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History Dependent Force Production in Single Skeletal Muscle Fibres

by

Eun-Jeong Lee

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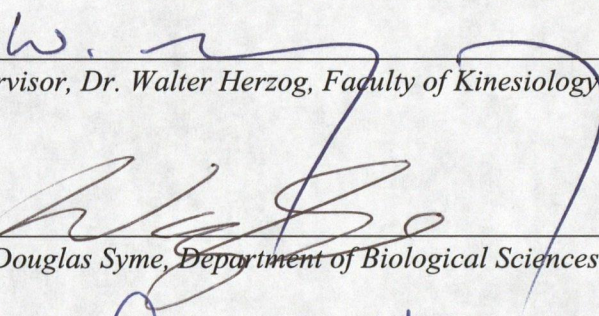
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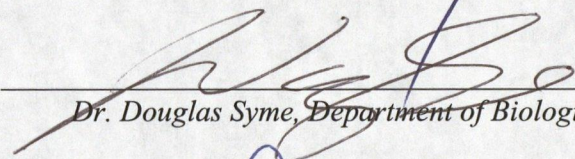
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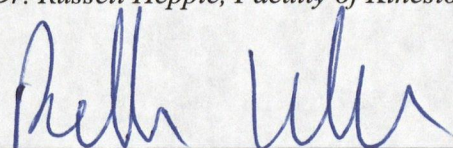
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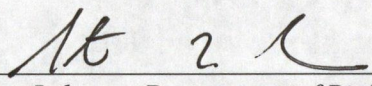
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## Abstract

Based on the cross-bridge theory of muscle contraction, the steady-state isometric force of a muscle is uniquely determined by the extent of actin-myosin overlap in a sarcomere, and thus muscle length. However, it has been shown that this prediction is not necessarily correct. Rather, it has been found that the steady-state isometric force at a given length can take many values depending on the contractile history preceding isometric contractions. Specifically, isometric forces following stretch of a muscle are greater and following shortening are smaller than the corresponding forces for purely isometric contractions. These properties of skeletal muscle contraction are referred to as (residual) force enhancement and force depression, respectively. Despite vast study of these history-dependent phenomena, the detailed mechanisms underlying force enhancement and force depression remain largely unknown. In this thesis, experiments with intact single fibres from frog (*Rana pipiens*) are described in an attempt to gain further insight into history-dependent properties and mechanisms of muscle contraction. Single fibres were prepared for mechanical analysis in an experimental chamber so that force and sarcomere length measurements could be made for controlled activation, contractile conditions, and temperature. Specifically, the steady-state isometric forces following a variety of stretch and shortening conditions were compared to the steady-state isometric forces following purely isometric reference contractions at the corresponding length. Force enhancement following stretch of activated fibres was shown to exceed the plateau of the force-length relationship, suggesting that force enhancement is caused by the recruitment of additional force that is not available for isometric contractions. Furthermore, force enhancement for specific conditions was also associated with an

increase in passive force, suggesting that passive structural elements might contribute to the observed force enhancement. Finally, when biasing cross-bridges to weakly or strongly bound attachment configurations, we observed that force enhancement was increased in preparations with cross-bridges that were initially biased towards weakly bound states, suggesting that force enhancement might be, in part, caused by a stretch-induced increase in the ratio of strongly to weakly bound cross-bridges. Force depression following shortening in normal fibres and fibres in which cross-bridge states were biased towards weakly bound configurations were associated with a decrease in fibre stiffness. This decrease in stiffness was related to the initial cross-bridge configurations, suggesting that force depression might be caused by a shortening-induced inhibition of the strongly bound cross-bridges.



## Preface

Chapters three through seven, respectively, are based on the following manuscripts:

- Chapter 3: Lee, E.-J., Herzog, W. (2008) Residual force enhancement exceeds the isometric force at optimal sarcomere length for optimized stretch conditions. *Journal of Applied Physiology (in press)*, Publishers ID JAP-01109-2006).
- Chapter 4: Rassier, D., Lee, E.-J., Herzog, W. (2005) Modulation of passive force in single skeletal muscle fibers. *Proceedings of Royal Society: Biology letters* **1(3)**, 342-345.
- Chapter 5: Lee, E.-J., Joumaa, V., Herzog, W. (2007) New insights into the passive force enhancement in skeletal muscles. *Journal of Biomechanics* **40(4)**, 719-727.
- Chapter 6: Lee, E.-J., Herzog, W. (2008) Effect of temperature on residual force enhancement in single skeletal muscle fiber. *Journal of Biomechanics (in press)*, Publishers ID BM-D-08-00125).
- Chapter 7: Lee, E.-J., Herzog, W. Shortening-induced force depression is primarily caused by cross-bridges in strongly bound states. (*in preparation*).

*This dissertation is based on a collection of stand alone manuscripts, and therefore has some redundancy in the introduction and methods sections of chapters three through seven.*

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*To my parents,  
Won-Shim and Do-Jin*



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## Epigraph

There is one great truth on this planet:  
whoever you are, or whatever it is that you do,  
when you really want something,  
it's because that desire originated in the soul of the universe.  
It's your mission on earth.

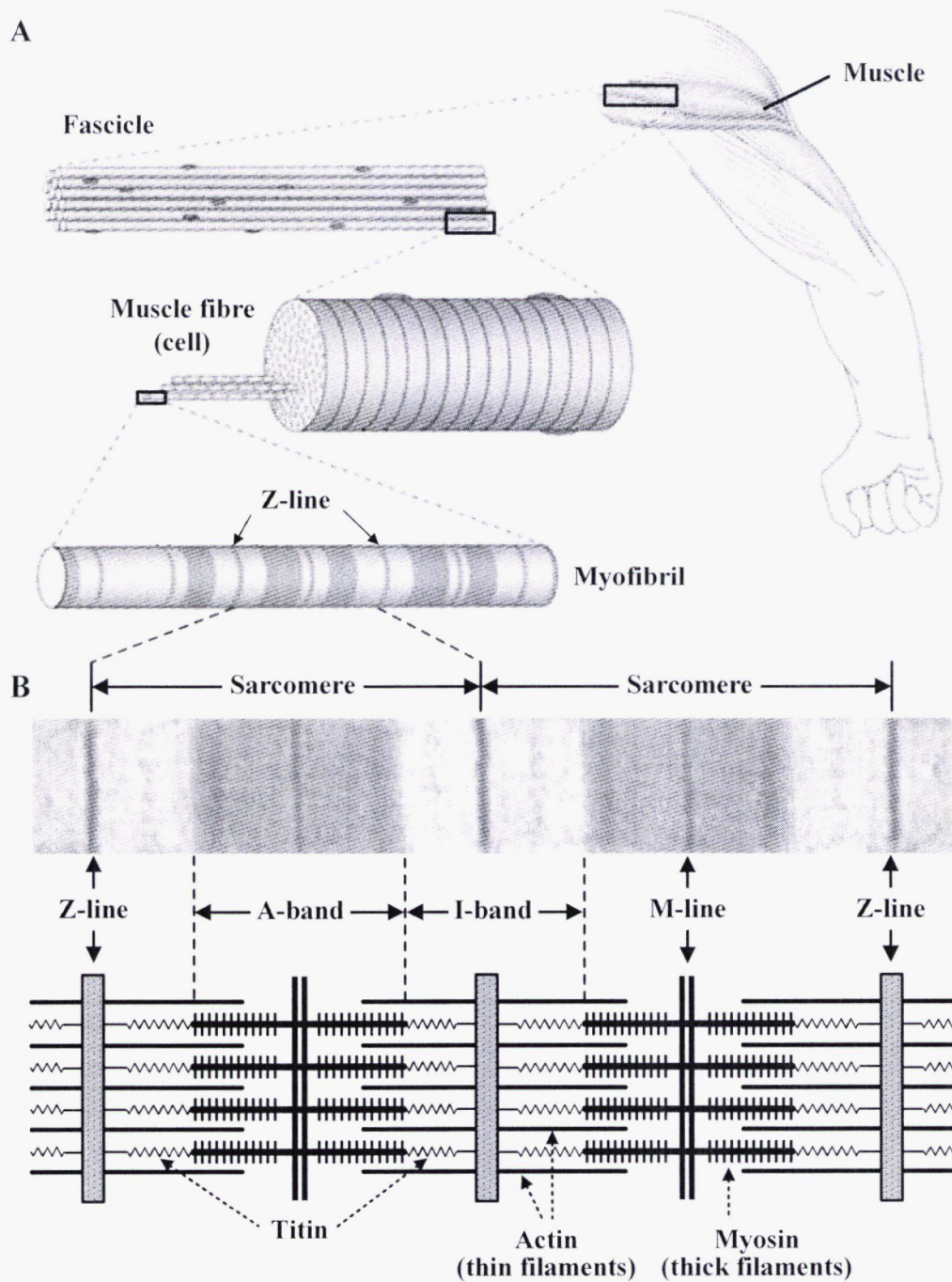
이 세상에는 위대한 진실이 하나 있어.  
무언가를 온 마음을 다해 원한다면, 반드시 그렇게 된다는 거야.  
무언가를 바라는 마음은 곧 우주의 마음으로부터 비롯된 때문이지.  
그리고 그것을 실현하는 게 이 땅에서 자네가 맡은 임무라네.

The Alchemist by Paulo Coelho

## **Chapter One: General introduction**

Skeletal muscle is a contractile tissue that produces force and causes movement. The basic control unit of a skeletal muscle is the motor unit, which is defined as a motor axon and all the fibres it innervates, while the basic contractile unit is a sarcomere. Skeletal muscle is divided into muscle bundles or fascicles, and each fascicle is comprised of cells or muscle fibres (Figure 1-1A). In turn, each muscle fibre (or cell) is made up of myofibrils, a sub-sarcomeric structure that consists of a string of sarcomeres perfectly arranged in series. (Figure 1-1B), and myofibrils are made up of the contractile proteins actin and myosin and a variety of structural proteins, most importantly for this work, titin, a giant protein that extends from the middle of the sarcomere (M-line) to the ends (Z-line) (Figure 1-1B).

The basic contractile unit of muscle, the sarcomere, is defined to extend from one Z-line to the next. Sarcomeres give skeletal muscle its structural band pattern that is visible in the light microscope as a dark-light repeat pattern. The dark area has been identified as the region of the myosin (or thick) filaments (the so-called anisotropic or A-band), whereas the light band has been identified as the area of the actin (or thin) filaments (the so-called isotropic or I-band) separated by the narrow, dark band representing the Z-line (Figure 1-1B).



**Figure 1-1: Schematic illustrations of skeletal muscle (A) and sarcomere (B) structure.** Arrangement of contractile proteins, actin and myosin filaments, and passive structural protein, titin, in a sarcomere is illustrated.



Prior to the 1950s, contraction of muscles and force production was primarily associated with a shortening of the A-band region, which was thought to occur due to a helix to coil transformation of the myosin filaments upon muscle excitation. However, Huxley and Niedergerke (1954) and Huxley and Hanson (1954) found independently that shortening, contraction and force production in single fibres and myofibrils, respectively, were associated with an essentially constant A-band length, therefore, A-band shortening could not be responsible for muscle contraction and force production. They proposed that muscle contraction and shortening occurred through a sliding of two sets of filaments (actin and myosin) relative to one another. This is now commonly referred to as the sliding filament theory of muscle contraction (see literature review for more details).

However, it has remained unclear to this day, how this sliding of actin and myosin filaments occurs. Huxley (1957) proposed a first mathematically defined interaction of actin and myosin and how muscle might contract. In short, he proposed that the myosin filaments had lateral extensions (now referred to as cross-bridges) that would cyclically interact with the actin filaments at precisely defined attachment sites. The interactions of actin and myosin were uniquely determined by a set of attachment and detachment rate constants that were thought to be exclusive functions of a distance “ $x$ ” which Huxley defined as the distance of the cross-bridge equilibrium point to the nearest actin attachment site. Huxley further postulated that this interaction was driven by Brownian noise (for attachment) and by the hydrolysis of a high energy compound (adenosine triphosphate ATP) for detachment: one ATP per cross-bridge cycle. This theory of

muscle contraction is referred to as the cross-bridge theory and it has been the paradigm of choice ever since, although modifications to the basic theory have been made (see literature review for more details).

A first modification was proposed by Huxley (1969), who argued that molecular interactions between actin and myosin in an environment of changing geometry (caused by muscle length changes and iso-volumetricity) could not be accomplished with a “fixed” cross-bridge, and thus suggested that cross-bridges were attached to the myofilament backbone via two hinge joints; one at the base of attachment of the cross-bridge to the myosin filament; the other between the myosin light chains and the heavy chains. Thus, the rotating cross-bridge model was born. Huxley and Simmons (1971) first formulated a mathematical description of a rotating cross-bridge with multiple attachment states (see literature review for more details).

A further modification of the cross-bridge model was based on completely novel information of the atomic structure of the cross-bridge head and the corresponding actin attachment site determined with x-ray diffraction and synchrotron radiation measurements (Rayment *et al.*, 1993a; Rayment *et al.*, 1993b). Rayment’s cross-bridge model showed the molecular interaction of ATP hydrolysis states with the corresponding mechanical cross-bridge states, and further suggested that the part of the cross-bridge head that attached to the actin was fixed, while just part of the cross-bridge head undergoes the rotational cross-bridge stroke (see literature review for more details).

Despite all these modifications, current cross-bridge models still rely on the idea that muscle contraction can be explained with a series of mechanical/biochemical states that are connected by thermodynamically consistent rate constants, that these rate constants depend exclusively on Huxley's "x-distance" and that force production is caused by the extension of an elastic element in the cross-bridge upon cross-bridge attachment and head rotation.

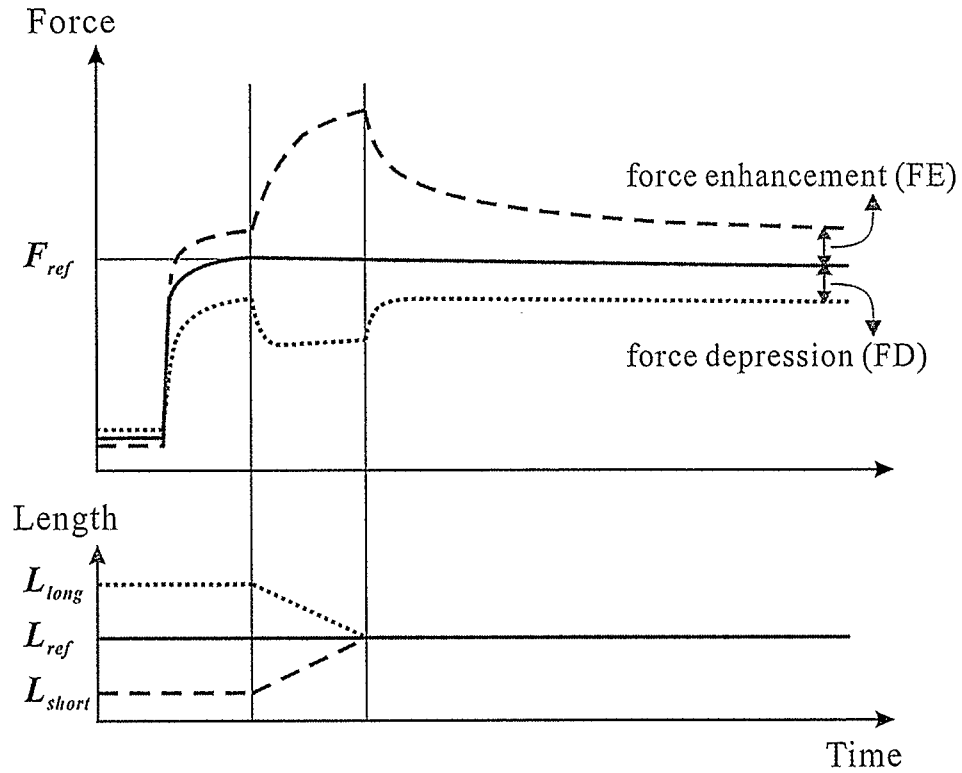
The mathematical description of the cross-bridge model (e.g., Huxley, 1957; Huxley and Simmons, 1971) are based on several non trivial assumptions. These include: (i) cross-bridges are uniformly arranged on the myosin filament, (ii) actin attachment sites are uniformly arranged along the actin filament, (iii) cross-bridges exert the same average force independent of location, and (iv) cross-bridges act independently of each other. Based on these assumptions, the maximal steady-state isometric force of a sarcomere is uniquely determined by the actin-myosin overlap, or, correspondingly, sarcomere lengths. Gordon *et al.* (1966) tested this prediction and found that the so-called descending limb of the force-length relationship was perfectly linear as predicted by the cross-bridge theory, thereby lending strong support to the theory (see literature review for more details).

However, the cross-bridge theory, although accounting perfectly for many properties of skeletal muscle contraction, cannot explain all properties. For example, the cross-bridge theory is not well suited for predicting the forces or metabolic cost of eccentric

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contractions (Huxley, 1957), and it cannot account for many of the history-dependent effects observed in muscle contraction (e.g., Abbott and Aubert, 1952). History-dependent properties have primarily been studied by measuring the steady-state isometric forces following stretch or shortening of muscles (fibres). Following stretch, these isometric forces are greater and following shortening they are smaller than the purely isometric reference forces at the corresponding length (Figure 1-2). These observations are typically referred to as residual force enhancement (following stretch) and force depression (following shortening). Residual force enhancement and force depression have been observed in all structurally identified preparations ranging from whole muscles to single myofibrils and isolated sarcomeres (Morgan *et al.*, 2000; Rassier *et al.*, 2003; Pinniger and Cresswell, 2007; Journaa *et al.*, 2008b). Although history-dependent force production has been studied systematically for more than half a century (Abbott and Aubert, 1952), the detailed mechanisms remain unknown.

The most accepted mechanism explaining force enhancement and force depression has been the so-called sarcomere length non-uniformity theory (Julian and Morgan, 1979; Morgan *et al.*, 2000). However, recent evidence (some of it reported in this thesis) suggests that this theory is likely not correct. In 2002, Herzog and Leonard described that force enhancement at long muscle length was associated with a distinct increase in passive force. However, this passive force enhancement was not observed for all contractile conditions and was always smaller than the total force enhancement, suggesting that a passive structural element might contribute to force enhancement but



**Figure 1-2: Schematic illustration of force enhancement and force depression.** Definition of residual force enhancement (FE; dashed line) and force depression (FD; dotted line). The steady-state isometric reference force ( $F_{ref}$ ) at the corresponding reference length ( $L_{ref}$ ) is shown (solid line). Activated muscles are stretched from shorter length ( $L_{short}$ ) to reference length ( $L_{ref}$ ) or shortened from longer length ( $L_{long}$ ) to reference length ( $L_{ref}$ ).

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could not be the single mechanism. A number of studies have been published since, confirming the existence of this passive force enhancement in other muscles than those described originally (Lee and Herzog, 2002; Pinniger and Cresswell, 2007), and also in single fibres (Rassier *et al.*, 2005) and myofibrils (Joumaa *et al.*, 2007). Another mechanism proposed to explain force enhancement is based on the idea that force enhancement is caused by a stretch-induced increase in the average force per cross-bridge (Sugi and Tsuchiya 1988). Specifically, it has been suggested that this increased average force is achieved by stretch-induced facilitation of transition from weakly to strongly bound cross-bridge states (Rassier and Herzog, 2004b).

As for force enhancement, the sarcomere length non-uniformity theory has been, and probably still is, the primary mechanism for explaining force depression. However, recent experimental observations revived a mechanism that had been originally proposed by Maréchal and Plaghki (1979), who suggested that force depression was caused by a shortening-induced inhibition of cross-bridge attachment to actin. Based on this theory, force depression is caused by a reduction in the proportion of attached cross-bridges, (Sugi and Tsuchiya, 1988). This theory had particular appeal as it has been shown that force depression is typically associated with a corresponding decrease in stiffness.

In summary, the sarcomere length non-uniformity theory has prevailed in explaining force enhancement and force depression. However, recent evidence suggests that this theory might not be suitable and alternative mechanisms have emerged.

The aim of this thesis was to obtain additional insight into history-dependent properties of muscle force production and elucidating the mechanisms underlying these phenomena.

The specific purposes of this thesis were as follows:

1. To determine if forces in the enhanced state can exceed the isometric steady-state forces on the plateau of the force-length relationship.
2. To quantify the effects of activation and stretch on the passive force–sarcomere length relationship.
3. To determine the origin of the passive force enhancement.
4. To test the hypothesis that force enhancement is caused by a stretch-induced increase in the ratio of strongly to weakly bound cross-bridges.
5. To test the hypothesis that force depression is caused by a shortening-induced inhibition of the strongly bound cross-bridges.

We used intact single fibres isolated from frog skeletal muscles to address these issues in a series of separate experiments. Intact single fibres have been used in many studies to identify properties of skeletal muscle (Gordon *et al.*, 1966; Edman *et al.*, 1982) and elucidating the molecular mechanisms of muscle contraction (Huxley, 1957; Huxley and Simmons, 1971). The single fibres used in this study were isolated from frog skeletal muscle. Frog fibres have the advantage that they are easy to isolate, they are very robust and they have been used more often for these types of experiment than any other muscle preparation.



The thesis continues with a review of the relevant literature (Chapter 2). This chapter provides the background of the classical theories of muscle contraction and history-dependent force production. Chapters 3 to 7 are based on five experiments addressing specifically the purposes mentioned above.

In Chapter 3, we address purpose 1, and will show that forces in the enhanced state can exceed the isometric forces on the plateau of the force-length relationship, suggesting that the sarcomere length non-uniformity theory cannot exclusively explain force enhancement.

In Chapter 4, we address purpose 2. We found that for a given sarcomere length passive forces following active stretch were substantially greater than those following purely isometric reference contractions, suggesting that a passive structural element likely contributes to force enhancement.

In Chapter 5, we address purpose 3. We found that passive force enhancement was increased with decreasing active force suggesting that passive force enhancement was linked to active force production.

In Chapter 6, we address purpose 4. In order to do this, residual force enhancement was measured at different temperatures as temperature affects the ratio of weakly to strongly bound cross-bridges. Greater force enhancement was observed at lower temperatures

suggesting that changes in cross-bridge binding states affect the magnitude of force enhancement.

In Chapter 7, we address purpose 5. Here, we observed that stiffness was decreased in the force depressed state compared to the corresponding isometric reference state and that the reduction in stiffness was smaller when fibres were exposed to a cross-bridge inhibiting agent such as 2,3-butanedione monoxime, suggesting that force depression is associated with an inhibition of cross-bridge attachment, particularly for cross-bridges that are initially in strongly-bound states.

Chapter 8 concludes this dissertation with a summary and general conclusion as well as recommendations for future research.

## **Chapter Two: Literature review**

This chapter is divided into two main parts. Chapter 2.1 contains a review of the mechanisms of muscle contraction and Chapter 2.2 provides information on properties and mechanisms of history dependent force production.

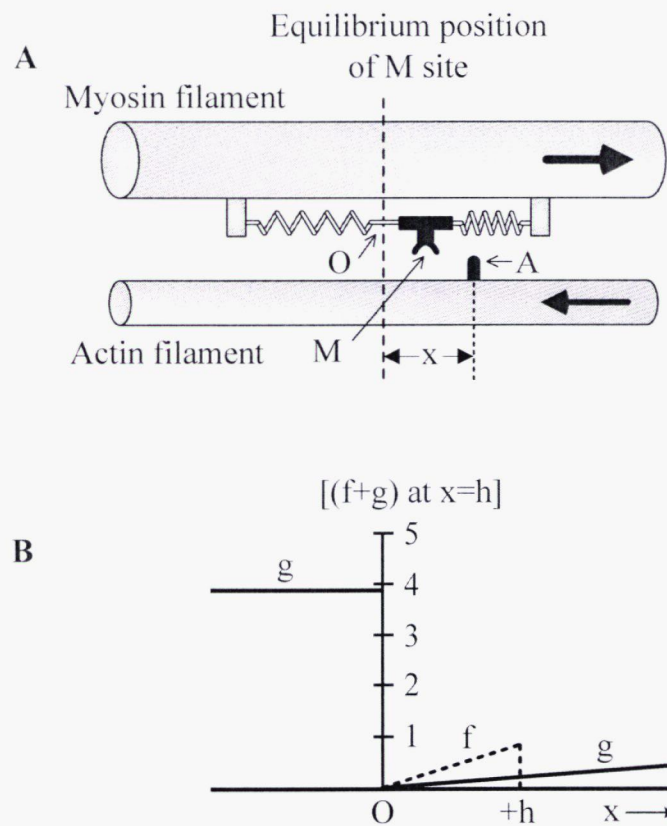
### **2.1 Muscle contraction – current mechanism**

In 1953, it was discovered, using electron microscopy that the A-bands and I-bands exist in series in myofilaments (Huxley, 1953) and myosin is located in the A-band (Hansen and Huxley, 1953; Hasselbach and Webber, 1953). A year later, Huxley and Niedergerke (1954) and Huxley and Hanson (1954) independently observed in single fibres and myofibrils, respectively, that the length of A-bands stayed constant during shortening, lengthening and isometric activation of muscle, and that the changes in fibre (myofibril) length corresponded to changes in length of the I-bands. This observation suggested that muscle contraction takes place through sliding of two kinds of filaments without appreciable changes in filament length. This is referred to as the sliding filament theory (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954).

Based on the idea of sliding filaments Huxley (1957) proposed the first quantitative model describing how myosin and actin filaments interact with each other and how a

muscle contracts and produces force. In this model, the so-called cross-bridge theory, it was proposed that there were side projections arising from the myosin filaments (referred to as cross-bridges) that were connected through an elastic element to the myosin filament. These cross-bridges (M in Figure 2-1A) were assumed to move around an equilibrium position (O) determined by the elastic attachment to the myosin filament, as a result of thermal agitation (Brownian motion). Cross-bridges were assumed to attach to specific attachment sites on actin (A), and the attachment/detachment kinetics were defined by a set of rate constants (Figure 2-1B). The rate constant of attachment ( $f$ ) was assumed to be a function of the distance from the cross-bridge equilibrium position (O) to the nearest attachment site ( $x$ ). Cross-bridges were assumed to detach based on a detachment rate function ( $g$  in Figure 2-1B) which was also defined as a function of  $x$ . One attachment/detachment cycle was assumed to be powered by the hydrolysis of one adenosine triphosphate (ATP). The force in each cross-bridge is thus given by the stiffness of the elastic element attaching M to the myosin filament and its extension from the equilibrium position.

