5-10% of the maximal active isometric force at optimal sarcomere lengths (Bartoo *et al.*, 1997). Following active stretching from 2.4 μm to 3.4 μm , as was done in this study, the passive forces (including the passive force enhancement (Herzog & Leonard, 2002)) were 18% of the maximum isometric forces at the plateau of the force-length relationship (Joumaa *et al.*, 2007). If it was assumed that a short sarcomere had no passive forces while a long one had the full 18%, an unlikely scenario, it still could not explain the force difference (up to 68% of the maximum isometric force) found here based on the observed sarcomere length non-uniformities.

Finally, one could argue that active force production just depends on one half of the sarcomere, while the other half produces much of its force passively. However, if that was the case then, in accordance with the myofilament overlap theory, all short (and therefore strong) half-sarcomere lengths should be the same, however they are not as shown in Figure 3.9. Furthermore, all long (and therefore weak) half-sarcomeres should be pulled beyond myofilament overlap, and would be expected to be at similar lengths, which they are not. In fact, for the six sarcomeres from a single myofibril shown in Figure 3.9, the biggest difference in half-sarcomere lengths is observed in sarcomere #3 and amounts to about 0.4 μm . Some of the half-sarcomeres shown in Figure 3.9 correspond to optimal myofilament overlap (e.g. short half-sarcomere #6) and they should be at a length of maximal isometric force capabilities, while other sarcomeres are much shorter (short half-sarcomere #4) or longer (long half-sarcomere #3), and their force capabilities based on the myofilament overlap theory (Gordon *et al.*, 1966b) should be about 69% and 75% of maximum. Therefore, we conclude that not only sarcomeres, but also half-sarcomeres, have force behaviour that cannot be explained with the theory of myofilament overlap (Huxley & Niedergerke, 1954; Huxley & Hanson, 1954).

In order to further characterize the mechanical behaviour of half-sarcomeres, we determined the modulus of elasticity for twelve half-sarcomeres in a myofibril with a clearly discernible Z-line pattern. We found that the modulus of elasticity, calculated as

the ratio of the change in stress and change in half-sarcomere strain, was greater in the long compared to the short half-sarcomeres (Table 3.1). Thus, differences in half-sarcomere lengths decreased after the stretch, and long half-sarcomeres seem to be endowed with a higher stiffness than short sarcomeres. This result is consistent with observations in single fibres (Edman *et al.*, 1982) and in isolated myofibrils (Telley *et al.*, 2006b) where it was suggested that stretch has a stabilizing effect on sarcomere dynamics on the descending limb of the force-length relationship. As a consequence, long half-sarcomeres resist stretch more than short half-sarcomeres and thus, they cannot be pulled easily beyond myofilament overlap as proposed by the sarcomere length instability theory (Morgan, 1990).

	Sarcomere 1		Sarcomere 2		Sarcomere 3		Sarcomere 4		Sarcomere 5		Sarcomere 6	
	L	S	L	S	L	S	L	S	L	S	L	S
HSL Before stretch (μm)	1.26	1.00	1.22	0.97	1.39	0.97	1.20	0.95	1.01	0.99	1.13	1.10
HSL After stretch (μm)	1.68	1.58	1.69	1.57	1.74	1.61	1.72	1.61	1.70	1.65	1.72	1.71
E (kN/m^2)	233.6	134.7	204.6	124.1	310.7	116.0	180.6	112.7	113.8	118.2	151.9	140.6

Table 3.1: Half-sarcomere length (HSL) before and after stretch in a myofibril composed of six sarcomeres. We noticed that half-sarcomeres before stretch were non-uniform. The difference in length between long (L) and short (S) half-sarcomeres in any given sarcomere is reduced following stretch. In most half-sarcomeres, the elasticity modulus (E) of long half-sarcomeres is higher than that of the short ones. This might be explained by differences in the proportion of two coexisting titin isoforms within half-sarcomeres.

Stiffness in active myofibrils can be attributed to the proportion of attached cross-bridges, and passive structures, most notably in myofibrillar preparations, the titin filaments. The proportion of attached cross-bridges would be expected to be greater at short compared to

long half-sarcomere length on the plateau and descending part of the force-length relationship, and thus is unlikely to explain the differences in stiffness, except if we assume that increased length favours cross-bridge attachment, an intriguing but as of yet unproven idea. Alternatively, the differences in stiffness could be caused by passive structural proteins. Rabbit psoas muscle expresses two isoforms of titin, which are also observed in single psoas fibres (Neagoe *et al.*, 2003). Furthermore, it has been shown that one half-sarcomere can coexpress different titin isoforms (Trombitas *et al.*, 2001), thus differences in half-sarcomere stiffness could be caused by changes in the ratio of coexpressed titin isoforms, an idea that needs independent evaluation.

3.5 Conclusion

From the results of this study, we conclude that there is force enhancement in single myofibrils and forces in the enhanced state are more than twice those at the plateau of the force-length relationship. Furthermore, there is residual force enhancement in all individual sarcomeres in a myofibril following active stretch; this force enhancement is derived from the sarcomere force length relationship (Gordon *et al.*, 1966b) and varies substantially between sarcomeres, is not associated with the magnitude of sarcomere stretch (except for sarcomeres in a given myofibril for which stretch magnitudes were small), but is significantly related to the sarcomere length prior to stretch. Finally, we confirm previous results that sarcomeres in a myofibril preparation are highly non-uniform but perfectly stable on the descending limb of the force-length relationship, and sarcomeres and half-sarcomeres, at different lengths (and therefore different actin-myosin overlap) produce the same amount of steady-state isometric force. The expected sarcomere force differences on the descending limb of the force-length relationship (Huxley, 1957a; Gordon *et al.*, 1966b) do not exist, and this result cannot be explained at present with differences in the number of contractile proteins across sarcomeres, passive forces, or systematic changes in the half-sarcomere dynamics.

Chapter Four: Force enhancement following stretch in a single sarcomere

4.1 Introduction

Our understanding of skeletal muscle contraction is based largely on the crucial works of A. Huxley and H. Huxley who demonstrated that contraction and active force production occurs through the sliding of two sets of filaments (actin and myosin) (Huxley & Hanson, 1954; Huxley & Niedergerke, 1954), and the cyclic formation of cross-bridges between them (Huxley, 1957a; Huxley, 1969). This so-called “cross-bridge theory” has been the accepted paradigm for muscle contraction and has been used successfully to explain a variety of experimental observations in muscle physiology and mechanics. One specific feature of the cross-bridge theory is that steady-state active forces are independent of the history of contraction (Walcott & Herzog, 2008), and it has been assumed tacitly that passive forces are too. However, it has been known for more than half a century that this is not the case (Abbott & Aubert, 1952). Specifically, it has been observed consistently that the steady-state isometric forces following active stretching of muscles (Morgan, 1994; De Ruiter *et al.*, 2000), single fibres (Edman *et al.*, 1978; Edman *et al.*, 1982) or isolated myofibrils (Rassier *et al.*, 2003a; Joumaa *et al.*, 2008a) are substantially greater than the isometric forces at the same length not preceded by stretch. This “residual force enhancement (Edman *et al.*, 1982)” has been a perplexing puzzle, sparking intense debate among muscle physiologists, specifically as to whether this force enhancement is associated with active or passive components of muscle (Herzog *et al.*, 2006). Cross-bridge forces provide the active component while non-cross-bridge forces which may arise from actin-titin interactions or calcium effects on titin may contribute to the passive component.

Four mechanisms have been proposed to explain this enhanced force following stretch. The first three presented here are the most often cited in the literature and can be considered the three primary mechanisms, with the fourth mechanism being used less frequently. The first proposed mechanism involves changes in cross-bridge kinetics (Linari *et al.*, 2000), the second requires the recruitment of a parallel elastic element (Edman & Tsuchiya, 1996; De Ruiter *et al.*, 2000), and the third utilizes a redistribution of the existing lengths of individual sarcomeres during muscle stretching (Morgan, 1994)

The fourth proposed mechanism arises from a model of force production which incorporates myosin shortening with connecting filament interactions and allows for active force production to occur in a manner that could explain different forces at the same sarcomere length (Pollack, 1990). Of these four mechanisms, the third, entitled the sarcomere length (SL) non-uniformity theory, has received the greatest acceptance and is inherently the easiest to reconcile within the frame work of the cross-bridge theory. The SL non-uniformity theory assumes that the descending limb of the force-length relationship is inherently unstable (Hill, 1953). Upon activation and stretch, some (few) sarcomeres are pulled to a length beyond actin-myosin filament overlap, thereby overstretching them (Morgan, 1994) while allowing the remaining sarcomeres to be placed at a shorter length where they can produce more force when compared to a muscle that was activated and held at a constant length (Figure 4.1).

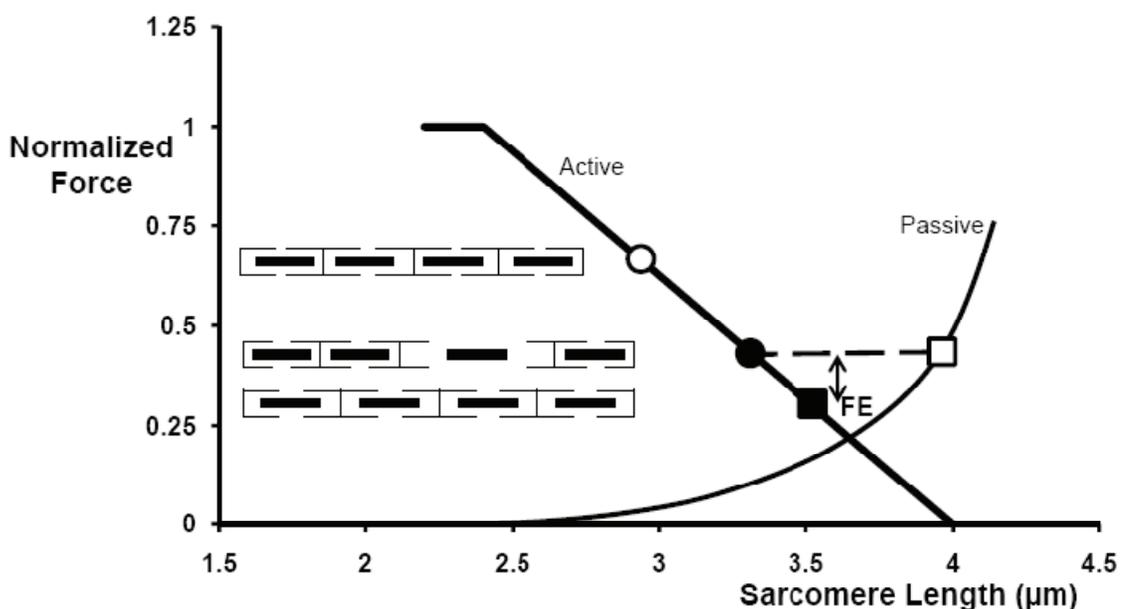


Figure 4.1: Schematic representation of force versus sarcomere length, with sarcomere disruption as the mechanism for force enhancement. Isometric reference force at a long SL (black square) produces less force than at a short SL (white circle). Stretching from the short to the long SL is thought to cause non-uniformities in SLs with some sarcomeres (white square) overstretching onto the passive portion of the curve while the other sarcomeres elongate less than expected (black circle) on the active curve and are able to produce force enhancement (FE). Insert shows schematic representation of a myofibril with 4 in-series sarcomeres. The two lower

drawings represent myofibrils of the same final length but one has uniform SL while the other shows one sarcomere which has overstretched to beyond actin-myosin filament overlap while the remaining three sarcomeres are shorter and stronger than the sarcomeres in the uniform myofibril.

Despite a lack of direct evidence, this theory remains well accepted. In an attempt to search for direct support of this theory, recent work on force-enhancement in single myofibrils (Rassier *et al.*, 2003a) has shown that SL non-uniformities do exist but that overstretching was not observed and that non-uniform sarcomeres exhibited perfectly stable behaviour on the descending limb of the force-length relationship (Rassier *et al.*, 2003a; Joumaa *et al.*, 2008a). We chose to continue this line of questioning and reduce a myofibrillar preparation to a single intact sarcomere (Pavlov *et al.*, 2009b) and subject it to both isometric and stretched contractions to determine whether force-enhancement is observed. If force-enhancement following stretch is the result of sarcomere length redistribution or overstretching, then it must not be observed in a single sarcomere preparation.

4.2 Methods

We isolated single sarcomeres mechanically, in a similar but not identical fashion, as has been shown previously (Pavlov *et al.*, 2009b), from isolated single myofibrils. We attached the outer ends of the Z-lines of a sarcomere to a relatively rigid glass needle and a pair of custom-fabricated nano-cantilevers (Pavlov *et al.*, 2009a; Joumaa *et al.*, 2008a; Joumaa *et al.*, 2008b; Leonard & Herzog, 2010). Sarcomere length was measured using an inverted microscope (Axiovert 200M, Zeiss, Germany) with a 100 x objective (N.A. 1.3) with a 2.5 optovar and a high resolution line scan camera (6.7 nm/pixel, model SK10680 DJR, Schafter & Kirschhoff, Germany). Sarcomeres from rabbit psoas were used for all testing (Joumaa *et al.*, 2008a). Strips of muscle were harvested from freshly euthanized animals and tied with silk suture to wooden sticks to maintain the *in-situ* fibre length. The samples were then stored in a rigor solution containing protease inhibitors (Complete®, Roche Diagnostics, Quebec, Canada) and glycerol and were stored for 10-14 days at -20°C. On the day of the experiment, the muscle strips were removed from the

freezer, homogenized and then placed in a low calcium relaxing solution containing ATP at 4°C. High calcium (pCa 3.5) and low calcium solutions (pCa 8) at 4°C were used for the activation-deactivation sequences (Joumaa *et al.*, 2007). Specimen length was controlled using a drawn glass pipette attached to a piezotube motor (Boston Piezo-Optics, Bellingham, MA, USA). Forces were measured using custom built silicon nitride cantilever pairs of known stiffness (Fauver *et al.*, 1998). The sarcomere was attached to one of the cantilever pairs using silicone adhesive (50:50 mixture of RTV 3140® and RTV 3145® Dow Corning, Midland, MI, USA). See Figures A1 and A2 in the Appendix of this thesis for further clarification. Displacement of one of the cantilever pairs with respect to the other was measured and the force estimated from the cantilever stiffness (350 nN/μm). Cantilever dimensions were width = 2.5 μm, length = 120 μm and nitride thickness = 0.745 nm. Forces (nN) were normalized by the sarcomere cross-sectional area (μm²) and reported as stress.

Ten sarcomeres were tested in the following sequence: Extended in a relaxed state to SL 3.4 μm, then activated, and then deactivated by placing it in a low calcium solution. Sarcomeres were then passively placed at 2.4 μm and reactivated. The activated myofibril was then stretched at 0.1μm/sec to 3.4 μm. Steady state forces were obtained after ≥ 20 seconds. The order of testing was designed so that sarcomere damage would tend to underestimate the true force enhancement. Cantilever compliance resulted in sarcomere shortening of about 4% for the 3.4 μm reference contraction and about 12% for the test contractions thereby resulting in a slight underestimation of the true force enhancement, if anything at all (Herzog & Leonard, 2000).

Wilcoxon signed rank testing with significance set at 0.05 was used for all analyses. A more complete treatment of the methods can be found in Appendix A of this thesis.

4.3 Results

The steady-state isometric reference contractions produced greater stresses at SLs of 2.4 μm compared to 3.4 μm (p=0.002), as expected based on the actin and myosin filament lengths of rabbit skeletal muscles (Page & Huxley, 1963;Herzog *et al.*, 1992). Following active stretching from SLs of 2.4μm to 3.4μm, the steady-state isometric stresses were

significantly higher ($p=0.002$) than the isometric reference stresses at the same SL ($3.4\mu\text{m}$) and were also significantly higher ($p=0.002$) than the isometric reference stresses at the optimal SL ($2.4\mu\text{m}$). This observation was made consistently for all sarcomeres (Figures 4.2, 4.3 and 4.4). The mean isometric stress at $2.4\mu\text{m}$ (prior to normalization) was $122.7\text{ nN}/\mu\text{m}^2 \pm 17.4\text{ nN}/\mu\text{m}^2$. Mean isometric stress at $3.4\mu\text{m}$ was $58.2 \pm 6.6\text{ nN}/\mu\text{m}^2$ and the mean steady state stress after stretch to $3.4\mu\text{m}$ was $168.8 \pm 32.0\text{ nN}/\mu\text{m}^2$.

