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The Origin of Force Increase in Actively Stretched Single Muscle Fibres

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The Origin of Force Increase in Actively Stretched Single Muscle Fibres

by

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A THESIS
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Abstract

Theoretically, at long sarcomere lengths (SLs) beyond myofilament overlap, only passive forces, produced by passive structural elements, are possible. However, previous studies showed a dramatic increase in force above the passive force when myofibrils were actively stretched beyond actin and myosin filament overlap. It has been suggested that titin might produce this increase in force, possibly by increasing its stiffness in a variety of ways. In this study, I used rabbit psoas single muscle fibres to investigate whether the increase in force observed after active stretch to a sarcomere length beyond myofilament overlap was caused by a passive component (titin) alone, or possibly some remnant cross-bridges that continue to contribute force in some unknown manner in muscle fibres. The results indicate that both passive and active components are contributing to this force increase in actively stretched single muscle fibres at an average sarcomere length beyond myofilament overlap.
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So the SL at $E = z + a + a + c = 3.91 \, \mu m$. $D = z + a + a + b = 2.43 \, \mu m$. $C = z + a + a = 2.26 \, \mu m$. $B = z + c = 1.75 \, \mu m$. (Adapted from Gordon et al., 1966).

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# List of Symbols, Abbreviations and Nomenclature

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<tr>
<td>RFE</td>
<td>Residual force enhancement</td>
</tr>
<tr>
<td>SL</td>
<td>Sarcomere length</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>BDM</td>
<td>2,3 butanedione monoxime</td>
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</table>
One never notices what has been done; one can only see what remains to be done.

Marie Curie
Chapter One: Introduction

Skeletal muscles produce force and human movement. Many basic muscle properties have been discovered in the last century. Arguably the key finding of the 20th century was the discovery that muscle contraction occurred via the sliding of two sets of filaments, actin and myosin (A. F. Huxley & Niedergerke, 1954; H. E. Huxley & Hanson, 1954), and associated interactions of cross-bridges, as described in the cross-bridge theory. A. F. Huxley (1957) was the first to provide a mathematical framework for the cross-bridge theory. A further conceptual step in the cross-bridge theory occurred when A. F. Huxley and Simmons (1971) proposed that cross-bridges rotate rather than translate and produce force and shortening with this rotation. Another conceptual development occurred with the discovery by Rayment et al. (1993), that the cross-bridge did not rotate around an attachment point on actin, but that cross-bridge attachment to actin was rigid, while part of the cross-bridge, the lever, rotated around approximately 45 degrees, to produce force and contraction of muscle.

Today, the cross-bridge theory is a well-accepted explanation for muscle contraction, and some mechanical properties of muscle can be directly derived from the theory. For example, the plateau and the descending limb of the force-length relationship are well explained with the amount of overlap between actin and myosin as muscle sarcomere lengths (SLs) change, and thus the number of potential interactions between myosin-based cross-bridges and actin attachment sites changes as well (Gordon, A. F. Huxley, & Julian, 1966). Also, the force-velocity properties of concentrically contracting muscles can be well captured with the cross-bridge theory, provided adequate rate functions of cross-bridge attachment and detachment are used.
However, some mechanical properties of skeletal muscles that are well documented and generally accepted, are not well captured by the cross-bridge theory. For example, the residual force enhancement (RFE) property of muscle, first described systematically by Abbott and Aubert (1952), eludes satisfactory explanation with the cross-bridge theory. RFE has been observed universally in all skeletal muscles, and manifests itself in entire muscles and single sarcomere preparations, and on any structural level between these two extremes.

RFE has been observed at all SLs (Abbott & Aubert, 1952; Edman, Elzinga, & Noble, 1982; Morgan, Whitehead, Wise, Gregory, & Proske, 2000; Joumaa, Leonard, & Herzog, 2008). Leonard and Herzog (2010) showed that RFE occurred even beyond actin-myosin filament overlap in single sarcomere and myofibril preparations. This latest observation is insofar important as cross-bridge-based forces are presumably eliminated at SLs beyond actin-myosin filament overlap, and thus cannot contribute to the enhanced force. In order to test if cross-bridge based forces contribute to RFE beyond actin-myosin filament overlap, rabbit psoas myofibrils that were actively stretched to an average SL of 5.0 µm length (actin-myosin filament overlap in these preparations is lost at SL of 3.9 µm) and then deactivated at the final length with no apparent loss of force (Leonard & Herzog, 2010). This result suggested that a passive structural element, such as titin, may produce this enhanced force in single myofibrils at very long SLs.

In single myofibril preparations, SLs and half-sarcomere lengths can be measured individually for each sarcomere, and loss of myofilament overlap in all sarcomeres can be verified optically. However, single fibres are arguably much more relevant preparations for furthering our understanding of the mechanics of muscle contraction and force production. In contrast to single myofibrils with all sarcomeres arranged strictly in series, single fibres contain sarcomeres in series and in parallel, and not all individual sarcomere and half SLs can be
measured. Furthermore, sarcomeres are connected by structural proteins within muscle fibres, and thus the mechanical system of forces in sarcomeres becomes redundant and cannot be solved without making non-trivial assumptions. In addition, it has been known for a long time that SLs in single fibres are highly non-uniform with fibres containing sarcomeres within a range of 1.8-4.2 µm (A. F. Huxley & Peachey, 1961, their figure 2). This extreme non-uniformity of SLs in fibres, combined with a structurally redundant system, may affect the mechanical properties in general and the RFE property of skeletal muscle specifically, to a great degree, and may differ substantially between single fibres (and thus presumably muscles) and single myofibrils.

The purpose of this thesis research was to investigate the force increase at steady-state after active stretch to SLs beyond myofilament overlap in single fibres, and test whether this increase in force is caused by a passive component (titin) alone, or possibly some remnant cross-bridges that contribute to force in some unknown manner in muscle fibres.

This thesis starts with a review of the relevant literature in Chapter 2, introducing muscle structure and hierarchy, muscle force regulation, RFE, force increase after active stretch beyond myofilament overlap, and the proposed mechanisms for this force increase. Chapter 3 describes the methods used in this study. Results are shown in Chapter 4, and in Chapter 5, my results are carefully discussed. In Chapter 6, I will briefly make some general conclusions about this research, and provide some ideas for possible future research in this area.
Chapter Two: Literature Review

2.1 Skeletal muscle structure

There are three types of muscle found in the human body: skeletal muscle, smooth muscle, and cardiac muscle. Skeletal muscles are the muscles that attach to bones through tendons. Skeletal muscles are responsible for force generation and movement production. Skeletal muscles are under voluntary control while smooth muscles and cardiac muscles are not.

Skeletal muscles are typically surrounded by multiple connective tissue sheaths, with the one closest to the muscle tissue called the epimysium. The epimysium penetrates into muscles dividing muscles into muscle bundles, and when doing so, the connective tissue is called perimysium. Muscle bundles contain muscle fibres, and muscle fibres individually are also surrounded by a connective tissue layer typically referred to as endomysium. Muscle connective tissue is primarily made up of collagen fibres, and the connective tissue provides the structure for the muscle and allows for force transmission between fibres, fascicles and even entire muscles (Borg & Caulfield, 1980; Rowe, 1981).

Muscle fibres are made up of myofibrils lying parallel to each other (Herzog, 1999, Figure 2.1). A network of proteins connects the myofibrils and connects myofibrils to other fibre organelles (Gautel & Djinović-Carugo, 2016, their figure 1). This network of proteins provides fibres with mechanical stability, elasticity, spatial organisation and the capacity for long range communication (Gautel & Djinović-Carugo, 2016). The protein network also prevents fibres from tearing during muscle contraction. In skeletal muscle, there are some intermediate filaments, such as desmin, synemin, and vimentin. These intermediate filaments wrap around the Z-line (the border of a sarcomere) and connect sarcomeres within a myofibril and myofibrils.
within a single fibre (Wang & Ramirez-Mitchell, 1983). Myofibrils are formed of sarcomeres arranged in series.

![Schematic illustration of skeletal muscle structure](image)

**Figure 2.1** Schematic illustration of skeletal muscle structure (Adapted from Herzog, 1999).

### 2.1.1 Sarcomeres.

The basic contractile unit in skeletal muscle is the sarcomere. A sarcomere is bordered by the so-called Z-lines (Figure 2.2). Sarcomeres contain the contractile filaments actin (or thin filament) and myosin (or thick filament), and structural proteins, such as desmin, nebulin and titin, all of which are thought to have distinct functional roles.
2.1.1.1 Thick and thin filaments.

The thick (myosin) filaments are in the centre of a sarcomere and form the so-called A-band (anisotropic) (Figure 2.2). Thick filaments are primarily composed of myosin II proteins. Each myosin molecule contains a long tail which helps form the thick filament, and a globular head, which extends outward from the thick filament and contains a binding site for attaching to thin filaments, and an enzymatic site for the hydrolysis of adenosine triphosphate (ATP). Muscle contraction requires energy which is obtained by splitting ATP into adenosine diphosphate (ADP) and a free phosphate (P). When a myosin cross-bridge attaches to actin, it will rotate, thereby pushing the thin filaments towards the centre of the sarcomere, assuming that the
external force resisting muscle shortening are smaller than the internal forces produced by the muscle (Herzog, 1999).