In the cross-bridge theory, it was further assumed that all cross-bridges exert the same average force, that cross-bridges act independent of each other and that the cross-bridges and the corresponding attachment sites are uniformly distributed along the myosin and actin filament, respectively. With these assumptions, the force of a maximally activated muscle became a linear function of the amount of actin and myosin overlap. Gordon *et al.* (1966) tested the idea that the active isometric force was linearly related to the amount of

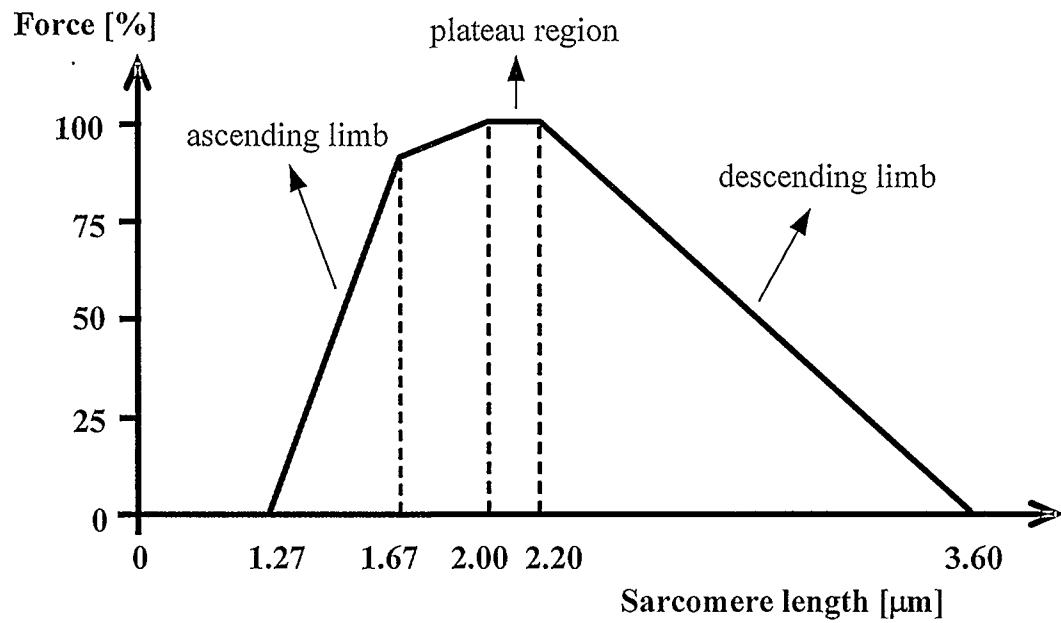


**Figure 2-1: The cross-bridge model proposed by Huxley (1957).** A) Schematic illustration of the cross-bridge model (Huxley, 1957). According to this model, cross-bridges (M) oscillate around an equilibrium position (O) and attach to specialized sites (A) on actin filament, B) Rate functions of cross-bridge attachment (f) and detachment (g) as a function of the distance from O to the attachment site (x) [Adapted from Huxley (1957)]

actin-myosin overlap. They performed experiments in which single fibres were isolated, and the average sarcomere length was controlled in a mid-segment of the fibre. Activating the fibres at different lengths, and thus different overlap between the actin and myosin filament, they demonstrated that the steady-state isometric force was predicted by the amount of actin-myosin overlap on the descending limb of the force-length relationship (Figure 2-2) in accordance with the predictions of the cross-bridge theory.

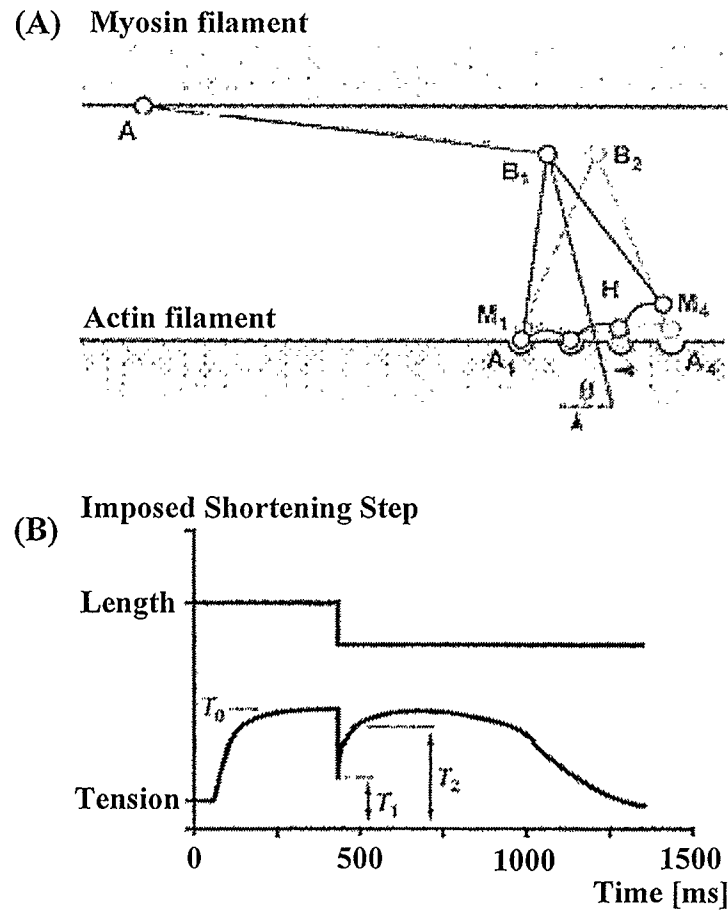
The 1957 cross-bridge model was a two state model allowing for one attached and one detached state. The next significant modification of the 1957 cross-bridge model was proposed by Huxley and Simmons (1971), who suggested that each cross-bridge had multiple attachment states (M1 to M4 in Figure 2-3A). This modification was introduced because Huxley and Simmons observed that force recovery following a quick shortening step in single fibres had two distinct time constants. A very quick ( $<1\text{ms}$ ) recovery immediately following the shortening step, and a much more gradual recovery ( $>100\text{ms}$ ) that followed the quick recovery (Figure 2-3B). These two distinct phases of force recovery could not be predicted with the 1957 two-state model but could be explained with the 1971 model of multiple attached states.

In the 1971 model, force production was assumed to occur through the rotation of the cross-bridge head about its attachment site on actin. This required that the entire orientation of the head underwent continuous changes. More recently, Rayment *et al.* (1993a) revealed the three-dimensional atomic structure of the myosin head and the actin



**Figure 2-2: The force- sarcomere length relationship of frog single skeletal muscle fibre.** The region of positive slope (sarcomere lengths ranging from 1.27 to 2.0μm ) is called the ascending limb, the region of zero slope (2.0–2.25μm) is called the plateau, and the region of negative slope (2.25-3.65μm) is called the descending limb of the force-length relationship [Adapted from Gordon *et al.* (1966)].



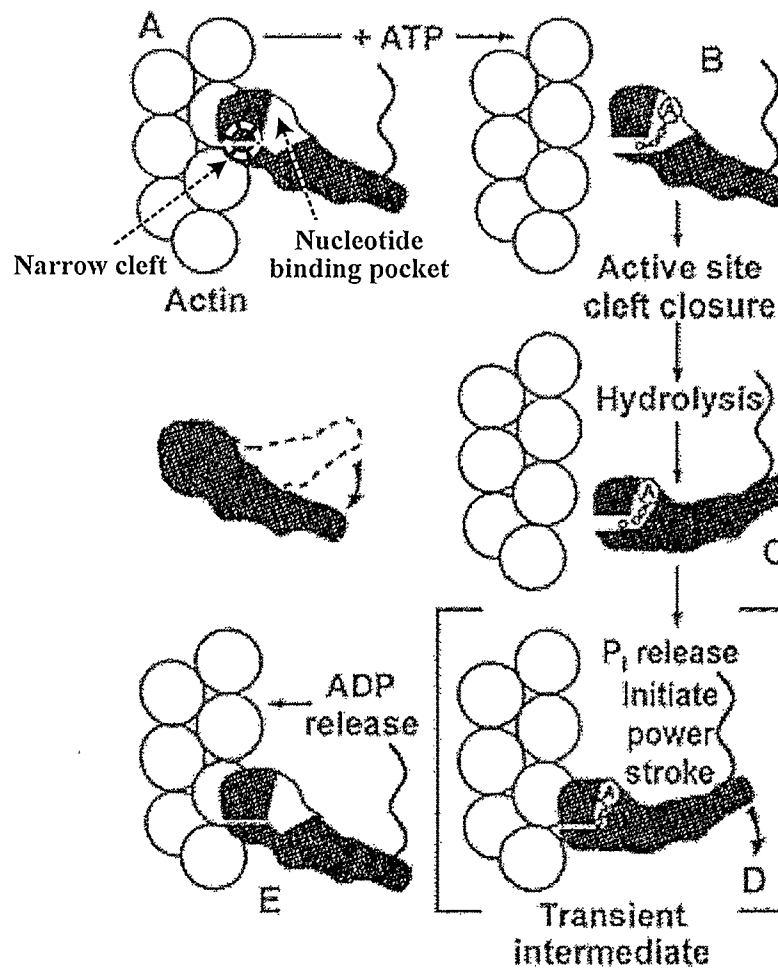


**Figure 2-3: Schematic illustration of the cross-bridge model proposed by Huxley and Simmons (1971).** A) The cross-bridge head is connected to the myosin filament via an elastic element (A-B) and has multiple attachment states ( $M_1$  to  $M_4$ ). A rotation of the cross-bridge head towards lower energy states produces increased tension through the stretch of the elastic element, B) Illustrations of the response of a fibre to an imposed shortening step.  $T_1$  is the minimal tension during a rapid shortening step of a muscle;  $T_2$

monomer using x-ray crystallography. Based on this detailed structural information, they proposed a cross-bridge model with multiple attached and multiple detached states, that provided detailed information on the coordination between the ATP hydrolysis steps and the associated mechanical interaction between actin and myosin (Figure 2-4).

This new proposed model of contraction suggested that the cross-bridge rotation might be achieved by a conformational change in the light-chain binding domain of the myosin molecule (Rayment *et al.*, 1993a; Rayment *et al.*, 1993b). A detailed description of this theory was as follow (Figure 2-4): actin and myosin molecules form the rigor complex (bind strongly) and the narrow cleft on the myosin is closed (A in Figure 2-4). Addition of ATP into the nucleotide-binding pocket of the myosin causes an opening of the narrow cleft, and interrupts the strong binding so that actin and myosin dissociate (B in Figure 2-4). Then, the nucleotide-binding pocket closes and causes a conformational change of myosin, and ATP is hydrolyzed (C in Figure 2-4). Myosin then rebinds to actin, followed by the closure of the narrow cleft which leads to strong binding of the actin-myosin complex. Release of phosphate ( $P_i$ ) initiates the power stroke (D in Figure 2-4). Following the power stroke, ADP is released, the nucleotide-binding pocket is opened and returns to its rigor position (E in Figure 2-4). The cross-bridge cycle as proposed by Rayment *et al.* (1993a) is currently the most accepted theory for how muscle contraction occurs.

In this model, there exist two different cross-bridge binding states, a weakly bound, and a



**Figure 2-4: Molecular mechanism of muscle contraction proposed by Rayment *et al.* (1993a).** A) Rigor conformation with actin attached to myosin; B) Binding of ATP to myosin and detachment of myosin from actin; C) ATP hydrolysis into ADP + Pi and a corresponding conformational change of the myosin molecule; D) Myosin rebinds to the actin, Pi releases, and the power stroke initiates; E) Myosin molecule performs the power stroke and undergoes a conformational change, returning it to the rigor state [Adapted from Rayment *et al.* (1993a)].

strongly bound state. Firstly, the weakly bound state occurs when the myosin molecule attaches to the actin (before the power stroke). Secondly, after the power stroke occurs, the strongly bound state is realized. Both states of cross-bridge attachment are believed to contribute to stiffness (Huxley and Simmons, 1971, Julian and Sollins, 1975); however, only the strongly bound cross-bridges are thought to contribute substantially to force production (Tesi *et al.*, 2002).

## **2.2 History dependent force production**

Based on the cross-bridge theory, the steady-state isometric force of a muscle on the descending limb of the force-length relationship is uniquely determined by the amount of actin and myosin overlap (Gordon *et al.*, 1966). However, some mechanical properties of muscle cannot be explained within the framework of the cross-bridge theory. These properties either require a distinct departure from current thinking or modification of the classical theory for muscle contraction.

One of those unexplained properties is history-dependent force production. It has been observed for more than 50 years (Abbott and Aubert, 1952) that the steady-state isometric forces following stretch or shortening during muscle contraction will be increased or decreased, respectively, compared to the steady-state isometric forces for a purely isometric contraction. The increased isometric force following lengthening is called residual force enhancement (FE in Figure 1-2) and the decreased isometric force

following shortening is called residual force depression (FD in Figure 1-2). Unfortunately, there is currently no clear mechanism to account for the history dependent force production. In this chapter, properties and several proposed explanations of force enhancement and force depression will be introduced.

### ***2.2.1 Properties of residual force enhancement***

Since the first observations of the residual force enhancement on entire muscles (Abbott and Aubert, 1952), many experimental results have confirmed this phenomenon at different structural levels. For instance, residual force enhancement has been observed in *in-vivo* human muscles (De Ruiter *et al.*, 2000; Lee and Herzog, 2002; Oskouei and Herzog, 2005), *in-situ* whole muscles (Morgan *et al.*, 2000; Herzog and Leonard, 2002), single fibres (Edman *et al.*, 1982; Granzier and Pollack, 1989; Rassier *et al.*, 2003), and recently in single myofibrils (Joumaa *et al.*, 2008b).

Residual force enhancement increases with increasing magnitudes of stretch (Abbott and Aubert, 1952; Edman *et al.*, 1978, 1982; Cook and McDonagh, 1995; Rassier *et al.*, 2003), and saturates at some threshold magnitude (Bullimore *et al.*, 2007). It is independent of the speed of stretch (Edman *et al.*, 1978; Sugi and Tsuchiya, 1988; Rassier *et al.*, 2003), and the rate of force decay after deactivation from a force enhanced state is decreased (Rassier and Herzog, 2005a; Lee *et al.*, 2007) compared to that observed in an isometric reference contraction. Stiffness in the force enhanced state seems to be about the same as in isometric reference contractions (Sugi and Tsuchiya

1988) or possibly slightly increased (Herzog and Leonard, 2000; Rassier and Herzog, 2005b). Force enhancement has been observed for maximal and sub-maximal voluntary and electrically elicited contractions (Oskouei and Herzog, 2005; Pinniger and Cresswell, 2007), thus implying that it may play a functional role in normal everyday movements.

Residual force enhancement has been observed consistently on the descending limb of the force-length relationship (e.g., Edman *et al.*, 1978; Morgan *et al.*, 2000; Rassier *et al.*, 2003), and the amount of force enhancement is increased for increasing length reaching peak values at sarcomere lengths of about  $3.0\mu\text{m}$  in frog skeletal muscles (e.g., Edman *et al.*, 1978, 1982). Force enhancement has also been observed on the ascending limb/plateau of the force-length relationship (Abbott and Aubert, 1952; Sugi, 1972; Cook and McDonagh, 1995; De Ruiter *et al.*, 2000; Herzog and Leonard, 2002; Rassier *et al.*, 2003; Peterson *et al.*, 2004; Pinniger and Cresswell, 2007), although the amount of force enhancement on the ascending limb/plateau part is typically smaller than that observed on the descending limb. In some studies, force enhancement was not observed on the ascending limb/plateau region of the force-length relationship (Brown and Loeb, 2000; Morgan *et al.*, 2000).

However, despite an abundance of observations on residual force enhancement, the mechanisms underlying this phenomenon remain unclear. Specifically, there is intense debate whether force enhancement can be explained with structural non-uniformities (i.e.

sarcomere length non-uniformity theory) exclusively, or if force enhancement is associated with the basic molecular mechanisms of contraction.

### *2.2.2 Proposed mechanism of force enhancement*

Among many proposed theories for the residual force enhancement following active stretch of muscle, the following three hypotheses are the most prominent: sarcomere length non-uniformity, engagement of a passive elastic element and increased proportion of the strongly bound cross-bridges. Detailed accounts of these three theories are given below.

#### *2.2.2.1 Sarcomere length non-uniformity theory*

The most accepted mechanism for residual force enhancement is associated with the development of sarcomere length non uniformities first proposed by Julian and Morgan (1979). The idea of this theory, the so-called sarcomere length non-uniformity theory, is based on an instability of sarcomeres on the descending limb of the force-length relationship (Hill, 1953), and it results in sarcomeres on the descending limb to elongate by different amounts upon stretch (A to B in Figure 2-5). That is, some weak sarcomeres are pulled beyond myofilament overlap (D in Figure 2-5) during an active muscle/fibre stretch on the ‘unstable’ (Hill, 1953) descending limb of the force-length relationship, and consequently these sarcomeres would ‘pop’ (Morgan, 1990, 1994). The ‘popping’ sarcomeres are supported exclusively by passive forces, while the remaining sarcomeres are stretched only by a small amount, if at all (C in Figure 2-5, (Julian and Morgan, 1979;

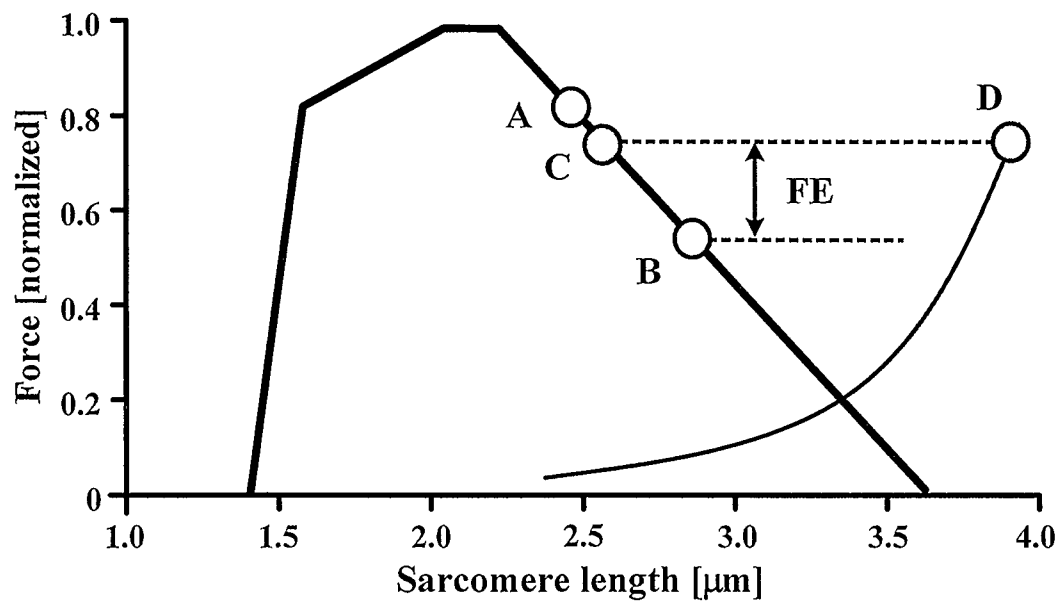
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Morgan *et al.*, 2000; Morgan and Proske, 2007)). Eventually the forces obtained by these two groups of sarcomeres will reach equilibrium, and the force in the enhanced state is given by the active force of the short, strong sarcomeres. This force is greater than that obtained for the purely isometric reference forces where sarcomere lengths are assumed to be relatively uniform (B in Figure 2-5, (Morgan, 1990; Morgan *et al.*, 2000)).

According to sarcomere length non-uniformity theory, several testable predictions can be made. The first prediction is that residual force enhancement should not occur on the 'stable' ascending limb of the force-length relationship. However, it has been shown by different laboratories and different muscle preparations that residual force enhancement may indeed occur on the ascending limb of the force-length relationship (Abbott and Aubert, 1952; Sugi, 1972; Cook and McDonagh, 1995; De Ruiter *et al.*, 2000; Herzog and Leonard, 2002; Peterson *et al.*, 2004; Pinniger and Cresswell, 2007). It must be noted that the amount of force enhancement on the ascending limb of the force-length relationship is typically small compared to that on the descending limb.

The second prediction is that residual force enhancement should not be greater than the purely isometric steady-state force obtained on the plateau of the force-length relationship (Morgan, 1994; Morgan *et al.*, 2000). However, it has also been observed that residual force enhancement does indeed exceed the plateau forces (Abbott and Aubert, 1952; De Ruiter *et al.*, 2000; Lee and Herzog, 2002; Rassier *et al.*, 2003; Peterson *et al.*, 2004; Schachar *et al.*, 2004).





**Figure 2-5: Schematic illustration of residual force enhancement (FE) based on the sarcomere length non-uniformity theory.** When muscle or fibre is stretched from A to B during activation, some sarcomeres are only stretched by a small amount (C), while others are ‘popped’ (D) and then are exclusively supported by passive force [Adapted from Morgan *et al.* (2000)].

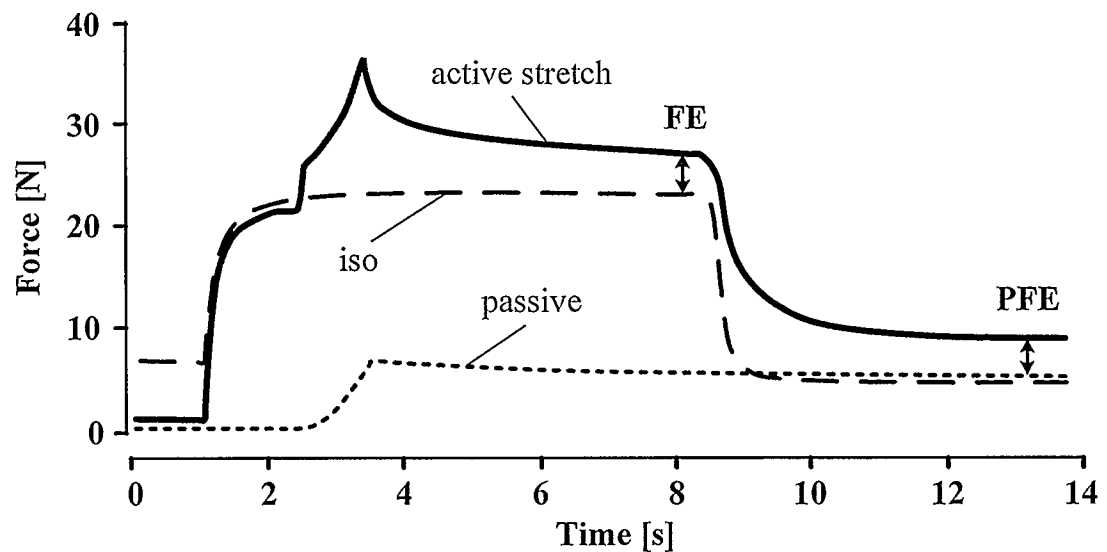
The third prediction is that residual force enhancement should be abolished when sarcomeres are maintained uniformly during and after active stretch. However, residual force enhancement persists under these conditions (Edman *et al.*, 1982). Thus, based on available evidence, the sarcomere length non-uniformity theory does not seem to explain the residual force enhancement observed experimentally.

#### 2.2.2.2 Engagement of a passive element

Edman *et al.* (1982) first suggested that residual force enhancement might be caused by the engagement of a passive element. This theory is based on the observation that residual force enhancement depends on the magnitude of stretch (Abbott and Aubert, 1952; Edman *et al.*, 1978, 1982), but not the speed of stretch (Edman *et al.*, 1978). This theory has been proposed by many studies (e.g., Noble, 1992; Edman and Tsuchiya, 1996; De Ruiter *et al.*, 2000; Herzog and Leonard, 2002; Herzog *et al.*, 2003; Rassier *et al.*, 2005). Edman *et al.* (1982) argued that if a passive element is engaged at the instant of activation and produces additional force when stretched, this additional force should not be apparent if the stretch is preceded by shortening of equal magnitude to the stretch. However, they found that force enhancement was unaffected by shortening preceding stretches, and thus argued that this theory could not be correct. However, when the same test was repeated by others (e.g., Herzog and Leonard, 2000; Lee *et al.*, 2001; Rassier and Herzog, 2004a), shortening preceding stretching reduced force enhancement in a dose dependent manner, suggesting that passive elements might play a role in causing the residual force enhancement.

Later, Herzog and Leonard (2002) (Figure 2-6) demonstrated that residual force enhancement was associated with a distinct increase in passive forces for some stretch conditions in the cat soleus. Since then, this “passive force enhancement” has been observed in other preparations (Herzog and Leonard, 2002; Lee and Herzog, 2002; Herzog *et al.*, 2003; Rassier *et al.*, 2005; Joumaa *et al.*, 2007), thereby providing further support for this idea that the residual force enhancement might be caused, at least in part, by a passive element. Passive force enhancement has been shown to increase with increasing stretch magnitudes and muscle lengths (Herzog and Leonard, 2002; Lee *et al.*, 2007). It is also long lasting (Herzog *et al.*, 2003) and positively correlated with the amount of residual force enhancement during activation (Herzog and Leonard, 2005; Bullimore *et al.*, 2007). Passive force enhancement was also found to decrease with increasing shortening magnitudes preceding stretch and can be by deactivation (Herzog *et al.*, 2003).

Passive force enhancement has also been observed in single myofibrils (Joumaa *et al.*, 2007, 2008a), suggesting that passive force enhancement originates from a passive structure in the sarcomere. The molecular spring titin (Figure 1-1B) has been proposed as a candidate for the passive force enhancement (Noble, 1992; Herzog and Leonard, 2002; Joumaa *et al.*, 2008a). Titin is the primary source for passive force in single myofibrils (Horowitz, 1992; Granzier *et al.*, 2000) and titin has been shown to change stiffness in a  $\text{Ca}^{2+}$ -dependent manner (Labeit *et al.*, 2003; Joumaa *et al.*, 2008a). In summary, residual force enhancement may in part be explained by the engagement of a passive element or a



**Figure 2-6: Exemplar of passive force enhancement.** Force-time histories of purely isometric reference contraction (iso, dashed), actively stretch contraction (active stretch, solid), and a passive stretch (passive, dotted) all finishing at the same final length (Herzog and Leonard, 2002). The amount of stretch for the active and passive condition is the same (9 mm or about 9% of optimal muscle length). Residual force enhancement (FE) and passive force enhancement (PFE) are indicated. *In-situ*, cat soleus muscle, temperature 35°C, stimulation frequency 30Hz [Adapted from Herzog and Leonard (2002)].

stiffening of an existing structure with activation. The specific nature of this passive force enhancement remains unclear. However, titin appears a viable candidate for this role.

#### 2.2.2.3 Increased proportion of the strongly bound cross-bridges

As discussed before, residual force enhancement has been shown to exceed isometric forces on the plateau region of the force-length relationship and has been observed on the ascending limb of the force-length relationship. Thus it seems likely that an additional contractile component might be somehow related to residual force enhancement. Additional contractile force could either be coming from an increased proportion of attached cross-bridges or an increased average force per cross-bridge following active stretch. Stiffness could be a good indicator of the proportion of attached cross-bridges (Huxley and Simmons, 1971; Julian and Sollins, 1975). However, force enhancement has been associated with little, if any increase in stiffness compared to the isometric reference contractions (Julian and Morgan, 1979; Sugi and Tsuchiya, 1988). Therefore, force enhancement does not appear to be caused by an increase in the proportion of attached cross-bridges.

If residual force enhancement is associated with an increased average force per cross-bridge, then on the average, the cross-bridge needs to be more stretched following stretching of an activated muscle compared to an isometric reference contraction. This could occur if stretch caused a bias from weakly towards strongly bound cross-bridges (Rassier and Herzog, 2004b). Rassier and Herzog demonstrated that residual force

enhancement was dramatically increased when single fibres were biased toward weakly bound cross-bridge states, thus supporting the idea of a stretch-induced bias towards strongly bound cross-bridges. Furthermore, lowering temperatures also biases cross-bridges towards weakly bound states and has been shown to result in greater force enhancement (Sugi, 1972). Thus, it appears that force enhancement might be caused by an addition of contractile force in the form of more force per cross-bridge.

