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COMMENTARY

The role of titin in eccentric muscle contraction

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ABSTRACT

Muscle contraction and force regulation in skeletal muscle have been thought to occur exclusively through the relative sliding of and the interaction between the contractile filaments actin and myosin. While this two-filament sarcomere model has worked well in explaining the properties of isometrically and concentrically contracting muscle, it has failed miserably in explaining experimental observations in eccentric contractions. Here, I suggest, and provide evidence, that a third filament, titin, is involved in force regulation of sarcomeres by adjusting its stiffness in an activation-dependent (calcium) and active force-dependent manner. Upon muscle activation, titin binds calcium at specific sites, thereby increasing its stiffness, and cross-bridge attachment to actin is thought to free up binding sites for titin on actin, thereby reducing titin's free-spring length, thus increasing its stiffness and force upon stretch of active muscle. This role of titin as a third force regulating myofilament in sarcomeres, although not fully proven, would account for many of the unexplained properties of eccentric muscle contraction, while simultaneously not affecting the properties predicted by the two-filament cross-bridge model in isometric and concentric muscle function. Here, I identify the problems of the two-filament sarcomere model and demonstrate the advantages of the three-filament model by providing evidence of titin's contribution to active force in eccentric muscle function.

KEY WORDS: Cross-bridge theory, Eccentric contraction, Force enhancement, Instability, Popping sarcomere hypothesis, Sarcomere, Titin

Introduction

Eccentric muscle contractions are defined as contractions in which an active muscle is stretched. Because muscles tend to shorten upon activation, eccentric contractions occur when the external forces acting on a muscle are greater than the forces produced by the muscle. Although eccentric contractions may be thought of as an anomaly, they occur during most everyday movements. For example, when descending a set of stairs, our knee extensor muscles are active to control the movement, but only to a degree that produces forces that are smaller than the forces acting on the muscle by gravity as we walk down the stairs.

While isometric (constant muscle length) and concentric contractions (muscle is actively shortening) are reasonably well described and explained by current theories of muscle force production (Huxley, 1957), eccentric contractions are not. Andrew Huxley, who first formulated the cross-bridge theory of contraction (the current paradigm for the molecular mechanism underlying muscle activation and force production), realized this when forces and energy requirements of eccentric contractions predicted by his

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theory vastly exceeded the experimentally observed values (Hill, 1938; Abbott and Aubert, 1951; Joumaa and Herzog, 2013). In his book 'Reflections on Muscle', while discussing eccentric contractions, Huxley (Huxley, 1980) stated that: 'Elongations of active muscle is something that happens in very many movements' and that 'I imagine that special features have been evolved which allow this elongation to take place without damaging the muscle' and further that 'these special features have little relation to the processes that take place during shortening.' He then went on to describe that eccentric muscle contractions comprise a wide and difficult field for investigation that will hold a number of surprises. Here, I will attempt to discuss some of the difficulties associated with eccentric muscle contractions, the special features that have evolved to limit damage, and explain some of the surprising features of actively lengthening muscles.

Mechanisms of contraction

Prior to the 1950s, it had been assumed that muscle activation and force production were associated with the shortening of the thick filaments in the centre of sarcomeres. It was thought that activation (calcium release) caused the myosin filaments that comprise the thick, A-band filaments to be transformed from a helix-like configuration into a coil-like configuration (Fig. 1), thereby causing muscle shortening and force production. Specifically, the long myosin filaments that were aligned in long strands were thought to undergo calcium-induced shortening at specific points, thereby producing muscle activation. However, Hugh Huxley hinted at the idea that thick filaments did not shorten upon activation (Huxley, 1953), and he and Andrew Huxley then published their seminal work on the sliding filament theory (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954) where they argued independently that thick filaments did not shorten. Rather, they suggested that muscle contraction occurred through the relative sliding of the thick and thin filaments (Fig. 1). Three years later, Andrew Huxley then provided the first molecular and mathematical framework of how this relative sliding of the two sets of filaments was supposed to occur: the cross-bridge theory (Huxley, 1957). His theory was based on the idea that there are uniformly arranged side projections (cross-bridges) on the thick filaments that interact cyclically with specific attachment points on the thin filaments, thereby pulling the thin past the thick filaments. These interactions by the cross-bridges were driven partially by Brownian motion and partially by the hydrolysis of adenosine triphosphate (ATP), one ATP per cross-bridge cycle. The theory proposed in 1957 had two cross-bridge states, one attached and one detached. Hugh Huxley then proposed that cross-bridge action likely involved rotation (Huxley, 1969), a concept adapted by Andrew Huxley and extended to multiple attachment states (Huxley and Simmons, 1971). This multiple-state model had the advantage that it could explain force transients following quick stretches or releases. Although cross-bridge models with more than 20 states have been described in the literature, commonly cross-bridge action and muscle contraction are described by models that

Glossary

Actin
Contractile protein in the sarcomere comprising the main component of thin filaments.

Active force
Force in muscle produced through the interaction of the contractile filaments actin and myosin, and powered by ATP.

A. F. Huxley
Muscle physiologist (1917–2012) who formulated the first mathematical description of the cross-bridge theory in 1957.

Ascending limb
The ascending limb of the force–length relationship describes the region of muscle function in which isometric force increases with increasing muscle length.

A. V. Hill
Muscle physiologist who popularized the force–velocity property of skeletal muscle and introduced the notion of muscle instability on the descending limb of the force–length relationship.

Butanedione monoxime
Inhibits cross-bridges from going from the weakly to the strongly bound state in the cross-bridge cycle.

Cross-bridge
Protein side piece emanating from the thick (myosin) filament that attaches to actin in a cyclic manner.