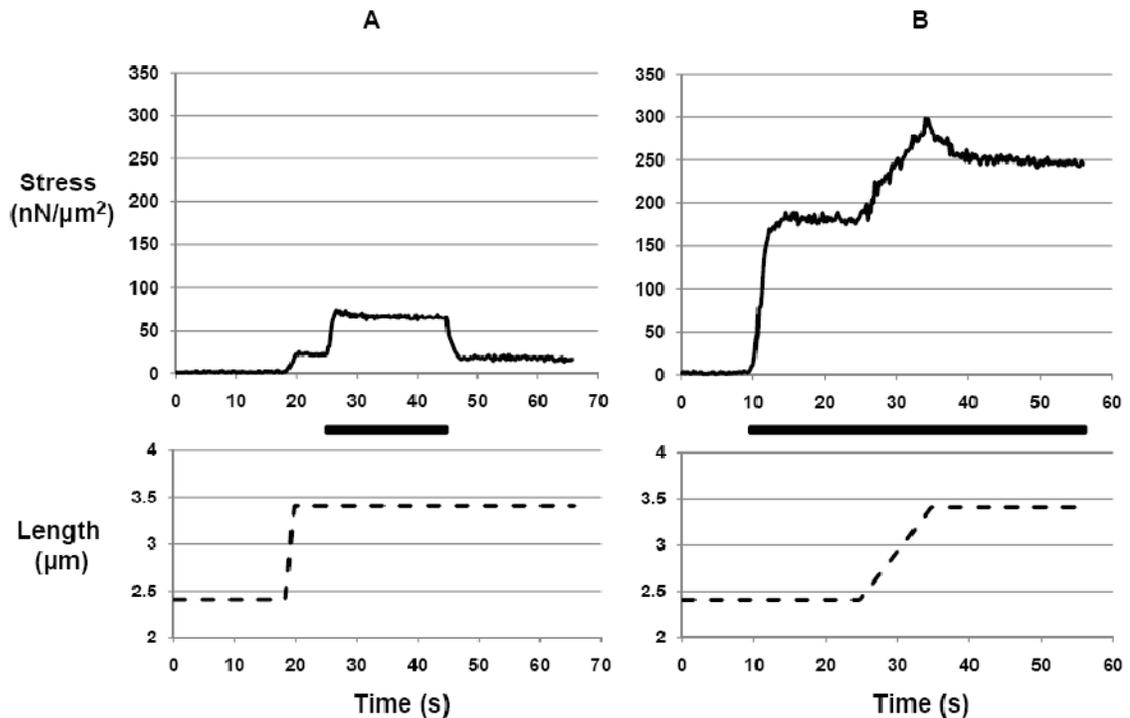


Figure 4.2: Exemplar data traces for one sarcomere. Stress-time (top) and length-time (lower) curves for an (A) isometric contraction at $3.4\mu\text{m}$ and (B) an isometric contraction at $2.4\mu\text{m}$ followed by active stretch and an isometric contraction at $3.4\mu\text{m}$. The black bar on the X-axis represents when the high calcium activating solution was present.

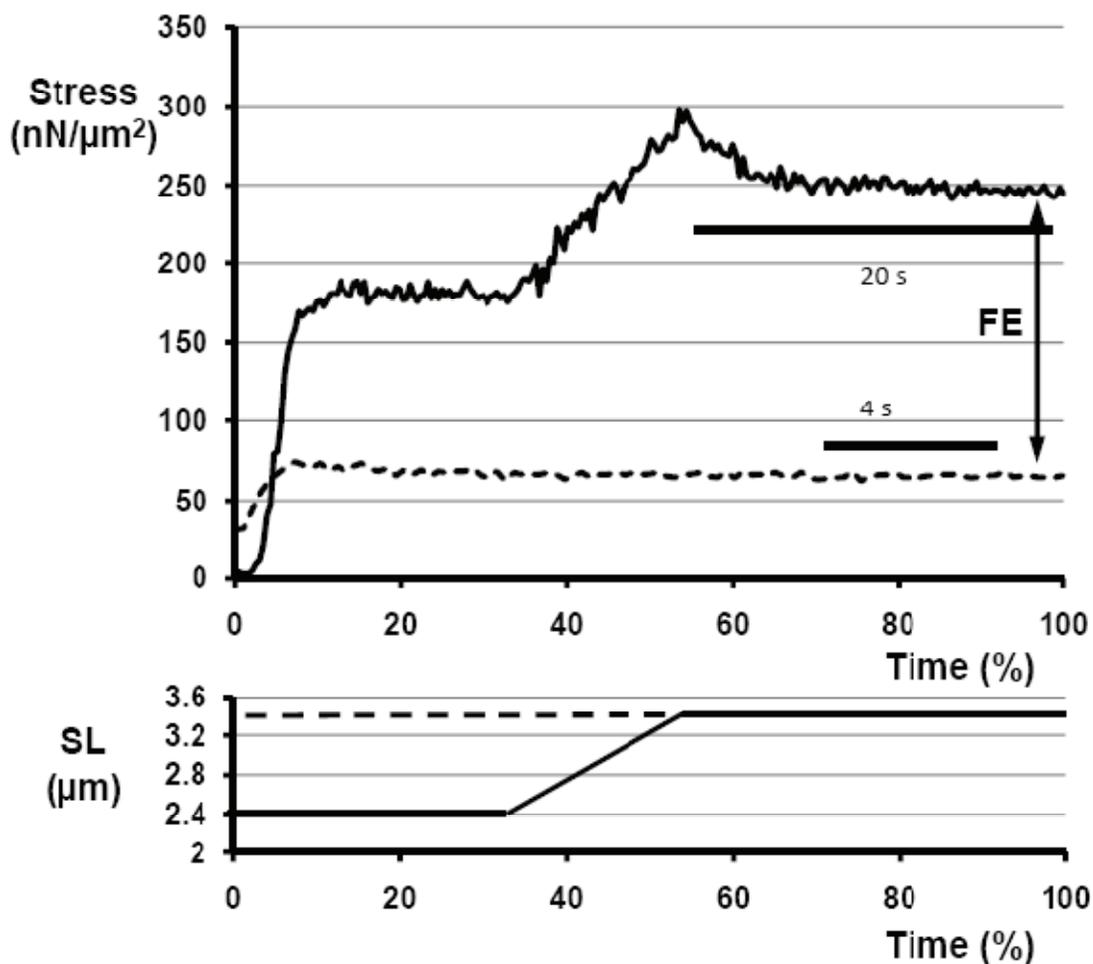


Figure 4.3: Exemplar data normalized for time of activation for one sarcomere. The same data as that used for Figure 4.2 is shown here and the enhanced force following the active stretch is measured at the time point in the figure representing 95% of the activation time. Solid traces represent stress (top panel) and length (bottom panel) for single sarcomere activated at 2.4 μm and then actively lengthened to 3.4 μm . Dashed traces are for (top) stress and (bottom) sarcomere length versus normalized time for an isometric contraction at 3.4 μm . Although the SL for the two conditions is the same after time at 55% and so presumably the amount of actin-myosin filament overlap is identical, the steady state stresses are significantly different. Solid bar gives actual time scale for the two test conditions.

4.4 Discussion

Recently, it has been suggested that the “half-sarcomere” rather than the entire sarcomere forms the smallest contractile unit of muscle (Telley *et al.*, 2006a). Therefore, we

explored the idea whether half-sarcomere length non-uniformities might explain the observed residual force enhancement in our single sarcomere preparations. However, this does not seem possible, as the enhanced forces always exceeded the steady-state isometric reference stresses at the optimal sarcomere length of 2.4 μm (Page & Huxley, 1963; Herzog *et al.*, 1992). Therefore, independent of the half-sarcomere non-uniformity, the short half of the sarcomere would not be able to produce the stresses observed here following active sarcomere stretching.

If we exclude (half-) sarcomere length non-uniformity as the cause for the observed force enhancement, three basic explanations remain. The first of these relates to the idea that force enhancement is caused by actin-myosin based cross-bridge forces. Within the framework of the cross-bridge theory, this could only occur if there was a stretch-induced increase in the proportion of attached cross-bridges, and/or an increase in the average cross-bridge force. An approximate three-fold increase in the proportion of attached cross-bridges would be required to explain our results (Figure 4.4) and would have to be associated with a substantial increase in stiffness. However, force enhancement in most experiments has been associated with either no change (Sugi & Tsuchiya, 1988), or only minute increases in stiffness (Morgan, 1994; Linari *et al.*, 2000), thereby rendering this idea unlikely (Edman *et al.*, 1978). However, to completely exclude this possibility, stiffness measurements in mechanically isolated sarcomeres will need to be performed in the future.

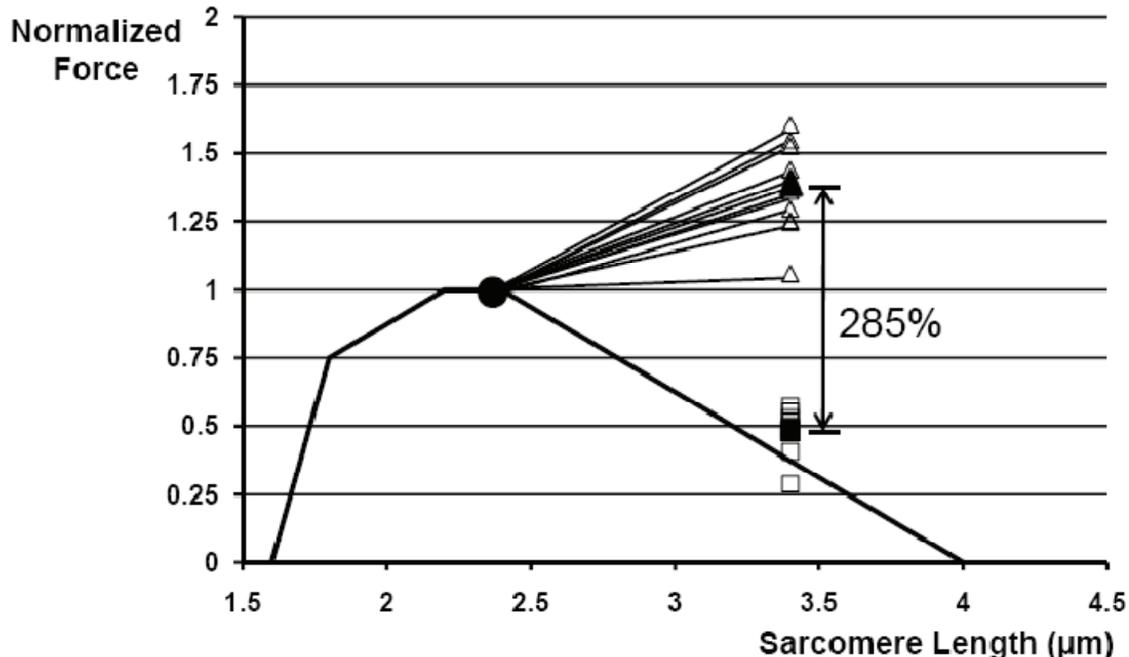


Figure 4.4: Force versus sarcomere length. Forces were normalized to the force obtained at optimal SL (black circle). Mean forces following active sarcomere stretching (black triangle) from SL of 2.4 to 3.4 μm (open triangles) were 285% greater than the corresponding mean isometric reference forces (black square) at the same (3.4 μm) SL (open square), and were on average 37% greater than the isometric reference forces at optimal (2.4 μm) SL.

Similarly, an increase in the average force per cross-bridge would require an enormous increase in stretch magnitude of the attached cross-bridges and would require that cross-bridges either remain attached for a very long time (seconds) or re-attach at very long length in the enhanced state, again an unlikely scenario (Huxley, 1969). However, the most compelling evidence against a solely cross-bridge based force enhancement explanation comes from experiments on single myofibrils stretched to very long sarcomere lengths (4-6 μm) where actin-myosin filament overlap is lost and cross-bridge action is absent. When stretched actively (saturated Ca^{2+} solution), forces at these long sarcomere lengths were 3-4 times greater than those observed when myofibrils were stretched passively (low Ca^{2+} concentration) (Leonard & Herzog, 2010). Thus, force enhancement is preserved even in situations when cross-bridge forces cannot contribute. Force enhancement measurements in isolated sarcomeres pulled actively and passively

beyond actin-myosin filament overlap ($>4.0 \mu\text{m}$ in rabbit psoas muscle) will need to be performed to test if the observations made in myofibrils also hold for isolated sarcomere preparations.

The second basic explanation for our results is based on the “engagement” of a structural component upon activation and stretch (Edman *et al.*, 1978). It has been shown previously that the structural protein titin is required for at least part of the observed force enhancement in isolated myofibrils (Joumaa *et al.*, 2008a). Titin has a propensity to bind to actin (Li *et al.*, 1995; Kulke *et al.*, 2001) and can increase its stiffness through binding of Ca^{2+} in the PEVK and Ig-domains (Labeit *et al.*, 2003b; Duvall & Herzog, 2009). Therefore, we suggest that the force enhancement observed here in single sarcomeres may at least in part be caused by force regulation through titin, specifically binding of titin to actin as the effect of Ca^{2+} binding to titin appears too small to explain the observed results (Joumaa *et al.*, 2008b). This hypothesis can be tested by labelling titin segments and quantifying their pattern of elongation in the reference and force enhanced states. If some titin segments bind to actin, then these segments would essentially become rigid and would not elongate when actively stretched, but would elongate when sarcomeres are stretched passively.

The third possibility is a mechanism which relies on the idea that cross-bridge mechanics are not the primary regulator of force in muscle but that there is a complex interaction between shortening of myosin filaments and connecting filaments which anchor myosin to the Z-disc (Pollack, 1990). This alternative mechanism of muscle contraction can account for different forces at the same sarcomere length but cannot readily explain the forces following stretch which are substantially above the forces observed at the optimal (plateau) sarcomere length.

4.5 Conclusion

Here, we performed experiments in mechanically isolated single sarcomeres and observed that residual force enhancement following active stretching occurs. Since our preparation utilizes a single sarcomere, a redistribution of the length of neighbouring sarcomeres to produce the higher force following stretch is, by design, precluded.

Further, the enhanced stresses in the single sarcomeres always exceed the isometric stresses on the plateau of the force-length relationship, thereby eliminating the possibility that our result might have been obtained because of a redistribution of half-sarcomere lengths. This work shows that force enhancement following stretch can occur in the absence of sarcomere instabilities and the development of sarcomere length non-uniformities. It is a property intrinsic to the sarcomere itself and may involve altered cross-bridge kinetics (Linari *et al.*, 2000) but likely is caused by the recruitment of a parallel elastic element (Joumaa *et al.*, 2008b;Edman *et al.*, 1978).

Chapter Five: Regulation of muscle force in the absence of actin-myosin based cross-bridge interaction

5.1 Introduction

The sliding filament based cross-bridge theory has been the paradigm of choice for muscle contraction and force production for the past half century (Huxley, 1969;Huxley, 1957a;Huxley & Simmons, 1971;Huxley & Hanson, 1954;Huxley & Niedergerke, 1954). According to this theory, contraction occurs through the interaction of myosin-based cross-bridges that attach cyclically to actin and tend to pull actin past the myosin filaments towards the centre of sarcomeres (Huxley, 1957a;Huxley, 1969;Huxley & Simmons, 1971). This produces muscle contraction and force. When a muscle is stretched, sarcomeres become longer and actin-myosin filament overlap decreases (Figure 5.1), thus decreasing the number of possible cross-bridge interactions and active force while passive forces increase (Huxley & Niedergerke, 1954;Gordon *et al.*, 1966b;Ramsey & Street, 1940;Huxley, 1957a;Huxley, 1957b).