### ***2.2.3 Properties of residual force depression***

Residual force depression was first reported systematically by Abbott and Aubert (1952), and since then, has been consistently observed in *in-vivo* human muscles (De Ruiter *et al.*, 1998; Lee and Herzog, 2003), *in-situ* whole muscles (Maréchal and Plaghki, 1979; Bullimore *et al.*, 2007), single fibres (Sugi and Tsuchiya, 1988; Granzier and Pollack, 1989; Edman *et al.*, 1993), and single myofibrils (Joumaa and Herzog, 2008). Residual force depression has been shown to be long lasting (Abbott and Aubert, 1952; Herzog *et al.*, 1998; Lee and Herzog, 2003), however it can be abolished immediately when muscle is deactivated and force is allowed to drop to zero (Abbott and Aubert, 1952; Herzog and Leonard, 1997; Morgan *et al.*, 2000).

Force depression increases with increasing magnitudes of shortening (Abbott and Aubert, 1952; Maréchal and Plaghki, 1979; Josephson and Stokes, 1999; Morgan *et al.*, 2000; Lee and Herzog, 2003; Bullimore *et al.*, 2007), decreases with increasing speeds of shortening (Sugi and Tsuchiya, 1988; De Ruiter *et al.*, 1998; Josephson and Stokes, 1999;

Ettema and Meijer, 2000; Herzog *et al.*, 2000; Morgan *et al.*, 2000; Lee and Herzog, 2003), and increases with the force during shortening (Herzog and Leonard, 1997; De Ruiter *et al.*, 1998; Lee and Herzog, 2003). Residual force depression has been found to be well described as a function of the mechanical work performed during the shortening phase (Herzog *et al.*, 2000; Josephson and Stokes, 1999; Granzier and Pollack, 1989). Residual force depression is also associated with a distinct decrease in stiffness in single fibres (Sugi and Tsuchiya, 1988) and whole muscles (Lee and Herzog, 2003).

#### ***2.2.4 Proposed mechanisms of force depression***

A number of mechanisms for force depression have been proposed, but the following three are considered the most accepted theories: the sarcomere length non-uniformity theory, the fatigue theory, the inhibition theory.

##### ***2.2.4.1 Sarcomere length non-uniformity theory***

The sarcomere length non-uniformity was first proposed by Julian and Morgan (1979). It originates from the idea of instability of the descending limb of the force-length relationship (Hill, 1953). When an active muscle shortens (A to B in Figure 2-7) on the descending limb of the force-length relationship, it is assumed that the majority of sarcomeres shorten very little (C in Figure 2-7), while a few, strong sarcomeres shorten more than average and fall onto the ascending limb of the force-length relationship (D in Figure 2-7, (Morgan *et al.*, 2000)). These two populations of sarcomeres reach a force equilibrium (D-C in Figure 2-7), and produce tension is smaller than the expected tension

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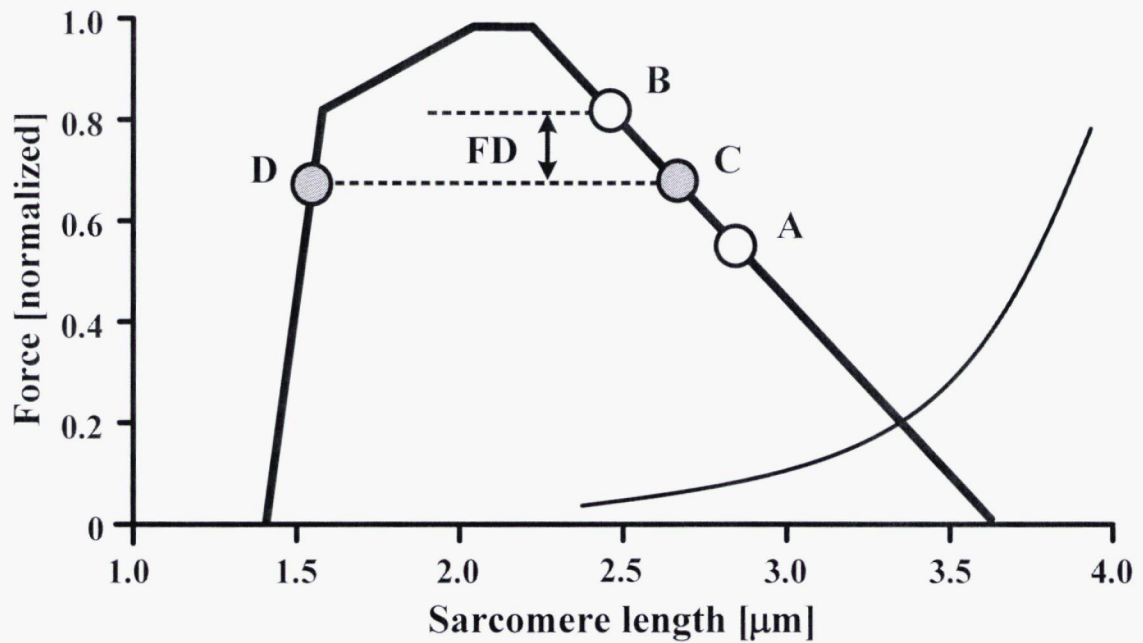
for isometric contractions where sarcomere lengths are assumed to remain similar (B in Figure 2-7, (Morgan *et al.*, 2000)).

The sarcomere length non-uniformity theory allows for the following predictions: force depression should not exist on the ‘stable’ ascending limb of the force-length relationship. However, small, but consistent amounts of force depression have been found on the ascending limb of the force-length relationship in different muscle preparations (Granzier and Pollack, 1989; Herzog and Leonard, 1997; De Ruiter *et al.*, 1998; Herzog *et al.*, 1998).

Secondly, force depression should not occur when the sarcomere lengths remain uniform. However, Granzier and Pollack (1989) observed force depression in single fibres whose sarcomere lengths were kept “constant” using a segment clamp approach. Also Edman *et al.* (1993) performing an identical experiment did not observe force depression once sarcomere length non-uniformities were eliminated.

Thirdly, stiffness of muscles would be expected to increase slightly in the force-depressed state compared to the purely isometric reference state (Morgan *et al.*, 2000). However, studies in single fibre preparation (Sugi and Tsuchiya, 1988) and human adductor pollicis (Lee and Herzog, 2003) showed clear decreases in stiffness in the force depressed compared to the isometric reference state. In summary, the sarcomere length non-uniformity theory seems an unlikely candidate explaining force depression.





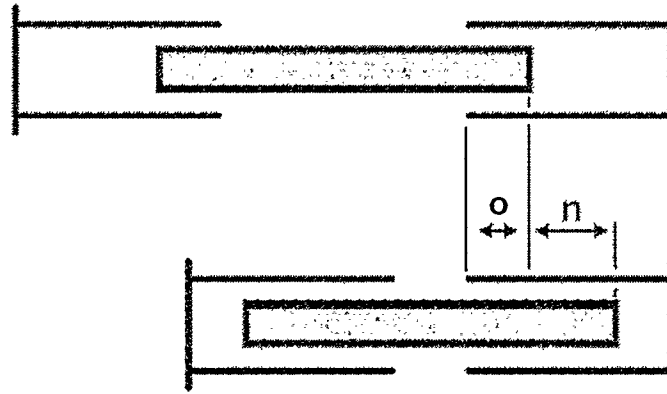
**Figure 2-7: Schematic illustration of the residual force depression (FD) based on the sarcomere length non-uniformity theory.** When an active muscle is shortened from A to B, some sarcomeres are assumed to shorten less than average (C) while others are assumed to shorten more than average (D) [Adapted from Morgan *et al.* (2000)].

#### 2.2.4.2 Fatigue hypothesis

The idea that fatigue products might accumulate during shortening of activated muscles and might cause the residual force depression was suggested by Granzier and Pollack (1989). They found that  $[H^+]$  and  $[P_i]$  concentrations were increased for repetitive shortening and could cause the decrease in force associated with force depression. If so, force depression should be long-lasting, and this was consistently observed experimentally (Abbott and Aubert, 1952; Julian and Morgan, 1979; Granzier and Pollack, 1989; Herzog *et al.*, 1998; Morgan *et al.*, 2000; Lee and Herzog, 2003). However, force depression was also found to be abolished instantaneously upon deactivation (Abbott and Aubert, 1952; Julian and Morgan, 1979; Herzog and Leonard, 1997; Herzog *et al.*, 2000; Morgan *et al.*, 2000) suggesting that force depression could not be associated with the accumulation of fatigue products as they take minutes to be removed.

#### 2.2.4.3 Inhibition theory

Maréchal and Plaghki (1979) proposed that a stress-induced inhibition of cross-bridge attachments caused by shortening may account for force depression. Stress produces elongation of actin filaments (Huxley *et al.*, 1994; Wakabayashi *et al.*, 1994; Higuchi *et al.*, 1995) and an associated distortion of the cross-bridge attachment sites (Daniel *et al.*, 1998). This was thought to inhibit cross-bridge attachment (Figure 2-8).



**Figure 2-8: Schematic illustration of proposed mechanism for residual force depression, the stress-induced inhibition of cross-bridges attachment hypothesis.** Old (o) and shortening-induced newly formed (n) actin-myosin overlap zones are indicated (Herzog, 2004).

Most experimental observations can be explained with this theory. One of the key predictions is that force depression should persist as long as stress on the myofilament is maintained, but should disappear immediately with release of stress. This behaviour has been observed in different experimental configurations (e.g., Abbott and Aubert, 1952; Julian and Morgan, 1979; Herzog and Leonard, 1997; Herzog *et al.*, 2000; Morgan *et al.*, 2000). Another testable prediction is that force depression should increase with increasing shortening magnitudes, increasing force, and increasing mechanical work performed during shortening, observations that have been made in abundance and are well accepted. Possibly, the most critical prediction of this theory is that force depression

should be associated with a decrease in stiffness which has also been observed (Sugi and Tsuchiya, 1988; Lee and Herzog, 2003). Therefore, it seems that the cross-bridge inhibition theory is a viable candidate for force depression.

## **Chapter Three: Residual force enhancement exceeds the isometric force at optimal sarcomere length for optimized stretch conditions**

### **3.1 Introduction**

The steady-state isometric forces following stretching of an active fibre or muscle exceed the steady-state forces obtained for purely isometric contractions at the corresponding length (Abbott and Aubert, 1952; Edman *et al.*, 1978; Julian and Morgan, 1979; Herzog and Leonard, 2002). This property of skeletal muscle has been called “steady-state” or “residual” force enhancement and has first been described systematically by Abbott and Aubert (1952). It is distinctly different from the increase in force during stretch which has been described by Hill (1938) and is explained by the actin-myosin cross-bridge kinetics (Huxley, 1957).

Residual force enhancement has been found to increase with increasing magnitudes of stretch (Abbott and Aubert, 1952; Sugi, 1972; Edman *et al.*, 1978, 1982; Cook and McDonagh, 1995), is thought to be independent of the speed of stretch (Edman *et al.*, 1978, 1982; Sugi and Tsuchiya, 1988), and has been observed consistently on the descending limb of the force-length relationship (Edman *et al.*, 1978, 1982; Rassier *et al.*, 2003). However, there is some controversy as to whether force enhancement exists on the

ascending limb and plateau of the force-length relationship. Force enhancement has been observed on the ascending limb/plateau in some whole muscle preparations (Abbott and Aubert, 1952; Cook and McDonagh, 1995; De Ruiter *et al.*, 2000; Herzog and Leonard, 2002), but not in others (e.g., Morgan *et al.*, 2000). However, the results on whole muscle preparations have been criticized because it is not known if some of the fibres might be operating on the descending part of the force-length relationship, thereby causing the observed force enhancement, while the majority of the fibres operate on the ascending part, and so produce increasing force with increasing muscle length (i.e. ascending limb behaviour). Similarly, force enhancement has been observed on the ascending limb/plateau in some single fibre preparations (e.g., Peterson *et al.*, 2004) but not in others (e.g., Edman *et al.*, 1982). The ascending limb/plateau in Peterson *et al.* (2004) was identified by an increase in isometric force with increasing fibre length, while the actual sarcomere lengths were not measured. Therefore, it could not be determined if the ascending limb/plateau corresponded to sarcomere lengths shorter/equal, respectively than those known to give optimal overlap between actin and myosin filaments (i.e. about 2.0-2.2 $\mu$ m for frog fibres; Gordon *et al.* (1966)).

Whether or not force enhancement exists on the plateau of the force-length relationship is crucial in terms of evaluating possible mechanisms for this phenomenon. The most accepted mechanism for force enhancement is associated with the idea that some weak sarcomeres are pulled beyond myofilament overlap by active stretching on the ‘unstable’ (Hill, 1953) descending limb of the force-length relationship, and that these sarcomeres

are supported exclusively by passive forces, while the remaining sarcomeres are stretched only by a small amount, if at all (Morgan, 1994; Morgan *et al.*, 2000; Morgan and Proske, 2007). Therefore, force in the enhanced state is given by the active force of the short, strong sarcomeres, and is greater than the corresponding purely isometric force for which sarcomere lengths are assumed to be relatively uniform (Morgan, 1990; Morgan *et al.*, 2000). According to this theory, hereafter referred to as the sarcomere length non-uniformity theory, force enhancement cannot exceed the purely isometric forces on the plateau of the force-length relationship (Morgan *et al.*, 2000; Morgan and Proske, 2007). However, there are no systematic studies in which residual force enhancement on the plateau of the force-length relationship has been investigated while optimizing stretch conditions and simultaneously measuring sarcomere length.

The purpose of this study was to test if there is residual force enhancement that exceeds the steady-state isometric force obtained at average optimal sarcomere length; i.e. on the plateau of the force-length relationship. The plateau was identified in single fibre experiments by finding the sarcomere length of maximal active isometric force production and was confirmed by average sarcomere length measurements across a mid-section of the fibre using a laser diffraction approach. Stretch magnitudes and sarcomere lengths were carefully chosen based on pilot work to maximize the probability of finding residual force enhancement exceeding the isometric forces at the plateau of the force-length relationship.

## 3.2 Methods

### 3.2.1 *Muscle fibre preparation*

Twelve single fibres of the lumbrical muscles (2-3mm in length, and approx. 80µm in diameter) from frogs, *rana pipiens*, were used for all experiments. Frogs were killed by decapitation and single fibres were isolated by mechanical dissection. Treatment of the frogs and all experimental procedures were approved by the University of Calgary committee for the ethical use of animals in research.

### 3.2.2 *Force and fibre length measurements*

After isolating a single fibre in a dissecting bath, the tendons at either end of the fibres were gripped with small T-shaped pieces of aluminium clips as close as possible to the fibres to avoid tendon compliance as much as possible. Fibres were then attached to a force transducer (Sensoror) at one end and a servomotor length controller (Aurora Scientific) at the other end. The experimental chamber containing the fibre was placed on an inverted microscope (Eclipse TE300, Nikon). The chamber was filled with physiological Ringer's solution (NaCl 115mM, KCl 3mM, CaCl<sub>2</sub> 3mM, NaH<sub>2</sub>PO<sub>4</sub> 2mM, NaHCO<sub>3</sub> 20mM, pH=7.5), and the temperature of the Ringer's solution was kept constant at 9°C by a controller (VWR scientific products).

Stimulation (Grass S88, Grass Instruments) of fibres was achieved through two platinum wire electrodes that were placed inside the chamber parallel to the muscle fibres. Square



wave pulses (0.4 ms duration) were delivered at an amplitude of 25% above the voltage (50-80V) that elicited maximal force. The frequency of stimulation was chosen individually for each fibre to induce a fused tetanic contraction at physiologically relevant frequencies (30-40Hz). Fibre lengths were measured before testing with a calibrated eyepiece (error <0.02mm).

### ***3.2.3 Sarcomere length measurement***

Average sarcomere lengths were measured with a laser diffraction technique (ter Keurs *et al.*, 1978; Rüdél and Zite-Ferenczy, 1980; Goldman, 1987) using a He-Ne laser beam (633nm wavelength, approx. 0.5mm diameter, Meredith Instruments). The laser beam was projected vertically onto a mid-section of the fibres, and the diffraction pattern was recorded by a fast single-array charge coupled device (CCD) camera (Line scan PL-2048EP, Pulnix) with 2048 pixels. Average sarcomere lengths were determined in real time based on the first order diffraction angle using a custom-designed detector (Stuyvers *et al.*, 2002).

Prior to each testing session, the sarcomere length detector was calibrated using two optical gratings (92 lines/mm and 110 lines/mm, Edmund Optics) and higher order diffraction patterns. The accuracy of the laser diffraction system was < 2% of sarcomere length within the range of 1.82 to 3.03 $\mu$ m.

### *3.2.4 Experimental procedures*

At the beginning of the experiment, stimulation parameters were determined with 1 s tetanic contractions, and fibres were then paced for 40 minutes with twitch contractions every 90 s. After pacing, fibres were inspected visually for damage, and were evaluated for any decrease in force with 1 s tetanic isometric contractions. If there was any visible damage or a decrease in isometric force, the fibre was discarded. The force-sarcomere length relationship was determined with 2 s tetanic isometric contractions (3 minute intervals) in order to identify the plateau and descending limb of the force-sarcomere length relationship. The length which gave the maximal active isometric force was then taken as the optimal fibre length.

One set of experimental tests consisted of five individual contractions: the first two contractions were isometric reference contractions at the final length (the length to which the fibre was stretched to) and the optimal length. The third contraction was the test contraction in which the activated fibre was stretched from some initial to the final length. The last two contractions were a repeat of the two isometric reference contractions at the optimal and final length. If the isometric reference force at the optimal length differed by more than 2% within a set, or decreased by more than 10% from its initial value at any time during testing, the fibre was discarded and the results were not included in the analysis.

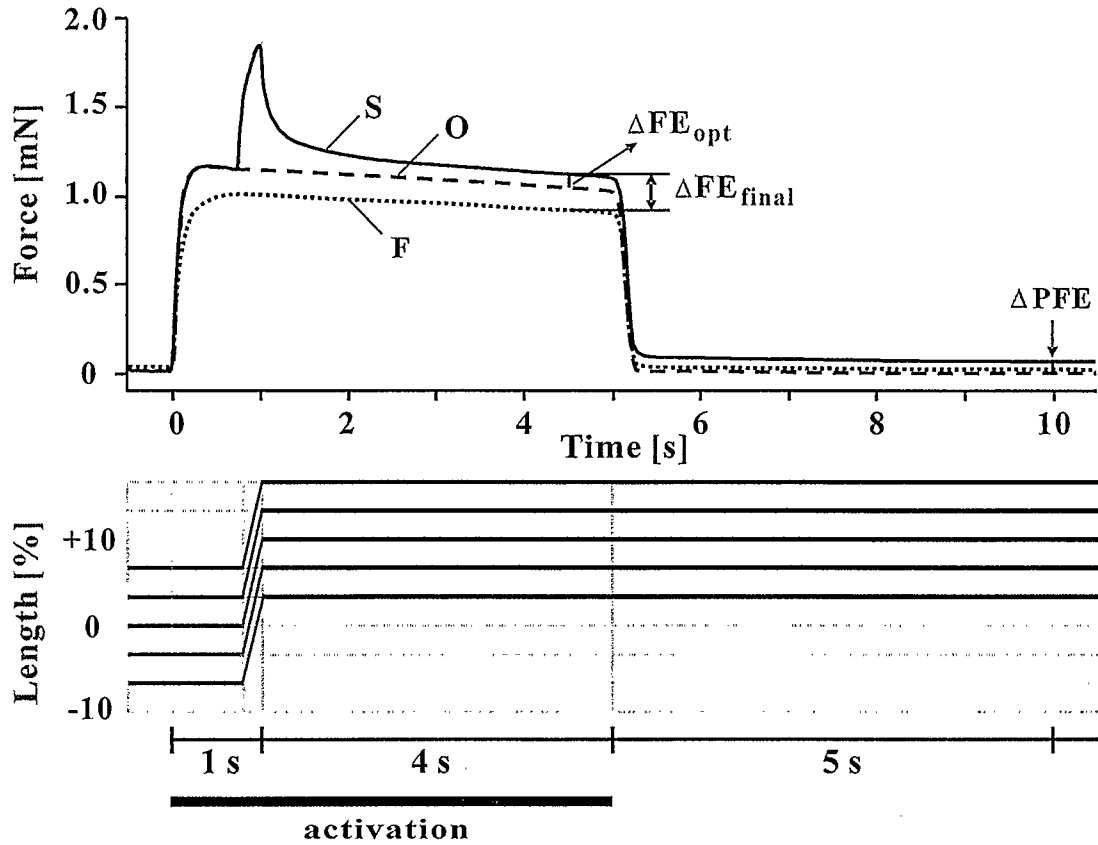
Five sets of experimental tests were attempted at different points along the force-length relationship as shown schematically in Figure 3-1. The first set started at the optimal sarcomere length (hereafter referred to as 0% length). The second and third set started at 3.3% longer and shorter, and the forth and fifth set at 6.7% longer and shorter than optimal length. All contractions lasted for 5 s and were separated by a 6 minute rest interval. Stretch magnitudes were 10% of the nominal optimal fibre length, and were performed at a nominal speed of 50% fibre length/s. These stretch conditions had been identified previously to produce forces in the enhanced state that exceeded the isometric reference forces at optimal fibre length and did not cause damage during repeat measurements as required here. Fibres were activated at the initial length for 800 ms prior to stretch, and were held isometrically at the final length for 4 s (Figure 3-1). Force, fibre length, and sarcomere length were recorded at a frequency of 1000Hz.

### ***3.2.5 Data analysis***

To obtain a mean ( $\pm 1$ S.E.) force-sarcomere length relationship, data from all fibres ( $n=12$ ) were grouped according to sarcomere lengths into bins of  $0.1\mu\text{m}$  ranging from  $1.85$  to  $2.75\mu\text{m}$ . Steady-state forces were approximated by measurements made at  $4.5$  s following the onset of activation. Force enhancement was defined as the increase in the steady-state isometric force following active fibre stretch compared to the steady-state isometric reference force at the corresponding final length obtained immediately prior to the test contraction ( $\Delta F E_{\text{final}}$  in Figure 3-1). Furthermore, the steady-state isometric force following active fibre stretch was also compared to the maximal isometric force at

optimal length obtained immediately prior to the corresponding test contraction ( $\Delta F_{E_{opt}}$  in Figure 3-1). This definition for force enhancement and force enhancement above the plateau was adopted so that any stretch-induced damage to the fibres or any fatigue accumulation during a given set of tests would decrease the observed force enhancement, and thus values obtained here, if anything at all, would underestimate the true force enhancement and also would underestimate the true force enhancement above the isometric plateau forces. In order to make comparisons across fibres, force enhancement was normalized with respect to the corresponding isometric reference force. Similarly, force enhancement above the isometric force at optimal sarcomere length was determined across fibres by normalizing all values relative to the corresponding isometric reference forces at optimal sarcomere length.

Passive forces were measured at 1 and 5 s after deactivation, and the passive force enhancement was defined as the increase in passive force following active stretch compared to the passive force following the corresponding isometric reference contraction ( $\Delta PFE$  in Figure 3-1 (Herzog and Leonard., 2002)). The passive force enhancement was normalized with respect to the maximal isometric force at the corresponding final length for comparison across fibres. The amount of active force enhancement was obtained by subtracting the passive force enhancement from the total force enhancement. In order to determine the mean total force enhancement and passive force enhancement, data from all fibres ( $n=12$ ) were grouped into bins of  $0.1\mu m$  of sarcomere length ranging from  $2.0$  to  $2.4\mu m$ .



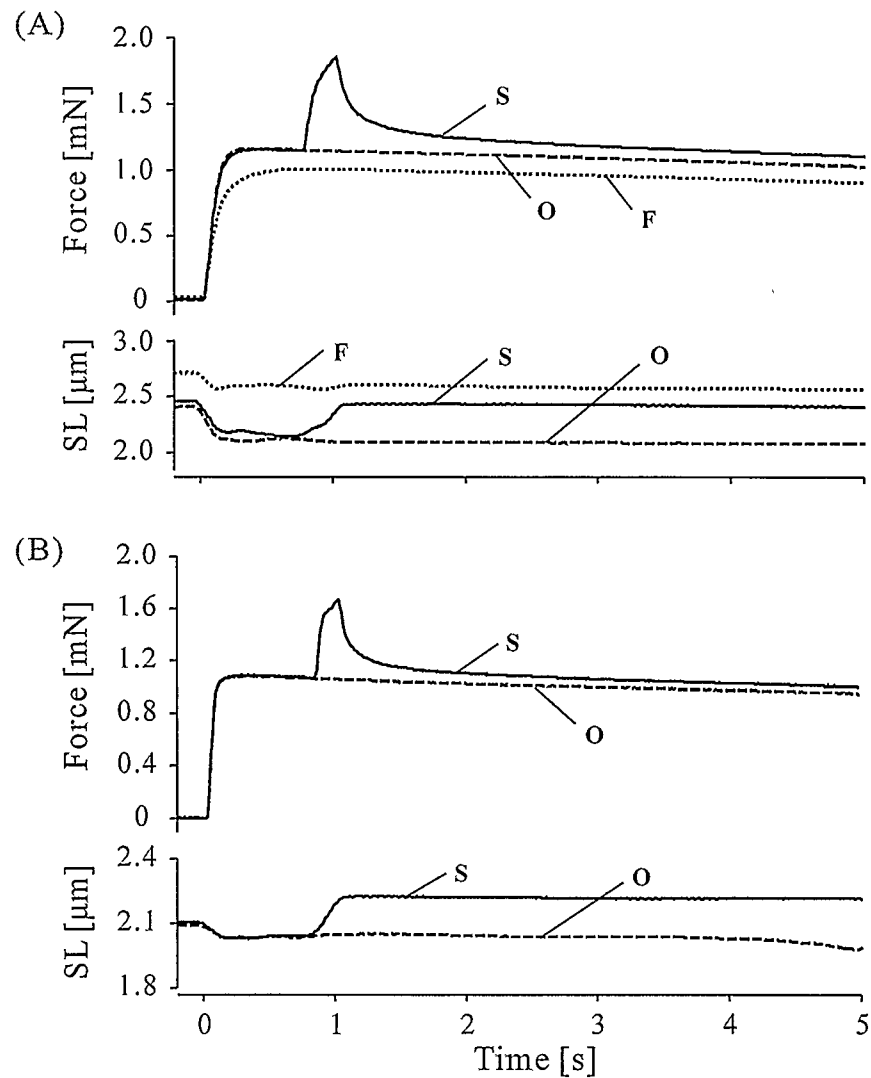
**Figure 3-1: Exemplar of force, length, and activation–time histories for isometric references and active stretch contractions.** Raw traces of force–time histories of one set of experimental trials consisting of an isometric contraction at the final length (F) and the optimal length (O), and an experimental stretch contraction (S) (top). The resulting force enhancements above the final length,  $\Delta FE_{final}$ , and above the plateau,  $\Delta FE_{opt}$ , are indicated. Similarly, the passive force enhancement is also identified at  $t=10s$  ( $\Delta PFE$ ), the measurement taken at 6s is not indicated for clarity. Also shown are a length–time (bottom) and an activation–time history (black bar) for the entire experimental protocol.