Thin filaments emerge on either side of the Z-line. The area just containing thin filaments is called the I-band (isotropic) (Figure 2.2). Two chains of linked actin globules (filamentous of F-actin) are the main components of the thin filaments. The two F-actins are wound around each other and form a helix (Doblare & Merodio, 2015). Actin globules have a diameter of about 5.4 nm. The thin filament also contains so-called regulatory proteins, most importantly the troponin complex and tropomyosin. The troponin complex is made up of troponin C (contains sites for calcium binding), troponin T (linked to tropomyosin), and troponin I (inhibits cross-bridge formation). Tropomyosin is a long filamentous protein running in the groove of the two F-actins that make up the thin filament.

Actin and myosin are thought to be the only contractile proteins in skeletal muscle, which are proteins that produce force and contraction by using chemical energy (through ATP hydrolysis). Troponin and tropomyosin are the regulatory proteins that regulate cross-bridge attachment through calcium binding (Herzog, 1999).

2.1.1.2 Titin.

Titin is a long, elastic protein that was discovered in the 1970s by Maruyama et al. (1977) and Wang et al, (1979). Titin is the largest known protein in animals with a molecular weight ranging from 3-4 MDa). It is the third most abundant muscle protein (Gao, Lu, & Schulten, 2001). Titin spans a half sarcomere from the Z-line to the M-line. Titin in the A-band region is thought to play a role in controlling the length of the thick filament during early muscle development (Labeit, Kolmerer, & Linke, 1997), maintaining the structural integrity of the
sarcomere (Horowits & Podolsky, 1987), and plays a role in protein signalling (Labeit, Kolmerer, & Linke, 1997). The I-band titin extends when the sarcomere is passively stretched and accounts for most of the passive force produced by single myofibrils (Granzier et al., 2000).

Figure 2.3 Schematic illustration of titin springs in a sarcomere (Powers et al., 2014). Titin in skeletal muscle is formed of the proximal Ig segment, N2A segment, PEVK segment, and distal Ig segment.

The extensible I-band component of titin has a modular structure. In skeletal muscle, titin’s extensible region includes the immunoglobulin (Ig) segments (proximal and distal tandem Ig domains), the P (proline) E (glutamate) V (valine) K (lysine) rich PEVK segment, and the N2A region (Granzier et al., 2000, Figure 2.3). The different segments of titin have different stiffness, therefore they extend at different forces, and thus different SLs. When the slack titin is stretched, the proximal Ig segment starts to straighten first and produces a small amount of passive force (Figure 2.4). When titin is further stretched, the PEVK segment elongates and generates increasing passive force (Granzier et al., 2000). It has been proposed that in skeletal muscle the PEVK region is the major source of titin extensibility and passive force generation (Granzier et al., 2000). The passive force produced by titin varies with the speed of stretching.
(Granzier & Labeit, 2007), and it has been proposed that the unfolding of Ig domains may be responsible for this viscoelasticity of titin (Minajeva, Kulke, Fernandez, & Linke, 2001).

**Figure 2.4** Schematic illustration of passive force generation from titin in skeletal muscle (Granzier et al., 2000). When titin is stretched, the proximal Ig domain starts to straighten. PEVK region is then extended. Finally, some Ig domains may unfold.

It has been shown that titin’s stiffness can be adjusted in physiological and pathological conditions, long term via altering via the expression of different titin isoforms, and short term by modulating the mechanical properties of the extensible I-band region of titin by mechanisms, such as phosphorylation (Somerville & Wang, 1988; Fukuda, Wu, Nair, & Granzier, 2005), calcium binding upon activation (Tatsumi, Maeda, Hattori, & Takahashi, 2001; Labeit et al.,
2003; Herzog, Powers, Johnston, & Duvall, 2015) and interaction with actin (Kellermayer, Smith, Granzier, & Bustamante, 1997; Linke et al., 1997; Niederländer, Raynaud, Astier, & Chaussepied, 2004).

2.1.1.2.1 Titin isoforms.

The influence of the titin isoform expressed by a muscle on its passive force was first reported in 1992 by Horowits. Horowits (1992) found that the passive forces of rabbit psoas and soleus muscles and various human muscles at the same SLs were different (Figure 2.5). Gel electrophoresis indicated that the titin isoforms in these muscles were different, while the concentration of titin relative to myosin and actin was constant between muscles. This indicated that titin isoform expression affected passive force; the bigger the titin isoform, the lower the titin based passive force. Similar results have been observed in human and other animal muscles and fibres (Granzier et al., 2000; Neagoe, Opitz, Makarenko, & Linke, 2003; Makarenko et al., 2004; Prado et al., 2005). Overall, it seems that skeletal muscle can tune its passive force by expressing a titin isoform of appropriate size. Each half myosin contains six titin molecules and this value appears to be constant despite widely varying titin isoforms (Granzier et al., 2000).
Figure 2.5 Schematic illustration of passive forces produced by rabbit psoas and soleus muscles and human fast and slow twitch muscles (Horowits, 1992). Passive force varies between muscles and this has been associated with the expression of different titin isoforms.

2.1.1.2.2 Phosphorylation.

Somerville and Wang (1988) showed that titin could be phosphorylated in rat diaphragm muscle. Yamasaki et al. (2002) showed that the phosphorylation in cardiac muscle by protein kinase A cause decreases passive force. Fukuda et al. (2005) also showed that titin can be phosphorylated and that phosphorylation decreases titin-based passive force (Figure 2.6). It was found that titin phosphorylation was associated with a greater decrease in force at short compared to long SLs.
Figure 2.6 Schematic illustration of titin-based passive force decrease after incubation in protein kinase A (Fukuda et al., 2005). The black trace shows the passive stress before phosphorylation, and the red trace after phosphorylation.

2.1.1.2.3 Interaction with calcium.

It has been suggested that the stiffness of titin can be influenced by calcium (Herzog, Powers, Johnston, & Duvall, 2015). One possible mechanism might be that calcium binds directly to titin upon muscle activation and renders titin more resistant to stretch. Tatsumi et al. (2001) showed that calcium binds to the extensible region of titin. Furthermore, Labeit et al. (2003) tested the effect of calcium binding on passive force in skinned fibres and in PEVK constructs. They found that passive force increased with increasing concentrations of calcium (Figure 2.7). Similar results were obtained by DuVall et al. in 2013 who evaluated calcium-dependent regulation of passive force in recombinantly produced cardiac Ig domains. When stretched in the presence of calcium, the Ig segments were stiffer and produced more force than
in the absence of calcium. Joumaa et al. (2007) and Leonard et al. (2010) observed increases in passive force of myofibrils when stretched in the presence of calcium, with cross-bridge formation inhibited either by troponin C depletion or by butanedione monoxime. Thus when a muscle is activated, calcium released into the sarcoplasm results not only in actin-myosin interactions, but appears to affect titin-based passive force.

![Figure 2.7](image.png)

**Figure 2.7** Schematic illustration of passive force increases with increasing calcium concentrations (Labeit et al., 2003).

2.1.1.2.4 Interaction with actin.

It has been proposed that titin’s stiffness may not be regulated by titin’s intrinsic elastic properties solely but also by possible titin-actin interactions which results in a change in titin’s free spring length. This suggestion is supported by reports showing titin-actin interactions in vitro using biochemical and mechanical techniques (Kellermayer et al., 1997; Linke et al., 1997; Niederländer et al., 2004). For example, Kellermayer and Granzier (1996) showed, using an in vitro motility assay, that native titin interacts with both actin and thin filaments. Titin-actin
interaction resulted in a decrease in actin/thin filament motility in the presence of titin. Furthermore, this interaction occurred in a calcium-dependent manner; the higher the calcium concentration, the higher the interaction between actin and titin. In 2010, Leonard and Herzog observed a force increase in myofibrils after active stretch to a SL beyond myofilament overlap compared to the force obtained for a corresponding stretch in a passive myofibril. They speculated that this force increase might be caused by titin-actin interaction which decreased the free titin length, and thus resulted in higher passive forces in the actively compared to the passively stretched myofibrils.

2.2 Muscle force regulation

2.2.1 Cross-bridge theory.

Prior to 1950s, the mechanism of muscle contraction was associated with the shortening of myosin filaments. However, in 1954, A. F. Huxley and Niedergerke and H. E. Huxley and J. Hanson published two independent papers in *Nature* showing that the A-band (myosin filaments) did not shorten for a variety of experimental conditions. However, during muscle contraction and muscle shortening, the I-band portion of sarcomeres changed. Combined, these results suggested a relative sliding of the actin past the myosin filaments: the sliding filament theory was then considered as the most likely explanation for muscle contraction.