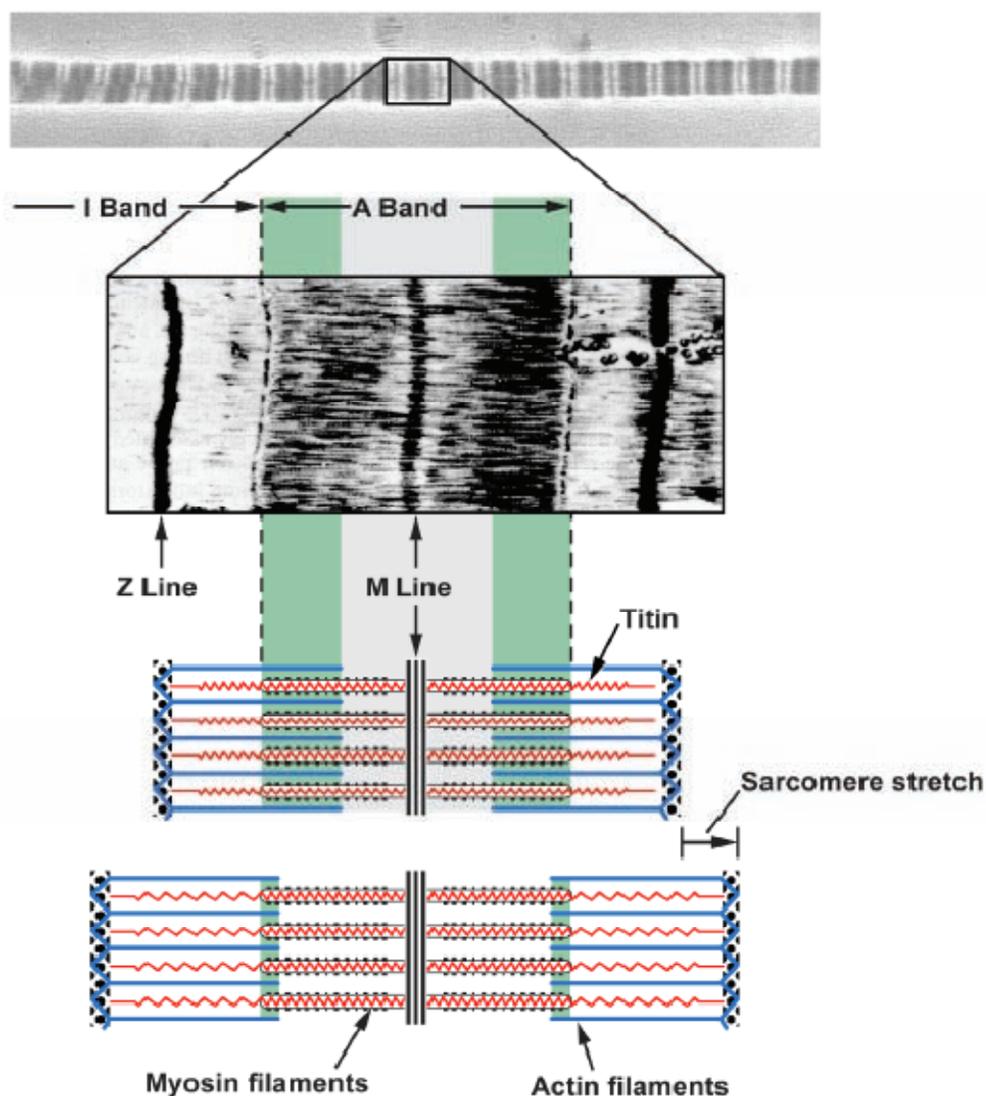


Figure 5.1: Active force is produced by cyclic interactions of myosin based cross-bridges with actin. A myofibril showing sarcomeres arranged in series (top panel). The dark (A-band) and light (I-band) striation pattern indicates the myosin and actin filament regions. An isolated sarcomere with Z-lines, M-line and A- and I-bands is shown in the second panel. The third panel shows a schematic illustration of a sarcomere with titin, myosin and actin filaments. The overlap area in the A-band (indicated by the dark green shaded region) is the area in which myosin cross-bridges can attach to actin and so produce sarcomere shortening and active force. In the fourth panel, the sarcomere is shown in a stretched position. The actin-myosin overlap area is near zero, thus active actin-myosin based force production is also near zero while titin is stretched and provides passive force at this length. Titin spans the half-sarcomere from the M line to the Z-line. It is symmetrically arranged in both halves of a sarcomere, and since it is attached to myosin filaments, it centres

these filaments in the middle of the sarcomere. The I-band region contains the extensible portion of titin which is thought to act as a molecular spring.

At sarcomere lengths where actin-myosin filament overlap ceases to exist, only passive forces are possible, and these are thought to be essentially invariant at a given muscle or sarcomere length when due account is given to transient viscous effects (Figure 5.2). However, recent pilot results suggest that passive forces in isolated myofibrils might be modulated substantially by active stretching (Leonard *et al.*, 2009). In myofibrils, passive forces are known to primarily originate from the structural protein titin (Tskhovrebova *et al.*, 1997;Granzier & Labeit, 2002;Prado *et al.*, 2005), the largest protein currently known in the natural world, and thus, we hypothesized that titin, in addition to actin-myosin, might be a strong regulator of force in actively stretched muscles. Therefore, the purpose of this study was to investigate the forces produced in myofibrils that were stretched to lengths too great to allow for actin-myosin interactions and to elucidate the role of titin in producing these forces. Although titin has been associated with force regulation through phosphorylation (Yamasaki *et al.*, 2002) and calcium binding (Labeit *et al.*, 2003b), these effects were assumed much too small to explain our pilot results (Labeit *et al.*, 2003a).

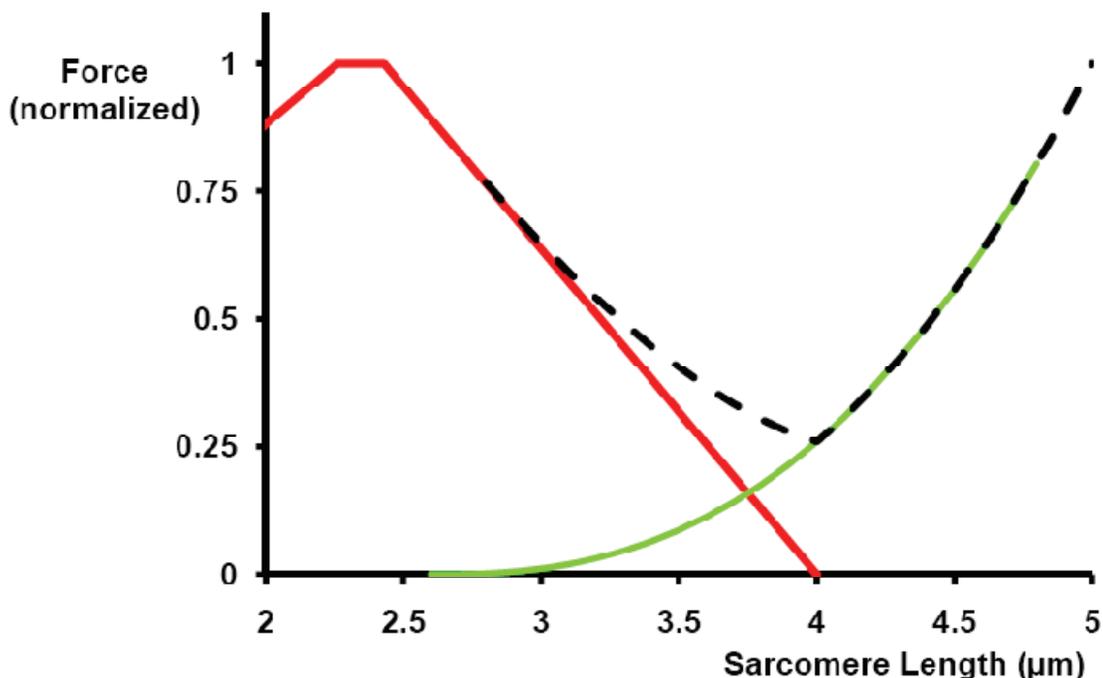


Figure 5.2: Representation of the force-length relationship (adapted from Gordon et al., 1966) showing active force (red) and passive force (green) contributions with the total force (black dashed). Up to sarcomere lengths of about 3.6 μm , active forces dominate, between 3.6 μm and 4.0 μm , passive forces dominate, and beyond 4.0 μm , the passive forces become the only source of force for rabbit psoas myofibrils.

5.2 Methods and Materials

5.2.1 *Sample preparation*

Strips of rabbit psoas muscle were taken from euthanized animals using Dumont # 3 forceps and tied to wooden sticks to preserve the in situ sarcomere length. These strips of muscle were then placed in a rigor/glycerol solution with protease inhibitors (Complete®, Roche Diagnostics, Montreal, Canada) and stored at -20°C for 10 to 14 days (Rassier *et al.*, 2003b). For experimentation, strips of muscle were placed in a $+4^{\circ}\text{C}$ rigor solution, homogenized and placed in the experimental chamber (20°C). Solutions used are

published elsewhere (Joumaa *et al.*, 2007; Tesi *et al.*, 2002). A more complete treatment of the methods can be found in Appendix A of this thesis.

Ethics approval was granted from the institutional Animal Ethics Committee.

5.2.2 *Testing protocol*

59 single myofibrils (with an aggregate total of 312 sarcomeres) from rabbit psoas were tested in these experiments and divided into 8 testing groups. Myofibrils with 3 to 8 sarcomeres in series were used (mean of 5.3) in these experiments due to the high magnification of the microscope (100X oil objective with a 2.5 Optovar) and the large magnitude of the stretches employed.

Group 1 myofibrils (n=12) were non-activated and stretched in a solution containing ATP.

Group 2 myofibrils (n=12) were activated in a calcium+ATP solution and then stretched.

Group 3 myofibrils (n=6) were treated with a mild (0.05 µg/ml) trypsin solution (for titin deletion) (Higuchi, 1992; Granzier & Irving, 1995; Funatsu *et al.*, 1990; Joumaa *et al.*, 2008b) and then kept non-activated and stretched in a solution containing ATP.

Group 4 samples (n=8) were also treated with trypsin in relaxing solution (containing 0.05 µg/ml) but then placed in the activating calcium+ATP solution (no trypsin) and then stretched.

Group 5 myofibrils (n=10) were placed in an activating (calcium+ATP) solution with 20 mM BDM (2,3-Butanedione monoxime), a cross-bridge inhibitor (Tesi *et al.*, 2002) and then stretched.

Group 6 (n=5) myofibrils were non-activated and lengthened to 3.4 µm and then activated in a calcium+ATP solution and then further lengthened.

Group 7 myofibrils (n=2) were non-activated and lengthened to approximately 5.0 µm, then activated and further lengthened to a mean sarcomere length of approximately 6.0 µm.

Group 8 myofibrils (n=4) were activated at optimal length (2.2 µm), lengthened to a mean sarcomere length of approximately 5.0 µm, and then deactivated by placing them in a relaxing solution.

Tests for all myofibrils were performed starting at sarcomere lengths between 2.0 μm - 2.4 μm (except for Group 6) and then lengthened at a speed of 0.1 $\mu\text{m}/\text{sarcomere}/\text{second}$ (which is approximately 5% of the initial sarcomere length per second).

5.2.3 *Sarcomere length and force measurements*

All tests were conducted using an inverted microscope (Zeiss Axiovert 200M, Germany) (Joumaa *et al.*, 2007) equipped with a 100X oil immersion objective (N.A 1.3) and a 2.5x optovar. Individual sarcomere lengths were measured using an ultra high resolution linear diode line scan camera (model SK10680 DJR, Schafter & Kirschhoff, Germany) with a resolution of 6.7 nm/pixel (Joumaa *et al.*, 2007). Sarcomere lengths were calculated from Z-line to Z-line of adjacent sarcomeres and only when this was not possible because the striation pattern tended to disappear with excessive stretching, average sarcomere length was determined by dividing the specimen length by the number of sarcomeres. A custom built piezo-tube motor with a drawn glass pipette was used to manipulate the length of the specimen with nanometer resolution. LabView® software (National Instruments Corp., Austin, Texas, USA) controlled the motor and data acquisition. Myofibril forces were determined using custom built nanofabricated silicon nitride cantilevers (Fauver *et al.*, 1998) with a stiffness of 22 pn/nm (for passive and titin deleted experiments) or 178 pn/nm (for all active experiments). Displacement of one lever attached to the myofibril relative to a reference lever was measured and forces were calculated from the measured displacement and the known lever stiffness (Joumaa *et al.*, 2007). Myofibrils were glued (Dow Corning® 3145)(Linke *et al.*, 1994) to one of the levers and wrapped around the lever to help prevent detachment. Forces were normalized to the cross-sectional area by measuring myofibril diameter (Linke *et al.*, 1994) and were expressed in units of stress ($\text{nN}/\mu\text{m}^2$).

5.3 Results

Activated and stretched myofibrils show much greater stress within the actin-myosin filament overlap zone (sarcomere lengths $<4.0\ \mu\text{m}$) than non-activated and stretched myofibrils, as one would expect (Figure 5.3A), but, completely unexpected, stresses in the activated and stretched myofibrils remain much higher, and increase more rapidly, than those of myofibrils that were lengthened while not activated, even at sarcomere lengths beyond $4.0\ \mu\text{m}$ (Figure 5.3A).

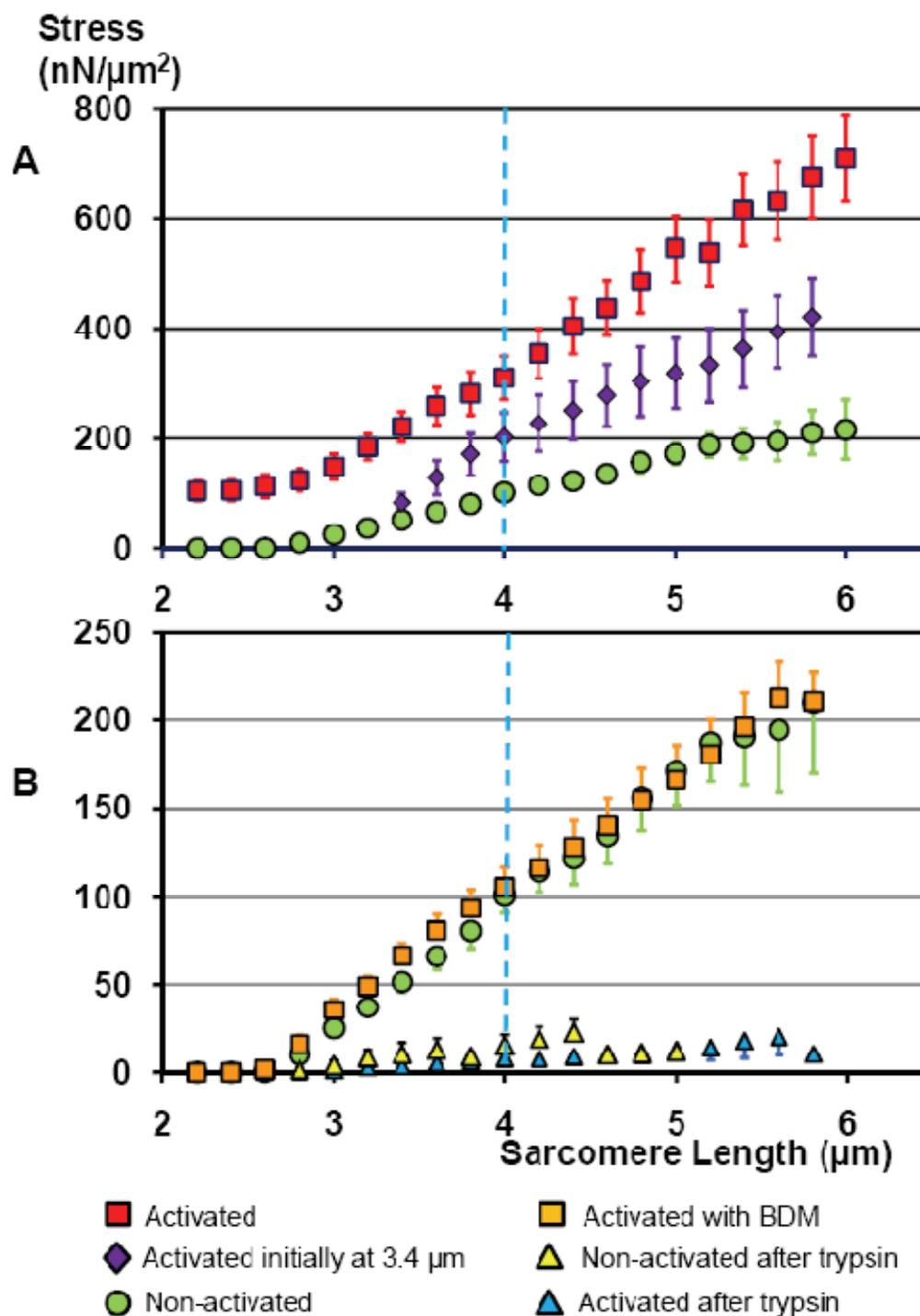


Figure 5.3: Stress as a function of sarcomere length. (A) Mean \pm s.e. of stress versus average sarcomere length for myofibrils stretched in a high calcium (activation) solution from an initial average sarcomere length of 2.4 μm (red squares) and 3.4 μm (purple diamonds), and myofibrils stretched in a low calcium (relaxation) solution (green circles). The stress-sarcomere length relationship curves for both