### 3.3 Results

Maximal active isometric forces were found to occur consistently at average sarcomere lengths ranging from 2.0 $\mu\text{m}$  to 2.2 $\mu\text{m}$ , thereby confirming that maximal forces were obtained near sarcomere lengths associated with optimal actin-myosin filament overlap in frog skeletal muscle fibres (Gordon *et al.*, 1966). The isometric forces were perfectly stable (that is, there was no creep as is sometimes observed in segment or sarcomere clamped preparations (Gordon *et al.*, 1966), and they were associated with a constant average sarcomere length throughout the isometric steady-state phases of the contractions (Figure 3-2).

The steady-state isometric force following active fibre stretch was greater than the purely isometric reference force at the corresponding final length for all fibres and each test contraction (Figure 3-2A and Figure 3-3A). The mean ( $\pm$ S.D.) force enhancement across all fibres and all sarcomere lengths was 10.4 ( $\pm$ 3.9) % reaching a peak value of 16.1% at the longest sarcomere length (Figure 3-3A). In contrast to the force obtained for the purely isometric reference contractions, there was no distinct decrease of the peak isometric forces following active fibre stretching beyond sarcomere lengths of 2.2 $\mu\text{m}$  (Figure 3-3).

Although force enhancement has been observed previously in single fibre preparations (Edman *et al.*, 1978, 1982; Sugi and Tsuchiya, 1988; Edman and Tsuchiya, 1996; Rassier *et al.*, 2003), novel to the literature is the result that forces in the enhanced state clearly



**Figure 3-2: Exemplar force-time and sarcomere length-time histories for isometric reference contractions and active stretch contraction.** Raw force (upper traces) and sarcomere length, SL, (lower traces) -time histories of active stretch (solid, S), isometric reference contractions at the final length (dotted, F) and at the optimal length (dashed, O) from two representative fibres. In panels (A) and (B), experiments from two representative fibres are shown.

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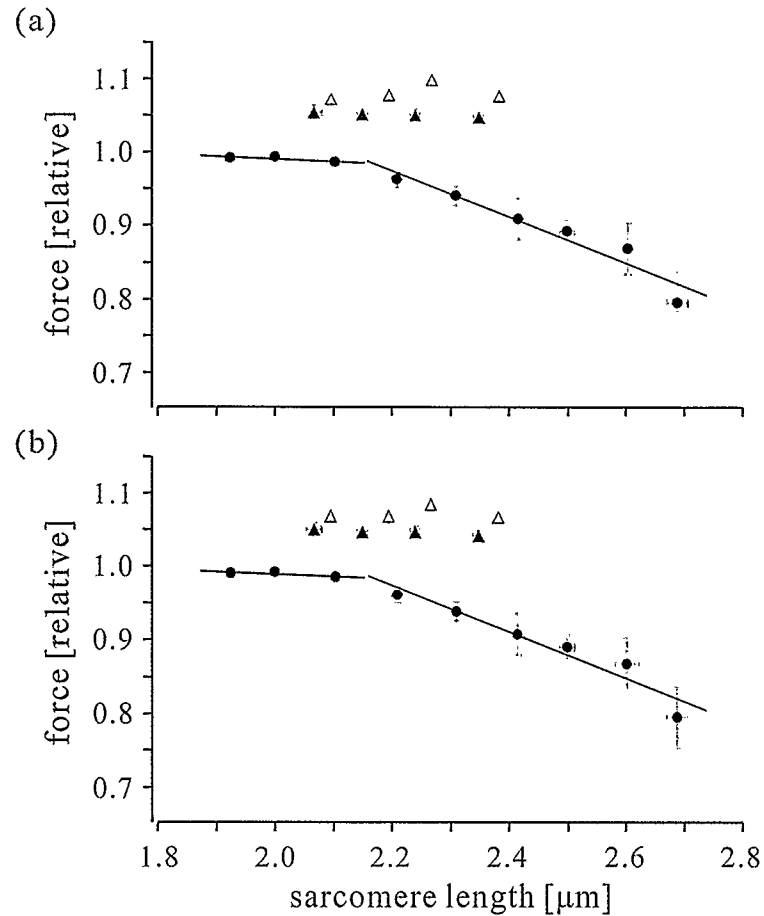
exceeded the isometric reference forces obtained at optimal sarcomere lengths ( $p < .001$ ), i.e. the plateau of the force-length relationship (Figure 3-2 and Figure 3-3). This result was obtained for all fibres and each stretch condition and for comparison with the isometric reference forces preceding the test contractions, therefore, any fatigue or damage to the fibre would have decreased the amount of force enhancement that was measured here. The mean ( $\pm$ S.D.) force enhancement above the isometric plateau forces was 4.8 ( $\pm$ 2.1)% with peak values reaching 10%.

There was a consistent, albeit small passive force enhancement averaging 0.7% (at 1s after deactivation) or 0.4% (at 5s after deactivation) of the active isometric reference forces. When subtracting this passive contribution from the total force enhancement, the remaining “active” component of the force enhancement was virtually unaffected, and more importantly, forces in the “active” enhanced state still clearly exceeded the peak isometric reference force obtained on the plateau of the force-length relationship (Figure 3-3B).

### 3.4 Discussion

Residual force enhancement has been observed for more than half a century (e.g., Abbott and Aubert, 1952) in a variety of muscles and structural preparations ranging from single fibres (e.g., Edman *et al.*, 1978, 1982; Julian and Morgan, 1979; Sugi and Tsuchiya, 1988; Edman and Tsuchiya, 1996) to *in vivo* human muscles (e.g., Cook and McDonagh,





**Figure 3-3: Force-sarcomere length relationship with total and active force enhancement.** Mean ( $\pm 1$ S.E.) force-sarcomere length relationship ( $\bullet$ ) and mean total force enhancement (A) and active force enhancement (B) (mean force enhancement,  $\blacktriangle$ ; peak force enhancement,  $\triangle$ ). Solid lines show linear regression approximations representing the plateau region and the descending limb of the force-sarcomere length relationship. All data were grouped into bins of  $0.1\mu\text{m}$  of sarcomere length ranging from  $1.85$  to  $2.75\mu\text{m}$  for the force-sarcomere length relationship and from  $2.0$  to  $2.4\mu\text{m}$  for the force enhancement results.

1995; De Ruiter *et al.*, 2000; Lee and Herzog, 2002). However, one crucial question that has eluded satisfactory explanation is how much force can there be in the enhanced state? More specifically, can force in the enhanced state (i.e. after active stretch) exceed purely isometric reference forces at sarcomere lengths associated with optimal actin-myosin filament overlap? According to the most accepted mechanism for force enhancement, the sarcomere length non-uniformity theory, forces in the enhanced state cannot exceed the purely isometric forces on the plateau of the force-length relationship (Morgan, 1990, 1994; Morgan *et al.*, 2000).

Edman *et al.* (1978, 1982) examined this question carefully and concluded that forces in the enhanced state did not clearly exceed the isometric reference forces at optimal sarcomere lengths in single fibres of frog skeletal muscle. Based on their results, they discussed that residual force enhancement was not a property of the cross-bridges, as had been suggested by Sugi and Tsuchiya (1988), and was not associated with the recruitment of additional contractile material. However, others have observed force enhancement above the isometric plateau forces in whole muscles (e.g., Abbott and Aubert, 1952) and single fibres (e.g., Rassier *et al.*, 2003; Peterson *et al.*, 2004) supporting our result presented here, but they were somewhat inconclusive as sarcomere lengths were not measured in these studies.

Here, we found the novel result that forces in the enhanced state consistently and systematically exceeded the purely isometric reference forces obtained at optimal

sarcomere lengths in single fibres of frog skeletal muscle. This observation was made in all fibres and for each test contraction, and the forces in the enhanced state exceeded the isometric reference forces at the plateau by  $4.8(\pm 2.1)\%$ , on average, with peak values reaching up to 10%. Although it has been shown that force enhancement is long lasting ( $\geq 30$  s; Abbott & Aubert (1952)), we, in agreement with the published literature, chose a specific instant in time for evaluation of the force enhancement (4.5 s after the onset of activation). At that instant in time, the force-time histories of the test and reference contractions were still converging, and thus, had we evaluated force enhancement at a later instant in time, it would have been smaller. In order to estimate the force enhancement at 6 s, as did Edman *et al.* (1982), we calculated what the force enhancement would have been at that time, assuming that the convergence of the two curves remained constant. At 6 s, the average force enhancement above the plateau would have been 4.6% (i.e., a mere decrease of 0.2% from the original value) and would have exceeded the isometric reference forces at the plateau for each test, thereby lending support to the idea that our results were not an artifact of early evaluation, but are different from the results obtained by Edman *et al.* (1982) at 6 s.

Average sarcomere lengths, as measured by laser diffraction, stayed constant in the steady-state phase following active fibre stretching, implying that average sarcomere lengths within the target zone remained approximately constant. These results are in agreement with those reported by Edman *et al.* (1982), who found that active stretching had a stabilizing effect on sarcomere lengths, and that force enhancement persisted even

in fibres that were stretched under conditions of segment length control. Similarly, Telley *et al.* (2006) demonstrated in single myofibrils of rabbit psoas that stretching provided a stabilizing effect on sarcomere lengths on the descending limb of the force-length relationship, and Herzog *et al.* (2006) also demonstrated that sarcomeres remained perfectly stable and at constant lengths immediately following active stretching of isolated myofibrils on the descending limb of the force-length relationship.

We used a laser diffraction approach to measure average sarcomere lengths, similar to many previously published papers on single fibre mechanics (Pollack *et al.*, 1977; ter Keurs *et al.*, 1978; Edman *et al.*, 1982; Sugi and Tsuchiya, 1988). This approach does not allow for the identification of individual sarcomere lengths, nor can it provide more than just a rough estimate of sarcomere length distribution within the target region, and sarcomere behaviour outside the target region remains unknown. Although these shortcomings of the laser diffraction approach have been pointed out in the literature (Kawai and Kuntz, 1973; Rüdel and Zite-Ferenczy, 1980; Lieber *et al.*, 1984; Goldman, 1987), and they present limitations for studies in which individual sarcomere lengths, sarcomere length distributions, sarcomere length control, or information of all sarcomeres of a fibre is required. However, these limitations are not relevant for the purposes of the current study because all we needed to know is that our measurements of force enhancement above the isometric plateau forces were made with some sarcomeres at optimal length, and it can be assumed fairly safely that if the center of the first order diffraction pattern is within 2.0-2.2 $\mu\text{m}$  (as it was in our study), some of the sarcomeres

are at those lengths too. Whether or not some sarcomeres were outside those lengths, or if some sarcomeres outside the target region behaved vastly different from those in the target region, does not matter, as the sarcomere length non-uniformity theory does not allow for force enhancement to be greater than those observed for purely isometric contractions at optimal sarcomere lengths (i.e. the plateau of the force-length relationship) for any sarcomere length distribution (Morgan, 1990, 1994; Morgan *et al.*, 2000).

In this study, we also observed a small but consistent passive force enhancement, as had been observed previously in whole muscle preparations (Herzog and Leonard, 2002). Therefore, the forces in the enhanced state exceeding the isometric plateau forces could have been caused by this passive force enhancement. However, when subtracting the passive component of the force enhancement, the residual forces remained greater than the isometric reference forces at optimal sarcomere lengths. Even when evaluating passive force enhancement at 1s after deactivation to maximize the passive contribution to the total force enhancement, the average active force enhancement was still  $4.1(\pm 1.7)\%$  above the plateau of the force-length relationship. The idea that the residual force enhancement might be caused by an increase in stiffness of a passive element upon activation was strengthened by the work of Labeit *et al.* (2003) who showed a statistically significant increase in passive forces when single fibres were stretched at a high ( $pCa=4.0$ ) compared to a low ( $pCa=9$ ) calcium concentration. This calcium-induced increase in passive force reached a peak value of about  $10\text{mN/mm}^2$  at a sarcomere length

of about  $3.0\mu\text{m}$ , but was only about  $1\text{mN/mm}^2$  at sarcomere lengths of  $2.0\text{--}2.4\mu\text{m}$ . This value is in the same range as the passive force enhancement measured in this study, and it is much too small to account for the entire force enhancement above the isometric plateau.

We found clear evidence that forces in the enhanced state exceeded the isometric reference forces at optimal sarcomere lengths (i.e. on the plateau of the force-length relationship). This result persisted when the passive force enhancement was accounted for, thus suggesting that force enhancement might be associated with the recruitment of additional contractile force. Since this additional force does not appear to be associated with a corresponding increase in fibre/muscle stiffness (Sugi and Tsuchiya, 1988), we propose that it might be caused by an increase in the average force per cross-bridge. This could potentially be explained by a change in the proportion of attached cross-bridges in different attached states. For example, we have observed that residual force enhancement is substantially increased, and may reach values of over 150% in fibre preparations whose cross-bridge kinetics are biased towards the weakly bound states by addition of 10mM 2,3-butanedione monoxime (BDM). However, and more importantly in the context of this study, not only did BDM cause an increase in the relative force enhancement, it also caused an increase in the absolute force enhancement of  $> 40\%$  in the 5 and 10mM BDM conditions compared to control (0mM) and low level (2mM) BDM conditions (Rassier and Herzog, 2004b). Therefore, it appears that force enhancement may be associated (at least in part) with a stretch-induced facilitation of transition of cross-bridges from the weakly to strongly bound states. Such a mechanism could cause force enhancement

without a corresponding increase in stiffness, but further research is needed to investigate this proposed mechanism more carefully.

The isometric force-sarcomere length relationship has a distinct change in slope at a sarcomere length of about  $2.2\mu\text{m}$  (Figure 3-3), indicating the change from the plateau to the descending limb region, as observed by Gordon *et al.* (1966). However, following active stretching, there was no change in slope to indicate that the descending limb of the force-length relationship had been reached (Figure 3-3). Rather, the isometric steady-state forces after stretching remained approximately constant between  $2.1\text{-}2.4\mu\text{m}$ , indicating that not only does active stretching provide for additional steady-state isometric force (i.e. force enhancement), but it also provides for a more extended plateau region than that obtained for purely isometric contractions, thereby offsetting the anticipated loss of force with a decrease in actin-myosin filament overlap. This result has important functional implications, as the “region of maximal force production (i.e. the plateau of the force-length relationship)” appears to cover a vastly greater sarcomere length range following active stretch compared to purely isometric contractions, thereby optimizing fibre and muscle function, force and work potential during everyday movements.

## Chapter Four: Modulation of passive force in single skeletal muscle fibres

### 4.1 Introduction

The passive force–length relationship in skeletal muscle has been investigated for decades, and it shows increasing levels of force at increasing muscle lengths. It has been proposed that most of the passive force in skeletal muscle is produced by the giant protein titin, that spans the half-sarcomere, connecting the A-band to the I-bands and Z-lines (Kellermayer *et al.*, 1997; Rief *et al.*, 1997). Titin consists of proline-glutamate-valine-lysine (PEVK)-rich domains and immunoglobulin-like (Ig) domains, that unfold as titin is stretched (Linke *et al.*, 1998; Trombitas *et al.*, 1998). Recent evidence shows that the PEVK segments of titin bind  $\text{Ca}^{2+}$  with high affinity (Tatsumi *et al.*, 2001), and that increasing  $\text{Ca}^{2+}$  concentration enhances force produced by titin at a given sarcomere length (Labeit *et al.*, 2003), suggesting a link between  $\text{Ca}^{2+}$ -induced activation and force produced by titin.

In studies conducted in our laboratory with single-muscle fibres (Rassier *et al.*, 2003; Rassier and Herzog, 2004a, 2004b), whole muscles (Herzog and Leonard, 2002; Herzog *et al.*, 2003) and human muscles (Lee and Herzog 2002), we observed that passive force is increased when activated muscles are stretched along the descending limb of the force–



length relationship, when compared with the passive forces following passive stretches or isometric contractions at the corresponding lengths. We refer to this phenomenon as passive force enhancement (Herzog and Leonard 2002; Herzog *et al.*, 2003; Rassier *et al.*, 2003; Rassier and Herzog, 2004a, 2004b). We have suggested that passive force enhancement might be caused by an increase in titin stiffness upon activation and stretch (Herzog and Leonard 2002; Herzog *et al.*, 2003; Rassier and Herzog 2004a). This mechanism would be consistent with the results of Labeit *et al.* (2003). However, it is still unclear if this phenomenon depends on muscle lengths and, consequently, if the entire passive force–sarcomere length relationship is affected by active muscle stretch. Further, previous experiments looking at passive force enhancement were performed without measurement of sarcomere length, and the optical measurement of fibre/muscle lengths before and after stretch may produce errors in length measurements.

In this paper, we evaluated the passive force– sarcomere length relationship following isometric contractions, passive stretches and active stretches. We observed that the passive force–sarcomere length relationship is shifted upwards on the force axis, when fibres are stretched while simultaneously activated, but activation or passive stretch alone do not produce any shift in the passive force–sarcomere length relationship.

## 4.2 Methods

### 4.2.1 *Muscle fibre preparation*

Experiments were performed with single-muscle fibre (n=6) dissected from lumbrical muscles of the frog, *rana pipiens* (2mm length). The tendons of the dissected fibre were gripped with small pieces of T-shaped aluminium foil close to the tendons. The fibre were mounted in an experimental chamber between a servomotor length controller (Aurora Scientific) and a force transducer (Sensomotor), bathed with a Ringer's solution (NaCl 115mM, KCl 3mM, CaCl<sub>2</sub> 3mM, NaH<sub>2</sub>PO<sub>4</sub> 2mM, NaHCO<sub>3</sub> 20mM, pH=7.5, temperature 9 °C). Stimulation (Grass S88, Grass Instruments) was given through two platinum wire electrodes placed in the chamber parallel to the muscle fibres, with square-wave pulses (0.4 ms duration) delivered at an amplitude of 25% above the voltage that gave maximal force production (range: 20–45V). The stimulation pattern was set individually for each fibre during 1 s tetanic contractions with the smallest frequency needed to produce a fused tetanic contraction (range in these experiments: 28–38 Hz) to avoid fatigue effects.

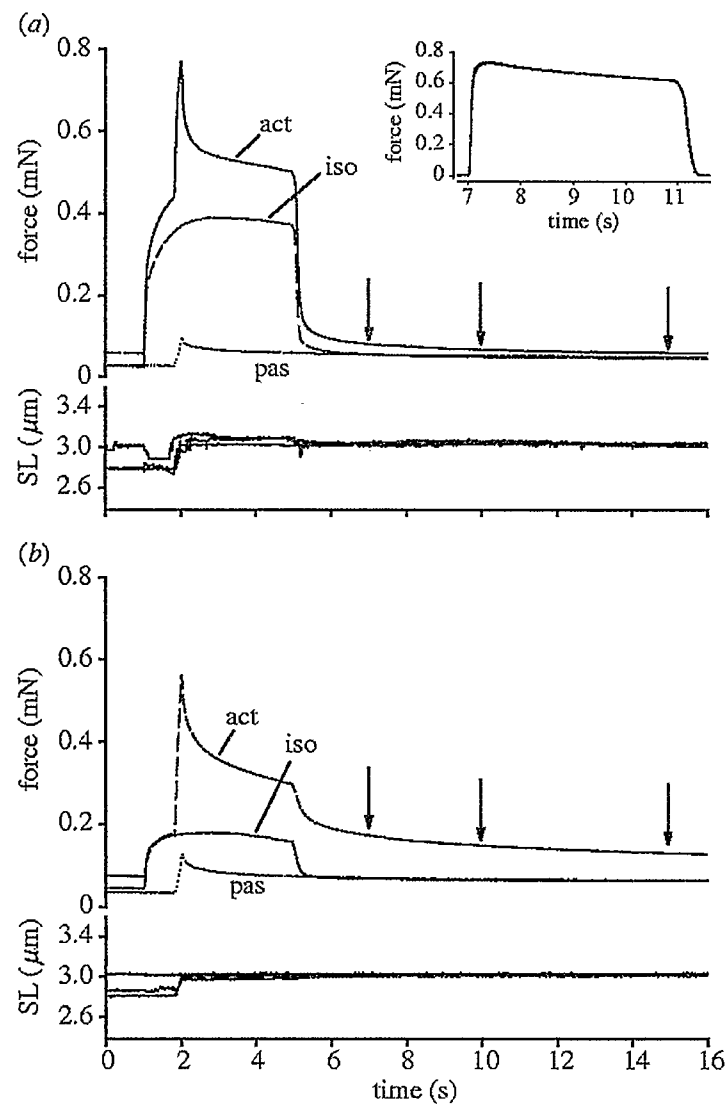
### 4.2.2 *Experimental procedures*

An active (total– passive) force-sarcomere length relationship was obtained from isometric tetanic contractions (2 s duration, 5 min intervals). Fibres were then placed at initial lengths varying from 5% to 40% beyond the plateau of the force-sarcomere length relation, and were activated to produce maximal isometric force. At 1000 ms after the

onset of activation, fibres were stretched ~10% of fibre length (~0.22  $\mu\text{m}$  per sarcomere) at a speed of 40% fibre length/s, and held isometrically at the final length (total contraction time = 4 s). Passive stretches with equivalent stretch amplitude and speeds were performed for comparison. Before and after the stretch contractions, isometric contractions were also performed at corresponding lengths. Forces were measured at 2, 5 and 10 s after deactivation of the fibres (i.e., passive force) for the active and the passive stretches.

### 4.3 Results

Figure 4-1(a) shows force-time and sarcomere length-time histories of active stretches, passive stretches, and isometric contractions at corresponding sarcomere lengths (after deactivation of the fibres). The total force after stretch of activated fibres was higher than the force produced during isometric contractions at the corresponding sarcomere length. Such active force enhancement has been observed in several studies by our group (Herzog and Leonard, 2002; Herzog *et al.*, 2003; Rassier *et al.*, 2003) and others (Julian and Morgan, 1979; Edman *et al.*, 1982; Sugi and Tsuchiya, 1988). Passive force after deactivation of the actively stretched fibres was higher than the passive force after a purely isometric contraction, and higher than the force produced when the fibre was stretched passively. Figure 4-1(b) shows contractions performed with the same fibre as in Figure 4-1(a), but after addition of 5 mM BDM to the Ringer solution. BDM inhibited active force production by biasing cross-bridges towards a weakly-bound state, but did



**Figure 4-1: Force and sarcomere length - time histories of active stretches, passive stretches, and isometric contractions at corresponding sarcomere lengths in normal ringer solutions (a) and after addition of BDM (b).** The inset shows isometric contractions performed at 2.0  $\mu\text{m}$  at the beginning and at the end of one typical experiment. Note that the isometric force did not decrease throughout the experiment, suggesting that the quality of the fibres was preserved after stretches and BDM treatment. act: active stretch; pas: passive stretch. iso: isometric contraction. Arrows show the times when passive forces were measured.

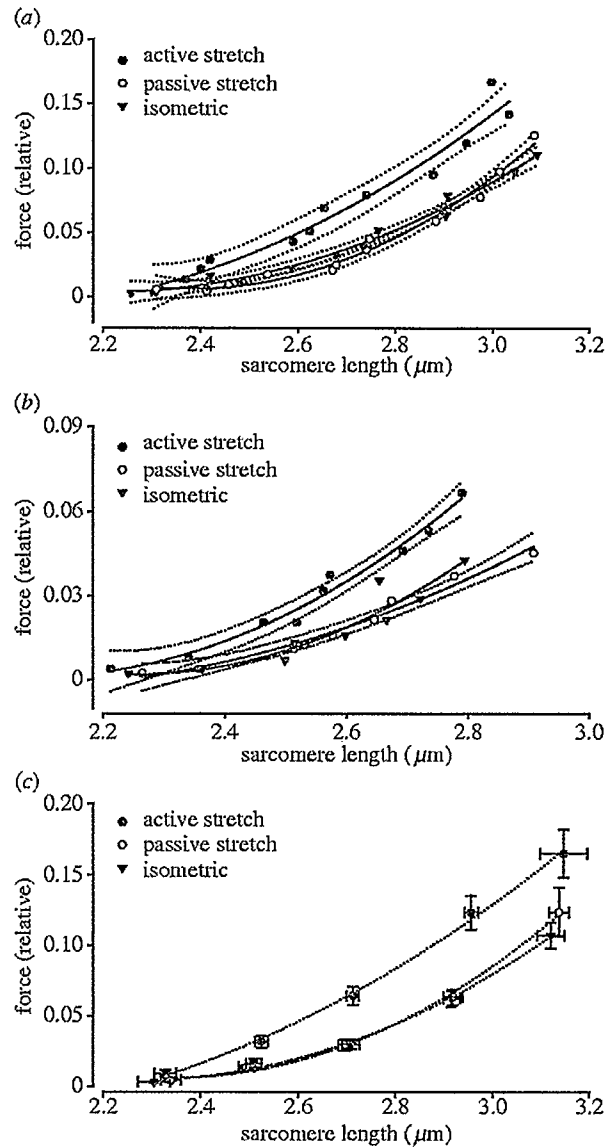
not inhibit the level of passive force enhancement. Isometric reference contractions performed throughout the experiments showed consistent force profiles (Figure 4-1(a), inset) indicating that fibres were not damaged.

Figure 4-2 shows the passive force-sarcomere length relationship for two representative fibres (Figure 4-2 (a) and (b)) and for all fibres following isometric contractions, active stretch and passive stretch. Passive force was increased for the actively stretched fibres. Passive force increased with increasing sarcomere lengths.

#### **4.4 Discussion**

We determined the passive force-sarcomere length relationship of single muscle fibres for three distinct situations: (i) following isometric contractions, (ii) following passive stretch, and (iii) following active stretch. Passive forces were increased following active stretch, suggesting that stretch and activation combined cause this increase in force, while activation alone (isometric contractions), or stretch alone (passive stretches) did not produce this effect. Passive force enhancement was length-dependent; it increased with increasing sarcomere lengths (Figure 4-2).

Conceptually, passive forces, and the passive force enhancement, could be caused by passive structures or by weakly-bound cross-bridges (Proske and Morgan, 1999; Campbell and Moss, 2002). Since passive force enhancement is increased with increasing



**Figure 4-2: Passive force-sarcomere length relationships after isometric contractions, active stretches and passive stretches.** All data were approximated with best-fitting second order polynomial equations. In panels (a) and (b), experiments from two representative fibres are shown, along with the best fit lines and 95% confidence intervals. Note that the data points for the actively stretched fibres do not overlap with those recorded after isometric contractions or passive stretches. In Panel C, mean( $\pm 1$  S.E.) values are shown from data that were grouped into intervals of 0.2  $\mu\text{m}$  (range: 2.2-3.2  $\mu\text{m}$ ). With the exception of a sarcomere length of 2.2  $\mu\text{m}$ , there is a significant increase ( $p < .05$ ) in the passive force following active stretch compared to the passive force following isometric contraction and passive stretch.

lengths, but filament overlap and the number of available cross-bridges for force production is decreased, it appears that cross-bridge mechanisms are not causing this phenomenon. Furthermore, we observed that muscle fibres treated with BDM, a drug that inhibits cross-bridge force production without altering  $\text{Ca}^{2+}$  transients significantly, did not decrease the passive force enhancement (Figure 4-1(b)), providing further evidence that passive force enhancement is not associated with cross-bridge mechanisms (Rassier & Herzog 2004b). Therefore, passive force enhancement is likely caused by a passive structural element in skeletal muscle.