Cross-bridge theory
The current paradigm of muscle contraction. It states that muscle contraction occurs through the interaction of myosin-based cross-bridges that cyclically attach to specific binding sites on actin, thereby producing a relative sliding of actin past myosin that is driven by the hydrolysis of ATP.

Concentric shortening
A concentric muscle contraction is one in which muscle shortens during contraction.

Descending limb
The descending limb of the force–length relationship describes the region of muscle function in which isometric force decreases with increasing muscle length.

Eccentric lengthening
An eccentric muscle contraction is one in which muscle is stretched while activated.

Fascicle
Segment of muscles containing fibres that is enclosed by a distinct connective tissue, the perimysium.

Fibre
An elongated, multinucleated cell.

Force–length relationship
Muscle property that describes the dependence of maximal, isometric force on the length of the muscle.

Force–velocity relationship
Muscle property that describes the dependence of maximal force at optimal muscle length on the speed of muscle contraction.

H. E. Huxley
Muscle physiologist (1924–2013) who first described the sliding filament theory in 1953.

Ig domain
Immunoglobulin domain of titin occurring at the proximal and distal ends of titin.

Isometric
Without change in length; an isometric muscle contraction is one in which muscle length does not change.

M-band/line
Protein band in the middle of two half-sarcomeres.

Myofibril
A sub-cellular organelle comprised of serially arranged sarcomeres.

Myofilament
Long muscle proteins in sarcomeres, such as actin, myosin and titin.

Myosin
Contractile protein in the sarcomere comprising the main component of the thick filament.

N2A region
A unique sequence within titin thought to be a possible binding site for titin with actin.

Passive force
Force in muscle not requiring energy (ATP) and primarily produced by the structural protein titin on the myofibrillar level and also by collagen-based connective tissue structures on the fibre, fascicle and muscle levels.

Passive force enhancement
Muscle property associated with the increase in passive force following active muscle stretching.

PEVK region
A unique sequence within titin that is rich in proline, glutamate, valine and lysine residues.

Plateau region
The plateau region of the force–length relationship describes the region of muscle function in which isometric force remains the same, and is maximal, for small changes in length.

Residual force enhancement
Muscle property that describes the increase in steady-state isometric force following active muscle stretching compared with the corresponding purely isometric force.

Sarcomere
Smallest structural unit of contraction in skeletal muscle.

Sliding filament theory
Theory of contraction that supposes that muscle contraction occurs through the relative sliding of the two contractile proteins actin and myosin.

Thick filament
Sarcomeric contractile filament consisting primarily of myosin.

Thin filament
Sarcomeric contractile filament consisting primarily of actin, troponin and tropomyosin.

Titin
Structural protein in sarcomeres spanning the half sarcomere from the Z-line to the M-band.

Troponin
Regulatory protein in sarcomeres.

Tropomyosin
Regulatory protein in sarcomeres.

'x' distance
The distance of the cross-bridge equilibrium position to the nearest eligible attachment site on actin in the cross-bridge theory.

Z-line/band
Protein band at the end of a sarcomere.

have approximately five states: two detached (pre- and post-ATP hydrolysis) and three attached (ADP·P, ADP and empty nucleotide binding site) (Rayment et al., 1993) (Fig. 2).

The cross-bridge theory has been the paradigm for explaining the mechanisms of contraction for the past half century. It predicts many experimentally observed phenomena extremely well,
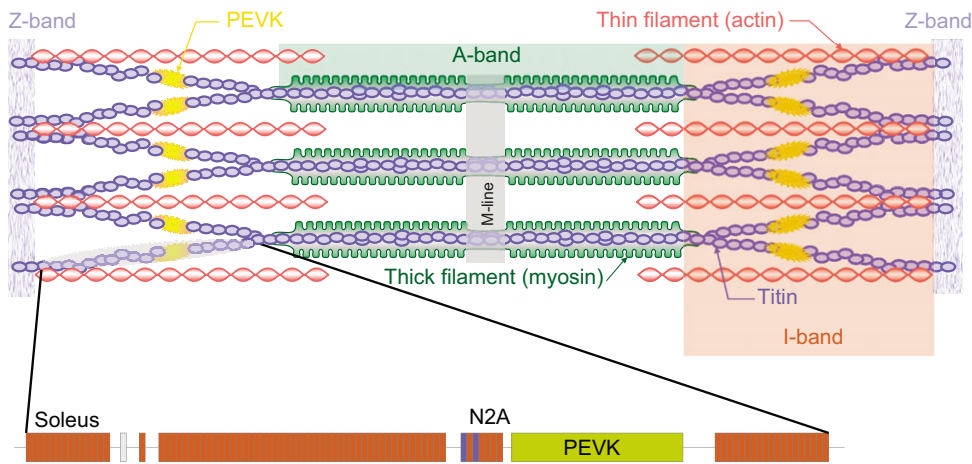
Fig. 1. Schematic figure of a sarcomere with the contractile proteins actin (thin filament) and myosin (thick filament), and the adaptable molecular spring titin, which stabilizes myosin in the centre of the sarcomere, provides most of the passive force in isolated sarcomeres and myofibrils, and changes its stiffness in an activation (calcium)- and force (cross-bridge attachment)-dependent manner.

particularly those involving isometric and concentric contractions, but fails to predict some of the basic and generally accepted properties of eccentrically contracting muscles (Pollack, 1990; Woledge et al., 1985). Some of these failures will be discussed in the next section.

Eccentric muscle contractions

Active lengthening of muscle (eccentric contraction) is associated with a variety of unique properties. Force is increased compared with isometric or concentric contractions (Hill, 1938), energy requirement is decreased, and forces following eccentric contractions are increased (Abbott and Aubert, 1952). Classic cross-bridge models have difficulties predicting the force and energy efficiency of eccentric contractions accurately (Woledge et al., 1985), and they cannot explain experimentally observed history-dependent properties of muscles (Walcott and Herzog, 2008).