2.2.1.1 1957 Model.

A. F. Huxley (1957) provided a mathematical model to describe the interaction between thick and thin filaments and force production during muscle contraction (Figure 2.8). The concept of cross-bridge formation, and the cross bridge theory were proposed. Cross-bridges
were thought to emanate from the thick filament and were proposed to be attached to the backbone of the thick filament via an elastic spring. Cross-brides were then thought to move around an equilibrium position (zero spring strain) through thermal agitation. This cross-bridge equilibrium position is designated by “O” in Figure 2.8, while “M” indicates the present position of the cross-bridge head. The attachment site on the actin filament is designated by “A” in Figure 2.8, and the distance between A and O is \( x \). During sarcomere shortening, an attached “M-A” cross-bridge will move towards the equilibrium position. The attachment and detachment kinetics of cross-bridges are designated by rate constants of attachment (\( f \)) and detachment (\( g \)) which are exclusively dependent on the \( x \)-distance (Figure 2.8).

**Figure 2.8** Schematic illustration of the cross-bridge theory model (Adapted from A. F. Huxley, 1957). Rate functions of the attachment (\( f \)) and detachment (\( g \)) of cross-bridges between actin
and myosin as a function of the distance (x) which is defined as the distance from the cross-bridge’s equilibrium position (O) to the nearest eligible attachment site (A) on the actin filament.

In the 1957 proposal of the cross-bridge theory, A. F. Huxley (1957) made some assumptions for ease of mathematical formulation of the theory. These include:

(i) All cross-bridges generated the same average force.
(ii) Each cross-bridge works independent the others.
(iii) Attachment sites are uniformly distributed along to the thin filament
(iv) Cross-bridges are uniformly distributed along the thick filament.
(v) Each cross-bridge cycle is associated with the hydrolysis of one ATP.

In 1971, A. F. Huxley and Simmons proposed some changes to the 1957 model in order to explain the quick recovery of muscle force after stretching or shortening which could not be explained by the model of 1957.

**2.2.1.2 1971 Model.**

In the 1971, A. F. Huxley and Simmons (Figure 2.9) extended the original cross-bridge model using a mathematical and an experimental approach. The 1971 model explained some experimental observations that could not be interpreted with the 1957 two-state model. In the 1971 model, the myosin (cross-bridge) head (H) is connected to the myosin filament by an elastic spring (AB), as it was in the 1957 model. However, instead of a single attachment configuration, the 1971 model contained multiple attached states for the myosin head (M₁ to Mₙ) on the actin filament (A₁ to Aₙ). One of the experimental observations that could not be explained by the 1957 theory was the quick recovery of tension observed after a quick shortening
step. However, with the 1971 model of multiple attachment states, the quick recovery of tension was explained by the rotation of the cross-bridge head from a position of high potential energy to a position of low potential energy that did not require detachment and re-attachment of the cross-bridge head. The slow tension recovery following the fast recovery was then explained by the regular detachment-attachment kinetics of the 1957 model (A. F. Huxley & Simmons, 1971).

![Figure 2.9](image)

**Figure 2.9** Schematic illustration of the 1971 cross-bridge model (A. F. Huxley & Simmons, 1971). The cross-bridge head is connected to the myosin filament via an elastic element (AB). There are multiple attachment sites on the actin filament (A₁ to A₄) for the myosin head (M₁ to M₄). A rotation of the cross-bridge head towards lower energy states produces increased tension through the stretch of the elastic element that links the cross-bridge head to the myosin filament.

In 1993, Rayment et al. developed a further conceptual model of the cross-bridge theory using information about the atomic structure of the cross-bridge and the actin attachment sites. Their model was based on multiple detached and multiple attached cross-bridge states. In the
model of Rayment et al. (1993, Figure 2.10), starting with the rigor state, ATP is required to allow for detachment of the cross-bridge from actin. Following detachment, ATP is hydrolyzed into ADP and a free phosphate, and the cross-bridge undergoes a movement that allows for re-attachment to actin. When reattached, phosphate is released from the myosin head’s nucleotide binding site and this is thought to trigger cross-bridge rotation, thereby producing force and sliding of actin relative to myosin. Following the power stroke, ADP is released from the myosin nucleotide binding site, and the cross-bridge goes into the rigor state, and the cross-bridge cycle starts anew. The cross-bridge model proposed by Rayment et al. (1993) contains two detached and three attached states and is the most commonly used cross-bridge model still today.
**Figure 2.10** Schematic illustrations on molecular mechanism of muscle contraction (Rayment et al., 1993).

### 2.2.2 Force-length relationship.

In the cross-bridge theory, the magnitude of force of a muscle is given by the number of attached cross-bridges and the average force per cross-bridge. For isometric, steady-state contractions, the average force per cross-bridge is constant, thus the force depends exclusively on the number of attached cross-bridges. The number of attached cross-bridges, in turn, depends directly on the amount of overlap between actin and myosin filaments, which in turn depends on the sarcomere length of the muscle. Therefore, the maximal, steady-state, isometric force of a muscle is proportional to the amount of overlap between the thick and thin filaments. This was experimentally verified by Gordon et al. (1966) (Figure 2.11). The sarcomere active force-length relationship can be divided into three parts: the ascending limb, the plateau, and the descending limb. Starting from a long sarcomere length where there is no overlap between actin and myosin filaments (position E in Figure 2.11), the descending limb (from E to D; Figure 2.11) starts. When the sarcomere shortens from this position, thick and thin filaments increasingly overlap more, and the active isometric force increases until it reaches a maximal value at maximal actin-myosin filament overlap (D, Figure 2.11). In rabbit psoas muscle, the descending limb region covers the SLs from 2.43 μm to 3.91 μm (Walker & Schrodt, 1974; Herzog, Kamal, & Clarke, 1992). The plateau region is characterized by a complete overlap between actin and the cross-bridge containing myosin region (from D to C; Figure 2.11). When the sarcomere shortens from point C, it will reach the ascending limb (CB and BA in Figure 2.11) of the force-length relationship. On the ascending limb, actin filaments start overlapping each other, they will reach
into the opposite half sarcomere, and at point B (Figure 2.11), the end of the sarcomere, indicated by the Z lines, will contact the myosin filament. All these events result in a reduction in the number of cross-bridges that can be formed between actin and myosin, and an associated decrease in active force. With further shortening of the sarcomere from B to A (Figure 2.11), the Z lines compress the myosin filaments which decreases active force even further (Figure 2.11).

Passive force starts to increase at a SL of 2.7 μm in rabbit psoas fibres (Horowits, 1992). The sum of the active force and passive force is the total force produced by the sarcomere. According to the force-length relationship, the force produced beyond myofilament overlap should be the passive force only.

**Figure 2.11** Force-length relationship of rabbit psoas muscle. Ascending limb (blue), plateau region (orange) and descending limb (green) are shown in the left graph and the black dashed line indicates passive force production. In rabbit psoas muscle, myosin length (c) is 1.65 μm. Actin filament length (α) is 1.08 μm. The length of the bare zone (b) is 0.17 μm, and the Z-line width (z) is 0.1 μm (Walker & Schrodt, 1974; Herzog et al., 1992). So the SL at E = z + α + α + c
= 3.91 μm. D = z + a + a + b = 2.43 μm. C = z + a + a = 2.26 μm. B = z + c = 1.75 μm. (Adapted from Gordon et al., 1966).

2.2.3 Sarcomere length non-uniformity during activation.

It has been shown that SLs in single fibres and myofibrils are highly non-uniform during activation (A. F. Huxley & Peachey, 1961; Bartoo, Popov, Fearn, & Pollack, 1993; Rassier, Herzog, & Pollack, 2003; Telley et al., 2006; Joumaa et al., 2008; Herzog et al., 2015; Johnston, Jinha, & Herzog, 2016). For example, Bartoo et al. (1993) observed SLs in active myofibrils ranging from 1.8 μm to 2.4 μm. Similarly, Rassier et al. (2003) showed SLs in myofibrils ranging from 2.1 μm to 2.5 μm (average SL was 2.38 μm) after activation at an average SL of 2.4 μm. Llewellyn et al. (2008) measured single SLs in a whole muscle preparation and found variations in SLs of up to 20%.

Our group also showed SL non-uniformities in myofibrils during activation and at steady state before and after stretch (Joumaa et al., 2008; Powers et al., 2014; Johnston, Jinha, & Herzog, 2016, Figure 2.12).
**Figure 2.12** Schematic illustration of SLs in two myofibrils (a and b) during purely isometric contraction and after active stretch (arrow). SLs were non-uniform during a purely isometric contraction and this non-uniformity remained after active stretch (Joumaa et al., 2008).

2.3 **History dependent properties in skeletal muscle: residual force enhancement**

The cross-bridge theory is the commonly accepted theory of muscle contraction, and the force-length relationship is a well-accepted and consistently observed property of muscle that is consistent with the cross-bridge theory. However, muscle contraction is a complicated dynamic process, and not all experimental observations can be explained by the cross-bridge theory.