The conclusion that a passive element may be engaged upon activation, and so may cause the passive force enhancement observed here, is strengthened by findings of an instantaneous increase in stiffness in single fibres upon activation that is independent of cross-bridge attachment (Bagni *et al.*, 1994; Bagni *et al.*, 2002). Bagni and colleagues called this observation “static tension”, and observed that it increased with increasing sarcomere length and amplitude of stretch, results that are consistent with those observed in this study. Also, Campbell & Moss (2002) showed that initial tension and stiffness, which are greater in activated compared to passive skinned muscle fibres, cannot be explained by cross-bridge structures.

Previously, we suggested that the structure responsible for the increase in passive force following stretch of activated fibres is titin (Herzog and Leonard, 2002; Herzog *et al.*, 2003; Rassier and Herzog, 2004a). Recent experiments by Labeit *et al.* (2003) indicate

that such an interpretation may be correct. They observed that skinned fibres, in which actomyosin interaction was prevented, showed an upwards shift in the force-sarcomere length relationship when  $\text{Ca}^{2+}$  concentration was increased. By performing additional experiments with titin molecules, the authors were able to associate the increase in the passive force with a specific, calcium-dependent conformational change in the PEVK segment of titin. Therefore, the following picture seems to emerge. Upon muscle activation, a rise in  $\text{Ca}^{2+}$  triggers actomyosin interactions, and increases titin stiffness. When an activated muscle is stretched, titin stiffness is increased, and passive force increases proportionally with the amount of stretch. The length dependence of the phenomenon may possibly be explained by assuming that the increase in fibre stiffness occurs at specific sites, as suggested by Labeit *et al.* (2003), and that functionally these stiffened sites only become important contributors once titin has reached a critical length.



## Chapter Five: New insights into the passive force enhancement in skeletal muscles

### 5.1 Introduction

When a muscle is actively stretched and then held at a constant length, its steady-state isometric force is greater than the corresponding force obtained without active stretching. This phenomenon has been first described in a systematic manner by Abbott and Aubert (1952) more than half a century ago. Recently, we demonstrated that this so-called “residual force enhancement” was composed of an active and a passive component (Herzog and Leonard, 2002). The passive component was only observed at relatively long muscle lengths, and like the residual force enhancement, it increased with increasing stretch magnitudes and stretch forces (Herzog and Leonard, 2002; Lee and Herzog, 2002; Schachar *et al.*, 2004), was decreased when active stretching was preceded by active shortening (Herzog *et al.*, 2003; Rassier and Herzog, 2004a), and increased with increasing muscle length, at least within the range observed in presently available studies (Schachar *et al.*, 2004; Herzog and Leonard, 2005; Rassier *et al.*, 2005).

Like for the residual force enhancement, there is no ready explanation as to the origin of the passive force enhancement, although the giant molecular spring titin has been proposed as a prime candidate (Noble, 1992; Edman and Tsuchiya, 1996; De Ruiter *et*

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*al.*, 2000; Herzog and Leonard, 2002). Specifically, it has been suggested that titin might be a calcium-dependent spring whose stiffness increases upon muscle activation when calcium is released from the sarcoplasmic reticulum into the sarcoplasm (Labeit *et al.*, 2003). However, there are many other explanations on how the passive force enhancement might be produced. For example, there is the possibility that cross-bridges might become “stuck” upon stretch and that these stuck cross-bridges might be released only slowly (over seconds or minutes) and so produce the increased passive forces that are observed following active muscle stretching at long lengths. These stuck cross-bridges would behave similar to cross-bridges in smooth muscle contractions that are in the so-called “latch-state” (Dillon *et al.*, 1981).

The purpose of this study was to determine if the passive force enhancement might be associated with a passive structural molecule or the contractile proteins, specifically actin and myosin interactions. The basic idea of this study was to stretch activated fibres in a normal Ringer’s solution and in solutions containing varying amounts of 2,3-butanedione monoxime (BDM). In the Ringer’s solution contractility of isolated fibres is normal, while in the BDM solutions active force is depressed in a dose-dependent manner as BDM is known to inhibit phosphate release (Herrmann *et al.*, 1992), and thereby bias cross-bridges toward the weakly-bound (pre-power stroke) state. We hypothesized that if the passive force enhancement was caused by a passive structural molecule alone, then it should be independent of the contractility and force production of the fibre. However, should the passive force enhancement be caused by the contractile proteins actin and

myosin and the kinetics of cross-bridge attachment/detachment, then we would expect the passive force enhancement to decrease with decreasing active forces, and therefore increasing concentrations of BDM.

## **5.2 Methods**

### ***5.2.1 Muscle fibre preparation***

Six single fibres from the lumbrical muscles (approx. 3mm in length, 100~150 $\mu$ m in diameter) of frogs, *rana pipiens*, were used for all experiments.

### ***5.2.2 Force and fibre length measurements***

Stimulation (Grass S88, Grass Instruments) was given through two platinum wire electrodes that were placed inside the chamber parallel to the muscle fibres. For maximal force production, square wave pulses (0.4 ms duration) at amplitude of 25% above the voltage that gave maximal force (about 50-70V) were given. The frequency of stimulation was chosen individually for each fibre to induce a fused contraction at the lowest possible frequency (20-30Hz). Fibre lengths were measured before testing with a calibrated eyepiece (error < 0.03mm).

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### 5.2.3 Sarcomere length measurement

Sarcomere lengths were determined with a laser diffraction technique (ter Keurs *et al.*, 1978) using a He-Ne laser beam (633nm wavelength, approximately 0.5mm diameter, Meredith Instruments). The diffraction pattern was recorded by a fast single-array charge coupled device (CCD) camera (Line scan PL-2048EP, Pulnix) with 2048 pixels. Sarcomere lengths were determined in real time using a custom-designed sarcomere length detector that calculated sarcomere length based on the first-order diffraction angle. Prior to each testing session, the sarcomere length detector was calibrated using two commercial gratings (92 lines/mm and 110 lines/mm, Edmund Optics). The accuracy of sarcomere length measurements was within 3% for sarcomere lengths ranging from 1.67 $\mu$ m and 3.33 $\mu$ m.

### 5.2.4 Experimental procedures

At the beginning of the experiment, the optimum voltage and frequency of stimulation were determined with 1 s tetanic contractions, and fibres were paced for 40min with twitch contractions every 90 s. After the 40min pacing, in order to identify the plateau region and the descending limb of the force-sarcomere length relationship, we determined the total, active and passive force-sarcomere length relationships of each fibre using 2 s tetanic isometric contractions (3min intervals) at different sarcomere lengths (range: 1.9-3.0 $\mu$ m,  $\sim$ 0.1-0.15 $\mu$ m steps). The sarcomere length showing the greatest active force was defined as optimal sarcomere length.

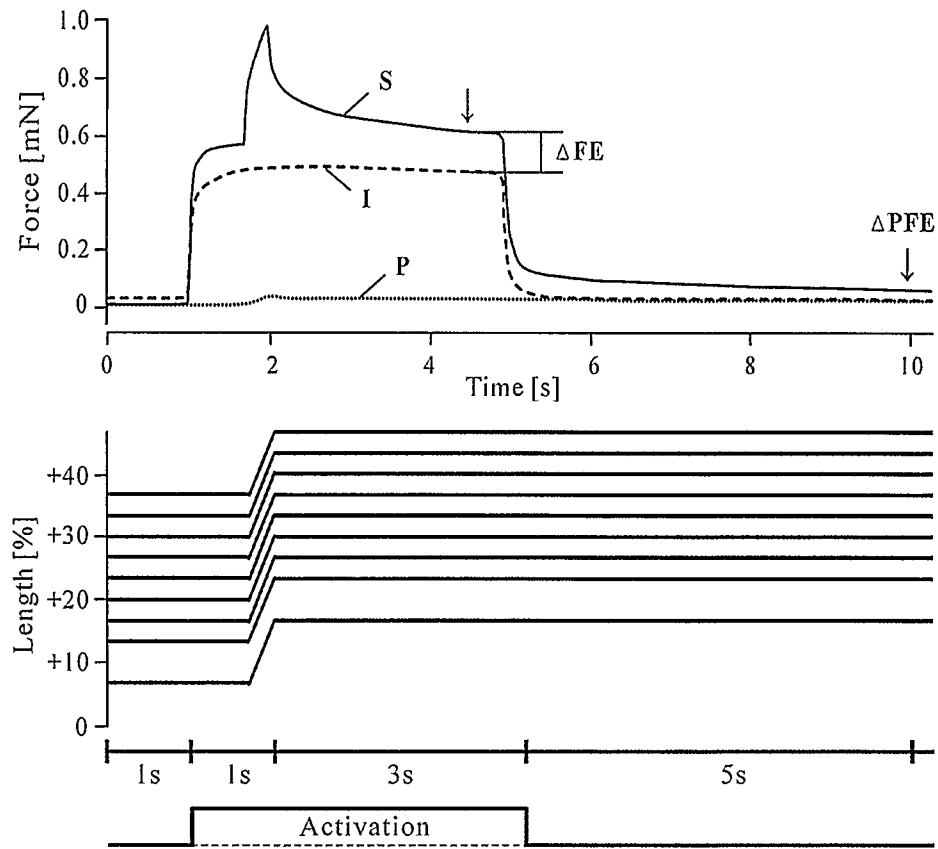
Just prior to starting the series of experimental tests, an isometric contraction was performed at optimal sarcomere length to check if force had decreased from its original value, and if it had, the fibre was discarded. Each experimental set consisted of three individual tests: the first test was a purely isometric reference contraction at the final length (i.e., the length to which the fibre was being stretched in the second test); the second test was a stretch contraction in which the fibre was stretched from an initial length to the final length; and finally the third test was the same as the second, except the fibre was stretched passively (without activation). All stretch contractions consisted of a fibre stretch of a magnitude corresponding to 10% of the optimal sarcomere length, and a speed of 50% of optimal sarcomere length. Contractions were separated by a 5 minute rest interval to avoid fatigue. The isometric force at optimal sarcomere length was checked after every two experimental sets, if it decreased by more than 10% of its initial value, testing was stopped, the fibre was discarded, and the corresponding results were not included in the analysis.

Nine sets of three experimental tests were performed with each fibre as shown schematically in Figure 5-1. The first and second set started at an initial length of 6.7 and 13.3% greater than the optimal sarcomere length (0%), and after stretching these fibres by 10% of optimal sarcomere length, they reached a final length of 16.7 and 23.3% greater than optimum. The remaining seven test sets were performed at initial lengths that were always 3.3% greater than the previous one, thereby covering a range of initial and final lengths from 16.7-36.7% and 26.7 and 46.7% of optimal length, respectively. Once

the fibres had undergone this protocol in a normal Ringer's solution, the exact same protocol was repeated twice more following the addition of 5mM and 15mM of BDM to the Ringer's solution. Each contraction lasted for 4s. For the stretch test contractions, fibres were activated at the initial length for about 800ms, then the stretch was imposed over about 200ms, and the final isometric length was maintained for the remaining 3s (Figure 5-1). For all tests, force, fibre length, and sarcomere length were recorded at 1000Hz, and stored on a computer for off line analysis.

#### ***5.2.5 Data analysis***

Force enhancement was measured as the difference in the isometric reference force and the isometric force following active fibre stretch at 3.5s after the onset of contraction ( $\Delta FE$  in Figure 5-1) when most of the transient force response caused by stretching had subsided. For comparison across fibres, force enhancement was normalized with respect to the isometric reference force. Passive force enhancement was defined as the difference in the passive force of the purely isometric and the stretch test contraction at 5s following deactivation of the fibres ( $\Delta PFE$  in Figure 5-1). Passive forces for the passive stretch experiments were also measured at the corresponding time. Half relaxation times were measured from the time of deactivation until force had dropped to 50% of the force at the instant of deactivation.



**Figure 5-1: Experimental procedures and representative force–time histories for isometric reference and active stretch contractions and passive stretch.** Exemplar force-time history of one set of experimental trials consisting of an isometric reference contraction (I), an active stretch test (S), and a passive stretch (P) (top). The resulting force enhancement,  $\Delta FE$ , and passive force enhancement,  $\Delta PFE$ , are indicated. Also shown are length (middle), and activation (bottom) –time histories for the entire experimental protocol. Vertical arrows indicate the time of measurement of the total residual force enhancement and the passive force enhancement. The protocol shown was repeated three times for all fibres: in normal Ringer’s solution, and after the addition of 5 and 15mM BDM.

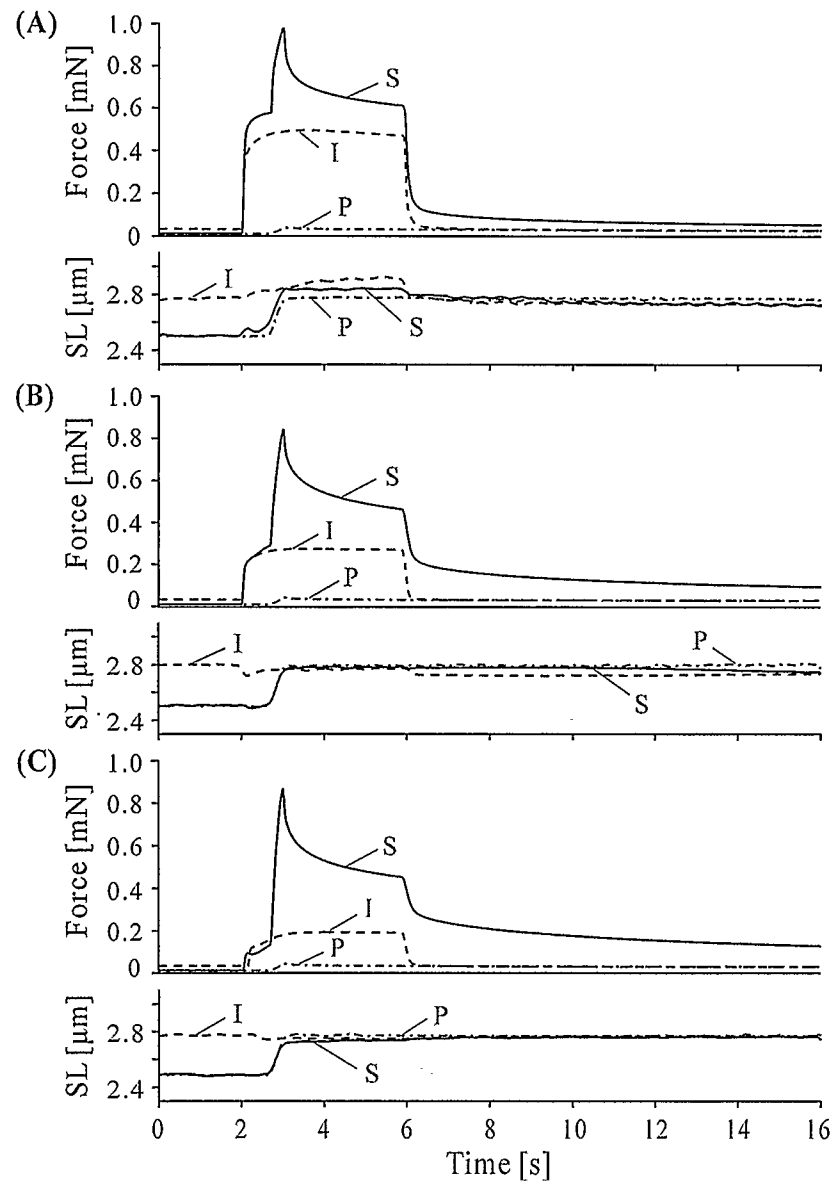
In order to compare force enhancement, passive force enhancement, passive forces, and half-relaxation times across the experimental conditions (Ringer's solution and 5 and 15 mM BDM), repeated measures ANOVA was used. When significant differences ( $p < 0.05$ ) were detected, contrasts were performed to elucidate the nature of the differences.

### 5.3 Results

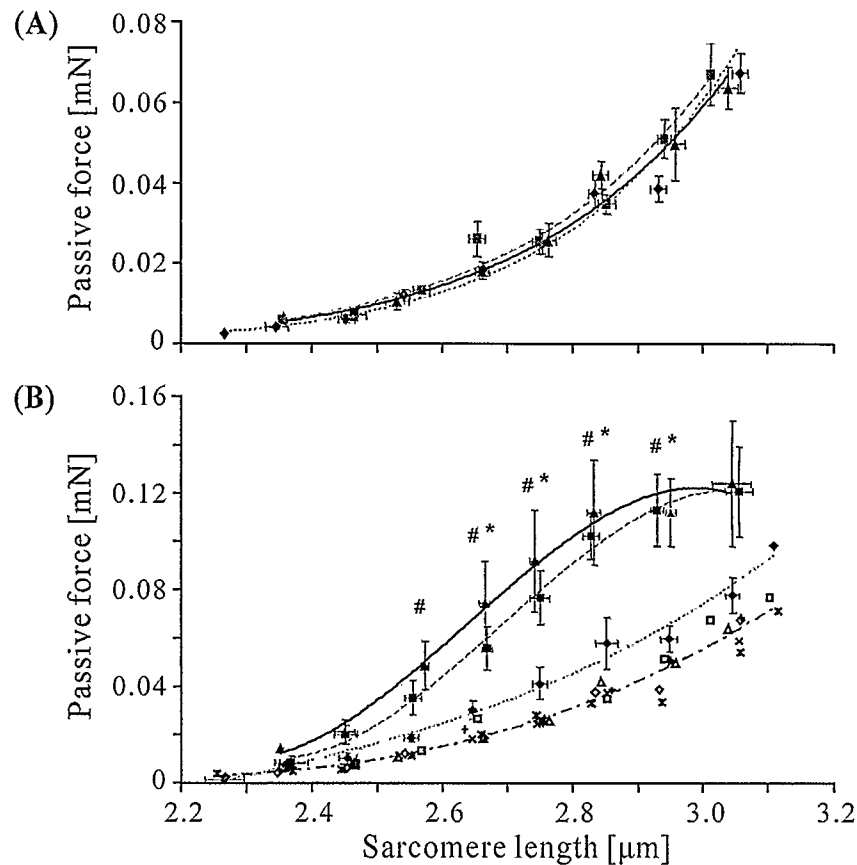
When fibres were exposed to 5 and 15mM of BDM, isometric forces decreased in a dose-dependent manner, compared to those obtained in normal Ringer's solution, thereby illustrating that BDM worked as had been observed previously (Horiuti *et al.*, 1988; Sun *et al.*, 2001; Rassier and Herzog, 2004b; Rassier and Herzog, 2005a, (Figure 5-2)). Fibres stretched actively on the descending limb of the force-length relationship all showed residual force enhancement and passive force enhancement independent of the experimental conditions (Figure 5-2). As shown previously, the normalized residual force enhancement increased with increasing concentrations of BDM (Rassier and Herzog, 2004b; Rassier and Herzog, 2005a). However, in contrast to expectation, the absolute passive force enhancement did not remain the same or decrease, but it increased with increasing BDM concentrations (compare Figure 5-2A-C)

The mean ( $\pm 1$ S.E.) passive force-sarcomere length relationships after passive stretching of single fibres were the same for the three experimental conditions (Ringer's and 5 and





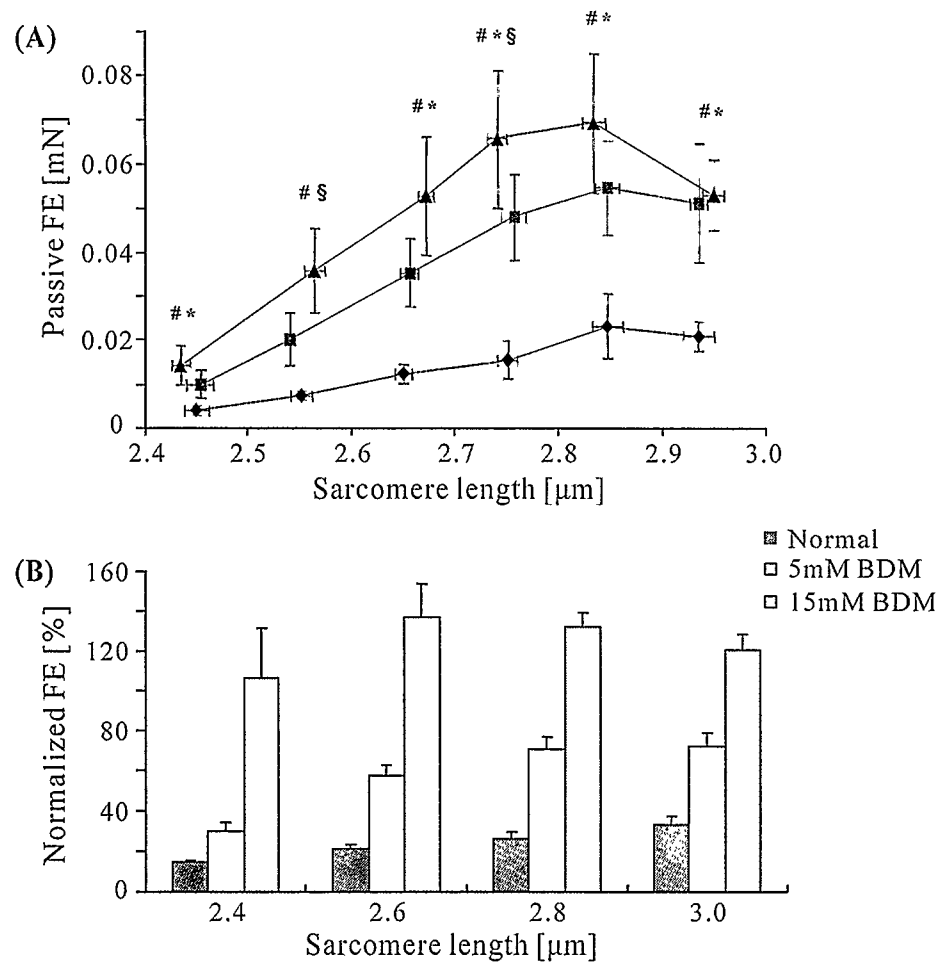
**Figure 5-2: Exemplar force-time and sarcomere length-time histories in different contractile conditions.** Force (upper traces) and sarcomere length, SL, (lower traces) – time histories of active stretches (solid, S), passive stretches (dashed-dotted, P), and isometric contractions (dashed, I) in a normal Ringer's solution (A), and after the addition of 5mM BDM (B), and 15mM BDM (C) from one representative fibre. Initial and final lengths were the same for all conditions.



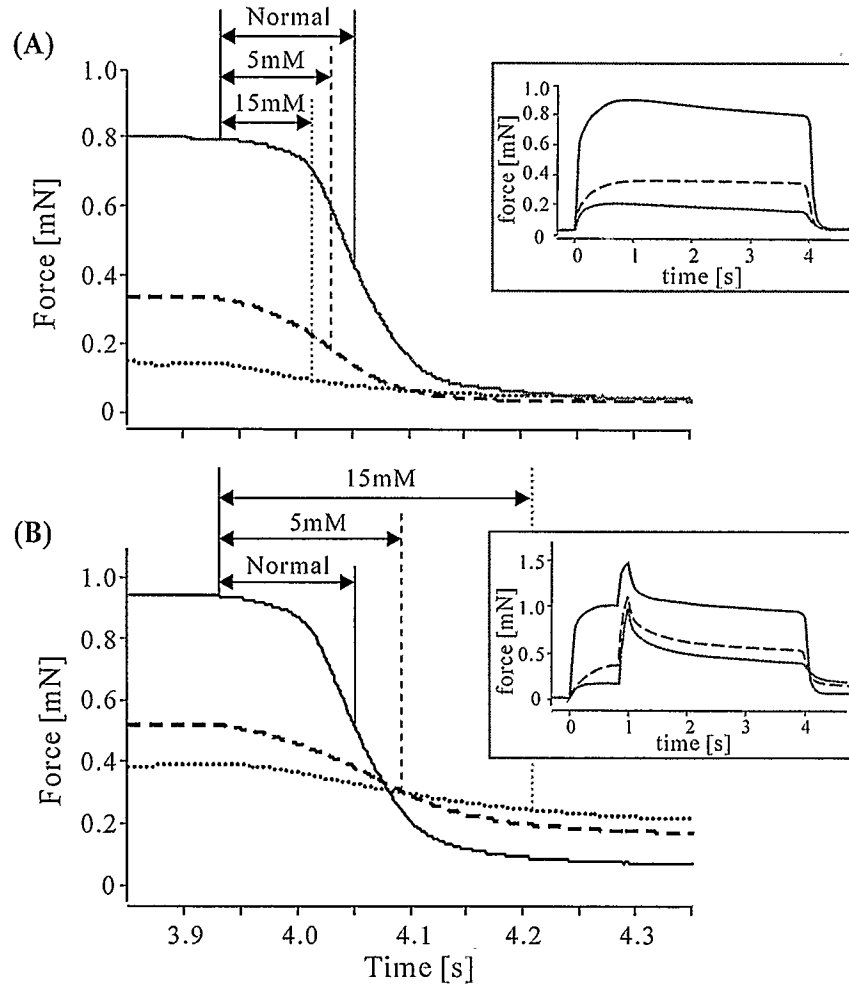
**Figure 5-3: Passive force-sarcomere length relationships following active and passive stretch and isometric contraction.** A) Mean( $\pm$ 1S.E.) passive force-sarcomere length relationship in a normal Ringer's solution ( $\diamond$ , solid), after the addition of 5mM BDM ( $\blacksquare$ , dashed), and 15mM BDM ( $\blacktriangle$ , dotted) along with the best-fitting lines (second or third order polynomial equations) following passive fibre stretch. B) Mean( $\pm$ 1S.E.) passive force-sarcomere length relationship after isometric contractions ( $\circ$  in normal;  $\square$  in 5mM BDM;  $\triangle$  in 15mM BDM) and passive ( $-$  in normal;  $+$  in 5mM BDM;  $\times$  in 15mM BDM) and active ( $\diamond$  in normal;  $\blacksquare$  in 5mM BDM;  $\blacktriangle$  in 15mM BDM) stretches were plotted. Data from isometric contractions and passive stretches were approximated with one best-fitting line (dashed-dotted). All data were grouped into intervals of  $0.1\mu\text{m}$  of sarcomere length from 2.2 to  $3.1\mu\text{m}$ . Statistical differences in passive force between normal and 15mM BDM (#) and between normal and 5mM BDM (\*) conditions,  $p < 0.05$ , were indicated.

15mM BDM; Figure 5-3A). However, after active stretching, the passive forces were increased for all experimental conditions, and these increases became greater with increasing concentrations of BDM (Figure 5-3B). Specifically, the passive forces following active stretching were greater for the fibres exposed to BDM than those stretched in the normal Ringer's solution. The passive force enhancement increased with increasing sarcomere lengths up to about  $2.8\mu\text{m}$  and then remained constant or decreased slightly (Figure 5-4A). The normalized total force enhancement increased steadily for the sarcomere length range studied here (about  $2.3\mu\text{m}$  to  $3.1\mu\text{m}$ ) in the normal Ringer's solution and after addition of 5mM BDM (Figure 5-4B), but peaked at about  $2.6\mu\text{m}$  for the 15mM condition, before gradually decreasing.