Cross-bridge models can be thought of as a series of mechanical and/or biochemical cross-bridge states that are connected by thermodynamically consistent rate functions (Howard, 2001; Walcott and Herzog, 2008). By definition, these rate functions are exclusively based on Huxley's so-called 'x' distance, the distance of a cross-bridge's equilibrium position to its nearest eligible attachment site on actin (Fig. 2A). The cross-bridge's equilibrium position is defined as the location at which the cross-bridge's elastic element has zero force (it is neither stretched nor compressed), while its nearest attachment site is defined as the attachment site closest to the cross-bridge's equilibrium position in the direction towards the Z-line (Fig. 2A).Therefore, following a short transient phase following muscle shortening or stretching, all history-dependence disappears and muscle force is independent of the contractile history. However, it is generally acknowledged, and uniformly observed, that the steady-state forces following active muscle lengthening are increased compared with the corresponding forces not preceded by stretching. This so-called residual force enhancement (Edman et al., 1982) has intrigued muscle physiologists, as it defies explanation with current cross-bridge thinking, and its molecular origins have eluded satisfactory explanation. Below, I will attempt to summarize the observations surrounding residual force enhancement, identify competing mechanisms, and propose a solution that explains all hitherto observed observations, but has yet to be proven correct.
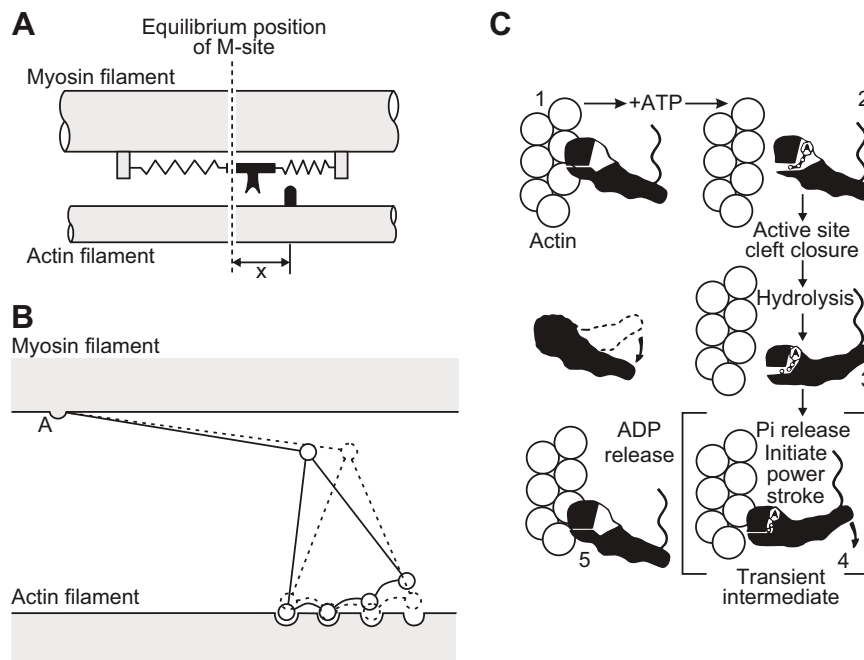

Fig. 2. Three classic cross-bridge models. (A) First cross-bridge model described by A. Huxley (Huxley, 1957) with two states, an attached and a detached state. (B) 1971 cross-bridge model with a rotating cross-bridge head and multiple attachment states (Huxley and Simmons, 1971). (C) Cross-bridge model proposed by Rayment et al. (Rayment et al., 1993) based on the atomic structure of the cross-bridge head and the actin attachment site. (Adapted and printed with permission). Pi, inorganic phosphate, i.e. the free phosphate released from ATP when ATP is hydrolysed to ADP.
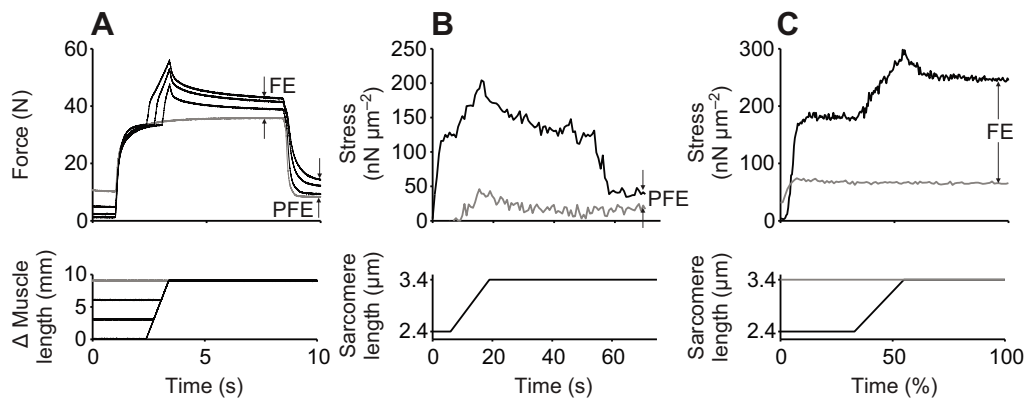
Fig. 3. Force enhancement on three structural levels of skeletal muscle. (A) Force enhancement (FE) in an entire muscle (cat soleus at 37°C), (B) in an isolated myofibril and (C) in a single, mechanically isolated sarcomere. Note also the passive force enhancement (PFE) (A,B). The grey trace in A represents the isometric reference force and length, while the black traces represent force enhanced states following three stretch magnitudes. The grey trace in B is a passive stretch while the black trace is an active stretch of a myofibril. Finally, in C, the grey trace is the isometric reference force and length, while the black trace represents the force-enhanced state following active stretching of a single sarcomere.