**2.3.1 Force increase after active stretch.**

According to the cross-bridge theory, the isometric, steady-state force at a given muscle (sarcomere) length and a given activation, is constant. During active muscle stretching, force increases. This transient increase in force depends on the speed and the amplitude of the stretch (Edman et al., 1982) and it can be explained by the cross-bridge theory. Following active stretching of a muscle, force decreases to reach a new steady-state. However, this new steady-state force following active muscle stretching is greater than the force obtained for an isometric contraction at the corresponding length and activation (Abbott & Aubert, 1952, Figure 2.13). This phenomenon is referred to as “residual force enhancement” (RFE) and has been observed in different muscle preparations ranging from *in-vivo* human muscles (De Ruiter, Didden, Jones, & De Haan, 2000; Lee & Herzog, 2002; Hahn, Seiberl, & Schwirtz, 2007), to *in-situ* whole muscles (Abbott & Aubert, 1952; Morgan, Whitehead, Wise, Gregory, & Proske, 2000; Herzog & Leonard, 2002), single fibres (Edman, Elzinga, & Noble, 1978; Sugi & Tsuchiya, 1981; Edman...

RFE increases with the magnitude of stretch (Abbott & Aubert, 1952; Edman et al., 1978; Herzog & Leonard, 2002) and appears to be independent of the speed of stretch (Edman et al., 1982; H.-D. Lee & Herzog, 2002). It is long lasting (more than 20s in cat soleus), but can be abolished by deactivating the muscle long enough for force to drop to zero (Herzog & Leonard, 2002; Morgan et al., 2000; Rassier & Herzog, 2004).

**Figure 2.13** Schematic illustration of force change after active stretch. An isometric contraction force (bottom trace) at reference muscle length and an isometric force after stretch to the reference length (top trace) are shown. The force difference (ΔF) indicates the additional force after active stretch (Herzog, 2001).
2.3.2 Force increase after active stretch beyond myofilament overlap.

Actin-myosin filament overlap in rabbit skeletal muscle is lost at a SL of 3.91 µm. Thus, theoretically, when skinned muscle fibres or myofibrils are stretched to a SL beyond 3.91 µm, there should be no active force, and all force should be associated with passive elements in the sarcomere only. However, previous studies showed a dramatic force increase above the passive force when myofibrils were actively stretched to SLs beyond the actin and myosin filament overlap (Leonard & Herzog, 2010; Powers et al., 2014). In 2010, Leonard and Herzog stretched rabbit psoas myofibrils from a SL of 2.4 µm to a SL of 6.0 µm. The results showed that the force after active stretch to a SL of 6.0 µm was much higher than the purely passive stretch force to the same SL (Figure 2.14 A). Similar results were obtained by Powers et al. (2014). Furthermore, when titin was degraded using low doses of trypsin, the total force increase after active stretch was abolished (Figure 2.14 B).
These results suggest that there is an additional force that comes into play when muscles are stretched beyond actin-myosin filament overlap. Since this force cannot come from cross-bridges, and since it can be seen in isolated myofibrils and single sarcomeres and since it is completely abolished when titin is eliminated from myofibril preparations, it appears that this additional force might originate in some manner from the structural protein titin.
2.4 Proposed mechanisms for residual force enhancement

2.4.1 Sarcomere-length non-uniformity theory.

The mechanism responsible for RFE is still a subject of debate. One of the early mechanisms proposed for RFE is the SL non-uniformity theory (Julian and Morgan; 1979). In the SL non-uniformity theory, it is assumed that SLs and forces are unstable on the descending limb of the force-length relationship (Hill, 1953). According to the SL non-uniformity theory, during active stretch of muscles (eccentric contractions) some sarcomeres are pulled beyond actin-myosin overlap, and these sarcomeres can only produce passive force. The remaining sarcomeres are assumed to be stretched by only a small amount and their (active) forces are in balance with the passive forces of the over-stretched sarcomeres. This development of SL non-uniformity is thus thought to result in greater forces than the forces obtained during purely isometric reference contractions where sarcomere lengths are assumed to remain relatively uniform (Morgan et al., 2000, Figure 2.15).

Even though the SL non-uniformity theory may be used to explain some aspects of RFE, there are some predictions of this theory that are not supported experimentally. For example, according to the SL non-uniformity theory, RFE should not occur on the ascending limb of the force-length relationship as this part of the relationship is thought to be stable, thus SL non-uniformities are not possible. However, it has been shown by different groups and across different structural preparations that RFE does occur on the ascending limb of the force-length relationship (e.g. Abbott & Aubert, 1952; Herzog & Leonard, 2002; Peterson, Rassier, & Herzog, 2004). Moreover, it has been shown that SLs are non-uniform in isometric contractions (and not uniform as assumed in the SL non-uniformity theory) across myofibrils (Rassier, Herzog, & Pollack, 2003; Rassier, Herzog, Wakeling, & Syme, 2003; Joumaa et al., 2008;
Johnston, Jinha, & Herzog, 2016), fibres (A. F. Huxley & Peachey, 1961), and muscles (Llewellyn et al., 2008; Moo, Fortuna, Sibole, Abusara, & Herzog, 2016). Overall the SL non-uniformity theory cannot fully explain the RFE. Finally, according to the SL non-uniformity theory, force in the force-enhanced state should never exceed the maximal isometric force achieved at optimal muscle lengths, but it has been shown for decades that forces in the enhanced state can easily exceed those obtained isometric ally at optimal muscle (e.g. Abbott & Aubert, 1952) and sarcomere lengths (e.g. Rassier et al., 2003; Peterson et al., 2004; E.-J. Lee & Herzog, 2008)

**Figure 2.15** Schematic illustration of RFE according to the SL non-uniformity theory. During stretch of a fibre from A to B, some strong sarcomeres are stretched by a small amount to C. However, other sarcomeres are stretched beyond actin-myosin filament overlap (they pop) (E) and are supported by passive forces exclusively (Rassier & Herzog, 2004).
2.4.2 Passive force enhancement.

It has been proposed that RFE might result from the engagement of a passive elastic element in a sarcomere upon activation and stretch (Edman et al., 1978, 1982; Noble, 1992; Forcinito, Epstein, & Herzog, 1998; Herzog & Leonard, 2002; Herzog, Lee, & Rassier, 2006; Joumaa et al., 2007; Herzog, 2014a, 2014b; Herzog et al., 2015). In a myofibril or sarcomere, titin is virtually the only structural element that can resist elongation and produce passive force (Horowits, Kempner, Bisher, & Podolsky, 1986). Therefore, it has been suggested that titin might also contribute to RFE observed on all structural levels of muscle including myofibrils (Herzog & Leonard, 2002; Joumaa, Rassier, Leonard, & Herzog, 2008). It has been shown that after deactivation of an actively stretched muscle, the passive force is higher than the purely passive force following an isometric contraction at the corresponding length or a passive stretch to the corresponding length (Herzog & Leonard, 2002; Joumaa, Rassier, Leonard, & Herzog, 2008, Figure 2.16). This property of muscle has been referred to as “passive force enhancement”. Passive force enhancement increases when the amplitude of stretch increases, it is independent of the speed of stretch, it occurs primarily at long muscle and sarcomere length, and it can be abolished instantaneously, by shortening (and re-stretching) the muscle (Herzog & Leonard, 2002; Herzog, Schachar, & Leonard, 2003). The origin of the passive force enhancement is unknown. However, titin has been suggested as a possible candidate (Rassier & Herzog, 2004). Titin has calcium binding sites (Tatsumi et al., 2001), and it has been shown that calcium binding to titin occurs upon muscle activation and increases titin’s stiffness (Labeit et al., 2003; Joumaa et al., 2008; DuVall et al., 2013). Therefore, it has been suggested that when a muscle is activated and calcium is released, titin’s stiffness might increase. It has also been suggested that titin might bind to actin upon muscle activation and stretch and so might shorten its characteristic
length, thereby decreasing its free spring length, increasing its stiffness and thus force upon stretch (Edman et al., 1982; Forcinito et al., 1998; Herzog, Joumaa, & Leonard, 2010; Leonard & Herzog, 2010; Herzog, Duvall, & Leonard, 2012; Herzog, Leonard, Joumaa, DuVall, & Panchangam, 2012; Herzog, 2014a, 2014b; Herzog et al., 2015; Herzog, Schappacher, DuVall, Leonard, & Herzog, 2016). Through these changes in mechanical properties, titin could potentially account for the passive force enhancement and greatly contribute to RFE.

**Figure 2.16** Schematic illustration of passive force enhancement (ΔP). An active stretch contraction (top trace), a passive stretch (bottom trace), and an isometric contraction (middle trace) are shown. After stretching, the muscle in all three conditions is at the same final length. Residual force enhancement is observed after active stretch (ΔF). After active stretch and deactivation, the passive force is greater than the passive force observed after the passive stretch and after deactivation of the purely isometric contraction (ΔP) (Herzog, 2001).
2.4.3 Force increase after active stretch beyond myofilament overlap.