The half-relaxation times after isometric contractions were similar in the normal Ringer's solution and the BDM conditions (Figure 5-5A), except at long sarcomere lengths, where the 15mM BDM conditions had the longest relaxation times (Figure 5-6A). However, after active stretching of the fibres, the half-relaxation times were greatest for the 15mM condition, followed by the 5mM and the normal reference tests (Figure 5-5B) for all but the shortest sarcomere lengths (Figure 5-6B). Mean ( $\pm 1\text{S.E.}$ ) half-relaxation times after isometric contractions for all six fibres remained virtually constant as a function of sarcomere length for the normal Ringer's solution while they gradually increased with increasing sarcomere length for the BDM conditions (Figure 5-6A). The corresponding mean ( $\pm 1\text{S.E.}$ ) half-relaxation times in the tests following active fibre stretching were



**Figure 5-4: Passive force enhancement (A) and residual force enhancement (B) at different sarcomeres in different contractile conditions.** A) Mean ( $\pm 1$ S.E.) passive force enhancement vs. sarcomere length for six fibres in a normal Ringer's solution ( $\blacklozenge$ ), and after addition of 5mM BDM ( $\blacksquare$ ), and 15mM BDM ( $\blacktriangle$ ). Data were grouped into intervals of  $0.1\mu\text{m}$  of sarcomere length from 2.4 to  $3.0\mu\text{m}$ . Statistical difference between normal and 15mM BDM (#), normal and 5mM BDM (\*), and 5mM and 15mM BDM conditions (§),  $p < 0.05$ , were indicated. B) Normalized total force enhancement vs. sarcomere length. Data were grouped into intervals of  $0.2\mu\text{m}$  of sarcomere length from 2.3 to  $3.1\mu\text{m}$ . At each sarcomere lengths, statistical differences among all three conditions were found,  $p < 0.05$ .



**Figure 5-5: Exemplar of half-relaxation times for isometric reference (A) and active stretch (B) contraction in normal and BDM conditions.** Example of half-relaxation times ( $50\%R_t$ ) after isometric reference contractions (A) and after stretch test contractions (B) for normal Ringer's solution (solid) and after addition of 5mM (dashed) and 15mM BDM (dotted) from one representative fibre. Arrows between vertical bars indicate  $50\%R_t$ . Inset: raw force-time traces of isometric contractions (A) and active stretch test contractions (B) for the three experimental conditions. Note that in the isometric contractions,  $50\%R_t$  tended to decrease with increasing BDM concentrations, while following the stretch test contractions  $50\%R_t$  increased with increasing BDM concentrations.

significantly greater than those obtained for the isometric reference contractions (compare Figure 5-6A and B). These half-relaxation times were essentially independent of sarcomere length for the normal Ringer's conditions but they increased with increasing sarcomere lengths for the two BDM conditions (Figure 5-6B).

#### **5.4 Discussion**

The purpose of this study was to test if the passive force enhancement was associated with a passive structural element (molecule) or might be directly associated with the kinetics of actin-myosin interaction through cross-bridge attachment. We hypothesized that by decreasing the active isometric force with BDM, the passive force enhancement would also decrease if it was linked to the cross-bridge kinetics, but would remain constant if it originated from a passive structural component. However, to our great surprise, the passive force enhancement was significantly increased when contractility was inhibited through BDM.

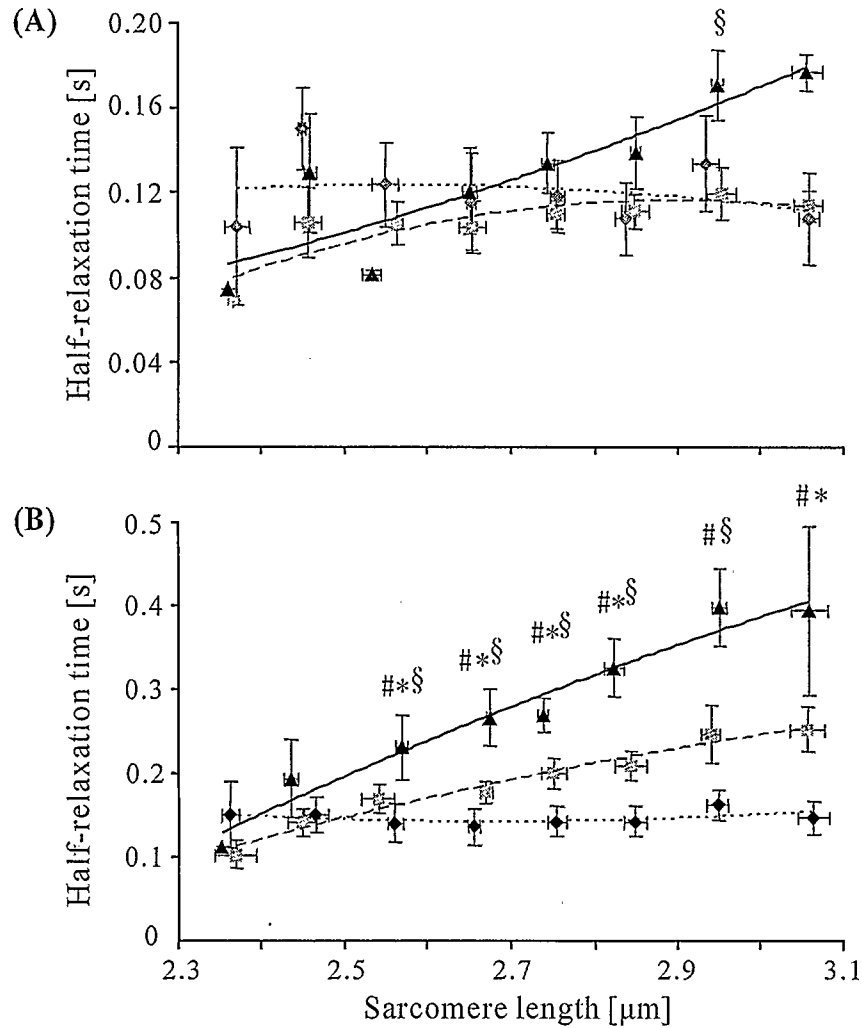
In order to check if BDM alone might have caused a change in the passive stiffness of the fibres, we compared the passive forces in the isometric reference contractions to the passive forces obtained after passive fibre stretching, and found that they were the same (Figure 5-3B; the dashed-dotted (lowest) best fitting line approximates all passive forces following passive fibre stretching and following isometric reference contraction in the

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three experimental conditions), thereby eliminating BDM alone as the cause for the observed increases in the passive force enhancement.

The half-relaxation times following active stretching of the muscle fibres were greater (i.e., the rate of force decrease after deactivation was slower) than those measured following the isometric reference contractions (compare Figure 5-6A and B). Furthermore, the addition of BDM to the Ringer's solution increased the half-relaxation times even further compared to the values obtained in the isometric reference contractions and also those obtained following stretch in normal Ringer's solution (Figure 5-6B). Also, following 5 and 15mM BDM addition, the passive force enhancement increased with increasing sarcomere lengths (Figure 5-4A), as did the half-relaxation times, thus part of the passive force enhancement in the BDM conditions might be caused by cross-bridges that remain attached for a long time after fibres are deactivated. It has been shown theoretically that such "stuck" cross-bridges could explain the residual and passive force enhancement (Walcott and Herzog, 2006), and such behaviour has been observed in cross-bridges of smooth muscles when they enter the so-called "latch state" (Dillon *et al.*, 1981).

We (Herzog and Leonard, 2002; Herzog *et al.*, 2003), and others (Bagni *et al.*, 2002; Labeit *et al.*, 2003; Bagni *et al.*, 2004), have suggested that the passive force enhancement might be caused by a structural protein that increases its stiffness with activation. The prime candidate for this role is titin, a giant molecule that spans half-



**Figure 5-6: Mean ( $\pm 1$ S.E.) half-relaxation times vs. sarcomere lengths for isometric contractions (A) and for the stretch test contractions (B) in normal Ringer's solution ( $\diamond$ , dotted) and after addition of 5mM ( $\blacksquare$ , dashed) and 15mM BDM ( $\blacktriangle$ , solid). Data were grouped for intervals of 0.1 $\mu\text{m}$  of sarcomere length from 2.2 to 3.1 $\mu\text{m}$ . Statistical differences were found between the normal and 15mM BDM conditions (#), the normal and 5mM BDM conditions (\*), and the 5mM and 15mM BDM conditions (§),  $p < 0.05$ .**



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sarcomeres from the middle of the A-band (the M-line) to the Z-disc (Kellermayer *et al.*, 1997; Rief *et al.*, 1997). There are many possibilities of how titin could increase its stiffness; one is by attaching to actin in a calcium-dependent manner, so that its resting length becomes smaller and its stiffness increases, another is by increasing its inherent stiffness in a calcium-dependent manner by strengthening specific molecular bonds that are relevant for its force elongation behaviour. However, if such a structural component was the only source for the observed passive force enhancement, and knowing that the addition of BDM did not increase the passive forces in fibres when passively stretched (Figure 5-3A), we would have expected the passive force enhancement to remain constant independent of the experimental conditions (Ringer's, 5, and 15mM BDM), which it did not (Figure 5-3B). However, since active stretching produced an increase in the passive forces beyond those measured in the isometric reference contractions, and since BDM increased these forces even further, it appears that the passive force enhancement is linked to active force and/or to cross-bridge attachment during stretch.

In unrelated experiments, the idea that the passive force enhancement requires either force, cross-bridge attachment, or both during stretch has received further support. When isolated myofibrils were stretched actively, there was a great amount of passive force enhancement, as observed in other preparations ranging from single fibres (Rassier *et al.*, 2003; Rassier *et al.*, 2005) to whole muscles (Herzog and Leonard, 2002; Herzog *et al.*, 2003). However, when these myofibrils were stretched in the activated state (by adding an activating solution with high calcium concentration), but cross-bridge attachment and

active force production was eliminated through the specific deletion of troponin from actin, the passive force enhancement was abolished, indicating that passive force enhancement does not only require activation (e.g., calcium release from the sarcoplasmic reticulum) but also active formation of cross-bridges and/or force production between actin and myosin (Joumaa *et al.*, 2008a).

The results of this study (increase in the passive force enhancement with the addition of BDM) were surprising and did not allow for a resolution of the detailed origins of the passive force enhancement. However, they provided new insights into the possible causes of the passive force enhancement. Passive force enhancement can increase while active isometric force decreases (through BDM application). Simultaneously, the active stretch of single fibres causes half-relaxation times to increase compared to those obtained for isometric reference contractions, indicating that maybe part of the passive force enhancement is associated with the slow release of stretched cross-bridges. These cross-bridges might become “stuck” as described theoretically for skeletal muscle (Smith and Geeves, 1995; Walcott and Herzog, 2006), and as observed experimentally for smooth muscle (Dillon *et al.*, 1981). The idea that the passive force enhancement does not only require activation, but also cross-bridge attachments and force production, is supported by unpublished results on single myofibrils, where it was shown that passive force enhancement only occurs when activation is coupled with force production and cross-bridge attachment. However, it is likely that a passive structural protein is also involved in the passive force enhancement, because if it was just a cross-bridge-related

phenomenon, we would expect the passive force enhancement to occur at all muscle and fibre lengths, whereas it only occurs at lengths where passive forces occur naturally (i.e. at relatively long lengths typically associated with the descending limb of the force-length relationship, Herzog and Leonard (2002)).

In summary, the results of this study suggest that the passive force enhancement observed following active stretch of muscles or single fibres might have two components, one that is associated with a passive structural component, as proposed previously, and one that requires cross-bridge attachment and force production. Of course, one might conceive a mechanism in which these two components are not independent but are working in an agonistic fashion. For example, if the passive force enhancement was caused by increased attachments of titin to actin, thereby decreasing its zero resting length, and if such attachments were only possible when actin filaments experience force and might undergo some structural change that enhances titin attachment, then a combined cause (one that is based on a passive structural protein but requires active force) is perfectly conceivable. These and other ideas about the origin of the passive force enhancement must be carefully tested in the future.

## **Chapter Six: Effect of temperature on residual force enhancement in single skeletal muscle fibres**

### **6.1 Introduction**

Residual force enhancement (or force enhancement for short) has been defined as the increase in steady-state isometric force following stretching of an active fibre or muscle compared to the steady-state force obtained for purely isometric contractions at the corresponding length (Abbott and Aubert, 1952; Edman *et al.*, 1982). Although this phenomenon has been observed consistently for muscle preparations ranging from in-vivo human muscles (De Ruiter *et al.*, 2000; Lee and Herzog, 2002) to single fibres (Edman *et al.*, 1978, 1982; Sugi and Tsuchiya, 1988), the underlying mechanisms remain unclear. Residual force enhancement is increased with increasing stretch magnitudes (Abbott and Aubert, 1952; Sugi, 1972; Edman *et al.*, 1978, 1982; Cook and McDonagh, 1995), is thought to be independent of the speed of stretch (Edman *et al.*, 1978; Sugi and Tsuchiya, 1988), and has been observed consistently on the descending limb of the force-length relationship (Edman *et al.*, 1978, 1982; Morgan *et al.*, 2000).

Although residual force enhancement had been associated at one point with the development of sarcomere length non-uniformities (e.g., Julian and Morgan, 1979) and/or

the engagement of passive forces (e.g., Edman *et al.*, 1982), it is unlikely that these mechanisms can account for more than just a small part of the total force enhancement (Herzog and Leonard, 2002; Herzog *et al.*, 2006). Stiffness measurements provide insight whether force enhancement is caused by an increase in the proportion of attached cross-bridges or an increase in the average force per cross-bridge (Huxley and Simmons, 1971, Ford *et al.*, 1986). Experimental evidence suggests that there is either only a small (Herzog and Leonard, 2000; Rassier and Herzog, 2005b) or no increase in stiffness (Julian and Morgan, 1979; Sugi and Tsuchiya, 1988), in the force-enhanced compared to the isometric reference state, suggesting that force enhancement is largely caused by an increase in the average force per cross-bridge.

An increase in the average force per cross-bridge can be achieved by increasing the ratio of strongly to weakly bound cross-bridges. Therefore, force enhancement may be caused by a stretch-induced increase in the ratio of strongly to weakly bound cross-bridges. 2,3-butanedione monoxime (BDM) is known to decrease the rate of phosphate release in attached cross-bridges (Herrmann *et al.*, 1992; Regnier *et al.*, 1995), thereby biasing attached cross-bridges towards weakly bound states (Horiuti *et al.*, 1988; Bagni *et al.*, 1992). We found that force enhancement was increased from 16-30% in a control preparation and 80-150% in a [10mM] BDM preparation (Rassier and Herzog, 2004b). Therefore, force enhancement may be caused by a stretch-induced facilitation of phosphate release causing a shift from weakly to strongly bound cross-bridge states.

If this theory is correct, any preparation that is biased initially towards weakly bound cross-bridge states should exhibit increased force enhancement. Lowering temperature is one way to increase the proportion of weakly to strongly bound cross-bridges (Coupland *et al.*, 2001; Wang and Kawai, 2001). Therefore, we hypothesized that a decrease in temperature is associated with an increase in residual force enhancement. The purpose of this study was to test the hypothesis that force enhancement is increased with decreasing temperature. Experiments were performed using single skeletal muscle fibres of frog and measuring force enhancement for given stretch conditions at temperatures of 7°C and 20°C.

## **6.2 Methods**

### ***6.2.1 Muscle fibre preparation***

Eleven single fibres (avg. 2.7mm in length, 0.02mm<sup>2</sup> in cross-sectional area) from the lumbrical muscles of frogs, *rana pipiens*, were used for all experiments.

### ***6.2.2 Force and fibre length measurements***

After isolating a single fibre in a dissecting bath, the tendons at either end of the fibre were gripped with small T-shaped pieces of aluminium clips as close as possible to the fibre to minimize compliance arising from the free tendon. Fibres were then attached to a force transducer (Sensoror) at one end and to a servomotor length controller (Aurora

Scientific) at the other end. The experimental chamber containing the fibre was placed on an inverted microscope (Eclipse TE300, Nikon). The chamber was filled with physiological Ringer's solution (NaCl 115mM, KCl 2.5mM, CaCl<sub>2</sub> 1.8mM, Na<sub>2</sub>HPO<sub>4</sub> 1mM, NaH<sub>2</sub>PO<sub>4</sub> 1mM, pH=7.2), and the temperature of the Ringer's solution was controlled at 7°C for the low temperature and at 20°C for the high temperature experiments (VWR scientific products).

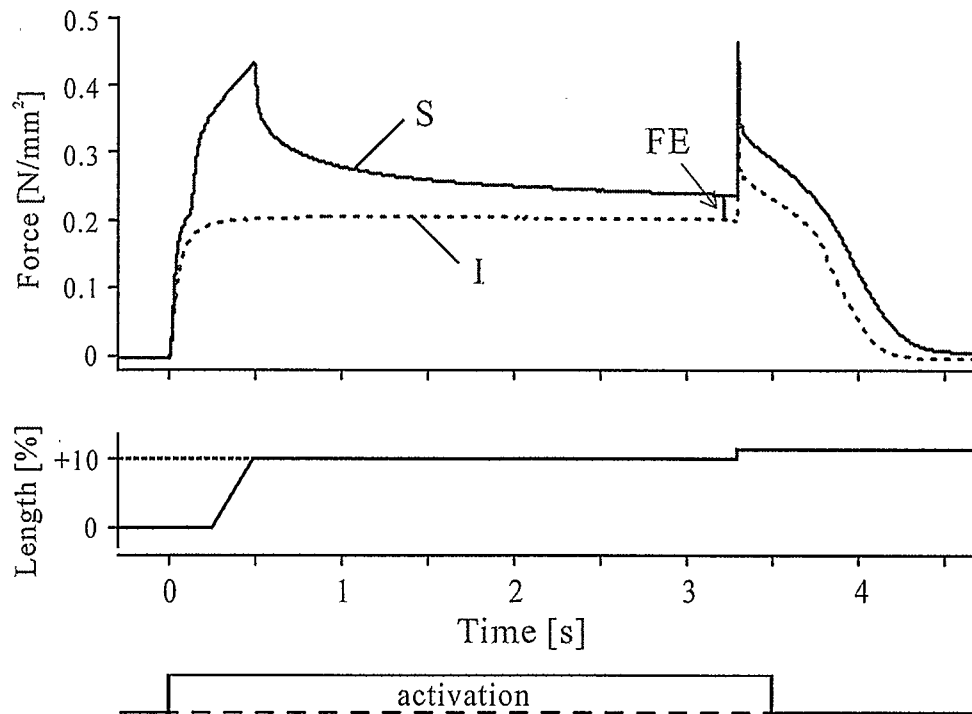
Stimulation of the fibres (Grass S88, Grass Instruments) was achieved through two platinum wire electrodes that were placed inside the chamber parallel to and at a distance of about 3mm from the muscle fibre. For complete activation, square wave pulses (0.4 ms duration) at amplitude of 25% above the voltage that gave maximal force (range 50-60V) were given. The frequency of stimulation was chosen individually for each fibre to induce a fused contraction at the lowest possible frequency (range 25-30Hz for 7°C and 40-50Hz for 20°C). Fibre lengths and diameters were measured before testing with a calibrated eyepiece (error < 0.02mm). In order to determine cross-sectional area, fibres were assumed ellipsoidal in cross-section. The two major axes of the ellipsoid were determined as the smallest and largest diameters measured by rotating the fibre. Cross-sectional areas were determined at five evenly distributed locations along the fibre, and the average of those five values was defined as the cross-sectional area of the fibre.

### *6.2.3 Experimental procedures*

At the beginning of the experiment, the optimum voltage and frequency of stimulation were determined with 1s tetanic contractions, and fibres were paced for 40min with twitch contractions every 90s. After the 40min pacing period, the plateau region and the descending limb of the force-length relationship were determined for each fibre using 1s tetanic isometric contractions (3min intervals) at different sarcomere lengths (range: 1.9-2.6 $\mu$ m,  $\sim$ 0.1-0.15 $\mu$ m steps). The sarcomere length showing the greatest active force was defined as optimal sarcomere length.

Each experimental set consisted of three individual test contractions: the first contraction was an isometric reference contraction at optimal length; the second contraction was a purely isometric reference contraction at the final length (i.e., the length to which the fibre was being stretched in the third test); and the third test was a stretch contraction in which the fibre was stretched from the initial length to the final length. The initial length for all tests was the optimal length. The stretch magnitude for all fibres was 10% of the optimal sarcomere length, and stretches were performed at a speed of 40% sarcomere length/s. For stiffness measurement in the isometric reference and the force-enhanced states, fibres were stretched by 20 $\mu$ m within 1ms just before deactivation (Figure 6-1). Each contraction lasted 3.5s, and contractions were separated by a 5 minute rest. For the stretch test contractions, the final isometric lengths were held for 3s after the end of the stretch (Figure 6-1). Experiments were performed at 7°C and 20°C in random order, and experiments at the initial temperature were repeated at the end to verify that the fibre was





**Figure 6-1: Experimental procedures and representative force–time traces for isometric reference and active stretch contractions.** Exemplar force–time history of one set of experimental trials consisting of an isometric reference contraction (I, dashed) and a stretch test contraction (S, solid) (top). The resulting force enhancement is indicated (FE). Also shown are a length–time (middle) and an activation–time history (bottom) for the experimental procedure. Fibres were stretched by  $20\mu\text{m}$  within  $1\text{ms}$  at  $0.2\text{s}$  before deactivation for stiffness measurement. Vertical bar indicates the time of measurement of force enhancement. Note that forces are normalized relative to the fibre cross-sectional area ( $\text{N}/\text{mm}^2$ ) and lengths are given relative to optimal length (0%).

still in good condition. If fibres showed any sign of damage or if the isometric force at optimal length decreased by more than 2% within a given set of tests or by more than 10% of its initial value throughout the entire experimental protocol, testing was stopped, and the fibre and all results were discarded. Force, fibre lengths, and sarcomere lengths were recorded at 10kHz, and stored on a computer for off line analysis.

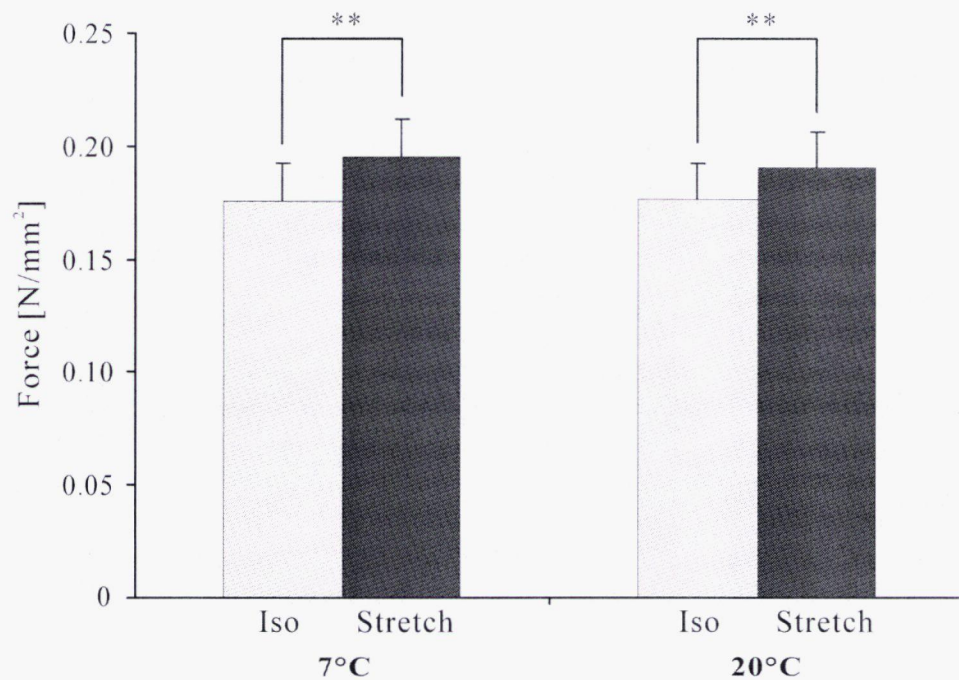
#### ***6.2.4 Data analysis***

Steady-state isometric forces were approximated by measurements made at 3.2s following the onset of activation (i.e. 10ms before the stiffness measurements) when the transient force response caused by stretching had subsided. Force enhancement was defined as the increase in the steady-state isometric force following active fibre stretch compared to the steady-state isometric reference force at the corresponding final length (FE in Figure 6-1). In order to make comparisons across fibres, force enhancement was normalized with respect to the corresponding isometric reference force at the final length.

All forces were normalized relative to the cross-sectional area of each fibre. Stiffness was calculated by dividing the increase in force by the corresponding stretch amplitude. In order to compare force enhancement, isometric forces, stiffness, and force/stiffness across the two temperatures, a repeated measures paired *t*-test was used to elucidate the nature of the differences. A paired *t*-test was also used to compare stiffness and force/stiffness in the force-enhanced and the isometric reference state. All data are presented as means  $\pm$  1S.E.

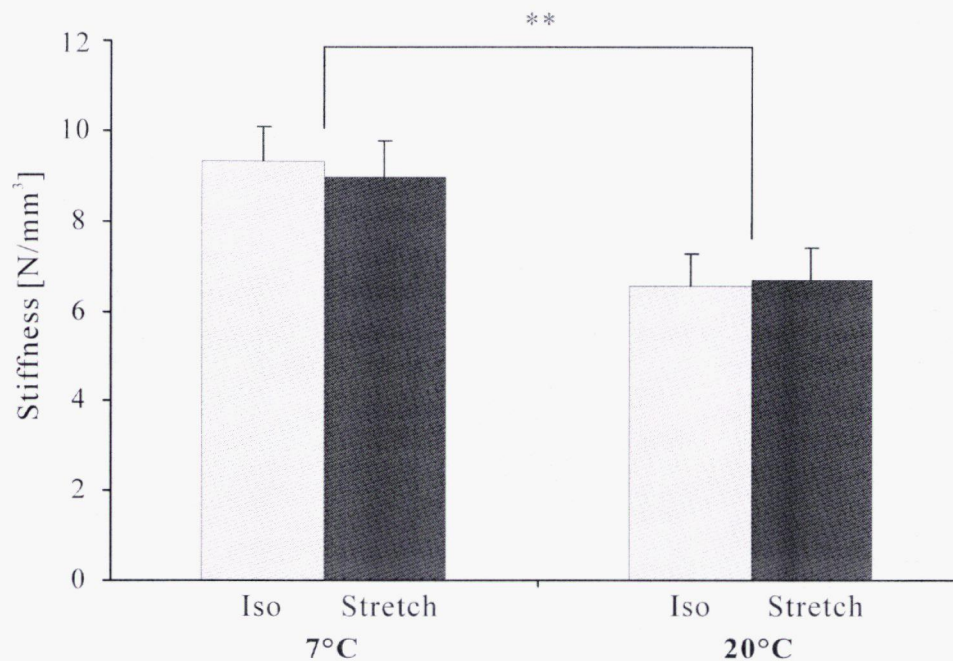
### 6.3 Results

The average isometric reference forces were the same at 7°C and 20°C (Figure 6-2). However, stiffness was significantly decreased at 20°C compared to 7°C, but for a given temperature, was the same for the isometric reference and the stretch test contractions (Figure 6-3). Force/stiffness was significantly greater at 20°C than that at 7°C (Figure 6-4).