Residual force enhancement

When a muscle, fibre, myofibril or sarcomere is actively lengthened, the steady-state force following lengthening will be greater than the corresponding force for a purely isometric contraction (Fig. 3). This residual force enhancement has been observed at all structural levels of muscles (Herzog et al., 2012c), it increases with the magnitude of stretch (Edman et al., 1982; Herzog et al., 2006), is independent of the speed of stretch (Edman et al., 1982), is greater on the descending than the ascending limb of the force–length relationship (Morgan et al., 2000; Herzog et al., 2012a), is accompanied by an increase in passive force (Herzog and Leonard, 2002), and can be eliminated instantaneously when a muscle is deactivated (Abbott and Aubert, 1952). Despite an abundance of consistent observations on force enhancement, and agreement on the properties associated with force enhancement, a convincing and generally accepted mechanism for force enhancement has not been identified. However, three basic ideas have emerged. The first has been associated with the active force producing cross-bridges, the second with the development of structural non-uniformities (sarcomere length non-uniformities) when muscles are actively stretched on the descending limb of the force–length relationship, and the third with the engagement of passive structural elements.

Cross-bridge mechanism for force enhancement

Force enhancement following eccentric muscle action could be caused by an increased force transmission by the cross-bridges between actin and myosin filaments. According to the cross-bridge theory, there are two ways to increase cross-bridge based forces: (1) by increasing the proportion of attached cross-bridges, or (2) by increasing the average force per cross-bridge (or a combination of the two).

If force enhancement was caused by an increase in the proportion of attached cross-bridges, then residual force enhancement would be associated with a corresponding increase in muscle stiffness (Ford et al., 1981). However, in the few studies that systematically evaluated muscle stiffness in the force-enhanced state, stiffness was either unchanged from isometric reference contractions or just marginally increased (Sugi and Tsuchiya, 1988; Rassier and Herzog, 2005), suggesting that force enhancement is likely not associated with a substantial increase in the proportion of attached cross-bridges. However, findings of increased residual force enhancement in muscle fibres either at cold temperatures or treated with the cross-

bridge inhibitor butanedione monoxime suggest that in those experimental circumstances, force enhancement might be caused in part by a conversion of weakly to strongly bound cross-bridges (Rassier and Herzog, 2005; Lee and Herzog, 2008a), which would be expected to result in an increase in cross-bridge-based force without a corresponding increase in stiffness.

Force enhancement could also be caused by an increase in the average force per cross-bridge while the proportion of attached cross-bridges remains constant. Because residual force enhancement is long lasting [minutes in myofibrils, where this can be tested without the confounding effects of fatigue (Leonard et al., 2010)], this idea is rather unlikely, except if one proposed that cross-bridges remain attached for minutes following active muscle lengthening, a proposal that cannot be fully rejected at this time, but that seems rather unlikely. Furthermore, because force enhancement has been shown to average 285% in single sarcomeres (Leonard et al., 2010), cross-bridges would need to be extended to almost three times of their original length to account for such an increase in force. Again, this scenario has not been scientifically eliminated but is highly unlikely. Therefore, it appears that residual force enhancement cannot be explained easily with cross-bridge action, in accordance with theoretical considerations (Walcott and Herzog, 2008).

Structural non-uniformity mechanism for force enhancement

For more than three decades, the primary mechanism for force enhancement was associated with the development of structural non-uniformities, specifically the development of sarcomere length non-uniformities during active muscle lengthening on the descending limb of the force–length relationship (Fig. 4). This idea originated from the proposal that muscle segments and sarcomeres were thought to be unstable on the descending limb of the force–length relationship (Hill, 1953; Gordon et al., 1966) because of the presumed softening behavior of muscle on that part of its working range. In the meantime, it has been shown that sarcomeres are perfectly stable on the descending limb of the force–length relationship (Rassier et al., 2003a; Rassier et al., 2003b), and that the apparent softening behavior of muscle was an artifact of the static, rather than the dynamic, evaluation of the stiffness of muscles on the descending limb of the force–length relationship (Allinger et al., 1996).

The idea of development of sarcomere length non-uniformities and their effects on force enhancement were insofar appealing as
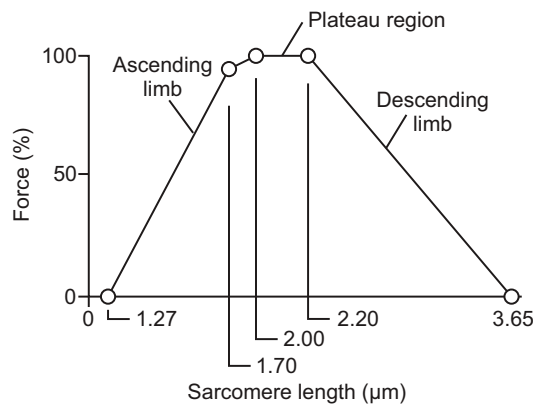
Fig. 4. Sarcomere force–length relationship, with the ascending and descending limb and the plateau region indicated. Adapted from Gordon et al. (Gordon et al., 1966) with permission.

this mechanism could explain force enhancement perfectly within the framework of the cross-bridge theory. Furthermore, the sarcomere length non-uniformity theory for force enhancement could be used to make testable predictions about muscle behavior following active lengthening. The most important of these predictions are that: (1) force enhancement is associated with the development of sarcomere length non-uniformities while isometric reference contractions are not; (2) force enhancement cannot occur on the ascending limb of the force–length relationship [as sarcomeres are stable in that region (Gordon et al., 1966; Hill, 1953)]; (3) forces in the enhanced state cannot exceed the maximal isometric forces at the plateau of the force–length relationship; and (4) force enhancement cannot occur in a single sarcomere, as by definition, multiple sarcomeres are required for sarcomere length non-uniformities to develop.