Leonard and Herzog (2010) found that the forces in actively (high calcium concentration) stretched myofibrils were much greater than the forces in passively (low calcium concentration) stretched myofibrils when myofibrils were stretched to lengths where all sarcomeres and half-sarcomeres were beyond actin-myosin filament overlap. In order to test that there were no remnant active cross-bridge based forces, myofibrils that were actively stretched to an average SL of 5.0 µm were deactivated, but deactivation did not result in a loss of force, suggesting that the additional force of the actively stretched (compared to the passively stretched) myofibrils had to come from a passive structural element (Figure 2.17). Since titin is the only substantial contributor to passive force in single sarcomeres and isolated myofibrils, titin was implicated as providing this additional force in active compared to passive stretching. If some of this additional force in actively compared to passively stretched myofibrils occurs because of titin’s spring length being shortened (for example by titin binding to actin for part of its proximal segment), then titin should elongate over its entire length in a passively stretched myofibril but should only elongate for part of its length (the distal part), while the proximal part of titin does not elongate (since it is bound “rigidly” to actin). If this scenario is correct, and titin indeed binds to actin upon muscle activation, then the stead-state force after an active stretch will be higher than the corresponding purely isometric force at the same length and same activation. Therefore, this mechanism could account for all the force enhancement properties observed in muscles at all structural levels.
Figure 2.17 Proposed mechanism of force regulation through titin-actin binding (Leonard & Herzog, 2010). A: Purely passively stretch, B: Active stretch contraction. In the passive stretch (A), titin is elongated along its entire length, while in the active stretch, titin is thought to bind some of its proximal segments to actin, thereby not allowing of those bound segments, and all the stretch would have to be accommodated by the distal segment of titin. Since this is equivalent to shortening of a mechanical spring, the stiffness of titin would be increased and its force upon stretch would also be greater in (B, active) compared to (A, passive).

The idea that the RFE property of skeletal muscle is explained by the engagement of a passive element upon activation is supported by findings that titin’s stiffness can be modulated by short term events, such as titin-actin interactions, calcium binding to titin, and titin phosphorylation (Yamasaki et al., 2002; Labeit et al., 2003; Granzier & Labeit, 2006; Anderson, Bogomolovas, Labeit, & Granzier, 2010).
2.5 Summary

Titin is a large protein responsible for most of the passive force in skinned muscle fibres, myofibrils, and isolated sarcomeres (Horowits, 1992; Granzier et al., 2000; Gao, Lu, & Schulten, 2001; Granzier & Labeit, 2007). Since forces in sarcomeres and single myofibrils that are stretched to SLs way beyond actin-myosin filament overlap, are much greater in actively compared to passively stretched preparations, it appears that titin might be responsible for this increase in force (Leonard & Herzog, 2010; Powers et al., 2014). It has been suggested that titin’s stiffness might increase, or characteristic length decrease, with activation because of calcium binding to titin and titin binding to actin upon muscle activation (Edman et al., 1982; Forcinito et al., 1998; Herzog et al., 2010; Leonard & Herzog, 2010; Herzog et al., 2012a, 2012b; Herzog, 2014a, 2014b; Herzog et al., 2015; Herzog et al., 2016). In a single myofibril preparation, SLs can be measured individually, and loss of myofilament overlap in all sarcomeres and half sarcomeres can be verified optically. Furthermore, the absence of any cross-bridge-based force at average sarcomere lengths of 5.0 µm in single myofibril preparations was tested by deactivating myofibrils after active stretch to these long SLs. In contrast to single myofibrils with all sarcomeres arranged strictly in series, single fibres contain sarcomeres in series and in parallel, and not all individual sarcomere and half SLs can be measured. In addition, it has been known for a long time that SLs in single fibres are highly non-uniform with passive fibres from frog skeletal muscles reported to contain sarcomeres within a range of 1.8-4.2 µm (A. F. Huxley & Peachey, 1961). This extreme non-uniformity of SLs in single fibre preparations have also been observed in entire muscles (Llewellyn et al., 2008; Moo et al., 2016) and single myofibrils (Johnston et al., 2016) and they may affect the mechanical properties in general, and the RFE property of skeletal muscle specifically. These properties may also differ between single
fibres (and thus presumably muscles) and single myofibrils because of the difference in the structural arrangement of the sarcomeres (purely in series vs. arranged in parallel and series simultaneously). Therefore, if we want to understand the role of sarcomere length non-uniformity in muscles and if we want to understand potential differences in the force enhancement properties across structural levels, it is necessary to study the role of titin and titin’s force enhancement properties in single skinned fibres in situations where actin-myosin based cross-bridge forces are eliminated. This can be achieved by pulling single fibres to lengths where the average sarcomere length is clearly beyond actin-myosin filament overlap and by inhibiting cross-bridge based forces artificially, for example with the use of 2,3 butanedione monoxime (BDM), a known cross-bridge inhibiting agent.

2.6 Purpose

The purpose of this thesis research was to investigate the force increase at steady-state after active stretch to SLs beyond myofilament overlap in single fibres, and test whether this increase in force is caused by a passive component (titin) alone, or possibly some remnant cross-bridges that contribute to force in some unknown manner in muscle fibres.

2.7 Hypothesis

We hypothesized that, similar to what had been observed in isolated myofibrils, actively stretched single fibres would have substantially greater forces than passively stretched fibres at SLs way beyond actin-myosin filament overlap, but unlike in myofibrils, this increased force would be comprised of a passive (titin-based) and active (cross-bridge-based) component. The active component could potentially originate from sarcomeres that were not stretched beyond
actin-myosin filament overlap (approximately 4.0 µm), despite the average SL being 5.0 µm. In order to test this hypothesis, we stretched the skinned fibres to an average SL of 5.0 µm, actively and passively, and then deactivated them using a deactivating solution (no calcium) and a cross-bridge force inhibitor (BDM). The deactivating solution would result in a loss of any remnant cross-bridge-based force, and any calcium-dependent titin force, as indicated by Labeit et al. (2003), Joumaa et al. (2008) and DuVall et al. (2013). The addition of BDM would leave any titin-dependent components unaffected, but would result in the loss of any remnant cross-bridge-based force.
Chapter Three: Methods

3.1 Skinned single fibre preparation

Psoas muscles were taken from New Zealand White Rabbits (Charles River Laboratories International, Inc.). Rabbits were euthanized by pentobarbital (2ml/4.5kg body weight, Euthanyl) through an intravenous injection. Strips of psoas muscle were taken by Dumont No.5 forceps (Fine Science Tools Ltd., Vancouver, BC, Canada) and a No.15 disposable scalpel blade (VWR Inc. Mississauga, Ontario, Canada). Then each small strip of muscle was tied (Black Braided Silk, Ref No.SP120, LOOK®, PA, USA) to a small wooden stick (Puritan Applicators, Ref # 807, Puritan Medical Products Co. LLC, Guilford, Maine, USA) before being stored in the 15ml tube (Falcon-BD part# 352099, VWR Inc., Mississauga, Ontario, Canada) with 12ml rigor solution (4°C). Muscles were transferred to a rigor overnight and glycerol (50:50, pH = 7.0) solution within 4 to 5 hours of collection (4°C). On the next day, fibres were moved into a rigor and glycerol (50:50, pH = 7.0) storing solution and kept at -20°C for 10 to 14 days for skinning before being used (Leonard & Herzog, 2010). Each solution contained one tablet of protease inhibitor (Complete®, Roche Diagnostics, Montreal, Quebec, Canada) per 50ml of solution. Ethics approval for all procedures was granted from the University of Calgary Animal Ethics Committee.

Skinning is a common method used in striated muscle fibre and myofibril experiments (Joumaa et al., 2007; Leonard & Herzog, 2010; Joumaa & Herzog, 2013). The purpose of skinning is to disrupt the connective tissue surrounding the muscle and increase the permeability of the fibre sarcolemma, to allow for free calcium diffusion into the myofilament (Wood, Zollman, Reuben, & Brandt, 1975; Cassens, Eddinger, & Moss, 1986). For rabbit psoas muscles, muscle structure changes within the first 24 to 48 hours in skinning solution, and there will be no
further changes. The structural changes mainly include the appearance of gaps in the cell membrane which allows for calcium activation of the isolated cells (Eastwood, Wood, Bock, & Sorenson, 1979).

On the day of the experiments, a small piece of muscle was cut from the skinned strip on the wooden stick, and transferred to a petri dish filled with relaxing solution with protease inhibitors. One single fibre was isolated under a dissection microscope (SMZ1500, Nikon Instruments Inc.) and tied at one end to a silk suture (size 5-0, Fine Science Tools Ltd., Vancouver, BC, Canada), which was used to transfer the fibre to the testing chamber without damaging it.