active myofibrils are significantly higher ($\alpha=0.05$) than that for the non-activated myofibrils within the myofilament overlap zone as one would expect (sarcomere length $< 4 \mu\text{m}$). However, completely unexpected is the 3-4 ($2.4 \mu\text{m}$) and 1.5-2 times ($3.4 \mu\text{m}$) higher stress of the actively compared to the non-actively stretched myofibrils beyond the myofilament overlap zone (sarcomere length $> 4.0 \mu\text{m}$, $p < 0.05$) where actin-myosin based cross-bridge forces are zero, and “passive” stresses were expected to be the same (area to the right of the vertical dashed blue line). Forces are normalized relative to the myofibrillar cross-sectional area and reported as stresses. (B) The stress-sarcomere length relationships for non-actively stretched myofibrils (green circles), for myofibrils stretched in a high calcium activation solution with an added cross-bridge inhibitor (20 mM BDM) (orange squares), after titin deletion in a low calcium relaxation solution (yellow triangles), after titin deletion in a high calcium activation solution (blue triangles). Values shown are means with \pm s.e. There is no isometric stress within the optimal myofilament overlap region ($2.26 \mu\text{m}$ to $2.43 \mu\text{m}$) for the active + BDM myofibrils indicating that BDM alleviated all cross-bridge related active force. Active + BDM treated myofibrils produced the same stress as the non-actively stretched myofibrils, indicating that calcium activation in the absence of actin-myosin based cross-bridge forces had no effect on passive stress during myofibril stretching. Titin deletion virtually abolishes all active or passive stresses indicating that titin is essential for passive stress production and the increased non cross-bridge based stresses observed in this experiment.

In order to elucidate the possible role of titin in the increased force of actively stretched myofibrils, we repeated the active and non-activated stretch experiments following depletion of titin. In the absence of titin, stresses during activated and non-activated stretching were essentially zero and did not increase systematically with increasing sarcomere lengths (blue and yellow triangles, Figure 5.3B), suggesting that titin is crucial for non-activated stress production, as suggested by others (Horowitz *et al.*, 1986; Tskhovrebova *et al.*, 1997; Granzier & Labeit, 2002; Prado *et al.*, 2005) and that titin is essential for the dramatic increase in stress observed here beyond myofilament overlap in the activated and stretched myofibrils (Figure 5.3A).

Activation of myofibrils has been associated with calcium binding to titin and an associated increase in titin's spring stiffness and force upon stretch (Labeit *et al.*, 2003b; Joumaa *et al.*, 2008b). These effects, however, have been thought to be minor but have only been studied within the region of actin-myosin filament overlap. Thus, in order to elucidate the role of calcium on titin's force and stiffness regulation in the

absence of actin-myosin based cross-bridge forces, we performed stretch experiments with calcium-activated myofibrils while inhibiting cross-bridge force through 2,3 butanedione monoxime (BDM) (Tesi *et al.*, 2002;Bagni *et al.*, 2002). Stretching “activated” myofibrils in BDM conditions was successful in abolishing all actin-myosin based stresses (orange squares, Figure 5.3B), and produced passive stresses that were essentially the same as those produced for purely non-activated (passive) myofibril stretching (green circles, Figure 5.3B). This result suggests that calcium activation, and calcium binding to titin has a negligible effect on myofibril force regulation, and cannot explain the results observed in Figure 5.3A. Thus, we hypothesized that actin-myosin based cross-bridge forces prior to stretching are essential to produce the observed force increase of actively compared to non-activated and stretched myofibrils.

In order to test this hypothesis, we performed a further set of experiments in which myofibrils were fully activated at different sarcomere lengths (2.4 μm and 3.4 μm /sarcomere) and then lengthened. The active stresses for myofibrils activated at 3.4 μm (purple diamonds, Figure. 5.3A) were about half of those activated at 2.4 μm (red squares, Figure 5.3A), and when stretched beyond actin-myosin overlap, the stresses remained smaller than those of the myofibrils activated and stretched from sarcomere lengths of 2.4 μm , but were greater than those of the non-activated and stretched myofibrils (green circles, Figure 5.3A). These results suggest that the force regulation observed here at sarcomere lengths beyond actin-myosin filament overlap depends on the active forces prior to stretching.

To test whether actin-myosin based cross-bridge forces could be produced at sarcomere lengths greater than 4.0 μm , we stretched non-activated myofibrils from sarcomere lengths of approximately 2.2 μm to a mean sarcomere length of approximately 4.5 μm to 5.0 μm , then activated them and stretched them further to sarcomere lengths of approximately 6.0 μm (Figure 5.4). Activating myofibrils at sarcomere lengths greater than 4.0 μm did not alter the stress-time curves from passive, indicating that cross-bridge interactions did not occur at these long lengths.

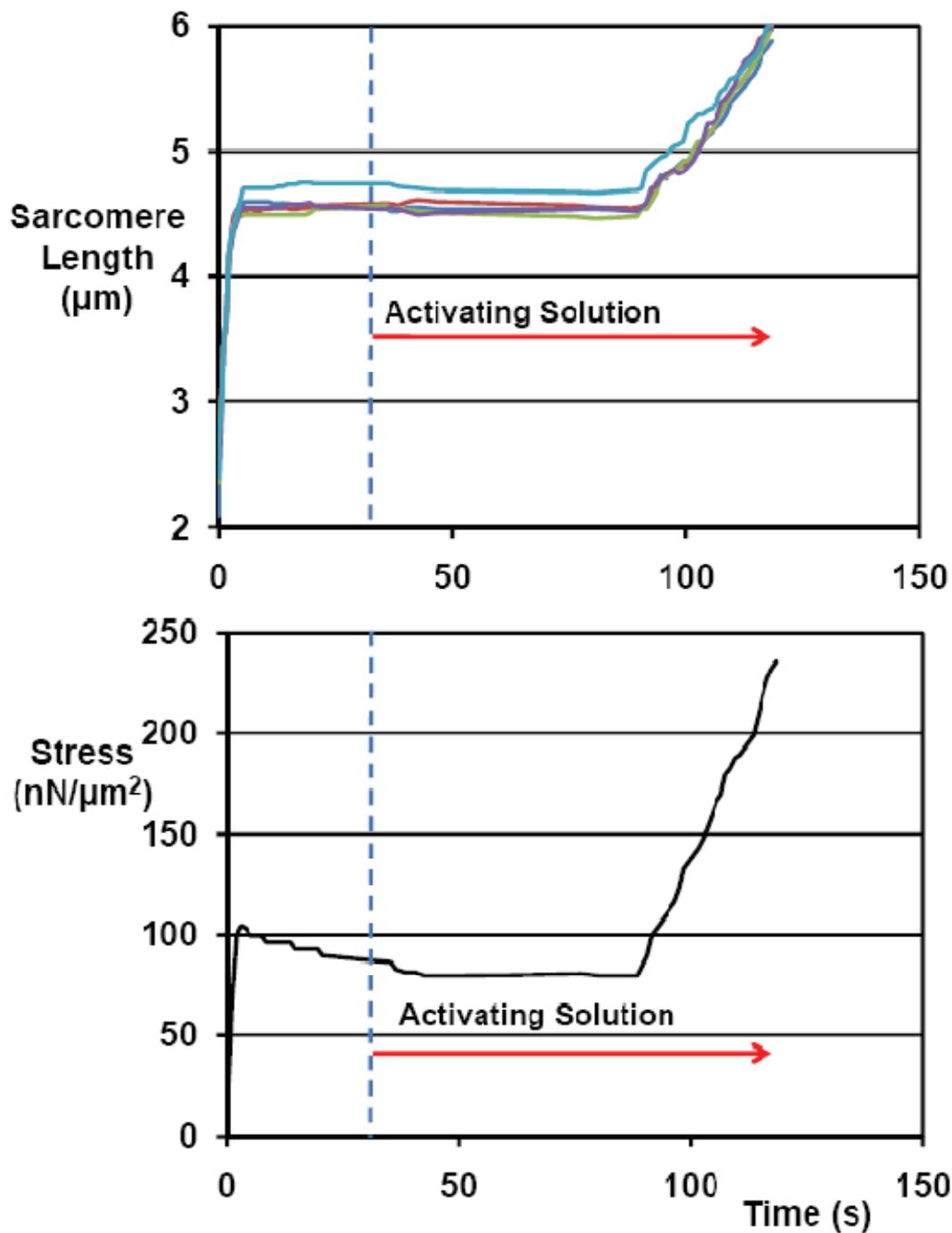


Figure 5.4: Individual sarcomere length-time curves and stress-time curve for one non-activated and lengthened myofibril (passive) from the Group 7 tests. All individual sarcomere lengths could be measured throughout the test, with all sarcomeres greater than 4.0 μm in length prior to the introduction of the activating solution; no change in stress is observed upon activation (at vertical dashed line)

indicating that cross-bridge interactions are not present at these sarcomere lengths. Further lengthening from a mean sarcomere length of 4.5 μm to approximately 6.0 μm in a high calcium activating solution produces stress that is essentially the same as that observed in purely passively lengthened myofibrils at the corresponding sarcomere length.

In order to confirm that the increased passive forces of actively compared to non-activated and stretched myofibrils were not caused by remnant cross-bridge attachments due to myosin filaments being elongated at their ends as suggested by previous work (Wang *et al.*, 1991) or torn from the A-band region due to the large stretch imposed in our testing, we stretched fully activated myofibrils from a mean sarcomere length of 2.4 μm to a mean sarcomere length of approximately 5.0 μm and then replaced the activating with a relaxing solution (Figure 5.5). We observed no effect on stress when the solutions were exchanged thereby confirming that the increase in stress in the absence of actin-myosin overlap was not due to “rogue” myosin filaments attaching to actin even at very long sarcomere lengths. Myofilament non-uniformity within the A-band could possibly occur if individual myosin filaments were to be forcefully dissociated from the A-band during eccentric contraction. It is possible that these myosin filaments would then be able to interact with the thin filaments and generate cross-bridge based force even though the sarcomere is nominally at a length where actin-myosin filament overlap is thought not to occur. These dissociated or “rogue” myosin filaments would need to “slip” or break from the ends of the A-band so as to be able to interact with the thin filament while at the same time somehow sufficiently resist being pulled completely out of the thick filament bundle during active force production.

Myosin filaments that have been pulled from the ends of the A-band would possibly do so because of the high passive forces generated at these long sarcomere lengths but it would be important that the titin associated with the end of the A-band does not rupture or pull anchored A-band titin into the I-band, but instead, leave the A-band titin in place (Wang *et al.*, 1991). Previous work has shown that the A-band region titin can be pulled from its anchor points on the thick filament at sarcomere lengths greater than 3.6 μm in frog muscle (Trombitas *et al.*, 1991) and this is the length at which actin-myosin filament overlap is likely lost (Gordon *et al.*, 1966b). An optical blurring of the edges of the A-

band as it transitions to the I-band could be an indicator of myofilament dislocation and non-uniformity but this could not be observed here. It is not known if this A-band myofilament non-uniformity occurs in the experiments contained within this thesis and further experiments using electron microscopy would be needed to verify this possibility.

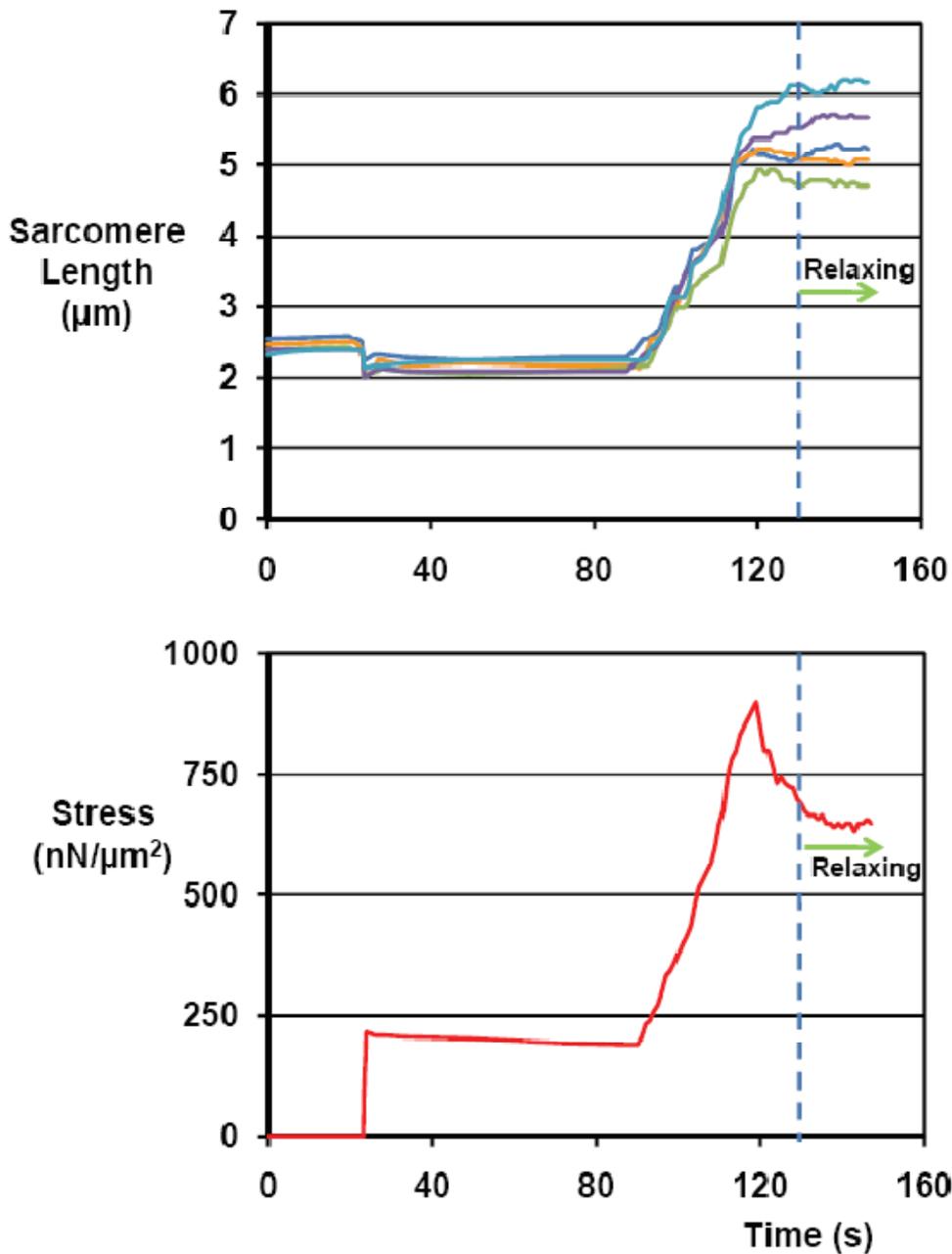


Figure 5.5: Individual sarcomere length-time curves and stress-time curve for an actively lengthened single myofibril composed of 5 sarcomeres in series from the Group 8 tests. There are distinct sarcomere length non-uniformities prior to activation and upon activation all sarcomeres shorten. The myofibril was then lengthened to a mean sarcomere length of approximately 5.0 μm . Sarcomere dispersion increases somewhat at very long sarcomere length but all sarcomeres are stretched beyond 4.0 μm at the final test length. Removing the high calcium activating solution and replacing it with a relaxing solution (area to the right of the vertical blue dashed line) did not alter the stress observed suggesting that there were no remnant actin-myosin cross-bridge interactions at these very long sarcomere lengths.

