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Titin Force is Enhanced in Actively Stretched Skeletal Muscle

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ABSTRACT

The sliding filament theory of muscle contraction is widely accepted as the means by which muscles generate force during activation. Within the constraints of this theory, isometric, steady-state force produced during muscle activation is proportional to the amount of filament overlap. Previous studies from our laboratory demonstrated enhanced titin-based force in myofibrils that were actively stretched to lengths which exceeded filament overlap. This observation cannot be explained by the sliding filament theory. The aim of the present study was to further investigate the enhanced state of titin during active stretch. Specifically, we confirm that this enhanced state of force is observed in a mouse model and quantify the contribution of calcium to this force. Titin-based force was increased by up to four times that of passive force during active stretch of isolated myofibrils. Enhanced titin-based force has now been demonstrated in two distinct animal models, suggesting that modulation of titin-based force during active stretch is an inherent property of skeletal muscle. Our results also demonstrated that 15% of titin's enhanced state can be attributed to direct calcium effects on the protein, presumably a stiffening of the protein upon calcium binding to the E-rich region of the PEVK segment and selected Ig domain segments. We suggest that the remaining unexplained 85% of this extra force results from titin binding to the thin filament. With this enhanced force confirmed in the mouse model, future studies will aim to elucidate the proposed titin-thin filament interaction in actively stretched sarcomeres.

Keywords: titin, skeletal muscle, force enhancement, cross-bridge theory, eccentric contractions

INTRODUCTION

The current, and generally accepted, theory which explains active force production in muscle is the sliding filament-based cross-bridge model. This model predicts that active force is produced when cross-bridges cyclically form between the thick and thin filaments in the sarcomere (Huxley and Simmons, 1971). Therefore, for a given level of activation, the isometric steady-state force generated by an active muscle should be proportional to the number of cross-bridges that can form, which corresponds to the amount of overlap between the thick and thin filaments (Gordon et al., 1966; Huxley and Niedergerke, 1954). This constitutes the force-sarcomere length relationship, which is generally adhered to during isometric contractions (Gordon et al., 1966). However, following eccentric, or lengthening, contractions the isometric steady-state force produced at a given sarcomere length exceeds the predictions of the force-length relationship (Abbot and Aubert, 1952; Edman et al., 1978; Edman et al., 1982; Herzog et al., 2006; Leonard and Herzog, 2010; Morgan, 1994). This property, termed residual force enhancement, provides a direct challenge to the sliding filament-based cross-bridge theory.

Residual force enhancement has been observed *in vivo* and down to the sarcomere level (Abbott and Aubert, 1952; Edman et al., 1982; Herzog and Leonard, 2002; Leonard et al., 2010; Rassier, 2012). There are three main filaments at the sarcomere level that contribute to force production in muscle: the thick (myosin), the thin (actin), and the titin filaments. The thick filament is comprised primarily of the protein myosin and the thin filament is comprised of actin and regulatory proteins. Interactions between the thick and thin filaments are understood to generate active force during muscle contraction. Titin is a spring-like protein that produces passive force when sarcomeres are stretched beyond a certain resting length. With increased stretch on the descending limb of the force-sarcomere length relationship, the contribution of titin to the total force produced increases exponentially (Granzier and Labeit, 2004). In an attempt to explain how sarcomeres can produce force beyond what is provided by the interactions between thick and thin filaments, it has been suggested that residual force enhancement may be the result of a passive element becoming ‘engaged’ during active stretch (Edman et al., 1982; Forcinito et al., 1998; Herzog and Leonard, 2002; Herzog et al., 2012a; Herzog et al., 2012c; Monroy et al., 2012; Nishikawa et al., 2012). Residual force enhancement exhibits properties that allude to a passive force contribution in the sarcomere. Residual force

enhancement increases with increased magnitude of stretch and is greater as the muscle is stretched further on the descending limb of the force-length relationship (Edman et al., 1982). Additional studies have observed enhanced passive force in muscles following active stretch (Herzog and Leonard, 2002; Joumaa et al., 2008; Labeit et al., 2003), alluding to a passive element in the property of force enhancement