3.2 Apparatus setup

The experimental chamber was placed under a microscope (Carl Zeiss, Germany) used for mounting fibres and observing them during testing (Figure 3.1). One of the eyepieces (Carl Zeiss, Germany) contained a graded grid which was used to measure fibre length and fibre diameter. Cross-sectional area of fibres were calculated from the fibre diameter with the assuming a cylindrical shape. Light was provided by a Fiber-Lite® High intensity illuminator (Series 180, Dolan-Jenner Industries Inc., US).
Figure 3.1 Illustration of the experimental set-up. A. Overview of the whole mechanical testing system. B. Under the microscope, the length controller is on the left side (blue circle), and the force transducer is on the right side (green circle). C. There are six baths for different solutions (red circle). From the front to the back, the chambers are numbered from 1 to 6. Chamber 1 is larger than the other baths. D. A single fibre (yellow circle) is attached between the hooks connected to the length controller and force transducer.

In Figure 3.1 D, the left (blue circle) hook is connected to a high-speed length controller (322C-I, Aurora Scientific Inc., ON, Canada) which was used to stretch the muscle fibre. The right (green circle) hook is connected to a force transducer (403A, Aurora Scientific Inc., ON, Canada) which measures the forces produced by the fibres during mechanical testing. The yellow
circle indicates a single attached fibre between the length controller and the force transducer. Following isolation, fibres were transferred into the first chamber containing the relaxing solution and were glued to the force transducer and the length controller using acetone and a small piece of cellulose acetate sheet (Sigma-Aldrich). The average SLs were measured by laser diffraction using a helium neon gas laser (1508P-1 Novette, JDSU, US) with a wavelength of 633 nm. SL was calculated as

\[ SL = \frac{\lambda x \sqrt{d^2 + x^2}}{x} \]

In this equation, \( \lambda \) indicates the wavelength of the laser (633 nm); \( d \) is the distance between the attached single fibre and the diffraction sheet, which was 55 mm in this set-up, and \( x \) is the distance between the zero and the first order diffraction line.

Fibres were activated and deactivated by transferring them between baths containing relaxing and activating solutions.

### 3.3 Solutions

**Rigor solution:** Tris (50 mM), potassium chloride (100 mM), magnesium chloride (2 mM), and ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA; 1 mM) at pH = 7.0.

**Rigor overnight solution:** Tris (50 mM), potassium chloride (2 mM), magnesium chloride (2 mM), sodium chloride (100 mM), and EGTA (1 mM) at pH = 7.0.

**Relaxing solution:** Potassium propionate (Kprop; 170 mM), 3-(N-morpholino)propanesulfonic acid (MOPS; 20 mM), magnesium acetate (2.5 mM), K₂EGTA (5 mM), and adenosine triphosphate (ATP; 2.5 mM) at pH = 7.0.
Activating solution: CaEGTA (5 mM), magnesium acetate (2.5 mM), Kprop (170 mM), MOPS (10 mM), and ATP (2.5 mM) at pCa = -\log_{10}(\text{Ca}^{2+}) = 4.2 and pH = 7.0.

BDM solution: BDM (100 mM) in the activating solution.

Solutions contained one protease inhibitor tablet (Complete, Roche Diagnostics, Quebec, PQ, Canada) for 50 ml of solution.

3.4 Mechanical testing protocol

Skinned single fibres were set at an average SL of 2.4 μm and then divided into three groups. Group 1 – passive fibres (n = 16): fibres were passively stretched in a relaxing solution without calcium ions (Ca^{2+}), to a nominal, average SL of 5.0 μm, which is beyond myofilament overlap (Walker & Schrodt, 1974; Herzog et al., 1992), and held at this SL until a steady-state force was reached. Group 2 – deactivation fibres (n = 14): fibres were activated using an activating solution containing Ca^{2+} at a SL of 2.4 μm, and then actively stretched to a nominal SL of 5.0 μm. After reaching a steady state force, fibres were deactivated by transferring them to a relaxing solution with no calcium. Group 3 – BDM fibres (n = 15): fibres were activated at a SL of 2.4 μm, and then actively stretched to a nominal SL of 5.0 μm. After reaching a steady state force, fibres were transferred to a BDM (100 mM) solution, then they were deactivated with a relaxing solution.
**Figure 3.2** Mechanical testing protocol for Group 1 – passive fibres. The duration of the passive stretch was 15s. The speed of the stretch was 7.2% of fibre length per second.

**Figure 3.3** Mechanical testing protocol for Group 2 – deactivation fibres. After active stretch, fibres were deactivated by transferring them into a chamber with relaxing solution. The duration of the stretch was 15s. The speed of the stretch was 7.2% of fibre length per second.
Figure 3.4 Mechanical testing protocol for Group 3 – BDM fibres. After active stretch, fibres were deactivated by transferring them into a chamber with BDM solution. After 30s in the BDM solution, fibres were deactivated by transferring them into a bath with a relaxing solution. The duration of the stretch was 15s. The speed of the stretch was 7.2% of fibre length per second.

Additionally, two groups of fibres were tested for evaluation of secondary outcomes. First, we wanted to test if fibres that were stretched passively to an average sarcomere length of 5.0 µm could produce force upon activation. In order to test this hypothesis, we stretched five additional fibres (Group 4 fibres) passively to a nominal SL of 5.0 µm. After reaching a steady state at this length, fibres were activated. Our expectation was that there should be no increase in force when fibres were activated at these super-long lengths. Second, we wanted to test if BDM affects the titin-based force and/or titin-calcium interactions. In order to test this hypothesis, we stretched six additional fibres (Group 5 fibres) passively to a nominal SL of 4.5 µm and after reaching a steady state at this long length, the fibres were activated. Fibres were then stretched to a nominal SL of 5.0 µm and exposed to BDM. We hypothesized that fibres at an average SL of 4.5 µm would not show an increase in force upon activation. Moreover, if titin stiffness increases
because of activation and the presence of calcium, then we hypothesize that force after active stretch to an average SL of 5.0 µm would not decrease following BDM exposure.

**Figure 3.5** Mechanical testing protocol for Group 4 fibres. After passive stretching to a nominal SL of 5.0 µm, fibres were activated. The duration of the stretch was 15s. The speed of the stretch was 7.2% of fibre length per second.

**Figure 3.6** Mechanical testing protocol for Group 5 fibres. Fibres were passively stretched to a nominal SL of 4.5 µm in 10s, and then activated. Following active stretching from 4.5 to 5.0 µm in 5s, fibres were exposed to a 100mM BDM solution.
3.5 Statistical analyses

Force produced during each experiment was recorded continuously. Forces were normalized to the cross-sectional area of the fibres and expressed as stress (mN/mm$^2$). Mann Whitney and Kruskal–Wallis tests were used to identify differences between the steady-state force after active stretch, force after deactivation, force after treatment with the BDM solution, and force after passive stretch, all at an average SL of 5.0µm. The level of significance was set at α=0.05 a priori.
Chapter Four: Results

4.1 Diameter and length of single fibres

The average length of fibres was 903±26 μm, and the average diameter was 75±3 μm (Table 4.1). The diameter and length of the fibres were similar between the experimental groups.

Table 4.1 Single skinned fibre lengths and diameters (mean±SE). There were no differences between groups.

<table>
<thead>
<tr>
<th></th>
<th>Fibre length (μm)</th>
<th>Fibre diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 - passive fibres</td>
<td>949±41</td>
<td>78±4</td>
</tr>
<tr>
<td>Group 2 - deactivation fibres</td>
<td>836±50</td>
<td>77±6</td>
</tr>
<tr>
<td>Group 3 - BDM fibres</td>
<td>916±42</td>
<td>67±4</td>
</tr>
<tr>
<td>Total</td>
<td>903±26</td>
<td>75±3</td>
</tr>
</tbody>
</table>

4.2 Forces and Stresses

Figure 4.1 shows stress and average SL as a function of time in a typical Group 1 – passive fibre and Group 2 – deactivation fibre experiment. In all fibres, stress after active stretch was higher than the stress obtained after passive stretch at an average SL of 5.0 μm (Table 4.2). The average stress of Group 2 fibres at steady-state after active stretch was 189±22 mN/mm². Stress decreased by 49±4% to 96±13 mN/mm² after deactivation in Group 2 fibres. However, the stress after deactivation of Group 2 fibres was still significantly greater than the passive stress of Group 1 fibres (Table 4.2).
Figure 4.1 Stress-time curves and SL-time curves for passively stretched Group 1 – passive fibres (grey line) and actively stretched then deactivated Group 2 – deactivation fibres (black line). The arrow shows the time point of deactivation of Group 2 fibres.