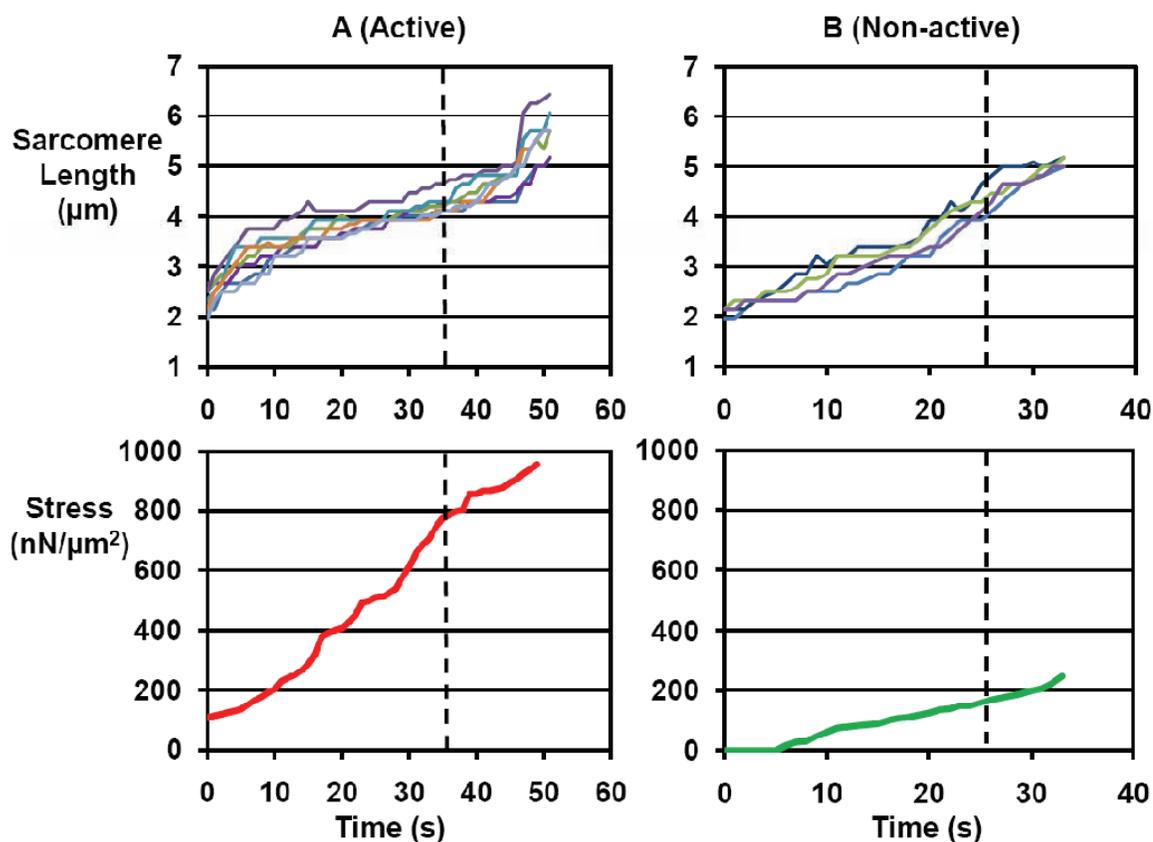


Figure 5.6: Two myofibrils lengthened from approximately 2.4 μm to a mean sarcomere length of approximately 5.0 μm , one actively (A) and one non-actively (passively) (B). The sarcomere length is shown for all sarcomeres in the two myofibrils and although non-uniformities are present initially and continue to be present throughout lengthening, all sarcomeres are eventually beyond myofilament overlap (demarcated by the vertical dashed line) and the stress of the actively stretched myofibril remains much greater than that for the non-activated and stretched myofibril at corresponding sarcomere lengths.

5.4 Discussion

The main result of this study is that in the absence of actin-myosin based cross-bridges, forces can be modulated significantly in skeletal muscles, thereby suggesting that there must be significant mechanisms of force production that are not explained by the cross-bridge theory and the traditional expectation of visco-elastic passive forces. The force regulation observed here is significantly greater in magnitude than the maximal actin-myosin based cross-bridge forces at optimal sarcomere length (Figure 5.3A).

Specifically, we show increases in stress of up to four times in actively (compared to non-activated) stretched rabbit psoas myofibrils at sarcomere lengths beyond actin-myosin filament overlap where active, cross-bridge based forces do not exist (Figure 5.4 and Figure 5.5). When eliminating titin, stresses in the activated and non-activated and stretched myofibrils remain nearly zero even at very long sarcomere lengths, suggesting that titin must be present for this force regulation to take place. Furthermore, since titin is well known to be the primary passive force producer in rabbit psoas myofibrils (Bartoo *et al.*, 1997; Horowitz, 1992), it is safe to assume that titin plays a crucial role in the force regulation observed here for the first time.

It has been known for some time now, that calcium binds to titin upon activation, and increases titin's resistance to stretch (Labeit *et al.*, 2003b; Joumaa *et al.*, 2007). However, this calcium-induced stiffening of titin, although well accepted, has been thought to be of small magnitude and thus functionally irrelevant. Nevertheless, in order to test if calcium binding to titin might play a role in the dramatic up-regulation of force during active elongation, we stretched myofibrils in the activated (activation solution with high calcium concentration) state but inhibited cross-bridge attachment with BDM (Tesi *et al.*, 2002). In these experiments, the stresses measured in the myofibrils were essentially identical to those measured for the purely passive stretches (in relaxing solution), thereby strongly suggesting that calcium activation of titin was not responsible for the observed increased stresses during activated myofibril stretching found in this study (Figure 5.3B; orange squares and green circles, respectively). Based on these results, we speculate that either

“active (actin-myosin based) force” or cross-bridge attachment to actin is required to produce the increased (non actin-myosin based) forces observed with activated myofibril stretching.

If active force or cross-bridge attachment was required for this phenomenon to occur, one would expect that the increase in non actin-myosin based forces was directly dependent on the magnitude of the cross-bridge based forces. In order to test this hypothesis, we performed another set of experiments where stretching of the activated myofibrils was not started at optimal (2.4 μm) sarcomere length, but at a longer (3.4 μm) average sarcomere length where active stresses would be reduced to approximately 40% of those at optimal length (Figure 5.3A). Stretching myofibrils with reduced active stress also produced stresses in the non-overlap zone (sarcomere lengths of greater than 4.0 μm) that were smaller than those obtained for the myofibrils for which active stress was greater, but produced stresses that were greater than those of non-activated and stretched myofibrils (Figure 5.3A). This result provides strong evidence that the increase in non actin-myosin based forces with active stretching is directly linked to active force or cross-bridge attachments to actin.

Before attempting to find explanations for a possible “titin” based force regulation, we need to make sure that more traditional explanations might not be possible. Probably the most frequently used explanation for force enhancement following muscle stretching is the so-called sarcomere length instability and non-uniformity theory (Morgan, 1990; Julian & Morgan, 1979a). According to this theory, sarcomeres are unstable on the descending part of the force-length relationship, and short sarcomeres remain short upon stretching, while long sarcomeres are pulled beyond overlap where they are rescued by passive forces (Hill, 1953). However, the sarcomere length non-uniformity theory is an unlikely explanation for our results for two reasons: first, if sarcomere length non-uniformity produced the dramatic increase in the non actin-myosin based cross-bridge forces, then the highest forces measured in our experiments should never exceed the active forces at optimal sarcomere length, as that would be the limiting force of the short (active force producing) sarcomeres. However, this result was not found, as the stresses of the activated and stretched myofibrils were approximately five times those measured at

optimal sarcomere length (Figure 5.3A). Even if we assume that the short sarcomeres were stretched, and further assume a maximal stretch force enhancement of 100%, we would still not be near the forces required to explain our measurements.

Second, if sarcomere length non-uniformity was responsible for the observed results, then the long (overstretched) sarcomeres should follow the passive force-extension curve once they are pulled beyond actin-myosin filament overlap. However, our experiments observed that non-activated and stretched myofibrils failed mechanically at sarcomere lengths of about $6.3 \mu\text{m}$ and at a stress level ($263 \text{ nN}/\mu\text{m}^2$) that was substantially less than the stresses obtained in the activated and stretched myofibrils, thus sarcomere length non-uniformity seems an unlikely explanation for the results observed here.

Nevertheless, in order to make absolutely sure that sarcomere length non-uniformity could not explain the current results, we measured the individual sarcomere lengths of the stretched myofibrils. Myofibrils are fairly fragile preparations, and one might expect that activation alone might produce a vast increase in sarcomere length non-uniformities, but this was not observed. Furthermore, and in contrast to the expectations of the sarcomere length non-uniformity and instability theory (Hill, 1953), we observed continuous elongation of all sarcomeres when myofibrils were stretched. Sarcomere lengths were not uniform, neither for non-activated and stretched nor for activated and stretched myofibrils, but based on the structural assumptions underpinning the force-length relationship, we assumed (without verification by a direct observational method like electron microscopy) that all sarcomeres were always pulled beyond actin-myosin filament overlap in all cases where such measurements were made (e.g. Figures 5.4, 5.5 and 5.6). Furthermore, activation of myofibrils with all sarcomeres at lengths greater than $4.0 \mu\text{m}$ did not produce an increase in stress (Figure 5.4), nor did deactivation in this same situation produce a decrease in stress (Figure 5.5), strongly suggesting that there were no actin-myosin based cross-bridge forces that affected (or caused) the observed increases in stress in the activated and stretched myofibrils at sarcomere lengths greater than actin-myosin overlap. From all these observations, we may safely conclude that whatever the cause for the dramatic increase in non cross-bridge based forces during active muscle stretching, it cannot be explained with sarcomere instability and the

associated development of sarcomere length non-uniformity. Classic studies by others (Edman & Tsuchiya, 1996; Linari *et al.*, 2003) have proposed the transverse cytoskeleton as a regulator of force during stretch. The transverse cytoskeleton produces lateral force transfer between myofibrils in single fibres (and presumably in bundles of myofibrils) but as our preparation is based on a single myofibril, this possibility of force increase during stretch of active myofibrils is precluded.

In the absence of an obvious traditional explanation for the observed force increases during active stretching beyond actin-myosin filament overlap (such as the cross-bridge or the sarcomere length non-uniformity theory), we must search for other possibilities. Reviewing the experimental evidence, it appears that titin must be present for the observed force increases, but calcium activation of titin alone is not sufficient. However, it is well known that titin can also change its spring stiffness (and thus resistance to stretch) by changing its free spring length in the I-band region by attaching selectively to actin. Here, we would like to tentatively propose, that titin binding to actin might be the cause for the dramatic increase in non cross-bridge based forces when muscles are stretched actively as compared to when they are stretched in the non-activated state.

Although the exact molecular details for such a mechanism need careful evaluation, we would like to suggest the following scenario. Upon active force production, titin preferentially attaches to actin, thereby shortening its spring length and increasing its resistance to stretch (Figure 5.7). In order to fully explain our observations, such titin-actin interactions must be modulated either by active force or by the number of attached cross-bridges. Force could be the modulator of titin-actin attachment through stretching of the actin filament thereby exposing “titin attachment sites” on actin, similar to the way stretching of the talin rod enhances binding of vinculin (del Rio *et al.*, 2009).

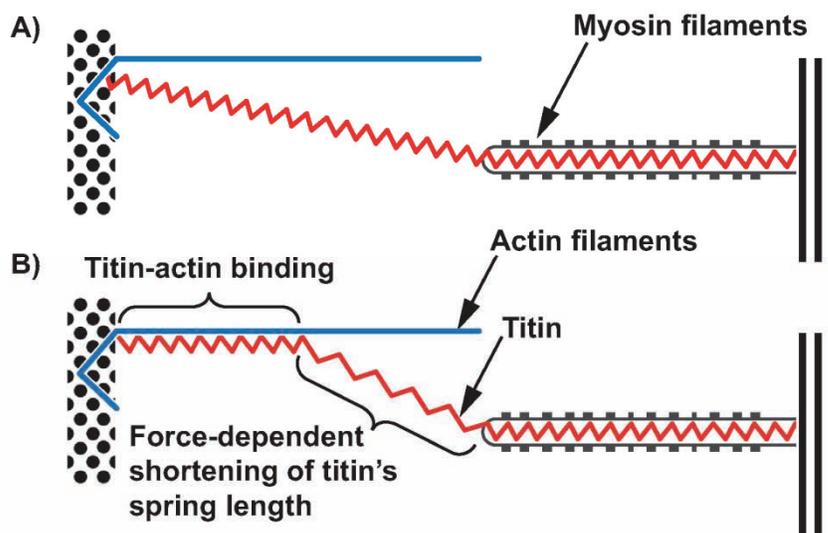


Figure 5.7: Proposed mechanism of force regulation through titin-actin binding. A) Passive myofibril lengthening shown with titin spanning the I-band region. No titin-actin interactions occur so titin is able to extend over its entire free length. B) Active force is thought to enhance interaction and binding of titin to actin thereby shortening titin's natural spring length. Upon stretch, strain in titin's free spring element is greater for a given absolute increase in sarcomere length, thereby increasing titin's force contribution. Thus, forces in stretched muscle are regulated not only through actin-myosin based cross-bridge forces but also through force-dependent interactions of titin with actin.

Alternatively, attachment of the cross-bridges to actin, and the associated movements of the regulatory proteins, troponin and tropomyosin, might free up previously covered titin attachment sites on actin. Whatever the detailed mechanisms, the force modulations produced by it must be as great as the actively produced muscle forces through actin-myosin binding by cross-bridges.

The idea about titin-actin binding in actively stretched myofibrils can be tested relatively easily. Imagine for example that titin is labelled at specific sites with a fluorescent marker so that lengths of individual titin segments can be carefully measured. If we now stretch a non-activated myofibril, we would expect all segments to elongate based on their constitutive stress-strain properties. However, if a myofibril is activated and stretched by the same amount and produces substantially more force as observed here, we would expect that some titin segments (those bound to actin) would not stretch (or stretch significantly less than in the passive state), while others (those not bound) would stretch more and thereby compensating for the “fixed” attached segments of titin. This then would produce greater stiffness and increased forces during stretching of activated muscle.

The proposed mechanism has the advantage that it is independent of actin-myosin filament overlap and would continue to be in operation at lengths greater than myofilament overlap (i.e. 4.0 μm in rabbit psoas). Therefore, this mechanism might provide powerful protection against stretch-induced muscle injuries, the most common mode of damage of skeletal muscles. Also, such a stretch-induced modulation of force would provide an elegant explanation for the observed stability of sarcomeres on the descending limb of the force-length relationship (Rassier *et al.*, 2003a; Joumaa *et al.*, 2008a) which has been thought (erroneously) to be unstable for more than half a century (Hill, 1953). Finally, the proposed mechanism might also offer a partial explanation for the so-called “residual force enhancement” of muscles following active stretching (Abbott & Aubert, 1952).

5.5 Conclusion

We conclude from the results of this study that there is a powerful mechanism for force regulation in muscles that is independent of actin-myosin based cross-bridges. This force modulation depends crucially on the presence of titin and active force. We tentatively suggest that force (or cross-bridge attachment) dependent titin-actin interactions causes the dramatic increase in force of activated and stretched compared to non-activated and stretched muscles.