All four of these basic predictions of the sarcomere length non-uniformity theory have been rejected by findings in experimental studies, suggesting that the development of sarcomere length non-uniformities during active muscle lengthening on the descending limb of the force–length relationship is not a major contributor to residual force enhancement. Specifically, regarding the four predictions above, the following observations have been made: (1) forces in the enhanced state have been found to be associated with more stable (Edman et al., 1982) sarcomere properties and with similar or smaller non-uniformities than the corresponding purely isometric reference contractions (Joumaa et al., 2008a), suggesting that sarcomere length non-uniformities are present not only in the force-enhanced but also in the isometric reference states; (2) force enhancement has been observed on the ascending limb of the force–length relationships in muscles (Abbott and Aubert, 1952; Morgan et al., 2000) and single fibres (Peterson et al., 2004); (3) forces in the enhanced state can easily exceed the maximal isometric forces at the plateau of the force–length relationship in muscles (Abbott and Aubert, 1952; Schachar et al., 2002), fibres (Lee and Herzog, 2008b), myofibrils (Joumaa et al., 2008a) and single sarcomeres (Leonard et al., 2010); and (4) force enhancement occurs in single sarcomeres and produced forces that exceeded the isometric reference forces by almost a factor of three (Leonard et al., 2010).

In recent years, it has been argued that maybe it is not the sarcomere lengths that matter but the half-sarcomere lengths, and that the development of half-sarcomere length non-uniformities might produce the observed residual force enhancement observed

consistently in fibre and muscle preparations (Campbell, 2009). However, measurement of half-sarcomere non-uniformities in isolated myofibril preparations suggests that if anything at all, half-sarcomere non-uniformities are smaller in the force-enhanced compared with the purely isometric reference state (Joumaa et al., 2008a), thus eliminating that possibility. However, in recent experimental work on half-sarcomere dynamics in isolated myofibrils, it was argued that half-sarcomere non-uniformity was a major contributor to residual force enhancement (Rassier, 2012). Rassier showed maximal half-sarcomere length non-uniformities of 12 nm in single sarcomeres and 70 nm in myofibrils in the force-enhanced state. These half-sarcomere length non-uniformities are associated with an increase in actin–myosin filament overlap (and thus expected increase in force) of ~1.3 and 8.8% for single sarcomeres and force-enhanced myofibrils, respectively, but the associated force enhancements were shown to be approximately 20 and 50% [see fig. 9 in Rassier (Rassier, 2012)], thus only a tiny percentage of the force enhancement could theoretically be accounted for by the half-sarcomere length non-uniformities. More importantly, however, although half-sarcomere non-uniformities were not systematically evaluated in the isometric reference contractions, the raw data plots suggest that they were equally, if not more pronounced in the reference compared with the force-enhanced states, as shown previously by others (Joumaa et al., 2008a), indicating that the half-sarcomere length non-uniformities are a regular occurrence of muscle contraction and not a special feature of eccentric contraction. Therefore, if sarcomere length non-uniformities were to contribute to force production, one would expect this contribution to be the same for purely isometric contractions and for isometric contractions following active muscle stretching.

In summary, the four major predictions regarding force enhancement based on the sarcomere length non-uniformity theory can be rejected based on experimental evidence. Furthermore, force enhancements of several 100% have been observed in sarcomere and serially arranged half-sarcomere preparations, suggesting that residual force enhancement is a true property of sarcomeres and half-sarcomeres. Sarcomere and half-sarcomere length non-uniformities appear to be a normal product of any muscular contraction, rather than a special event that only occurs when muscles are actively stretched on the descending limb of the force–length relationship, thus alternative theories must be found to explain this evasive property.

Passive mechanism for force enhancement
In 2002, Herzog and Leonard made the observation that force enhancement in the cat soleus was associated with a passive component that persisted when the muscle was deactivated (Herzog and Leonard, 2002). This observation was termed 'passive force enhancement' (PFE; Fig. 3A,B) and was subsequently observed in single-fibre and myofibril preparations (Joumaa et al., 2008b; Lee et al., 2007), indicating that this passive component of force enhancement was a sarcomeric property. We suggested that this PFE might originate in the structural protein titin (Fig. 1), that it might contribute substantially to the total residual force enhancement (Herzog et al., 2012b; Herzog et al., 2012c), and that titin might play a role in muscle contraction that goes beyond its purely passive structural role. In the next few paragraphs, I will discuss the observations of titin as the origin of passive and total force enhancement, and then propose a new model of force regulation that includes titin as a 'contractile' protein working in tandem with the contractile proteins actin and myosin.

Following the discovery of PFE in skeletal muscles (Herzog and Leonard, 2002), we soon focused on the filamentous spring titin as a likely candidate for this property. The reasons for this choice were initially purely intuitive. Titin is a long filament that spans half-sarcomeres inserting into the Z-band at one end and the M-line at the other end (Fig. 1), with known spring-like properties in the I-band region (Kellermayer et al., 1997). Titin is also known to provide approximately 90–95% of all passive force in single myofibrils (Linke, 2000), and a substantial amount of passive force in skeletal and cardiac muscle (Horowits, 1992). Finally, titin's passive force depends directly on sarcomere and muscle length (Herzog et al., 2012a), is arranged in parallel with the cross-bridge based forces, becomes strong when cross-bridge based force becomes weak, and provides stability to sarcomeres and myosin filaments in the centre of sarcomeres (Horowits and Podolsky, 1987). Therefore, titin seems uniquely placed to play a role in muscle force regulation. Even prior to our discovery of PFE, titin had already been suggested as a candidate for residual force enhancement (Noble, 1992), as the magnitude of force enhancement increases with increasing amounts of active muscle lengthening (Edman et al., 1982), it is independent of the speed of lengthening (Edman et al., 1982), and it is partly offset by active shortening of muscle immediately preceding stretch (Lee et al., 2001; Rassier and Herzog, 2004), all properties that hint at a passive structural element that is engaged upon muscle activation and produces increased force upon stretch.