Figure 4.2 shows the stress and average SL as a function of time for Group 1 and Group 3 fibres. Stress decreased by 44±3% to 90±9 mN/mm² after adding the BDM solution, which was not significantly different from the steady-state stress after deactivation of Group 2 fibres. The steady-state stress after BDM treatment was still significantly greater than the purely passive stress at an average SL of 5.0 μm (Table 4.2).
Figure 4.2 Stress-time curves and average SL-time curves for passively stretched Group 1 fibres (grey line) and actively stretched then transferred to BDM Group 3 fibres (black line). The arrow shows the time point of transferring into the BDM solution.

Table 4.2 Stress of passively stretched fibres, actively stretched fibres that were subsequently deactivated, and actively stretched fibres that were subsequently treated with BDM and then deactivated, all at an average SL of 5.0 μm.

<table>
<thead>
<tr>
<th></th>
<th>Group 1 - passive fibres</th>
<th>Group 2 - deactivation fibres</th>
<th>Group 3 - BDM fibres</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress at a SL of 5.0 μm after stretch (mN/mm²)</td>
<td>55±5</td>
<td>189±22*</td>
<td>164±9*</td>
</tr>
<tr>
<td></td>
<td>Group 1 - passive fibres</td>
<td>Group 2 - deactivation fibres</td>
<td>Group 3 - BDM fibres</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------------------</td>
<td>--------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Stress after adding BDM (mN/mm²)</td>
<td>-</td>
<td>-</td>
<td>90±9*‡</td>
</tr>
<tr>
<td>Stress after deactivation (mN/mm²)</td>
<td>-</td>
<td>96±13*†</td>
<td>88±6*‡</td>
</tr>
</tbody>
</table>

Stress (mean±SE) produced by fibres after passive stretch (Group 1 – passive fibres), active stretch and deactivation (Group 2 – deactivation fibres) and active stretch, treatment with BDM and deactivation (Group 3 – BDM fibres) at an average SL of 5.0 μm. p<0.05,

* significant difference compared to stress at a SL of 5.0 μm after stretch in Group 1 – passive fibres.
† significant difference compared to stress at a SL of 5.0 μm after stretch in Group 2 – deactivation fibres.
‡ significant difference compared to stress at a SL of 5.0 μm after stretch in Group 3 – BDM fibres.

Figure 4.3 shows the stress and average SL as a function of time for Group 4 fibres. After passive stretching of the fibres to a nominal SL of 5.0 μm, they were activated. There was no increase in force with activation, indicating that there is no cross-bridge-based force when fibres are passively stretched to an average sarcomere length of 5.0 μm, which is beyond actin-myosin filament overlap. Figure 4.4 shows the stress and average SL as a function of time for Group 5 fibres. Fibres were passively stretched to a SL of 4.5 μm, and then activated. There was no increase in force with activation, suggesting that there is no cross-bridge-based force at this
sarcomere length. Fibres were then stretched in the activated state to a SL of 5.0 μm. After reaching a steady state, fibres were exposed to a 100 mM BDM solution. Force did not decrease with BDM exposure. However, force after an active stretch from an average SL of 4.5 μm to an average SL of 5.0 μm was not different from the purely passive force at that same SL. This result indicates that either titin stiffness never increased upon activation at an average SL of 4.5 μm and thus any effect of BDM on a “stiffened” titin molecule would not be observed, or that titin stiffness increased upon activation at a SL of 4.5 μm and exposure of the fibres to BDM after an active stretch to a SL of 5.0 μm indeed had no effect on titin stiffness and force.

**Figure 4.3** Stress-time and average SL-time curves for Group 4 fibres. Fibres were passively stretched, and then activated. The arrow shows the time point of activation.
Figure 4.4 Stress-time and average SL-time curves for Group 5 fibres. Fibres were passively stretched and then activated. Fibres were then actively stretched and exposed to a 100 mM BDM solution. The grey arrow shows the time point of activation, and the black arrow shows the time point of exposure to the BDM solution.
Chapter Five: Discussion

5.1 Purpose

The purpose of this study was to investigate the force increase for steady-state conditions after active compared to passive stretch at SLs beyond myofilament overlap in single fibres, to test whether this increase in force is caused by a passive component (titin) alone, or possibly some remnant cross-bridges that contribute to force in some unknown manner. A SL of 5.0 µm is beyond actin-myosin filament overlap in rabbit psoas fibres, and thus, theoretically speaking, there should be only passive forces acting at that length, independent of the activation conditions, and thus forces would be expected to be the same for the actively and passively stretched fibres. However, our results indicate that the steady-state force after an active stretch is clearly greater than after passive stretch, even after deactivation and BDM exposure of the actively stretched fibres. However, since deactivation and BDM exposure resulted in a decrease in force for actively stretched fibres, we suggest that the increase in force in actively compared to passively stretched fibres has an active, cross-bridge based, and a passive, titin based component.

5.2 Force after active stretch beyond myofilament overlap

According to the active force-length relationship of rabbit skeletal muscle (Joumaa et al., 2008), the end of the descending limb for rabbit psoas fibres occurs at a SL of 3.91 µm. Thus, theoretically, when rabbit psoas fibres were stretched to an average SL of 5.0 µm, there should be no overlap between the actin and myosin filaments, and force should be produced by passive elements exclusively. However, we found more than a threefold increase in stress after an active stretch compared to a passive stretch to an average SL of 5.0 µm (Figure 4.1). Previous studies (Leonard & Herzog, 2010; Powers et al., 2014) showed increases in force by a factor of 3 to 4
above the purely passive force in actively stretched rabbit and mouse myofibrils to average SLs beyond actin and myosin overlap. In single myofibrils, this additional force could be explained with increased forces in titin (Leonard & Herzog, 2010; Powers et al., 2014), and elimination of titin eliminated all forces in actively and passively stretched myofibrils.

It has been shown that calcium binds to titin upon activation and increases titin’s stiffness (Labeit et al., 2003). However, the calcium-dependent increase in titin’s stiffness has been found to be of small magnitude (Labeit et al., 2003; Joumaa et al., 2008; DuVall, Gifford, Amrein, & Herzog, 2013), and when stretching myofibrils in the presence of calcium, but preventing actin myosin cross-bridge interactions, the difference in force between actively and passively stretched myofibrils was merely 10-20%. In 2010, Leonard and Herzog provided a titin-actin interaction based mechanism of the force increase after active stretch. They suggested that upon muscle activation, titin attaches to actin thereby shortening its spring length and increasing its resistance to stretch when muscles are stretched actively compared to when they are stretched passively (Leonard & Herzog, 2010).

The titin-actin interaction mechanism appeared to be the most feasible explanation for their results, as titin is the primary and almost exclusive passive force producing element in myofibrils. Furthermore, all sarcomeres and half-sarcomeres were confirmed to be at lengths beyond actin-myosin filament overlap, and deactivation of the actively stretched myofibrils at the long lengths did not result in a measurable decrease in force (Leonard & Herzog, 2010). However, in single fibre preparations as used in this study, individual sarcomere and half-sarcomere lengths cannot be measured for all sarcomeres, great SL non-uniformities are known to exist in single fibres, and sarcomeres in single fibres are connected in parallel, which allows for force transfer between sarcomeres of neighbouring myofibrils. A. F. Huxley and Peachey
systematically quantified SL non-uniformities in single fibres of frog muscles. They observed active force in single frog fibres at an average SL of 4.0 μm, a length at which there is no overlap between actin and myosin (Gordon et al., 1966). Similar results were obtained in experiments performed in frog muscles by Carlsen et al. (1961; 1965) in which active force was not abolished at the end of myofilament overlap but remained in evidence at SLs as long as 4.5 μm. Therefore, force after active stretch to a SL beyond myofilament overlap may not necessarily originate from titin alone in single fibres, but cross-bridges might still be involved due to the non-uniformities in SLs.

In order to investigate if cross-bridges could still be involved in force production beyond myofilament overlap, fibres were stretched actively and passively to a SL of 5.0 μm, which is clearly beyond the lengths at which rabbit psoas sarcomeres loose myofilament overlap (about 3.91 μm). Following stretching to this long lengths, they were either deactivated or treated with a cross-bridge inhibitor, BDM. If the increase in force after active stretch beyond myofilament overlap is caused by titin exclusively, then fibre force should not decrease substantially after deactivation and treatment with BDM.

5.3 Force after active stretch beyond myofilament overlap and deactivation

Deactivation decreased force after active stretch beyond myofilament overlap by about 49%. This result could be explained by calcium release from titin and titin unbinding from actin upon deactivation and the associated decrease in titin stiffness and force, or with the unbinding of remnant cross-bridges even at these long SLs. To differentiate between these two possible mechanisms, we treated actively stretched fibres with BDM. BDM exposure would release active force producing cross-bridges but should not cause an unbinding of calcium from titin or an
unbinding of titin from actin. Thus, BDM exposure should provide an exclusive estimate of the cross-bridge based forces that might be present in single fibres stretched actively to very long SLs.