Chapter Six: An Activatable Molecular Spring Reduces Muscle Tearing During Extreme Stretching

6.1 Introduction

When we fall on ice, or prior to impact in a car collision, humans instinctively brace themselves by tensing their muscles in an attempt to avoid injury. This appears to be a good strategy, as active muscles can absorb more energy than passive muscles. However, when muscles are stretched to extreme lengths, this advantage quickly disappears, as contractile filament overlap is lost (Cutts, 1988), and actin-myosin based cross-bridge forces become zero (Gordon *et al.*, 1966b). The loss of actin-myosin filament overlap and active force results in some sarcomeres being supported by purely passive structures and these overstretched sarcomeres are disrupted and damaged (Talbot & Morgan, 1996; Proske & Morgan, 2001; Morgan, 1990). Previous work has also shown that injury to muscle fibres occurs during eccentric contractions or during single burst movements (Brooks *et al.*, 1995) but not during concentric or isometric contractions (McCully & Faulkner, 1985). It would be beneficial if low passive forces could be reinforced during active stretching to prevent injury and tearing of muscles in accident situations. Here, we demonstrate that such a mechanism exists by showing that forces in actively stretched myofibrils are much greater than in passively stretched myofibrils at lengths beyond contractile filament overlap. The corresponding energy to failure is also much higher in actively compared to passively stretched myofibrils. These results suggest a mechanism of force production in actively stretched muscles that increases with muscle length when actin-myosin based active forces decrease to zero.

6.2 Materials and Methods

Muscle from rabbit psoas was harvested and stored at -20°C with protease inhibitors (Complete®, Roche Diagnostics, Quebec, Canada). On the day of the experiment, muscle was homogenized and placed in the testing chamber. Single myofibrils were attached to a glass needle/piezo motor assembly at one end and to one cantilever of a pair with known stiffness (154 pN/nm or 22 pN/nm) at the other end. An inverted microscope

(Zeiss-Axiovert 200M, Germany) with a high resolution (6.7 nm/pixel) line scan camera (Shafter & Kirchhoff, Germany) was used for sarcomere length measurements. Myofibrillar forces were measured by the deflection of the one cantilever attached to the myofibril relative to the free arm of the cantilever (Fauver *et al.*, 1998). Stress (kPa) was calculated by dividing force (nN) by the cross-sectional area (μm^2) of the myofibril (Linke *et al.*, 1994). All myofibrils were at an initial sarcomere length of approximately 2.2 μm and were stretched actively (high calcium solution, n=12, pCa = 3.5), passively (low calcium solution, n=12, pCa = 8), actively (n=8) and passively (n=6) after a titin deletion protocol using trypsin shown to cleave only titin and result in no changes to other proteins (Fukuda *et al.*, 2001;Granzier & Irving, 1995;Higuchi, 1992), and actively (n=10) with cross-bridge inhibition using 20mM of BDM (Tesi *et al.*, 2002). Stretch speed for all conditions was 0.1 $\mu\text{m}/\text{sarcomere}/\text{second}$ until mechanical failure occurred. Myofibrils which detached from the cantilever or the needle prior to failure were not analyzed. The solutions used are published elsewhere (Joumaa *et al.*, 2007;Tesi *et al.*, 2002;Granzier & Irving, 1995). A more complete treatment of the methods can be found in Appendix A of this thesis.

6.3 Results

Failure was identified when the slope of the stress-extension curve became negative (Tidball *et al.*, 1993); an example of an activated and lengthened myofibril stretched to failure is shown in Figure 6.1.

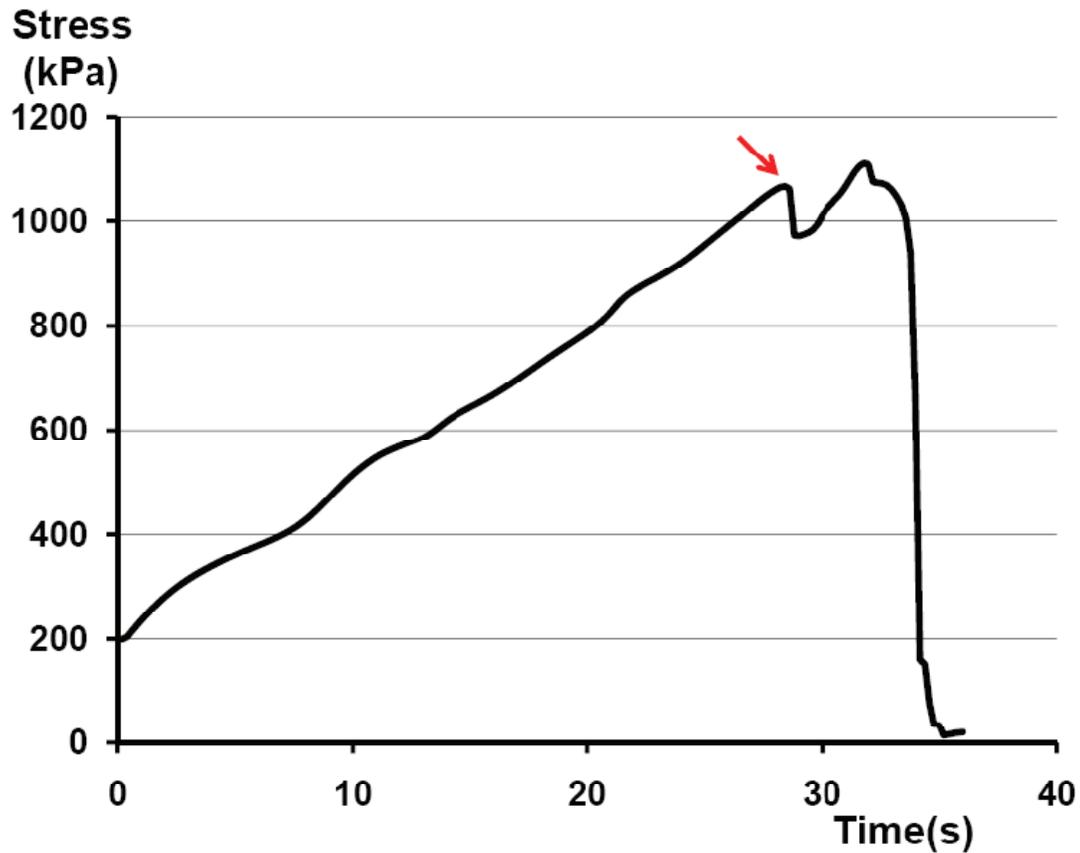


Figure 6.1: Stress versus time for a single activated myofibril stretched to failure. The myofibril was activated at a mean sarcomere length of $2.2 \mu\text{m}$ and then stretched to mechanical failure (arrow, where slope of the stress-time curve first turns negative) and eventual rupture.

Actively and passively stretched myofibrils failed at similar mean sarcomere lengths (Figure 6.2), but failure stresses were significantly greater ($\alpha=0.05$) in the active (1027 ± 164 kPa) compared to the passive myofibrils (262 ± 55 kPa) as were the failure energies (2.32 ± 0.79 picoJoules/ μm^2 active vs. 0.53 ± 0.17 picoJoules/ μm^2 passive), even though cross-bridge forces were presumably zero at failure. Sarcomere lengths were non-uniform during activation and during the subsequent stretching of the myofibrils, as has been observed previously (Rassier *et al.*, 2003a; Telley *et al.*, 2006b; Joumaa *et al.*, 2008a) but at failure, all individual sarcomeres were longer than $4.0 \mu\text{m}$, the length at

which rabbit psoas is known to be beyond myofilament overlap (Huxley & Peachey, 1961; Herzog *et al.*, 1992) and cannot produce cross-bridge force.

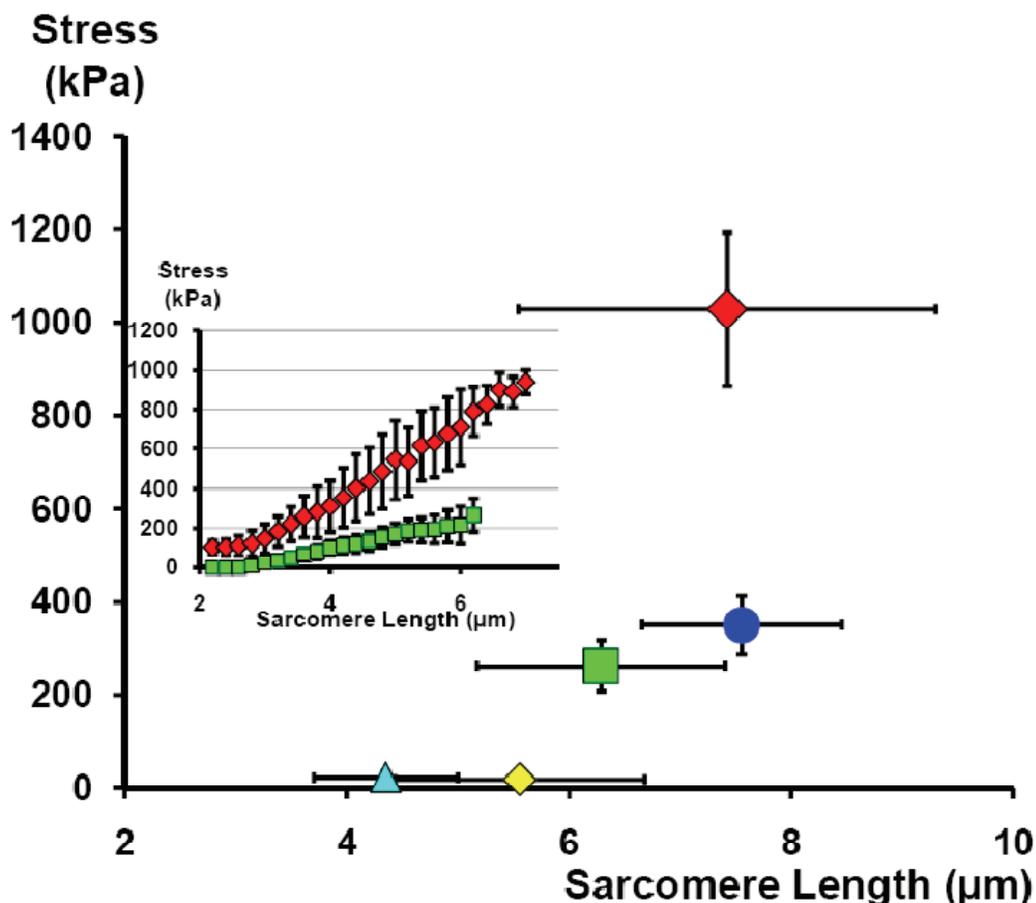


Figure 6.2: Mean failure stress ($\pm 1SD$) versus mean failure sarcomere length ($\pm 1SD$) for actively and passively stretched myofibrils. Failure stresses of actively stretched myofibrils (red diamond) were much greater than for passively stretched myofibrils (green square). Myofibrils stretched in a BDM and high calcium concentration solution (blue circle) failed at slightly higher stresses and sarcomere lengths than the passively stretched myofibrils. Titin deleted myofibrils (active-blue triangle and passive-yellow diamond) failed at very low stresses. Insert panel shows stresses of actively (red) and passively (green) stretched myofibrils as a function of sarcomere length. Insert data are for single active or passive lengthening tests on individual myofibrils pulled to failure. Values are means and $\pm 1SD$ at discrete sarcomere lengths.

Titin deletion reduced failure stresses in actively and passively stretched myofibrils to nearly zero (Figure 6.2), suggesting that titin is essential for active and passive force

transmission across sarcomeres. Inhibition of actin-myosin based cross-bridge forces with BDM in the active state (high calcium) increased failure stresses (350 ± 62 kPa) and energies ($1.06\pm .28$ picoJoules/ μm^2) beyond the levels of the passively stretched myofibrils ($p<0.05$), but this increase was modest compared to that observed between passively and actively stretched myofibrils (Figure 6.2). Therefore, it appears that stress and/or cross-bridge attachment in the physiological range is required to evoke the mechanism that provides the large additional force at muscle lengths when myofilament overlap is lost and cross-bridge forces become zero.

6.4 Discussion

The primary finding here is that actively stretched myofibrils resist stretching to a much greater extent than passively stretched myofibrils at extreme lengths where actin-myosin based cross-bridge forces cannot contribute to force production (Herzog *et al.*, 1992; Huxley & Peachey, 1961). Therefore, there must be a mechanism of force production in the absence of cross-bridge forces. The force produced by this mechanism is potent as it results in a stress difference at failure between actively and passively stretched myofibrils that is in excess of 700 kPa, which corresponds to approximately seven times the maximum cross-bridge based stresses (104 kPa) observed in these myofibrils at optimal sarcomere lengths (Figure 6.2, insert panel, active forces (red diamonds) at $2.4\ \mu\text{m}$).

In whole muscle, passive force is supported by many structures including connective tissues (Kovanen *et al.*, 1984), the sarcolemma (Rapoport, 1973), and the giant protein titin (Bartoo *et al.*, 1997; Horowitz *et al.*, 1986; Granzier *et al.*, 2000). However, in single myofibrils, titin is the primary and almost exclusive source of passive force (Cazorla *et al.*, 2000; Linke *et al.*, 1996). Our results support the passive force role of titin in myofibrils by showing that titin deletion essentially eliminates all stresses in actively and passively stretched myofibrils, and calcium activation in the presence of a cross-bridge inhibitor (BDM) essentially reproduces the passive failure stresses (Figure 6.2). Based on these observations, it appears that titin is a strong regulator of force in actively stretched myofibrils. We propose that titin acts as a molecular spring that

increases its stiffness upon actin-myosin based force production, thereby providing strong protection against injury, especially at extreme sarcomere lengths where cross-bridge forces are zero. The failure stress of the BMD (and high calcium) lengthened myofibrils is significantly higher than the passively lengthened myofibrils (Figure 6.2, blue circle and green square, respectively) but the effect of calcium alone appears to be rather modest, as expected, based on reported small increases in calcium based titin stiffness (Labeit *et al.*, 2003b; Joumaa *et al.*, 2008b).

The large difference in stress production between actively and passively stretched myofibrils might arise from force dependent titin interactions with actin. The conventional view of titin is that it attaches myosin to the Z-disc (Granzier & Labeit, 2007) but titin-actin interactions have been proposed as a method for altering passive stiffness (Yamasaki *et al.*, 2001) and while the bulk of the research into actin-titin interactions has been performed using cardiac titin (Jin, 1995; Linke *et al.*, 1997; Linke *et al.*, 2002), some titin-actin interactions have been observed in skeletal muscle (Kulke *et al.*, 2001). Sarcomere length where failure occurs in actively and passively stretched myofibrils (about 6 μm to 7 μm) is physiologically and functionally irrelevant except perhaps in injury situations. However, sarcomere overstretching, which is the stretching of sarcomeres beyond actin-myosin filament overlap, is thought to be a regular occurrence in eccentric muscle contraction (Morgan, 1990). In fact, sarcomere overstretching has been implicated as the cause for the substantial loss in force that is observed when muscles or fibres are quickly stretched on the descending limb of the force-length relationship (Lieber *et al.*, 1996; Macpherson *et al.*, 1997; Morgan & Proske, 2004), and has been suggested to be the cause for muscle injury and damage in eccentrically contracting muscles (Proske & Morgan, 2001; Morgan & Proske, 2004). However, our results suggest the opposite: active sarcomeres pulled beyond actin-myosin filament overlap (overstretched sarcomeres) are very strong (rather than weak), and thus, if anything, would tend to increase the force in a muscle rather than decreasing it. The lack of a negative slope of the stress-sarcomere length data for activated and stretched myofibrils (Figure 6.2 insert) further suggests that the origin for sarcomere overstretching, the “unstable” “negative” slope of the descending limb of the force-length

relationship (Gordon *et al.*, 1966b), only exists for static conditions (isometric contractions), but is abolished for active stretching.

The site of failure has not been investigated in a single myofibrillar preparation, but work on whole frog muscle has shown that in passively lengthened muscle, failure occurs within the Z-disc and is presumed to originate with titin or desmin filaments (Tidball *et al.*, 1993; Macpherson *et al.*, 1997). Z-line streaming in damaged muscle is likely to involve desmin or titin (Proske & Morgan, 2001) but in myofibrils no proteins external to the sarcomere are present and so in our preparation, desmin can be discounted. We do not know the site of failure in our experiments but the deletion of titin precludes the generation of the high stresses we observed at failure.