Titin is an entropic spring molecule with virtually elastic properties at short sarcomere lengths (Kellermayer et al., 1997) and at long sarcomere lengths if immunoglobulin (Ig) domain unfolding in titin is effectively prevented (Granzier et al., 1997; Herzog et al., 2012a; Kellermayer et al., 1997). Titin, like any other spring molecule, can alter its stiffness in essentially two ways: (1) by changing its inherent stiffness, or (2) by shortening its functional spring length. The first convincing evidence for a role of titin beyond its passive force contribution came when it was shown that titin binds calcium upon muscle activation and that this increases titin's stiffness, and thus its force when muscles are stretched (Labeit et al., 2003). This initial result, performed in skinned muscle fibres, was confirmed when calcium activation was shown to increase passive forces in myofibrils in which cross-bridge based forces were eliminated through depletion of troponin C from the actin filaments (Joumaa et al., 2008b). Finally, we showed that Ig domain unfolding in titin requires ~20% more force in the presence of physiologically relevant amounts of calcium (DuVall et al., 2012). All these studies suggest that upon activation, titin binds calcium at specific locations, thereby increasing its stiffness, and thus its force when stretched. This is a mechanism that could explain residual force enhancement in skeletal muscle contraction (Herzog et al., 2012c). However, the increases in titin stiffness and force observed in these studies were merely a fraction of the enhanced forces measured following active muscle lengthening (Herzog et al., 2006). Therefore, an additional mechanism explaining the entire magnitude of the residual force enhancement was needed.

In order to identify the true increase in passive force when a muscle is actively stretched compared with when it is passively stretched, we elongated single myofibrils from rabbit posas muscles actively (high calcium concentration) and passively (low calcium concentration) to lengths (6–7 μm per sarcomere) much beyond actin–myosin filament overlap (~3.8 μm per sarcomere) where active, actin–myosin-based cross-bridge forces were completely eliminated, and we found that actively stretched myofibrils had passive forces three to four times greater than passively stretched
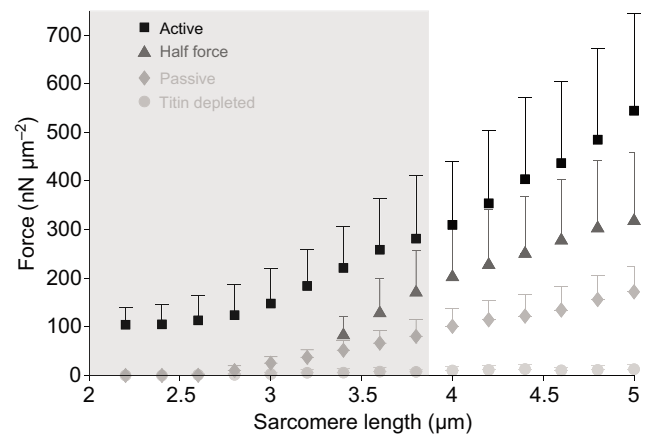

Fig. 5. Myofibril force (stress) as a function of sarcomere length obtained with slow stretches (0.1 μm s⁻¹ sarcomere⁻¹) of single myofibrils beyond actin–myosin filament overlap (lengths greater than 3.8 μm sarcomere⁻¹; non-shaded area). Active refers to myofibrils that were stretched while fully activated. Half force refers to experiments in which myofibrils were fully activated at ~3.4 μm sarcomere⁻¹ where active forces are small because of substantial (~70%) loss of actin–myosin filament overlap. Passive refers to myofibrils that were stretched passively. Titin depleted refers to passive and active myofibril stretching when titin's function was eliminated by short exposure to trypsin. Note that the actively stretched myofibrils have much greater passive forces (beyond actin–myosin filament overlap) than passively stretched myofibrils, indicating a substantial increase in force in the absence of actin-myosin based cross-bridges. Note further that once titin's function is eliminated, neither passive nor active force production is possible in these myofibril preparations. The shaded area indicates the region where actin and myosin filaments overlap; the non-shaded area indicates the region of sarcomere lengths where actin–myosin filament overlap is lost and only passive forces are possible [adapted from Leonard et al. (Leonard et al., 2010), with permission].

myofibrils (Fig. 5). Elimination of titin from these preparations reduced passive forces to a few percent of original and eliminated any force differences between actively and passively stretched myofibrils, indicating that titin was indeed responsible for these differences (Leonard and Herzog, 2010). Stretching the same myofibrils in a high calcium concentration solution (active stretching) but inhibiting cross-bridge attachments through butanedione monoxime reduced the force differences between 'actively' and 'passively' stretched myofibrils to a few percent (result not shown), supporting the idea that calcium binding to titin is just a small part of the enhanced force observed in actively stretched muscle, and that somehow, cross-bridge based forces are required to increase the titin-based passive forces when a myofibril is actively stretched. Finally, when reducing the active forces to approximately half of maximum, the passive forces upon myofibril stretching were also reduced to approximately half of those obtained when maximum cross-bridge forces were allowed (Fig. 5).

Combined, these results suggest that titin is a molecular spring whose stiffness, and thus force upon stretch, is increased by calcium binding upon activation, and that cross-bridge binding and force production increases titin's stiffness dramatically. It is not clear how this latter force increase is achieved, but the simplest explanation is that titin's proximal regions (those close to the Z-band) attach to actin upon force production, thereby reducing titin's free spring length, thus increasing its stiffness and force when stretched. Binding of titin to actin at the N2A region or near the PEVK region (Fig. 1) (Herzog et al., 2012b; Herzog et al., 2012c) or winding of titin onto a rotating thin filament (Monroy et al., 2012; Nishikawa et al., 2012) have been proposed (but by no means proven) as

possible mechanisms for force and stiffness modulation of titin in actively contracting muscles.