5.4 Force after active stretch beyond myofilament overlap and BDM

BDM is a chemical used as a cross-bridge inhibitor in muscle experiments (Herrmann, Wray, Travers, & Barman, 1992; E.-J. Lee, Joumaa, & Herzog, 2007). BDM inhibits phosphate release and keeps cross-bridges at a pre-power stroke, and thus non-force producing state (Herrmann et al., 1992; Tesi, Colomo, Piroddi, & Poggesi, 2002). When fibres actively stretched to a SL beyond overlap were treated with BDM, force decreased by about 44%, a similar decrease in force to that observed after active stretch and deactivation. Since the force decrease after exposure to BDM is associated exclusively with cross-bridge inhibition, and since it is similar to that when myofibrils were deactivated, we suggest that deactivation and BDM exposure of actively stretched fibres resulted in a force loss exclusively associated with remnant cross-bridge forces, and not with any titin-associated mechanism.

It has been known for a long time that the steady-state force produced by a muscle following active stretch is greater than the corresponding purely isometric force and that this stretch-induced RFE is often accompanied by an increase in passive force after deactivation (Herzog & Leonard, 2002; Herzog, Schachar, & Leonard, 2003). This phenomenon is termed “passive force enhancement”. Passive force enhancement is believed to originate from the molecular spring titin whose stiffness and characteristic length may be modulated by interactions with actin and calcium upon activation and stretch (Edman et al., 1982; Forcinito et al., 1998; Bagni, Cecchi, Colombini, & Colomo, 2002; Bagni, Colombini, Geiger, Palmini, & Cecchi,
2004; Joumaa et al., 2008; Herzog et al., 2010; Leonard & Herzog, 2010; Herzog et al., 2012a, 2012b; Herzog, 2014a, 2014b; Herzog et al., 2015; Herzog et al., 2016). Here, force after deactivation and BDM treatment decreased by about 49% and 44% respectively and was still significantly higher than the force following passive stretch to an average SL of 5.0 μm.

Based on our results, it appears that the force increase after active stretch beyond myofilament overlap in single fibres has an active and a passive component. The active component is likely associated with actin-myosin based cross-bridge forces. The passive component appears independent of the cross-bridges and is likely associated with titin, as has been found in single myofibrils (Leonard & Herzog, 2010; Powers et al., 2014).

Although active force production beyond myofilament overlap has not been extensively studied, some related results reported in the literature support our finding that cross-bridge-based forces could be produced at average SLs beyond myofilament overlap. In experiments carried out by Carlsen et al. (1961; 1965) on frog muscles that loose actin-myosin filament overlap at a SLs of about 3.6 μm, active force development persisted until the sarcomeres had been stretched to an average SL of 4.5 μm (Carlsen et al., 1961; 1965). Similar results were obtained by Walcott and Dewey (1980). Furthermore, A. F. Huxley and Peachey (1961) showed that active force in frog muscles decreased with activation at increasing SLs. However, active force persisted up to average SLs of 4.0 μm, while myofilament overlap was lost at 3.6 μm in their single fibre frog skeletal muscle preparations.
5.5 How can cross-bridge-based force be produced at sarcomere lengths substantially beyond myofilament overlap?

A. F. Huxley and Peachey (1961) speculated that their observation of substantial active forces at sarcomere lengths substantially beyond actin-myosin filament overlap might be due to non-uniform lengths of sarcomeres in fibres. They showed that sarcomeres in the middle of single frog fibres that were stretched to lengths exceeding 4 μm were accompanied by sarcomeres at the ends of the fibres that were as short as 1.8 μm, and thus were well within the actin-myosin overlap zone. These short sarcomeres can produce active force even with the average SLs being well above 3.6 μm, and thus beyond contractile filament overlap. Since these short sarcomeres in a fibre are connected in parallel to other myofibrils in the fibres and adjacent sarcomeres, these short sarcomeres can produce active force that is transmitted along the entire fibre. Similar observations have been made by others in entire muscles (Llewellyn et al., 2008; Moo et al., 2016), single fibres, and mechanically isolated myofibrils (Rassier et al., 2003; Joumaa et al., 2008; Johnston, Jinha, & Herzog, 2016). Here, we suggest that the development of SL non-uniformities is partially responsible for the remnant cross-bridge based forces that we observed at average SLs beyond myofilament overlap.

In contrast to the single fibre results, deactivation or BDM treatment did not result in a loss of force in single myofibrils that were stretched to lengths beyond actin-myosin filament overlap. However, in single myofibrils, in contrast to single fibres, all sarcomeres are mechanically arranged in series with each other with no parallel force transmission, and all sarcomere (and half-sarcomere) lengths can be checked individually to ensure that no actin-myosin filament overlap exists in any (half-) sarcomere.
Another mechanism for the persistence of cross-bridge interactions at SLs beyond myofilament overlap could be that selected myosin filaments are pulled out of the structural arrangement of the A-band, and so might provide cross-bridge interactions even though the majority of actin and myosin filaments do not overlap anymore. Although theoretically possible, there is no direct evidence that such structural damage might occur in highly stretched sarcomeres.
Chapter Six: Conclusion and Future Directions

6.1 Conclusion

In summary, our study showed that the steady-state force produced by rabbit psoas fibres after active stretch to SLs beyond actin-myosin overlap is significantly greater than the purely passive force at the corresponding length. In contrast to the force increase in single myofibrils, which seems to be passive in nature only, the force increase in fibres has an active cross-bridge based component that is eliminated when the preparation is deactivated, and a passive component that persists even after deactivation. The passive component may be associated with the structural protein titin, as suggested in the past. The molecular details of how titin might accomplish this increase in force needs further investigation.

6.2 Future directions

This study was aimed to investigate the steady-state force increase after active stretch to a SL beyond myofilament overlap. Force increases substantially after active compared to passive stretching, and we speculate that this force increase is associated with a passive and an active component.

The active component is related to the cross-bridges which may be due to the SLs non-uniformity in single fibres during activation and active stretch. If single fibres after active stretch at an average SL of 5.0 μm can be fixed during activation, and imaged under microscope, we can measure the SL non-uniformities in the fixed specimens.

Since it has been proposed that calcium might influence the active and passive components of force increase, it might be of interest to perform these experiments with different calcium concentrations (in the sarcoplasm), in order to change the magnitude of the active and
passive forces. We hypothesize that both the active and passive components of the force increase after active stretch are calcium-dependent.

We can also measure the energy cost in the actively stretched and passively stretched single fibres respectively at a SL of 5.0 μm. Since cross-bridges contribute to the force after active stretch at an average SL of 5.0 μm, we hypothesize that there should be some energetics in the actively stretched fibres. However, in the passively stretched fibres, there should be no energy cost. Moreover, if energy cost can be measured from the actively stretched fibres, we can also estimate whether the remaining energetics is compatible with the force decrease after deactivation.

We found that a passive component is likely contributing to the force increase after active stretch to a sarcomere length beyond myofilament overlap. Since titin is the passive element responsible for most of the passive force in skinned fibres and myofibrils, it would be of importance to test the effect of different titin isoforms on the force increase after active stretch beyond overlap. In the current study, we used rabbit psoas muscle fibres. In the future, soleus and diaphragm muscle fibres, known to express a relatively long form of titin isoforms, may be used to investigate the relationship between titin isoforms and force increase after active stretch.


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https://doi.org/10.1152/japplphysiol.00653


APPENDIX A: RAW DATA

Group 1 – Passive Fibres (n = 16)

Figure A.1 The raw data of Group 1 – passive fibres. The dots show the stress (mN/mm²) of each fibre at each time point: after passive stretch to an average SL of 5.0 μm, one minute and two minutes after stretch to an average SL of 5.0 μm.
Group 2 - Deactivation fibres (n = 14)

**Figure A.2** The raw data of Group 2 – deactivation fibres. The dots show the stress (mN/mm$^2$) of each fibre at each time point: activated at an average SL of 2.4 μm, after active stretch to an average SL of 5.0 μm, one minute after active stretch to an average SL of 5.0 μm, and deactivated at an average SL of 5.0 μm.
Group 3 – BDM fibres (n = 15)

Figure A.3 The raw data of Group 3 – BDM fibres. The dots show the stress (mN/mm²) of each fibre at each time point: activated at an average SL of 2.4 μm, after active stretch to an average SL of 5.0 μm, one minute after active stretch to an average SL of 5.0 μm, after adding BDM solution at an average SL of 5.0 μm, and deactivated at an average SL of 5.0 μm.
Group 4 (n = 5)

Figure A.4 The raw data of Group 4 fibres. The dots show the stress (mN/mm²) of each fibre at each time point: after passive stretch to an average SL of 5.0 μm, one minute and after activation at an average SL of 5.0 μm.
Group 5 (n = 6)

Figure A.5 The raw data of Group 5 fibres. The dots show the stress (mN/mm$^2$) of each fibre at each time point: after passive stretch to an average SL of 4.5 μm, after activation at an average SL of 4.5 μm, after passive stretch to an average SL of 5.0 μm, and after treating with BDM solution.