6.5 Conclusion

The purpose of this study was to determine failure stresses and failure lengths of actively and passively stretched myofibrils. As expected, myofibrils failed at average sarcomere lengths (about 6 μm to 7 μm) that vastly exceeded sarcomere lengths at which actin-myosin filament overlap ceases to exist (4.0 μm) and thus actin-myosin based cross-bridge forces are zero at failure. Surprisingly, however, actively stretched myofibrils had much greater failure stresses and failure energies compared to passively stretched myofibrils, thereby providing compelling evidence for strong force production independent of actin-myosin based cross-bridge forces. Follow up experiments in which titin was deleted and cross-bridge formation was inhibited at high and low calcium concentrations point to titin as the regulator of this force, independent of calcium. The results of this study point to a mechanism of force production that reduces stretch-induced muscle damage at extreme length and limits injury and force loss within physiologically relevant ranges of sarcomere and muscle lengths.

Chapter Seven: Summary and Future Research

7.1 Summary of the work in this thesis

The three goals of the research conducted in this thesis were:

1. To test whether force enhancement was observed in isolated myofibrils and whether it was associated with sarcomere length non-uniformity and instability.
2. To test whether force enhancement was observed in a mechanically isolated sarcomere.
3. To test whether force enhancement was observed in the absence of actin-myosin based cross-bridge forces.

7.1.1 *Force enhancement and sarcomere length non-uniformity and stability*

Force enhancement has been observed at a variety of structural levels but not at the myofibrillar level which offers the unique ability to determine the length and force sustained by each sarcomere in a single myofibril. Previous work on force enhancement following stretch at the myofibrillar level showed non-uniform sarcomeres and enhanced forces following active stretching, but measured sarcomere length and force in different preparations separately rather than simultaneously (Rassier *et al.*, 2003a). Therefore, in order to uniquely answer the question of force enhancement in single myofibrils and sarcomeres, and elucidate the origin of this mechanical property of skeletal muscle, it was necessary to determine if residual force enhancement was associated with sarcomere length non-uniformities and instability.

Myofibril experiments performed using rabbit psoas and isometric contractions at sarcomere length (SL) of 3.4 μm showed less force than myofibrils isometrically activated at 2.4 μm , as expected, since the longer SL at 3.4 μm placed the myofibrils down the descending limb of the force length relationship where actin-myosin filament overlap is reduced and the number of potential cross-bridge interactions is lessened (Gordon *et al.*, 1966b). Experiments were conducted to investigate force enhancement following stretch by comparing the predicted force for isometric contractions at a long SL

to stretch experiments which started at a short SL but ended at the long (same as the isometric reference) SL. In all cases, the forces observed for the lengthening experiments were significantly higher than the predicted force for an isometric contraction at the same length and residual force enhancement was observed in all sarcomeres. All sarcomeres were analyzed for these experiments and while non-uniformities in SL were observed, they displayed stable behaviour (not having negative stiffness) (Zahalak, 1997) and did not continuously lengthen even though these sarcomeres were on the descending limb of the force-length relationship. Stable but non-uniform sarcomeres in a single myofibril on the descending limb of the force-length relationship is a profound observation because individual sarcomeres in-series must support the same steady state force but, according to the cross-bridge theory, cannot do so actively because of the differing amounts of actin-myosin filament overlap. For all sarcomeres on the descending limb, long sarcomeres should not be able to produce as much active force as short sarcomeres. Based on the work shown here, **sarcomere length non-uniformity and instability do not provide the mechanism underlying residual force enhancement**. One possible scenario is that a passive structure may come into play which supports the additional force required in long and therefore weak sarcomeres. Since titin is the primary (if not sole) source of passive force in myofibrils (Horowitz, 1992; Linke *et al.*, 1994; Granzier *et al.*, 2000; Maruyama, 1997; Freiburg *et al.*, 2000), this result points to titin as a critical component in both sarcomere stability and force enhancement.

7.1.2 *Force enhancement in a mechanically isolated sarcomere*

The most popular explanation for force enhancement following active stretch has been the sarcomere length non-uniformity (overstretching sarcomere) theory (Morgan, 1994). This theory has one attractive feature in that it does not require any changes to the assumptions (e.g. same force per cross-bridge) underlying the sliding filament (Huxley & Hanson, 1954; Huxley & Niedergerke, 1954) and cross-bridge theories (Huxley, 1957a) to explain force enhancement. Essentially, the overstretched sarcomere theory allows for the redistribution of sarcomeres in-series within a myofibril to generate the extra force. This theory then provides a mechanism for force enhancement which is based on a

structural realignment of sarcomeres rather than an inherent property of the individual sarcomere. The aim of this research was to test if force enhancement could exist within a single mechanically isolated sarcomere, thereby providing insight into the origin of force enhancement.

Experiments were performed on single, mechanically isolated skeletal muscle sarcomeres that were activated isometrically at 3.4 μm and then deactivated. This gave the isometric reference force for the sarcomere at a long (final) SL. The same sarcomere was then activated at optimal SL length (2.4 μm), then stretched to 3.4 μm and held until steady state force had developed. This steady state force was then compared to the force produced during the isometric reference contraction at 3.4 μm . Two key observations were made: First, the steady state force after the active stretch to 3.4 μm was significantly greater than the isometric reference force. The enhanced forces exceeded the isometric reference forces by 285%, on average. This result provides compelling evidence that great amounts of force enhancement can be obtained in a single sarcomere, and thus, in the absence of sarcomere length non-uniformities. Another key finding was that the enhanced force after stretch always exceeded the isometric forces at the optimal sarcomere length of 2.4 μm , thereby eliminating the idea that half-sarcomere non-uniformities might explain the observed force enhancement. Taken together, **this research provides strong evidence that force enhancement following stretch is not due to sarcomere overstretching and is intrinsic to the sarcomere.** Combined with other findings (Herzog & Leonard, 2002; Joumaa *et al.*, 2008b; Joumaa *et al.*, 2007; Rassier *et al.*, 2005; Lee *et al.*, 2007; Rassier *et al.*, 2003c; Hisey *et al.*, 2009; Hahn *et al.*, 2007) that force enhancement has an important passive component, it seems possible that the structural protein titin is a contributor to force enhancement following stretch.

7.1.3 *Force enhancement in the absence of actin-myosin based cross-bridge forces*

Force enhancement following active stretch has been associated with two components: an active and a passive component (Herzog & Leonard, 2002). The source of the active component is still a hotly debated subject and could involve altered cross-bridge kinetics (Walcott & Herzog, 2008). The passive component has been observed at several

structural levels (Herzog & Leonard, 2002; Rassier *et al.*, 2005; Rassier & Herzog, 2004) including single myofibrils (Joumaa *et al.*, 2007). Given that titin is recognized as the primary (if not sole) source of passive force in myofibrils (Horowitz, 1992; Linke *et al.*, 1994; Maruyama, 1997; Freiburg *et al.*, 2000), it is likely that the passive force enhancement arises, at least in part, in titin. When muscle is continuously lengthened beyond optimal length (Gordon *et al.*, 1966a; Bornhorst & Minardi, 1970), the relative contribution of cross-bridge generated force becomes less (Gordon *et al.*, 1966b) while the role of passive structures increases. If force enhancement arises in passive structural elements, we would expect this to become apparent when comparing actively stretched and passively stretched myofibrils pulled to lengths beyond actin-myosin filament overlap. The specific aim of this research was to determine whether forces in actively stretched myofibrils were the same or greater than those observed in passively stretched myofibrils at lengths where actin-myosin based cross-bridge forces cannot contribute to force production (i.e. at lengths of $>4.0 \mu\text{m}$).

Experiments were performed on myofibrils that were stretched either actively or passively from optimal SL ($2.4 \mu\text{m}$) to beyond actin-myosin filament overlap and ultimate mechanical failure. At SL where actin-myosin filament overlap was predicted to be lost ($4.0 \mu\text{m}$) based on the linear descending limb of force-length relationship (Gordon *et al.*, 1966b) and rabbit myofilament lengths (Huxley & Peachey, 1961; Herzog *et al.*, 1992), it was observed that actively lengthened myofibrils produced much more force than passively lengthened myofibrils. Removing titin from myofibrils eliminated all active and passive myofibril forces, indicating that **titin is necessary to maintain a structural framework for active and passive force transmission**. The effect of calcium on the stiffness of titin was assessed by blocking active force production using BDM while still using a high calcium activating solution. Using BDM, no significant differences in force were observed between actively stretched (calcium+BDM) and passively stretched myofibrils (no BDM or calcium), indicating that the increased forces in the active tests at SL greater than $4.0 \mu\text{m}$ did not arise from changes in the titin stiffness due to calcium effects. Activating and stretching myofibrils from a SL greater than optimal ($3.4 \mu\text{m}$) produced less active force as expected (due to SL half way down

the descending limb of the force length relationship) and less force at SL beyond actin-myosin filament overlap ($4.0 \mu\text{m}$), but more force than at comparable SL for the passive tests. This result suggests that either active force production or cross-bridge attachments to actin are initially necessary to produce increased myofibril forces at SL beyond actin-myosin filament overlap. This in turn suggests that titin stiffness is increased in the tests where high active forces were present early on in the tests and that the stiffness of titin depends on the amount of active force at SL less than $4.0 \mu\text{m}$ in a dose dependent manner. We propose that titin may become bound to the thin filament through the creation/exposure of force-dependent binding sites on actin or because of changes in configuration of the regulatory proteins troponin and tropomyosin on actin upon cross-bridge binding. There is a precedent for the former scenario for vinculin which can bind to talin through force-induced exposure of binding sites on the talin rod (del Rio *et al.*, 2009). A strong affinity of titin to actin has been established for cardiac (Kulke *et al.*, 2001; Linke *et al.*, 2002; Li *et al.*, 1995; Granzier *et al.*, 1997), but not so much for skeletal muscle (Bianco *et al.*, 2007; Kellermayer & Granzier, 1996; Nagy *et al.*, 2004). To summarize, we propose that **actin-titin interactions may be the mechanism for augmented passive force at all sarcomere lengths**, although compelling evidence is only provided here for sarcomere lengths beyond $4.0 \mu\text{m}$.

7.2 Future Research

7.2.1 *Role of titin-actin interactions in residual force enhancement*

The results of the experiments contained within this thesis strongly suggest that titin plays a crucial role in the enhanced forces observed following active muscle stretch. The proposed mechanism revolves around the shortening of the free region of titin (I-band region) so as to modulate the spring stiffness of the molecule. The proposal is that titin interacts with binding site regions of the thin filament that are made available only when force is applied to the thin filaments or when cross-bridges attach to actin. The enhanced force during the active stretch may deform or otherwise affect the thin filament in a manner that uncovers or makes available sites that will interact with some part of the free

region of titin. Also, cross-bridge attachment on actin is known to move regulatory proteins in the vicinity of actin and so might make binding sites on actin (or possibly even the regulatory proteins) available for titin-thin filament interactions. Titin-actin interactions have been found previously in cardiac preparations (Granzier *et al.*, 1997), and it was proposed then that these interactions would affect the unfolding of specific regions of titin during stretch and therefore modulate passive force. The free region of titin is composed of several distinct domains, including immunoglobulin repeat regions (Ig domains) as well as the PEVK region. Figure 7.1 shows a schematic representation of the half-sarcomere with titin and the behaviour of the molecule with increasing sarcomere length.

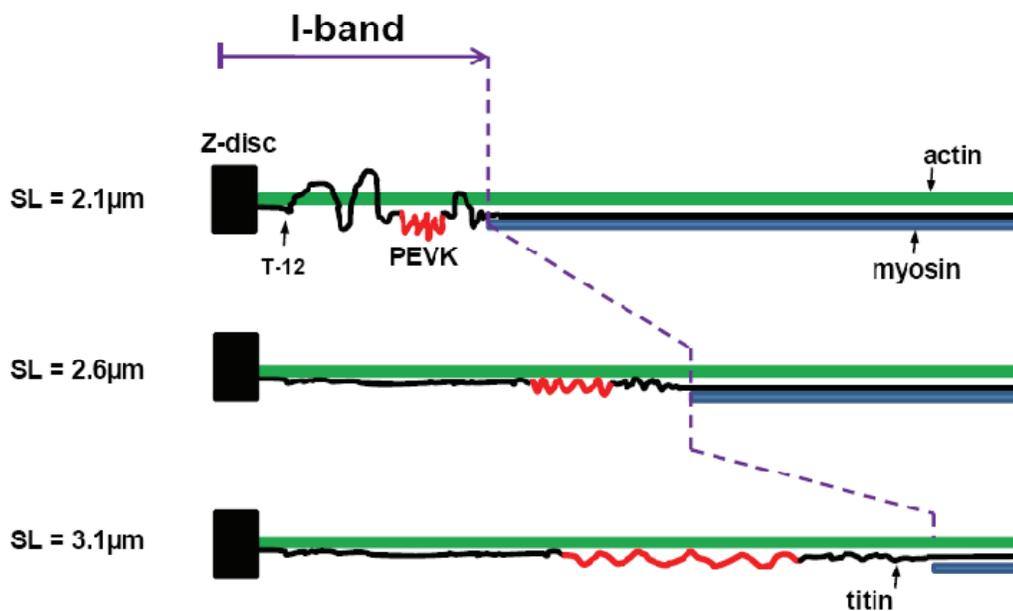


Figure 7.1: Schematic representation of titin behaviour at different sarcomere length. At a SL that is optimal for force production (2.1 μm), titin is at a slack length and no passive force is observed. With increasing SL proximal and distal Ig domains elongate and straighten out and the PEVK region partially unravels. With further SL increase the Ig domains are fully extended and the PEVK completely unravelled. The I-band region of titin from the T12 epitope to the Z-line is bound to actin and is inextensible. Adapted from Linke *et al.*, *J. Cell Science*, 1998.

The I-band region of titin is extensible (Wang *et al.*, 1991;Granzier & Wang, 1993;Linke *et al.*, 1997;Trombitas *et al.*, 1991;Furst D.O. *et al.*, 1988) with some exceptions; specifically the 100 nm region near the Z-line as well as a 50 nm region near the myosin filament (Furst D.O. *et al.*, 1988;Trombitas *et al.*, 1991;Trombitas & Pollack, 1993). The titin molecule associated with the A-band region is tightly bound to the myosin and is essentially inextensible (LeWinter & Granzier, 2010). It has been generally agreed that the extension and unfolding of titin follows a prescribed order as sarcomere lengths increase: initially the Ig domains straighten out, followed by the unravelling of the PEVK region and finally at very long SL, the Ig domains unfold (Linke *et al.*, 1996;Linke *et al.*, 1998;Granzier *et al.*, 1996). However, recent work (Grutzner *et al.*, 2009) has shown that using antibody conjugated Qdot labels on the proximal Ig domains of rabbit psoas myofibrils, under physiologically relevant SL, Ig domains unfold simultaneously with PEVK extension rather than after. The Qdot label protocol offers an opportunity to visualize specific regions of titin and it is possible, at least in theory, to measure titin's extension in active and passive stretch experiments with Ig domain Qdot labels. This approach would allow for observing the specific elongations of isolated regions of titin in active and passive conditions, and thus might provide evidence of titin binding to actin. If titin-actin interactions are absent during active stretching, we would expect increasing displacement between adjacent Qdots as well as increasing movement away from the Z-line. If regions of titin interact during active stretching of myofibrils (as we have proposed here), the Qdots associated with these bound or locked segments would not move with respect to the Z-line nor would the distance between locked Qdots change during lengthening of the myofibril. This would be strong evidence that titin-thin filament interactions take place and render the remaining I-band region of titin shorter and therefore stiffer.

Results obtained in this thesis suggest that titin is a force-dependent spring where titin interacts with the thin filament in a dose-dependent manner. Force-dependent protein interactions have been observed before (del Rio *et al.*, 2009), and if titin did indeed bind to actin in a force-dependent manner, titin would need to be considered a strong force regulator with actin and myosin.