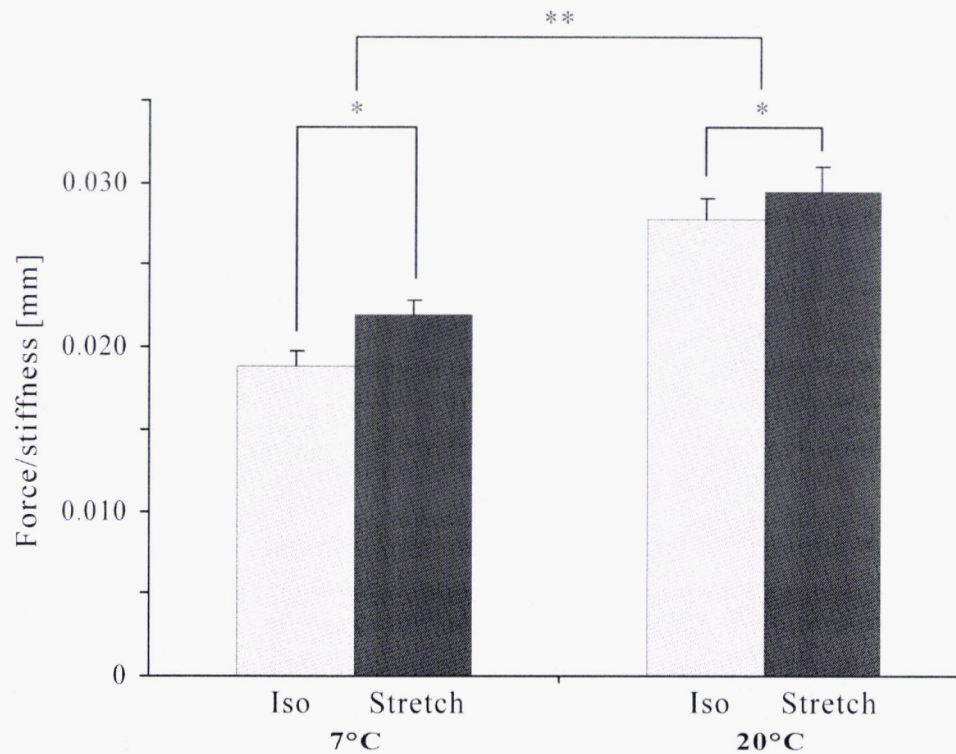


**Figure 6-2: Forces for isometric reference and active stretch contractions at 7°C and 20°C.** Mean values ( $\pm$ 1S.E) of normalized force (Force) for purely isometric reference contractions (iso) and stretch test contractions (stretch) at 7°C and at 20°C. The isometric reference forces at 7°C and 20°C were the same, while the isometric forces following stretch were greater than the purely isometric reference forces at a given temperature (\*\*,  $p < 0.01$ ).

Force enhancement was observed consistently for all active stretch tests (Figure 6-5 and Figure 6-6), and was significantly greater at 7°C compared to 20°C (Figure 6-6). Values of force/stiffness were significantly greater in the force-enhanced (stretch) than the isometric reference (iso) state (Figure 6-4), and enhancement of this variable, that is the percent increase in the enhanced compared to the isometric reference state, was significantly greater at 7°C compared to 20°C (force/stiffness enhancement in Figure 6-6).



**Figure 6-3: Stiffness for isometric reference and active stretch contractions at 7°C and 20°C.** Mean values ( $\pm 1$ S.E) of stiffness (normalized force/elongation) were significantly (\*\*,  $p < 0.01$ ) greater at 7°C compared to 20°C for both the purely isometric reference (iso) and the stretch test contractions (stretch). However, stiffness, at a given temperature, was the same for the isometric reference and the stretch test contractions.



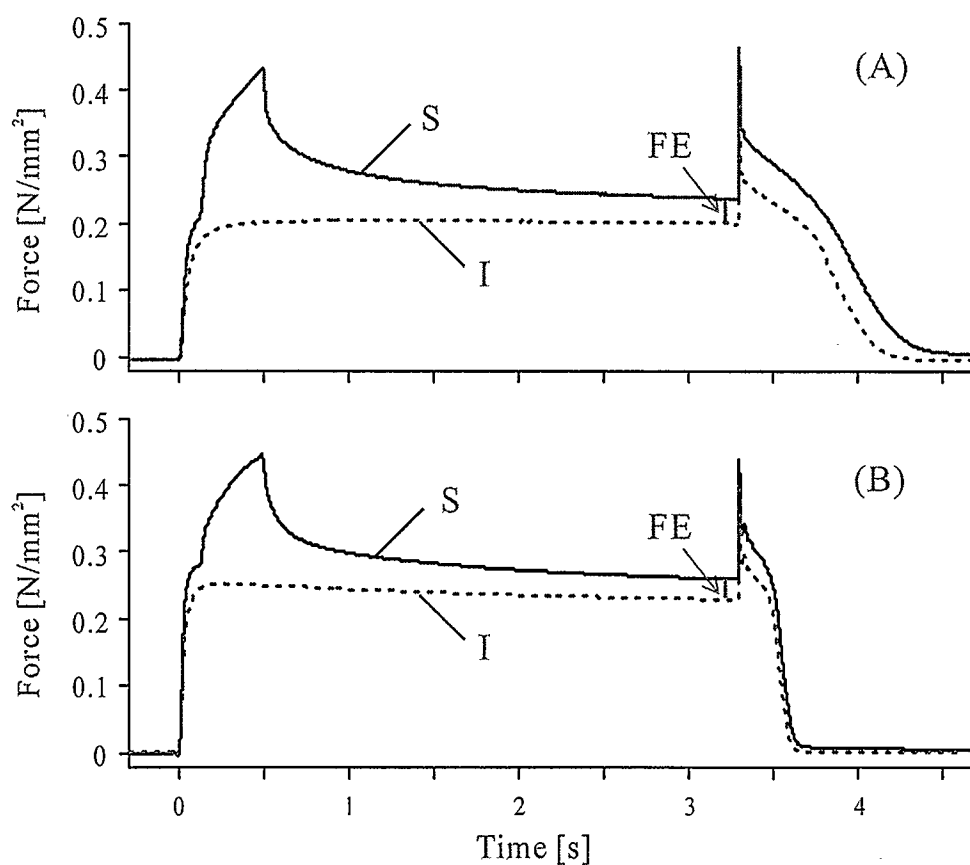
**Figure 6-4: Force/stiffness for isometric reference and active stretch contractions at 7°C and 20°C.** Mean values ( $\pm 1$ S.E) of force/stiffness (normalized force/stiffness) for purely isometric reference contractions (iso) and stretch test contractions (stretch) at 7°C and at 20°C. Force/stiffness was greater for the stretch test contractions (stretch) than the isometric reference contractions (iso) at a given temperature (\*,  $p < 0.05$ ). Also force/stiffness was greater at 20°C than at 7°C for both the isometric reference (iso) and stretch test (stretch) contractions (\*\*,  $p < 0.01$ ).

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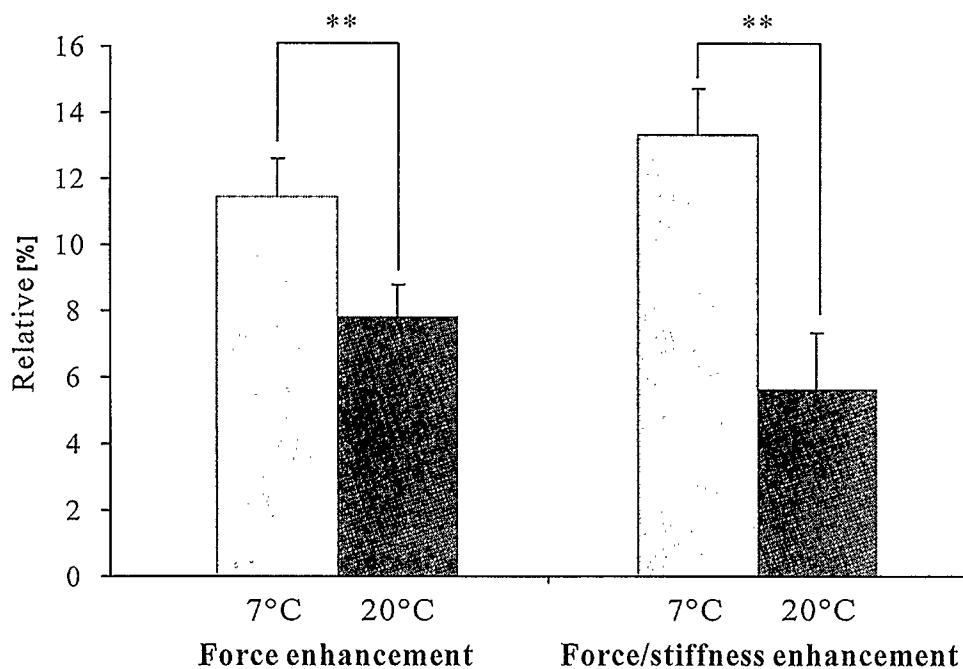
## 6.4 Discussion

Residual force enhancement has been shown to increase with increasing stretch magnitudes (Abbott and Aubert, 1952; Sugi, 1972; Edman *et al.*, 1978, 1982; Cook and McDonagh, 1995), increasing sarcomere lengths (on the descending limb of the force-length relationship; Edman *et al.* (1982)), and increasing proportion of weakly bound cross-bridges at the initial length prior to stretch (Rassier and Herzog, 2004b; Lee *et al.*, 2007). However, force enhancement does not depend on the speed of stretch (Edman *et al.*, 1978; Sugi and Tsuchiya, 1988) or the development of sarcomere length non-uniformities (Herzog *et al.*, 2006), nor is it associated with an appreciable change in muscle stiffness (Julian and Morgan, 1979; Sugi and Tsuchiya, 1988). Furthermore, force enhancement has been shown to occur on the ascending limb of the force-length relationship (Peterson *et al.*, 2004), and exceed the purely isometric forces on the plateau of the sarcomere force-length relationship (Joumaa *et al.*, 2008b). Force enhancement has been shown to be long lasting (Abbott and Aubert, 1952), but can be abolished instantaneously by deactivation (Abbott and Aubert, 1952; Morgan *et al.*, 2000). It is also observed in the presence of stable sarcomere lengths on the descending limb of the force-length relationship (Herzog *et al.*, 2006).

Together, these properties suggest that residual force enhancement is not associated with the development of sarcomere length instabilities on the descending limb of the force-length relationship (Hill, 1953) and the associated redistribution of sarcomere lengths following active muscle stretching (Julian and Morgan, 1979). Furthermore, although



**Figure 6-5: Exemplar force-time histories for isometric reference and active stretch contractions at 7°C and 20°C.** Force-time traces of isometric reference contractions (I) and stretch test contractions (S; 10% stretch magnitude, 40% fibre length/s stretch speed) at 7°C (A) and at 20°C (B). Force enhancement (FE) was observed to be greater at 7°C than at 20°C. Note that forces are normalized relative to the fibre cross-sectional area ( $\text{N}/\text{mm}^2$ ).



**Figure 6-6: Mean values ( $\pm 1$ S.E) of force enhancement and force/stiffness enhancement at 7°C and at 20°C.** Force enhancement and force/stiffness enhancement are the percent increases in force and force/stiffness relative to the corresponding isometric reference values. Force enhancement and force/stiffness enhancement were greater at 7°C than at 20°C (\*\*,  $p < 0.01$ ).



force enhancement has been associated with an increase in passive forces (passive force enhancement; Herzog and Leonard (2002)), and this passive force enhancement can be explained in part by an activation (calcium) induced increase in titin stiffness (Labeit *et al.*, 2003; Joumaa *et al.*, 2008a), force enhancement has been observed in the absence of passive force enhancement (Abbott and Aubert, 1952; Edman *et al.*, 1978; Julian and Morgan, 1979; Morgan *et al.*, 2000). If present, passive force enhancement only accounts for a small amount of the total force enhancement (Joumaa *et al.*, 2008a). Therefore, neither sarcomere length non-uniformities nor passive forces can account for the residual force enhancement.

Since force enhancement is not associated with a corresponding increase in muscle stiffness, we hypothesize that it is caused by an increase in the average force per attached cross-bridge. Furthermore, since the greatest force enhancement has been observed for fibre preparations treated with BDM, which causes a bias towards weakly bound cross-bridges, we further hypothesize that the increase in the average force per cross-bridge in the enhanced state is achieved by a stretch-induced facilitation of transition from weakly to strongly bound cross-bridges.

#### **6.4.1 Temperature effects**

Previous studies have shown that twitch and tetanic forces typically increase with increasing temperatures, while stiffness remains nearly the same (Coupland *et al.*, 2001; Wang and Kawai, 2001; Piazzesi *et al.*, 2003; Linari *et al.*, 2005). Therefore, it can be

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assumed that the average force per cross-bridge increased in these studies, possibly because of a shift from weakly to strongly bound cross-bridges (Piazzesi *et al.*, 2003). Here, we found that force was not increased with increasing temperature but stiffness was for the low compared to the high temperature. Therefore, our results are conceptually different from most others, but they lead to the same conclusion: the average force per cross-bridge is greater at higher temperatures, likely because of a shift towards strongly bound cross-bridges. However, our observations are not novel. Ishii *et al.* (2004) and Renaud and Stevens (1981) also found essentially constant forces in frog skeletal muscles across a large range of temperatures. Frogs in these latter studies, like ours, were kept in a cold environment (about 5°C) prior to testing, and it appears that frog muscles adapt to cold environments such that force can be kept constant over a large range of temperatures (Ishii *et al.*, 2004).

Force enhancement was increased at 7°C ( $11.5 \pm 1.1\%$ , Figure 6-6) compared to 20°C ( $7.8 \pm 1.0\%$ , Figure 6-6) in our study, thereby supporting the hypothesis that force enhancement is caused by a facilitation of the transition from weakly to strongly bound cross-bridges. If this hypothesis was correct, one would also expect that force/stiffness would be increased more at 7°C ( $13.3 \pm 1.4\%$ , Figure 6-6) compared to 20°C ( $5.6 \pm 1.7\%$ , Figure 6-6), which it was.

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#### 6.4.2 *Stretch effects*

Although stretch increased the force, and thus produced the expected residual force enhancement, stretch did not change stiffness (Figure 6-3). This result suggests that the proportion of attached cross-bridges, albeit different at the two temperatures, was not changed by stretch. Therefore, the residual force enhancement appears to be caused by an increase in the average force per cross-bridge rather than an increase in the proportion of attached cross-bridges. Probably the simplest explanation for this result is that stretch may produce a shift in the ratio of weakly to strongly bound cross-bridges towards the strongly bound states. Since all cross-bridges (weakly and strongly bound) are thought to contribute to stiffness (Huxley and Simmons, 1971, Julian and Sollins, 1975), but only the strongly bound cross-bridges are thought to contribute to force (Tesi *et al.*, 2002), such a stretch induced shift towards strongly bound cross-bridges could explain the results observed here, and those observed previously in fibre preparations exposed to BDM.

A limitation of the current study is that weakly and strongly bound cross-bridge states cannot be observed directly but are inferred from mechanical measurements of force, stiffness and the value of force/stiffness. However, independent of the indirect nature of these observations, and the detailed characteristics of various attached cross-bridge states, it is well accepted that force and stiffness can vary independently suggesting that there are cross-bridge states that contribute to either force (primarily), stiffness (primarily) or both. If so, the results of this study and the associated interpretations hold, independent of

the precise nature of these cross-bridge states. Furthermore, the results are also consistent with previous studies on BDM treated fibres (Rassier and Herzog, 2004b), which are also thought to be biased towards weakly bound cross-bridge states (Horiuti *et al.*, 1988; Bagni *et al.*, 1992).

#### **6.4.3 Conclusion**

The results of this study suggest that residual force enhancement is caused, at least in part, by a stretch-induced shift in the attached cross-bridges from weakly to strongly bound states. We speculate that this shift is achieved by a stretch-induced facilitation of phosphate release in pre-power stroke cross-bridges.

## **Chapter Seven: Shortening-induced force depression is primarily caused by cross-bridges in strongly bound states**

### **7.1 Introduction**

Force depression has been defined as the decrease in the steady-state isometric force following shortening of an active muscle compared to the steady-state force obtained for purely isometric contractions at the corresponding length (Sugi and Tsuchiya, 1988; Edman *et al.*, 1993; Josephson and Stokes, 1999; Ettema and Meijer, 2000; De Ruiter and De Haan, 2003). Force depression has been observed on various structural levels such as *in-vivo* human muscles (De Ruiter *et al.*, 1998; Lee and Herzog, 2003), *in-situ* muscles (Maréchal and Plaghki, 1979; Bullimore *et al.*, 2007), single fibres (Granzier and Pollack, 1989; Sugi and Tsuchiya, 1988; Edman *et al.*, 1993), and single myofibrils (Joumaa and Herzog, 2008).

Force depression increases with increasing magnitude of shortening (Abbott and Aubert, 1952; Maréchal and Plaghki, 1979; Josephson and Stokes, 1999; Morgan *et al.*, 2000; Bullimore *et al.*, 2007), increasing force (De Ruiter *et al.*, 1998; Herzog and Leonard, 1997), and decreasing speed of shortening (Sugi and Tsuchiya, 1988; De Ruiter *et al.*, 1998; Josephson and Stokes, 1999; Ettema and Meijer, 2000; Herzog *et al.*, 2000;

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Morgan *et al.*, 2000). It is closely related to the amount of mechanical work performed in the shortening phase (Josephson and Stokes, 1999; Herzog *et al.*, 2000).

Despite consistent observations for more than half a century (Abbott and Aubert, 1952), the mechanism(s) underlying force depression remain unclear. Maréchal and Plaghki (1979) suggested that force depression may be caused by a stress-induced inhibition of cross-bridge attachments in the newly formed filament overlap zone during active shortening. Specifically, it was hypothesized that during active shortening, stress produces elongation of actin filaments (Huxley *et al.*, 1994; Kojima *et al.*, 1994; Wakabayashi *et al.*, 1994; Higuchi *et al.*, 1995) and an associated distortion of the actin attachment sites (Daniel *et al.*, 1998). Thus cross-bridge inhibition may be caused by actin deformation or deformation of the regulatory proteins, tropomyosin and/or troponin. This mechanism can account for many previously described observations, and it can predict correctly that force depression is eliminated instantaneously upon deactivation when force drops to zero (Abbott and Aubert, 1952; Herzog and Leonard, 1997; Morgan *et al.*, 2000). It can also predict that force depression is related to a proportional decrease in the number of attached cross-bridges, and thus muscle or fibre stiffness (Sugi and Tsuchiya, 1988; Lee and Herzog, 2003). Therefore, force depression has been thought to be caused by an inhibition of cross-bridge attachment following shortening during contractions (Maréchal and Plaghki, 1979). However, it is not clear if all cross-bridges (weakly and strongly bound states) contribute to force depression equally, or if force depression affects specific states in the cross-bridge cycle.

The purpose of this study was to investigate whether force depression affects weakly and strongly bound cross-bridges to the same degree or different degree. Since force depression is known to depend on the force during shortening (Herzog and Leonard, 1997; De Ruiter *et al.*, 1998), we hypothesized that active shortening of fibres inhibits cross-bridge attachments, primarily for cross-bridges that are initially in strongly-bound states. In order to address this specific hypothesis, tests were performed with normal fibres and fibres exposed to 2,3-butanedione monoxime (BDM) which biases cross-bridges towards the weakly bound state (i.e. increased ratio of the weakly to strongly bound cross-bridges, (Horiuti *et al.*, 1988; Bagni *et al.*, 1992)). Hereafter, single fibres tested in Ringer's solution with addition of BDM are called BDM exposed fibres, while fibres in a normal Ringer's solution are called normal fibres. If our hypothesis is correct, then the amount of force depression (relative to the isometric reference forces corresponding to fibre conditions) should be the same in the normal and BDM exposed fibres, whereas decrease in stiffness should be smaller for the BDM exposed fibres compared to the normal fibres.

## 7.2 Methods

### 7.2.1 Muscle fibre preparation

Nine single fibres of the lumbrical muscles (avg. 2.4mm in length, 0.02mm<sup>2</sup> in cross-sectional area) from frogs, *rana pipiens*, were used for all experiments.

### *7.2.2 Force and fibre length measurements*

Stimulation of the fibres (Grass S88, Grass Instruments) was achieved through two platinum wire electrodes that were placed inside the chamber parallel to and at a distance of about 3mm from the fibre. For complete activation, square wave pulses (0.4 ms duration) at an amplitude of 25% above the voltage that gave maximal force (range in this experiment 50-80V) were given. The frequency of stimulation was chosen individually for each fibre to induce a fused contraction at the lowest possible frequency (30-40Hz). Fibre lengths and diameters were measured before testing with a calibrated eyepiece (error<0.02mm). In order to determine cross-sectional area, fibres were assumed ellipsoidal in cross-section. The two major axes of the ellipsoid were determined as the smallest and largest diameters measured by rotating the fibre. Cross-sectional areas were determined at five evenly distributed locations along the fibre, and the average of those five values was defined as the cross-sectional area of the fibre.

### *7.2.3 Experimental procedures*

The initial procedures were identical to those described in Chapter 6 of this thesis.

Each experimental set consisted of three individual test contractions: the first contraction was an isometric reference contraction at a final length corresponding to the length following the shortening step in the shortening contractions; the second contraction was a shortening contraction in which the fibre was shortened from an initial length (i.e. the length before the fibre shortening) to the final length; and the third contraction was a

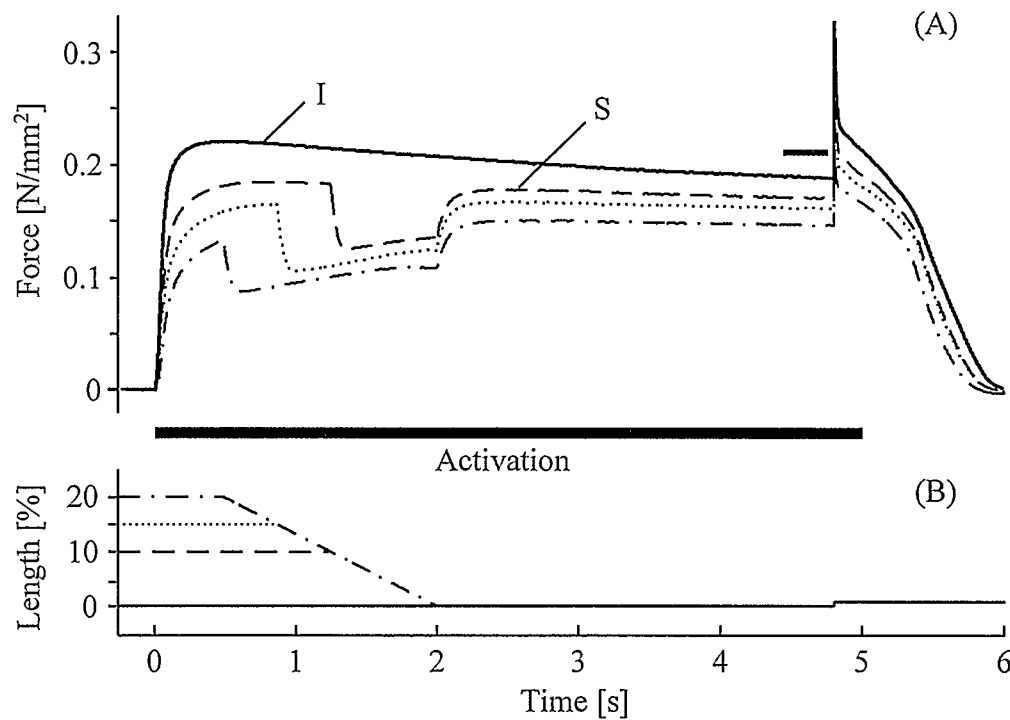


repetition of an isometric reference contraction at the final length. The final length for all tests in the present study was the optimal length. The shortening magnitude was 10% of optimal fibre length, and shortening speed was 13% fibre length/s. Following these tests, a second set of experiments, identical to the first one, except with a shortening magnitude of 15%, was performed (Figure 7-1). A third set with a shortening magnitude of 7.5% (n=3) or 20% (n=2) was performed for selected fibres.

Stiffness in the isometric reference and the shortening test contractions was determined with a quick stretch (20 $\mu$ m in 1ms) just before deactivation (Figure 7-1). Each contraction lasted for 5s, and contractions were separated by a 6 minute rest interval. For the test contractions, fibres were held isometrically at the final lengths for 3s following shortening (Figure 7-1). Once fibres had been tested in normal Ringer's solution, the protocol was repeated in Ringer's solution containing 7.5mM BDM, and then again in normal Ringer's solution. If fibres showed any sign of damage or if the isometric force at optimal length decreased by more than 10% from its initial value at any time, testing was stopped, and the fibre and all results were discarded. Force, fibre length, and sarcomere length were recorded at 10kHz, and stored on a computer for off line analysis.

#### ***7.2.4 Data analysis***

Steady-state forces were approximated as the median force over a 0.3s period starting at 4.5s following the onset of activation when most of the transient phase caused by shortening had subsided. Force depression was defined as the percent decrease in the



**Figure 7-1: Experimental procedures and force–time histories of the isometric reference and active shortening contractions for one representative fibre.** Exemplar force–time histories of experimental trials consisting of an isometric reference contraction (I, solid) and shortening test contractions (S, dashed, 10%; dotted, 15%; dash-dotted, 20% shortening amplitude) (A). The thick horizontal bar (short) in (A) indicates the 0.3s period over which force were measured. Also shown is a length–time history (B) for the experimental procedures. Quick stretches of fibres by  $20\mu\text{m}$  within 1ms were performed at 0.2s before deactivation for stiffness measurement. Note that forces are normalized relative to the cross-sectional area of fibre ( $\text{N/mm}^2$ ) and lengths are shown as relative length to optimal length (0%).

steady-state isometric force following shortening of activated fibres compared to the steady-state isometric reference force for purely isometric contraction at the corresponding final length. Stiffness depression was defined as the relative decrease in stiffness measured in the force-depressed state compared to that measured in the isometric reference state.

Force was normalized relative to the cross-sectional area of each fibre. Average stiffness was calculated as the change in force (i.e. normalized force) divided by the change in fibre length. A paired *t*-test was used to compare the isometric reference force and stiffness between normal and BDM exposed fibres. A two-way repeated measures ANOVA was used to determine the effect of BDM on force depression and stiffness depression with BDM and shortening magnitudes as main factors. All data are presented as means ( $\pm 1$ S.E.).