In this last section, I will propose a possible model for active and passive force regulation in skeletal muscle that incorporates the traditional cross-bridge theory, but adds titin as a third filament with force-regulating properties to the sarcomere. Although this is merely a proposal for a new mechanism of muscle contraction and force production that needs rigorous testing, it has the advantage that, in contrast to all cross-bridge models, it can predict muscle properties in concentric, isometric and most importantly in eccentric contractions, and incorporates all findings in the area of residual force enhancement that have escaped a consistent molecular explanation.

Proposal for a new theory of muscle contraction and force production

A revolution in science is described as the replacement of an existing scientific paradigm with a new and more powerful paradigm. The new paradigm needs to explain a whole series of thus far unexplained phenomena while maintaining the successful predictions of the old paradigm (Kuhn, 1962). Here, I am proposing an inherently new paradigm of muscle contraction that, in contrast to the cross-bridge theory, not only explains force regulation in isometric and concentric contractions, but also explains force regulation, stiffness and energetics for eccentric muscle contractions.

Instead of two contractile proteins, actin and myosin, force in this paradigm is regulated by three sarcomeric proteins, actin, myosin and titin. While actin and myosin play their usual role and interact via myosin-based cross-bridges that cyclically attach to specific sites on actin and are driven by ATP hydrolysis (Huxley, 1957), titin acts as a spring that binds calcium upon activation and binds to actin upon cross-bridge attachment. Calcium binding to identified regions, such as the E-rich region in the PEVK domain (Labeit et al., 2003) and selected Ig domains of titin (DuVall et al., 2012), increase titin's stiffness and thus its force when stretched actively (Leonard and Herzog, 2010b). Selective binding of titin upon cross-bridge attachment has not been demonstrated directly, but has strong support from cross-bridge-dependent increases in titin force (Leonard and Herzog, 2010b) observed in myofibrils stretched beyond actin–myosin filament overlap under passive and various active force conditions (Leonard and Herzog, 2010b) (Fig. 5). Specifically, the following three-filament theory of muscle force regulation can explain all experimental observations in isometric and concentric muscle contractions, and adds a simple explanation to many phenomena observed in eccentric muscle contraction, such as the residual force enhancement and the decreased energy requirement of eccentric contractions.

The three-filament model of muscle contraction

In the three-filament model, activation not only causes cross-bridges to attach to actin filaments, but also will cause titin to bind calcium at specific binding sites and bind (at a hitherto unknown site) to actin (Fig. 6). Calcium binding to actin has been identified for at least two
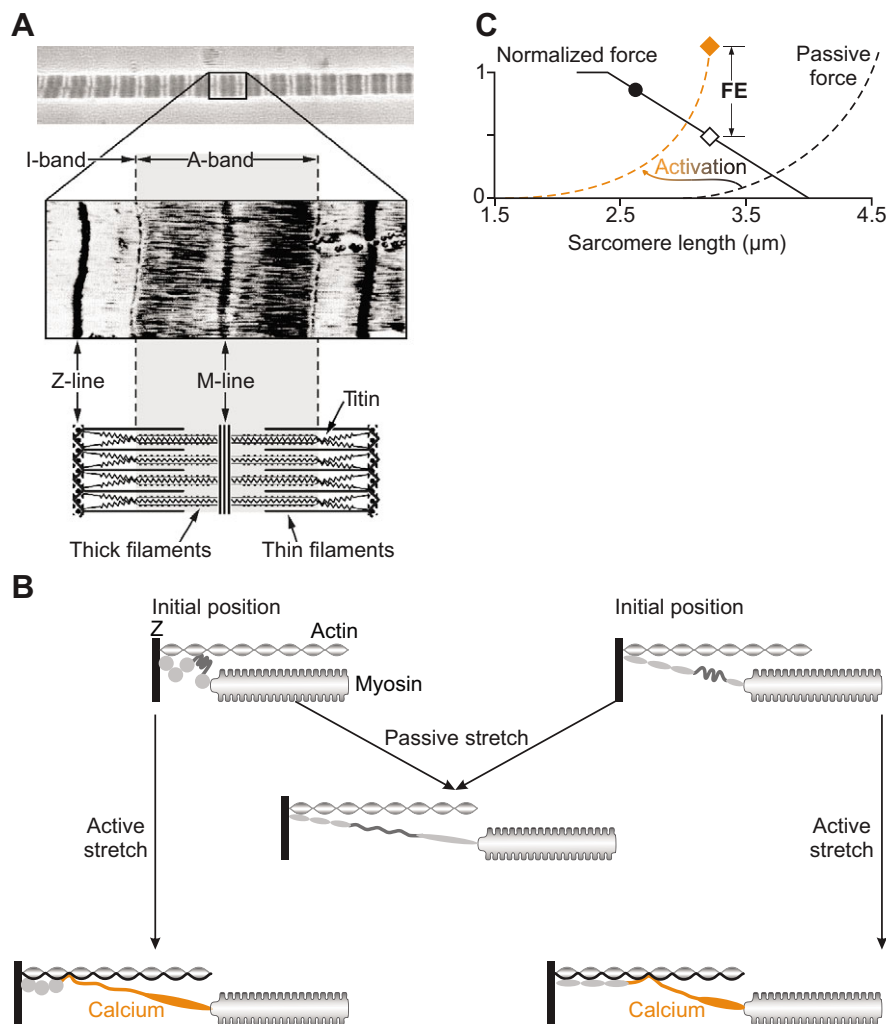