7.2.2 *Sarcomere length non-uniformity and titin*

Experiments in this thesis have shown and confirm previous work describing the presence of sarcomere length non-uniformities in actively lengthened myofibrils (Rassier *et al.*, 2003a; Telley *et al.*, 2006b). However, it was shown here that individual sarcomeres with distinctly different lengths on the descending limb of the force-length relationship can support the same active force not only when actin-myosin filament overlap is present, but also when it is not. This implies that titin plays a role in supporting the force in sarcomeres that were not able to do so actively. Injury and damage in eccentric muscle contraction has been associated with extreme sarcomere length non-uniformity and the overstretching of sarcomeres (Talbot & Morgan, 1996; Morgan & Proske, 2004; Armstrong *et al.*, 1983). It has also been shown that active force imbalances, which are the result of non-uniformities in SL, can be supported in whole muscle fibres when titin and the costamere systems are intact (Claflin & Brooks, 2008). It has been proposed in an MDX mouse model of Duchenne muscular dystrophy, that overstretching of titin was a possible source of the damage to the fibre seen during incomplete activation (Claflin & Brooks, 2008). This potential relationship between muscle damage during eccentric contraction and the disruption of titin seems plausible but more work is needed, specifically, to look at how titin's ability to regulate force may affect the development of localized extreme sarcomere lengths in muscle.

Experiments in which selected regions of an activated fibre were deactivated (Miura *et al.*, 2008; Wakayama *et al.*, 2005; ter Keurs *et al.*, 2006) have been performed and this approach could possibly be utilized in single myofibrils to observe (in-)stability and force regulation of sarcomeres in view of local deactivation. A small pipette with a diameter of 3 μm to 5 μm could deliver a jet of relaxing solution to one or two adjacent sarcomeres in an activated myofibril to observe the associated length changes in the relaxed sarcomeres and the changes in myofibrillar force associated with local sarcomere deactivation. If these deactivated sarcomeres dramatically lengthen it would be interesting to investigate if the amount of lengthening is proportional to the isometric force achieved during initial isometric activation. If titin binding to actin is force dependent as we have proposed,

then higher initial cross-bridge based force would result in a less dramatic overstretching in the few deactivated sarcomeres since titin would be bound to actin with the free portion of the molecule being stiffer and the sarcomere less likely to lengthen uncontrollably. The functional implications of this could be that weaker sarcomeres might be protected from uncontrollable lengthening (and damage) during eccentric contractions because of the increased titin stiffness.

7.3 Summary of future research

These proposed experiments investigating whether the extensible region of titin binds to the thin filament in a force-dependent manner as well as the experiments investigating local force-dependent sarcomere length (in-) stability could help elucidate a mechanism for some of the results observed in this thesis and help translate these findings to a functional and physiologically relevant mechanism of injury in lengthened and activated muscle. They could also provide further support for viewing titin not purely as a passive molecular spring but rather as a third myofilament capable of making important contributions to active force regulation in skeletal muscle.

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APPENDIX A: METHODS OVERVIEW

All experiments contained in this thesis were performed on skeletal muscle myofibrils and share the same general experimental methods. These methods are duplicated throughout the chapters which were written as manuscripts for publication. This summary is designed to collect the various methods sections contained within this thesis into one location but individual chapters will have contained in their methods sections, specific alterations or additions above and beyond what is contained in this method overview.

A.1. Tissue

Ethics approval for these experiments was granted by the Life and Environmental Sciences Animal Care Committee of the University of Calgary. Rabbit psoas muscle was used in all myofibril preparations. Six month old female New Zealand White rabbits (Riemens, Fur Ranches Ltd., Saint Agatha, Ontario, Canada) were euthanized by an I.V. injection of sodium pentobarbital (MTC Inc., Cambridge, Ontario, Canada). Psoas muscle strips were freed using Dumont # 3 forceps (Fine Science Tools Ltd., Vancouver, B.C., Canada) and #15 scalpel blade (VWR Inc., Mississauga, Ontario, Canada). Strips of muscle were tied to wooden sticks to preserve the *in-situ* muscle length. Once tied, the ends of the attaching muscle were cut and the specimen was placed in a 15 ml centrifuge tube (Falcon-BD part# 352099, VWR Inc., Mississauga, Ontario, Canada) containing 14 ml of rigor solution and protease inhibitor (Complete®, Roche Diagnostics, Quebec, Canada). These samples were stored at +4 °C for 8 hours. They were then transferred to a new 15 ml tube containing fresh rigor solution (50% rigor solution with protease inhibitor and 50% glycerol) and stored at +4 °C for 18 hours. They samples were then placed in a new tube containing fresh rigor solution (50% rigor solution with protease inhibitor and 50% glycerol) and stored at -20 °C for 10-14 days. On the day of experimentation, the muscle sample (still tied to the wooden stick) was placed in a glass Petri dish (on ice) in 20 ml of fresh rigor solution for 1 hour. Then, a small (1 mm thick by 3 mm long) piece was taken from the middle region of the sample (between the sutures that were used to

maintain the *in-situ* length) using a # 15 scalpel blade and placed in a plastic tube (part# 72-1327, Harvard Apparatus Inc, St-Laurent, Quebec, Canada) with 1 ml fresh rigor solution and homogenized (Model PRO250, Pro Scientific, Oxford, CT, USA). The tissue sample was homogenized using the following protocol:

10,000 (revolutions per minute) rpm for 10 seconds, repeated twice, then
13,200 rpm for 5 seconds, repeated four times, then
16,000 rpm for 2 seconds, repeated four times, then
18,800 rpm for 2 seconds, repeated once.

The homogenate was kept in the plastic homogenization tube and the tube was placed in ice at 0 °C.

A.2. Solutions

Solutions used in all experiments fall under three classifications, rigor, relaxing and activating and have been reported previously (Joumaa *et al.*, 2007; Tesi *et al.*, 2002; Rassier *et al.*, 2003a). A custom written computer program was used to calculate the pCa and allow for ligand/EGTA effects on the final calcium concentration, in a similar way to what has been reported previously (Colomo *et al.*, 1997; Fukuda *et al.*, 1996). All experiments in this thesis used the identical three formulations. All solutions were pH adjusted using either 10 M potassium hydroxide or 40% hydrochloric acid and a TRIS compatible pH meter and electrode (Corning Pinnacle 530, Corning Inc, Corning, NY, USA).

Rigor solution (storage and homogenizing): 50 mM 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris), 100 mM sodium chloride, 2 mM potassium chloride, 2 mM magnesium chloride, 10 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA) all adjusted to pH = 7.0.

Relaxing solution (passive tests): 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 64.4 mM potassium propionate, 9.45 mM sodium sulphate, 5.23 mM magnesium propionate, 10 mM EGTA, 0.188 mM calcium chloride, 7 mM adenosine triphosphate (ATP) at pCa = 8.0 and adjusted to pH = 7.0.

Activating solution (active contraction tests): 10 mM MOPS, 45.1 mM potassium propionate, 5.21 mM magnesium propionate, 9.27 mM sodium sulphate, 10 mM EGTA, 9.91 mM calcium chloride, 7.18 mM ATP, 10 mM creatine phosphate at pCa = 3.5 and adjusted to pH = 7.0.

Where applicable, trypsin was used to degrade titin. The trypsin (part# T8003, Sigma-Aldrich Ltd., Oakville, Ontario, Canada) concentration used (0.05 µg/ml) in the titin deletion experiments was based on previous work by Joumaa et al. (2008b), but here the time allowed for of digestion of the titin was increased from 5 minutes to 8 minutes. Previous titin deletion using trypsin by others (Granzier & Irving, 1995;Fukuda *et al.*, 2005;Funatsu *et al.*, 1990;Higuchi, 1992) had used much higher concentrations of trypsin when compared to the work presented here. It has been shown previously that 0.05 µg/ml of trypsin for 8 minutes was sufficient to selectively remove titin (Joumaa *et al.*, 2008b) and the work by others at the higher concentration demonstrated convincingly that the other proteins (actin and myosin) remained after the trypsin treatment.

Where applicable, BDM (part# B-0753, Sigma-Aldrich Ltd., Oakville, Ontario, Canada) was used in the activating solution at a concentration of 20 mM, estimated to be sufficient to inhibit all cross-bridge based force (Tesi *et al.*, 2002).

A.3. Microscope and testing apparatus

A custom-built testing chamber with solution input and removal ports was placed on the stage of an inverted microscope (Zeiss Axiovert 200M, Zeiss, Germany). The microscope had four Zeiss objectives which were used in sequence from lowest magnification to highest to locate suitable single myofibrils for testing. The objectives used were 5x (N.A. 0.15), 20x (N.A. 0.50), 40x (N.A. 0.75) and 100x oil immersion (N.A. 1.3). The microscope was also equipped with a 2.5x optovar and all tests were conducted using phase-contrast illumination so as to allow for visualization of the I- and A-bands of the sarcomeres. The microscope was equipped with a 60 Hz video camera (model# XC-ST50, Sony Corp., Japan) and a high-resolution line-scan camera (linear

array) with 10,680 elements (model# SK10680 DJR, Schafer & Kirschhoff, Germany) with a theoretical resolution of 6.7 nm/element at 250x magnification and a sample rate of 5 Hz. The video images were captured on video tape (model# W625CF, Toshiba Corp, Japan). Radial piezo-tubes (part# PZT-5H with 90° quadrants, Boston Piezo-Optics Inc, Bellingham, MA, USA) were used, coupled to pulled 5 μ l glass pipettes (part# 53432-706, VWR Inc., Mississauga, Ontario, Canada) to control the myofibril specimen length. Pipettes were pulled to a fused, sharp tip on a pipette puller (Model # 720, Kopf Instruments Ltd., Tujunga, CA, USA). Forces were estimated using custom-built silicon nitride cantilevers (Fauver *et al.*, 1998). Cantilevers were fabricated using LPCVD silicon nitride coated 4 inch silicon wafers using photolithographic and reactive ion etching processes in a class 100 clean room facility. These devices were fabricated at the Cornell Nano-scale Facility of Cornell University (Ithaca, NY, USA). The displacement of one cantilever with respect to the second lever of a pair and knowing the stiffness of the device allows for calculating the forces generated during the experiments. Frequency response of the cantilevers in fluid is greater than 1 kHz which is sufficient for the tests performed here. Micromanipulators (model# MMN-1, Narishige Inc, Japan) were used for each of the piezo-tube motor assembly and the cantilevers so that myofibrils could be attached to both the drawn glass pipette and to one of the cantilever pairs. The glass pipette and one arm of the cantilever pair were attached to the I-band region (but as close as possible to the Z-line) of the end sarcomeres in a myofibril, or in the case of the single mechanically isolated sarcomere tests, to the I-band regions of the adjacent sarcomeres to the single sarcomere being isolated. Motor length control and data collection from the line-scan camera were controlled by custom written software (LabVIEW®, National Instruments Corp, Austin TX, USA). For the single mechanically isolated sarcomere, the method is similar to that used previously (Pavlov *et al.*, 2009b) however, we used a silicon nitride cantilever to measure displacement rather than a glass pipette needle, the advantage of this being that the properties and dimensions of the cantilevers are known beforehand and force can be calculated directly, whereas each glass pipette must be calibrated following the experiment. A mechanically isolated single sarcomere is shown in Figures A1 and A2.

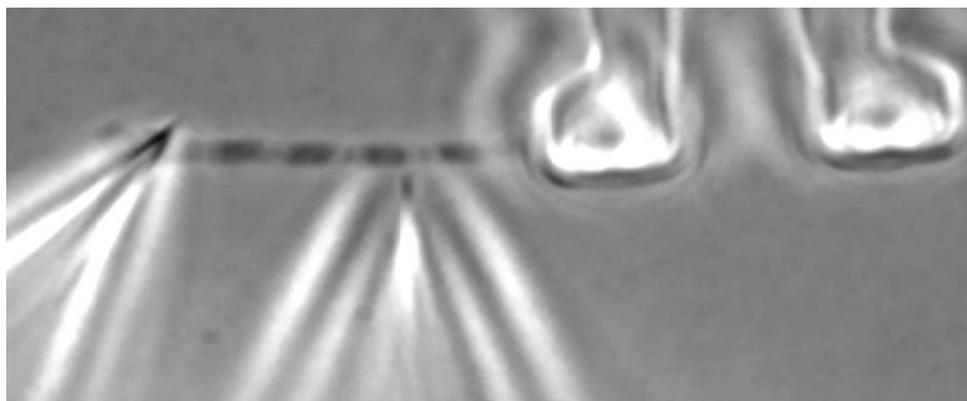


Figure A1: Photograph of a mechanically isolated single sarcomere. The cantilever and glass needle (motor) are attached to the I-band region of the adjacent sarcomeres, thus preventing damage to the sarcomere of interest. The remainder of the myofibril (three sarcomeres to the left) is then shortened and placed to the side and slightly below for testing purposes.

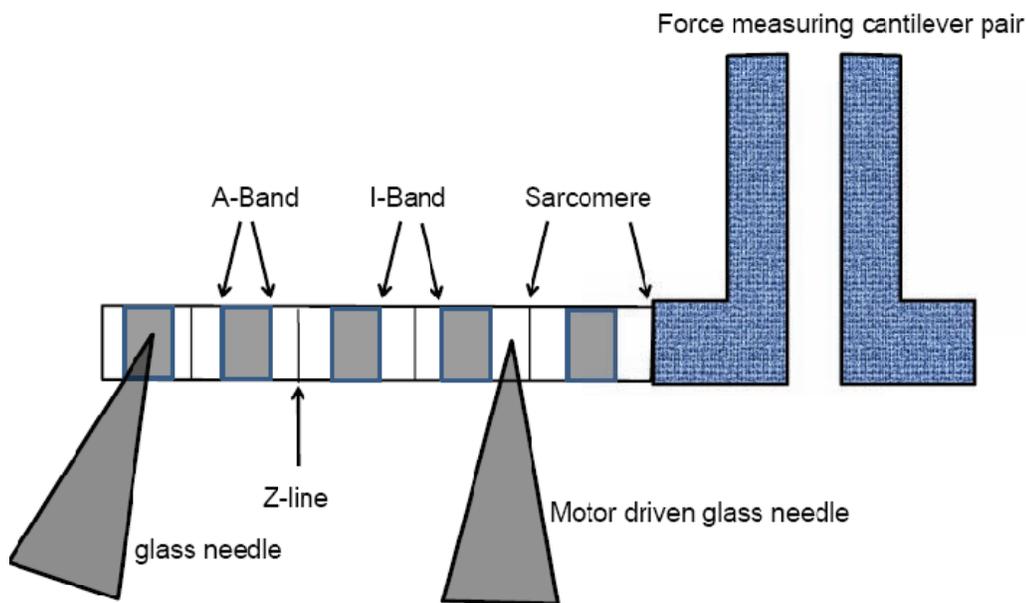


Figure A2: Diagram showing a single mechanically sarcomere at the end of a myofibril with four sarcomeres in-series. The ends of the glue coated cantilever and the tip of the motor driven glass needle are attached to the sample outside of the sarcomere of interest, as close to the Z-line as possible but within the I-band of the adjoining sarcomere. In this way, the sarcomere of interest lies between the cantilever and the motor driven glass needle. One sarcomere is lost from view since it is glued to the cantilever and is not visible.

Fluid exchange was by using two 10 ml plastic syringes (part# CABD309604, VWR Inc., Mississauga, Ontario, Canada), one for addition and one for simultaneous removal of the solutions in all experiments. All experiments were conducted at 20-22 °C with the exception of the single sarcomere force enhancement experiments, which were conducted at 4 °C.

A.4. Analysis

Data from the line-scan camera was analyzed using custom written software in MATLAB® (The Mathworks Inc., Natick, MA, USA). Sarcomere length was determined using Z-line to Z-line distances and when Z-lines became difficult to determine (typically when sarcomere lengths which exceeded 4 μm), sarcomere length was determined by measuring the distances between the centers of adjacent A-bands.

Statistical testing was done using Stata software (Stata 8.2, Stata Corp., College Station, TX, USA).