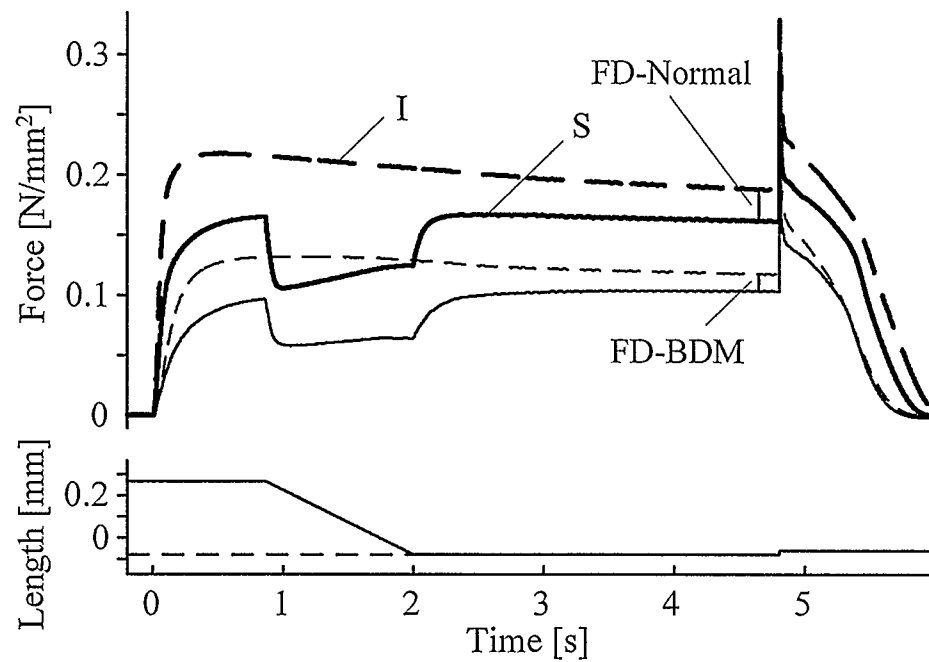
### **7.3 Results**

Exposure of fibres to BDM led to a decrease of 42% in isometric force and 25% in stiffness compared to values in the normal fibres (Table 7-1). Force depression was observed in all fibres, for every shortening magnitude, and in normal and BDM exposed fibres. Force depression increased with increasing magnitudes of shortening (Figure 7-1). Force depression was 12( $\pm 2.0$ )% and 11( $\pm 0.8$ )% in the normal and BDM exposed fibres, respectively (Figure 7-2 and Figure 7-3A).

Stiffness in the force depressed state was decreased compared to the isometric reference state for the both normal and BDM exposed fibres, however, this stiffness depression was smaller in the BDM exposed ( $6.7 \pm 1.5\%$ ) than the normal ( $17 \pm 1.6\%$ ) fibres (Figure 7-3B).

**Table 7-1: Mean( $\pm 1$ S.E.) force and stiffness for isometric reference contractions in normal and BDM exposed fibres.** Force and stiffness were significantly smaller in the BDM treated compared to normal fibres (\*;  $p < .01$ ). Percent decreases in force and stiffness measurements for the BDM exposed compared to the normal fibres were presented (change). The decrease in force was significantly greater than the decrease in stiffness (#;  $p < .01$ ).

Condition	Normal	BDM	Change <sup>#</sup> [%]
Force* [N/mm <sup>2</sup> ]	$0.13 \pm 0.01$	$0.08 \pm 0.01$	$42.2 \pm 1.4$
Stiffness* [N/mm <sup>3</sup> ]	$9.0 \pm 0.4$	$6.8 \pm 0.3$	$25.1 \pm 1.2$

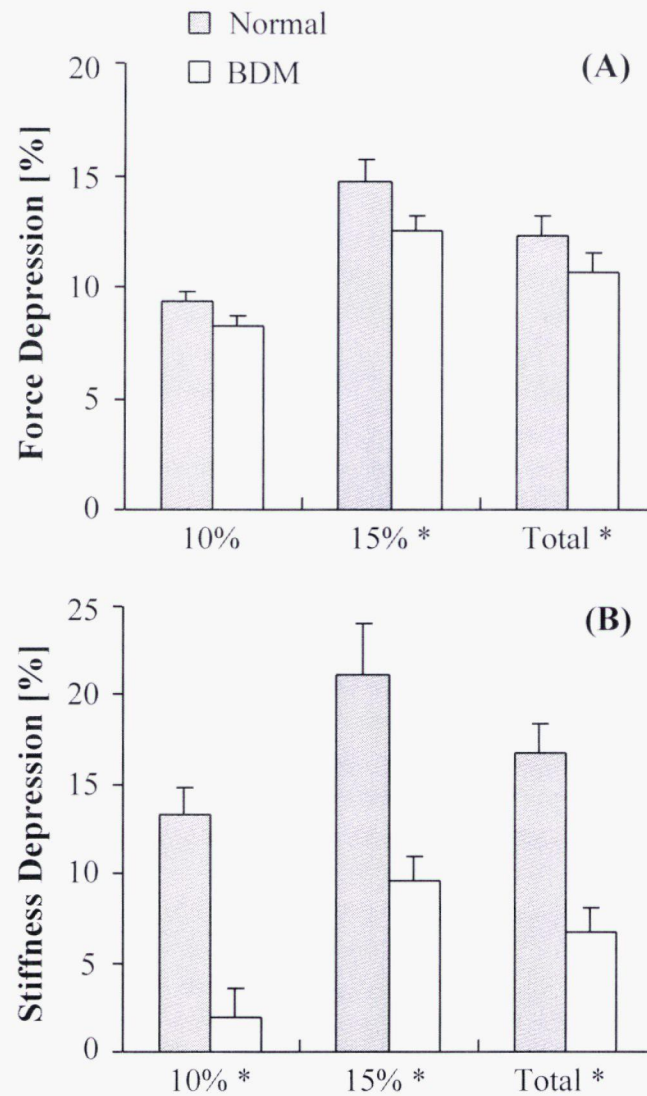


**Figure 7-2: Raw force and length vs. time traces of the isometric reference and active shortening contractions in the normal and BDM exposed fibres.** Force (top) and change in length (bottom) time histories of active shortening (solid, S) and isometric reference contractions at the corresponding final length (dashed, I) in the normal (thick) and BDM (thin) exposed condition for one representative fibre. Force depression in normal (FD-Normal) and BDM exposed fibres (FD-BDM) is indicated.

#### 7.4 Discussion

In this study, we investigated a residual force depression following shortening of single fibres during activation in the normal Ringer's solution and after the exposure to BDM. BDM is known to inhibit a phosphate release in the cross-bridge cycle (Herrmann *et al.*, 1992; Regnier *et al.*, 1995), and thus cross-bridges are delayed in their transition to strongly bound state. This gives a bias toward the weakly-bound (i.e. pre-power stroke) state. Therefore, the ratio of the weakly to strongly bound cross-bridges is increased in the BDM exposed fibres compared to the normal fibres. Since the strongly bound cross-bridges are thought to contribute primarily to active force production (Tesi *et al.*, 2002), active force was expected to be lower in the BDM exposed fibres compared to the normal fibres. Isometric force was decreased by  $42(\pm 1.4)\%$  while stiffness was decreased by  $25(\pm 1.2)\%$  in the BDM exposed fibres compared to the normal fibres (Table 7-1). Thus, these results confirmed our assumption that the ratio of the weakly to strongly bound cross-bridges is greater in the BDM exposed fibres than that in the normal fibres.

Although active force production was depressed by 42% in the BDM exposed fibres, force depression following active shortening in the normal (12%) and BDM exposed fibres (11%) were very similar (Figure 7-2 Figure 7-3A). This small difference between the relative force depression in normal and BDM exposed fibres was observed systematically for all nine fibres, and therefore, the observed decrease in force depression for the BDM exposed compared to normal fibres was statistically significant. In spite of



**Figure 7-3: Force depression and stiffness depression following active shortening in the normal and BDM exposed fibres.** A) Force depression at 10% and 15% of shortening magnitudes, and total were shown. At 15% of shortening magnitude and total, statistically significant decreases in BDM exposed fibre (white) compared to normal (grey) fibre were shown (\*,  $p < .01$ ), B) Stiffness depression for normal (grey) fibres was significantly greater than that for BDM (white) exposed fibres (\*,  $p < .01$ ) at 10% and 15% of shortening magnitudes, and total.

the statistically significant difference, it should be noted that BDM caused a much greater difference in the active force production, compared to its effect on force depression.

Stiffness were measured in the force-depressed and isometric reference states in order to determine whether force depression is associated with a decrease in the proportion of attached cross-bridges or a decrease in the average force per cross-bridges. Two previous studies (e.g., Sugi and Tsuchiya, 1988; Lee and Herzog, 2003) have concluded that stiffness was decreased in parallel with the amount of force depression. Specifically, the result of Lee and Herzog (2003) using human *adductor pollicis* showed that about 10% of force depression was accompanied by a decrease of about 15% in stiffness (Figure 12. in Lee and Herzog, 2003). In this study, stiffness was  $17.0(\pm 1.2)\%$  and  $6.7(\pm 1.1)\%$  lower in normal and BDM exposed fibres, respectively (Figure 7-3B). Since both normal and BDM exposed fibres showed stiffness depression, our results confirmed the idea that residual force depression might be associated with a decrease in the proportion of attached cross-bridges. This is a key prediction of the proposed mechanism for force depression by Maréchal and Plaghki (1979) which is that force depression may be caused by shortening-induced inhibition of attached cross-bridges.

Furthermore, here, it was observed that stiffness depression was much less in BDM exposed fibres ( $6.7\pm 1.1\%$ ) than that in normal fibres ( $17.0\pm 1.2\%$ ). Together with our assumption that the BDM exposed fibres have a greater proportion of the cross-bridges in the weakly bound state compared to the normal fibres, force depression might be related



more to the strongly bound cross-bridges than weakly bound cross-bridges. Therefore, if this shortening-induced inhibition of cross-bridge attachment theory is correct to account for the residual force depression, our finding suggests that active muscle shortening might inhibit cross-bridge attachment not to the same degree for the both weakly and strongly bound cross-bridges. It might primarily affect the strongly bound cross-bridges while having less affect on the weakly bound cross-bridges.

## **Chapter Eight: Summary and future direction**

### **8.1 Summary**

History-dependent force production is a property that is observed on all structural levels of skeletal muscle. However, the cross-bridge theory of muscle contraction cannot predict these stretch or shortening effects on force production. Despite a wealth of literature on residual force enhancement and force depression dating back more than half a century, the mechanisms underlying these properties remain unknown. There are a few mechanisms aimed at explaining the history-dependent properties of skeletal muscle, but there is no clear, well-defined, and comprehensive mechanism that is accepted by most of the scientists working in this area.

The general purpose of this thesis was to obtain additional insight into history-dependent properties of muscle force production and elucidating the mechanisms underlying these phenomena.

The specific purposes of this thesis were as follows:

1. To determine if forces in the enhanced state can exceed the isometric steady-state forces on the plateau of the force-length relationship.
2. To quantify the effects of activation and stretch on the passive force–sarcomere length relationship.
3. To determine the origin of the passive force enhancement.

4. To test the hypothesis that force enhancement is caused by a stretch-induced increase in the ratio of strongly to weakly bound cross-bridges.
5. To test the hypothesis that force depression is caused by a shortening-induced inhibition of the strongly bound cross-bridges.

In Chapter 3 of this dissertation, a critical prediction of the sarcomere length non-uniformity theory (force enhancement can not exceed the force on the plateau region of the force-length relationship) was tested. Average sarcomere lengths were measured simultaneously with force in order to identify the plateau region. Fibres were stretched 10% of fibre length at or near the plateau of the force-length relationship. Forces in the enhanced state were found to exceed the isometric reference forces on the plateau of the force-length relationship by, on average, 5%. The prediction of the sarcomere length non-uniformity theory, thus, was rejected and force enhancement was thought to be at least in part associated with the recruitment of additional contractile force.

It is also accepted by now that a passive structural component contributes to the residual force enhancement. However, neither the origin nor the mechanism of this passive contribution is understood. In chapter 4, the passive forces and sarcomere lengths were measured for three experimental conditions: (i) following purely isometric contractions, (ii) following active stretch, and (iii) following passive stretch. Passive forces following the isometric contractions and passive stretching were identical, while they were greater following active stretching, thereby providing evidence that the passive force

enhancement is associated with a structural element whose stiffness was changed by activation and stretch.

Passive force enhancement has been shown to be partly associated with an increase in the stiffness of the structural protein “titin”. However, it also appears that not all of the passive force enhancement can be explained in this way. In Chapter 5, an attempt was made to test if the passive force enhancement was also linked to the contractile proteins, Active force production was altered by using different concentrations of BDM. Passive force enhancement was found to increase with decreasing active forces, suggesting that passive force enhancement is related to the active component of force production.

As shown in Chapters 3, 4, and 5, it is likely that neither the sarcomere length non-uniformity theory, nor the engagement of passive elements, is able to exclusively explain residual force enhancement. Maybe a new or more developed explanation of the origin of force enhancement is required. Therefore, in Chapter 6, the idea of additional contractile force was explored, specifically the idea that force enhancement was achieved by an increase in the average force per cross-bridge. One way to achieve this could be by a stretch-induced shift towards strongly bound cross-bridge states. In order to test this idea, fibres with different ratios of strongly to weakly bound cross-bridges were tested with the expectation that if the initial state was biased towards weakly bound cross-bridges, force enhancement would be greater. Lowering the temperature of fibres is one way to affect the ratio of weakly to strongly bound cross-bridges, therefore, force enhancement was

determined for fibres kept at 7°C and 20°C. It was found that force enhancement was about 50% greater at 7°C than at 20°C, supporting the idea, that force enhancement is caused by a stretch-induced increase in strongly bound cross-bridges.

In Chapter 7, experiments were performed to gain further insight into force depression following active shortening. Maréchal and Plaghki (Maréchal and Plaghki, 1979) had suggested that force depression might be caused by an inhibition of cross-bridge attachment to actin. Since force depression is known to depend on the force during shortening, we hypothesized that force depression might be caused by inhibition of the strongly bound cross-bridges exclusively. The ratio of weakly to strongly bound cross-bridges was modified with BDM which inhibits the formation of strongly bound cross-bridges in a dose-dependent manner. Normal fibres and fibres exposed to BDM had the similar force depression, and this force depression was accompanied by a decrease in stiffness. However, the decrease in stiffness was much lower in the BDM exposed fibres compared to the normal fibres. Hence, it seems likely that strongly bound cross-bridges might be inhibited, and so contribute to the observed force depression, while weakly bound cross-bridges remain largely unaffected.

## **8.2 General conclusion**

Based on the results obtained in this thesis, it appears unlikely that the sarcomere length non-uniformity theory plays a major role either in force enhancement or force depression. Rather, we conclude that force enhancement is caused by an active and a passive

component. The passive component, likely, is associated with an increase in force by titin, while the active component is more likely to be associated with an increase in the average force per cross-bridge. Force depression is associated with a decrease in stiffness and thus appears to be caused by a decrease in the proportion of attached cross-bridges. We suggest that this inhibition preferentially affects strongly bound cross-bridges. It was found in this thesis that force enhancement has both an active and a passive component and therefore force enhancement may not be explained by a sole mechanism rather at least two mechanisms may be required to account for force enhancement.

As shown in Chapter 3, a critical prediction of the sarcomere length non-uniformity was rejected and sarcomere lengths in the force-enhanced state were stable. Previous literature has shown a number of studies that demonstrate that the force enhancement is found on the ascending limb of the force-length relationship (e.g. Peterson *et al.*, 2004). Therefore, the sarcomere length non-uniformity theory might not be applicable to the active mechanism for force enhancement. In Chapter 3 of this thesis, it was found that force enhancement was observed to be greater than the maximal isometric force at the plateau of the force-length relationship and suggested that an additional contractile force has been recruited. Thus this study implied that force enhancement may be a property of the cross-bridge action rather than the sarcomere or muscle structural level. In addition, the above conclusions are strengthened by a recent publication that showed force enhancement in single myofibrils and single sarcomeres (Joumaa *et al.*, 2008b).

At the cross-bridge level, there are two conceptual possibilities to cause the increase in force; increased number of cross-bridges and increased average force per cross-bridge. As indicated in previous literature (Sugi and Tsuchiya, 1988) and in Chapter 6 of this thesis, stiffness in the force-enhanced state is same as one in the isometric reference state. The results indicate that there is no increase in the number of attached cross-bridges in the force-enhanced state compared to the isometric reference state; the force enhancement seems to be caused by an increased average force per cross-bridge. Increasing the proportion of the strongly bound cross-bridges is one way to increase average force per cross-bridge if everything else remains constant. It is proposed that an increase in the proportion of the strongly bound cross-bridges could be achieved if the active stretch facilitated a transition from weakly to strongly bound cross-bridges.

Initial evidence supporting the theory described above was that the force enhancement was increased with an increasing concentration of BDM which inhibited the active force production in a dose-dependent manner through blocking phosphate release (Rassier and Herzog, 2004b). That is, greater force enhancement was observed when the ratio of weakly to the strongly bound cross-bridges was increased. In Chapter 6, the observation showing that force enhancement was increased at lower temperatures supports the mechanism described above because it is assumed that by lowering temperatures the cross-bridges are biased towards the weakly bound state.

In Chapter 5 of this thesis, it was shown that the half-relaxation time was increased in the force-enhanced state compared to the isometric reference state. Also, results in this chapter indicated that increased force enhancement was closely related to an increase in half-relaxation time which implies a slower cross-bridge detachment after deactivation. Since the cross-bridge detachment rate is thought to be greater for the cross-bridge in the strongly bound state compared to the weakly bound state, this result would suggest that a greater proportion of strongly bound cross-bridges might be induced by active stretch.

A detailed mechanism to explain how stretch facilitates a transition from a weakly to a strongly bound cross-bridge is not yet known. It could be speculated that the stretch might induce phosphate release from the actomyosin complex, thus leading a transition from a weakly to a strongly bound cross-bridge. As mentioned in the literature review of this thesis, the phosphate release step initiates the conformational change of cross-bridges (Rayment *et al.*, 1993), which then will switch into a strongly bound state. However, there is no direct evidence to support this theory, therefore further investigation is needed.

An additional mechanism has been used to explain the increase in the proportion of strongly bound cross-bridge, namely that it is the decrease in the rate of adenosine diphosphate (ADP) released from the actomyosin complex. It could be speculated that a stretch during muscle activation may lead to a slower ADP release from the actomyosin complex, thus allowing the cross-bridges to increase the time spent in the strongly bound state. Therefore, the proportion of strongly bound cross-bridges might increase following



active stretch. It was proposed that the ADP release may be kinetically blocked under strain, thereby decreasing this ADP release rate (Smith and Geeves, 1995; Geeves *et al.* 2000; Nyitrai and Geeves, 2004). It was also suggested that the strain induced slower ADP release might prolong the duty ratio occupied by the strongly bound cross-bridges, thereby contributing to the economical force production (Cremo and Geeves, 1998; Khromov *et al.*, 2004). Also the greater duty ratio was observed with mechanical strain in smooth muscle myosin (Veigel *et al.*, 2003). An increased duty ratio of cross-bridges indicates a prolonged cross-bridge attachment time relative to the total cross-bridge cycle time. These studies support the idea of the stretch-induced decrease in the ADP release rate which might cause an extended cross-bridge attachment time in the strongly bound state.

Back in 1964, Infante *et al.* observed with frog muscle that ATP hydrolysis in contractions with stretch is less than that in an isometric tetanus. Thus they concluded that the effect of stretch is to reduce the rate of ATP hydrolysis. Recently the ADP release rate appears to determine the rate of ATP hydrolysis and the maximum velocity of movement by the molecular motors (Siemankowski *et al.*, 1985; Weiss *et al.*, 2001). Together, the reduced ATP hydrolysis rate through active stretch might be caused by the delay of ADP release from the actomyosin complex.

This concept might explain, at least in part, experimental results in Chapter 5 of the thesis. The half-relaxation time after deactivation was increased in the force-enhanced state

compared to the isometric reference state. Furthermore, it appeared that the half-relaxation time was positively related with the amount of force enhancement and passive force enhancement (i.e., the half-relaxation time was increased when BDM was treated). If there are more cross-bridges in the strongly bound state, it would take more time for relaxation after deactivation. Therefore, an observation of a longer relaxation time following active stretch might be explained by the stretch-induced decrease in the ADP release rate.

In summary, it could be suggested through this thesis that force enhancement might be caused by the increase in the proportion of strongly bound cross-bridges. However, this thesis was unable to provide a detailed mechanism since the different states of cross-bridges were not observed directly and there is no direct measurement of the proportion of weakly and strongly bound cross-bridges.

In conjunction with previous literature (e.g. Herzog and Leonard, 2002), our observations in Chapter 4 and 5 of this thesis show an increase in passive force following stretch of activated muscle or fibre. It is likely a part of force enhancement and might originate from passive elements. However, as shown in Chapter 3, total force enhancement was much greater than the observed passive force enhancement, thus this passive mechanism would cover only small portion of total force enhancement, especially at the sarcomere length range at or near the plateau of the force-length relationship. In contrast, the contribution of the passive mechanism might be increased with increasing

sarcomere lengths since a substantial increase of passive force enhancement was observed at longer sarcomere lengths in Chapter 4 and 5 of this thesis.

With the passive mechanism, force enhancement might be explained by the increase in stiffness of the molecular spring titin through stretch during activation. The PEVK region of titin was shown to have an elastic property like a spring, and this elastic property seems to be stiffer by the activation or an increase in calcium concentration (Labeit *et al.*, 2003). An observation of passive force enhancement in the myofibril preparation strongly supports this mechanism because in this preparation except for titin most of the passive elements were removed (Joumaa *et al.*, 2007). However it was also found that the existence of passive force enhancement required not only high calcium concentration but also active force production or cross-bridge interaction (Joumaa *et al.*, 2008a). Therefore, a new concept of the passive mechanism through this thesis appears that at least a part of passive force enhancement might be explained by the remaining attached cross-bridges in the strongly bound states or the so-called ‘latch’ states after deactivation.

Overall, both active and passive mechanisms might play a role for force enhancement; however, there would be more of an effect of the active mechanism for force enhancement at shorter sarcomere lengths ranges where the proportion of attached cross-bridges is relatively greater. While at the range of long sarcomere lengths where a substantial amount of passive forces are naturally occurring, the passive mechanism might play a more important role than the active mechanisms.

Sarcomere length non-uniformity theory was used trying to explain force depression as well as force enhancement. However the decrease in stiffness in the force-depressed state observed in Chapter 7 of this thesis was controversial according to the prediction of this theory. Based on previous literature and findings in this thesis, in general, force depression might be associated with a decreased proportion of attached cross-bridges rather than a decreased average force per cross-bridge. The distortion of the actin filaments during active shortening might inhibit, at least in part, the cross-bridge attachment, but it primarily affect the cross-bridge in the strongly bound states. The results observed here and in previous literature support this idea as the force and work during shortening were closely related with the amount of force depression. This implied that cross-bridges in the force-generating step might play a significant role in determining the amount of force depression, thus the mechanism for force depression. However, as only one study had been done in this thesis regarding force depression, it is limited informing a general conclusion from theses results.

### **8.3 Future direction**

In order to have a comprehensive understanding of the mechanism of muscle contraction and force production, the mechanism which causes history-dependent force production in skeletal muscle must be considered. Despite an abundance of literature regarding this property of muscle contraction, the mechanisms explaining these phenomena are poorly understood. In this dissertation, some of the currently proposed mechanisms for force enhancement and force depression were evaluated, and further insights into the possible explanation of these phenomena were presented. Although the current studies in this

dissertation suggest a mechanism for force enhancement and depression, further studies are required to assess current theories and to clearly identify the correct mechanism.

Overall, it was unable to confirm the proposed mechanism in this thesis because direct observations of the proportion of attached cross-bridges or the ratio of the weakly to strongly bound cross-bridges were missed. However it is difficult to measure the distribution of different states of cross-bridges in experiments at the single fibre or myofibril level. The X-ray diffraction study in single fibre preparation provides information about structural changes, specifically; this could indicate whether myosin heads have a more perpendicular tilt to the filament axis (Linari *et al.*, 2000; Reconditi *et al.*, 2004). Therefore, to detect this structural change in cross-bridges may give some evidence of the transition of cross-bridge states. The X-ray diffraction pattern in the force-enhanced or depressed state can be compared with that from the purely isometric contraction to know if force enhancement or depression might be caused by the changes in the ratio of strongly to weakly bound cross-bridges. For example, in the case of force enhancement, it is expected that the intensity of the axial M3 reflection increases which indicates that the myosin heads have moved towards the centre of the sarcomere (i.e. strongly bound cross-bridges). Although this study cannot give a direct measure of the proportions of weakly and strongly bound cross-bridges, evidence based on structural changes would be helpful to understand the molecular mechanism of force enhancement and force depression.

Instead of BDM, other myosin inhibitors used to alter the ratio of weakly to strongly bound cross-bridges can be used to verify the observations in this thesis. For example, blebbistatin is known to slow down phosphate release in skeletal muscle myosin II, and thus induce the decrease the isometric forces (Kovacs *et al.*, 2004). While BDM and blebbistatin are non-competitive myosin inhibitors, vanadate is a phosphate analog that inhibits force by preventing cross-bridge transition into strongly bound states, thus increasing the proportion of cross-bridges in the pre-power stroke position (Dantzig and Goldman, 1985; Goodno, 1982; Martyn *et al.*, 2007). To strengthen the general conclusion made here, the amount of force enhancement should increase with increasing concentrations of those myosin inhibitors as found in experiments using BDM. Furthermore, using the myosin inhibitors, the isometric force may be completely depleted while all cross-bridges are assumed to be in the weakly bound state. In this situation, if stretch during activation induces force enhancement, this would be strong evidence that force enhancement may be caused by a transition from weakly to strongly bound cross-bridges through active stretch.

In addition further studies should aim to provide evidence of the stretch-induced decrease in ADP release from cross-bridges because it was speculated that force enhancement might be caused by the delay of ADP release. Therefore the strain-dependent rate constant of ADP or phosphate release has to be investigated in the single skeletal myosin II interaction. As speculated, in order to test that active stretch causes cross-bridges to remain in the strongly bound state longer or leads to some kinds of ‘larch’ state of cross-

bridges, dissociation time of a single myosin molecule from actin should be studied when the attached cross-bridges are pulled compared to one without a pulling.

It has been suggested that some cross-bridges are stuck in the new cross-bridge state which produces force but will not to detach easily (i.e. so-called 'latch' state). Thus it was thought that those cross-bridges affect part of the increased passive force after deactivation. To support this argument, stiffness has to be measured in the passive state to estimate the proportion of detached or attached cross-bridges after deactivation, although it will still not provide direct evidence. Regarding studies using BDM, it was largely understood that BDM has little influence on  $\text{Ca}^{2+}$  release from sarcoplasmic reticulum in intact skeletal muscle fibres (Horiuti *et al.*, 1988; Sun *et al.*, 2001). In contrast, it was observed that  $\text{Ca}^{2+}$  concentration was suppressed when [10mM] BDM was applied (Horiuti *et al.*, 1988). Therefore, to identify any effect of  $[\text{Ca}^{2+}]$  with stretch it would be better to measure  $\text{Ca}^{2+}$  transient using aequorin injection into a fibre. In order to implicate the role of titin in passive force enhancement and increase the physiological relevance of the findings, the increased force-extension curve at higher  $\text{Ca}^{2+}$  concentrations compared to lower  $\text{Ca}^{2+}$  concentrations is expected when purified whole titin molecule is stretched.

Lastly, this thesis concluded that the sarcomere length non-uniformity is not sole mechanism for either force enhancement or force depression. Thus, to completely rule out the sarcomere length non-uniformity theory, examination of the behaviour of individual sarcomeres simultaneously with force measurement should be performed.

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