Fig. 6. Proposed new model of muscle contraction incorporating titin as the third force-regulating filament besides actin and myosin. (A) Electron micrograph of a single myofibril (top panel) with a sarcomere isolated (middle panel) and a schematic illustration of the three-filament sarcomere (bottom panel). (B) Schematic proposal of muscle contraction including titin as a force-regulating protein. In the top panel we have two (half) sarcomeres with a short (left) and a long (right) initial length. If passively stretched from these two initial configurations, the initial sarcomere length and passive force is the same (middle panel; passive stretch). If, however, the sarcomeres are activated first at the short and long lengths, respectively (top panel), titin will bind to actin at a more proximal (short initial length) or a more distal site (long initial length), thus experiencing more stretch for the remnant free spring when the initial sarcomere length is short than when long. Simultaneously, calcium binds to specific sites on titin upon activation, providing an additional increase in stiffness to the remnant free spring, thereby adding even more titin-based force when sarcomeres are stretched actively compared with when they are stretched passively. (C) Active and passive sarcomere force–length relationships. Note that in this model, the passive (titin-based) force increases upon activation because of the calcium binding to titin and the reduction of titin's free-spring length. The shift of the passive force curve upon activation depends crucially on the initial sarcomere length, where activation occurs that will determine where titin binds to actin. FE, force enhancement.

parts of the protein, the E-rich portion of the PEVK region (Labeit et al., 2003), and specific immunoglobulin domains (DuVall et al., 2013), while binding of titin to actin upon force production has not been experimentally verified *in situ*. However, because we know that titin's contribution to active force requires cross-bridge binding and actin–myosin-based force [rather than just calcium activation (Leonard and Herzog, 2010b)], a possible explanation might include a mechanism whereby the regulatory proteins troponin and tropomyosin free up titin binding sites on actin upon strong cross-bridge binding, thus allowing titin's free-spring length to shorten in the presence of cross-bridge forces but not in the presence of activation (calcium) alone. For eccentric contractions, the increase in stiffness in titin will result in an increased force upon stretch, which comes at virtually no additional energy cost. The actual force transmitted by titin will depend primarily on the binding site of titin to actin. The more distal this titin binding site, the shorter the free-spring length, and the greater titin's stiffness and force upon stretch.

Fig. 6 illustrates this scenario. In the top row, two half-sarcomeres with an actin, myosin and titin filament are shown for a short passive (left) and a long passive (right) half-sarcomere. If passively stretched (no titin binding to actin) to a certain length (middle row), the elongation of titin for both initial sarcomere lengths is the same, and the force transmitted by titin is the same as well. However, if the two half-sarcomeres are stretched actively (bottom row), titin will have attached at a more proximal (closer to the Z-line) location to actin for the initially short half-sarcomere compared with the initially long half-sarcomere. Therefore, if both half-sarcomeres are stretched actively to the same length, the stretch of the remnant free-spring titin segments is greater for the initially short than the initially long half-sarcomere, and thus will produce a greater titin force. However, if titin indeed binds to actin in a force-dependent manner, where this binding occurs, and whether this binding can occur at multiple locations or occurs in an altogether different way (Nishikawa et al., 2012; Herzog et al., 2012b; Herzog et al., 2012c), needs careful study, but will likely be known within the next 3 years through ongoing titin segment labeling work in our laboratory and in the laboratory of Dr Nishikawa at Northern Arizona University.

If the proposal of titin's force regulation in a calcium- and force-dependent manner is confirmed, it would explain several unaccounted properties of skeletal muscle contraction. For example, the following observations would have a ready explanation: (1) the residual force enhancement observed following active muscle stretching; (2) the energetic efficiency of eccentric muscle contraction; (3) the stability of sarcomeres and muscle segments on the descending limb of the force–length relationship; and (4) the stability of myosin in the centre of the sarcomere.

However, aside from these properties, the proposed contribution of titin to active force production would also have some intriguing implications for active and passive force regulation in skeletal muscles and associated properties. The primary examples are threefold. First, it would allow for passive muscle elongation against little resistance, while providing great passive (titin-based) resistance at virtually no energy cost in eccentric contractions. Titin's active role, therefore, might be similar to that observed in the catch mechanism of molluscan muscle by the titin-like protein twitchin, which connects myosin with actin and establishes a long-lasting force state (Butler et al., 2001) at essentially no energy cost. Second, it would protect muscles against muscle injuries on the descending limb of the force–length relationship. Third, it would provide increases in force when the actin–myosin-based cross-bridge forces decrease, thereby allowing for a greater working range of sarcomeres with useful force contributions.

In summary, the cross-bridge theory has been the paradigm of choice for muscle contraction mechanisms for the past half century (Huxley, 1957). It relies on force regulation through the contractile proteins actin and myosin. However, it does not account for many properties of muscles observed during eccentric contractions. By studying one of these unexplained properties, residual force enhancement, we stumbled across the force-regulating role of titin, and although titin's role is by no means understood in detail, it is now accepted that titin can change its stiffness in an activation (calcium)-dependent manner. The molecular details of this force regulation will need further elucidation, and thus the model proposed here for contraction mechanisms and force regulation in skeletal muscles (Fig. 6) needs to be seen as such – a proposal rather than a fully established theory. However, the beauty of this proposal is that it incorporates and asserts the cross-bridge theory, and merely adds a third myofilament, titin, to the contractile mechanism.

A great advantage of this three-filament model is that it endows muscle with some elegant properties: an increase in force for one system (titin) when force in the other system (actin-myosin) decreases at long muscle lengths, elongation of passive muscle against little resistance and elongation of active muscle against great (passive) resistance that comes at no extra (energetic) cost, and an explanation for the residual force enhancement, a property that has been identified prior to the formulation of the cross-bridge and sliding filament theories (Abbott and Aubert, 1952), but has eluded convincing explanation for decades.

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