Titin is the primary contributor to passive force at the myofibrillar level (Wang et al., 1991). Titin is a dynamic protein that contributes to many different aspects of muscle function, including passive force production (28), myofibrillar assembly (Gregorio et al., 1999), centering of the thick filament (Horowits and Podolsky, 1987), sarcomere stability (Herzog et al., 2012c), and various signaling events (Granzier and Labeit, 2004). Titin is functionally separated into a structural A-band and an extensible I-band region (Fig. 1). Titin's I-band region is comprised of a proximal Ig and PEVK spring, linked by the N2A segment (Wang et al., 1991). The distal Ig domain connects the segment to the A-band. During stretch, elongation of titin's I-band, particularly the PEVK segment, produces force (Gautel and Goulding, 1996). The spring properties of I-band titin can be rapidly adjusted in response to mechanical demands on the sarcomere (Granzier and Labeit, 2004). Phosphorylation (Hidalgo et al., 2009; Krüger et al., 2009), small heat shock protein infiltration (Kötter et al., 2014) and disulfide bonding (Alegre-Cebollada et al., 2014) are among the many processes by which the stiffness of I-band titin can be modulated to protect the protein from damage during stretch. While the contribution of titin-based stiffness to muscle force has traditionally been limited to passive stretch, this conventional view of titin is now being challenged with numerous studies which also demonstrate tuning of titin's spring properties during calcium activation (Bianco et al., 2007; Campbell and Moss, 2002; Labeit et al., 2003; Leonard and Herzog, 2010; Monroy et al., 2007; Tatsumi et al., 2001). The configuration of titin's extensible I-band is changed in the presence of calcium (Tatsumi et al., 2001). Specifically, it has been shown that the stiffness of titin's I-band region increases when calcium ions bind to the PEVK region and Ig domains (DuVall et al., 2013; Labeit et al., 2003). In addition to intrinsic changes to the stiffness of titin, calcium-dependent interactions between titin and the thin filament have also been observed (Kellermayer and Granzier, 1996). These observations support the speculation that titin may be engaged during active stretch (Edman et al., 1982; Herzog et al., 2006; Herzog et al., 2012a; Herzog et al., 2012c; Leonard and Herzog, 2010; Nishikawa et al., 2012).

Recently, Leonard and Herzog (2010) investigated modulation of titin-based force during active stretch beyond filament overlap. The findings of that study indicated that the increase in titin-based stiffness must employ an additional mechanism other than calcium to produce the increase in force that was observed since no effect of calcium activation, in the absence of cross-bridge-based active forces, was observed. They speculated that modulation of titin-based force may occur when titin binds to the thin filament during strong cross-bridge binding to actin (Leonard and Herzog, 2010). While titin binding to the thin filament has been previously suggested (Edman et al., 1982; Herzog et al., 2006; Leonard and Herzog, 2010; Nishikawa et al., 2012), a mechanism by which this may occur remains to be discovered.

Recent experiments (Monroy et al., 2012; Nishikawa et al., 2012) using muscles from mice with a mutated titin protein may provide insight into the region of titin that modulates titin-based stiffness during active stretch. The muscular dystrophy with myositis (*mdm*) model is a genetic mutation in the mouse genome which results in a deletion of amino acids from the N2A region of titin (Garvey et al., 2002). The deletion of a predicted 83 amino acids from distal N2A (9 of which are deleted from proximal PEVK) results in progressive degeneration of skeletal muscle in mice (Garvey et al., 2002). This is a modest deletion considering the ~33,000 amino acids encompassing the titin protein. Nevertheless, the *mdm* deletion has profound effects on the musculoskeletal system of mice (Garvey et al., 2002; Huebsch et al., 2005). Despite the visible differences in phenotype between *mdm* and wild-type mice, it remains unclear how the *mdm* mutation affects titin functionally. However, whole muscle experiments demonstrate that modulation of titin-based stiffness does not occur in *mdm* soleus during calcium activation (Nishikawa et al., 2012), thereby suggesting the possibility that modulation of titin-based force occurs within the deleted region of *mdm* titin. We would like to investigate this possibility. However, prior to embarking on such experiments, the properties of titin-based force enhancement in actively stretched muscles need to be confirmed in the mouse model, and the contribution of calcium activation in the absence of cross-bridge-based active forces on titin's stiffness and force need to be carefully determined.

The purpose of this study was to determine whether modulation of titin-based force during active stretch occurs in the wild-type, or normal, mouse and to quantify the effects of calcium activation on titin stiffness to further elucidate the mechanism of titin-based force

enhancement during active stretch. Enhanced titin-based force with active stretch has only been directly observed in experiments using rabbit psoas myofibrils (Leonard and Herzog, 2010) and needs to be confirmed in another animal model, as was previously suggested (Granzier, 2010). In addition, the experiments by Leonard & Herzog were unable to resolve an effect of calcium on the increase in stiffness of titin, possibly due to the high variation of their data. This is surprising as calcium has been shown to increase the stiffness of titin (DuVall et al., 2013; Joumaa et al., 2008; Labeit et al., 2003). Thus, this study specifically aimed to quantify the contribution of calcium effects to titin's enhanced force. We hypothesized that titin-based stiffness would be increased in actively stretched mouse skeletal muscle and that the increase in stiffness would not be explained entirely by the effects of calcium on titin stiffness.

RESULTS

The steady state force following stretch was greater in active than passive experiments (Fig. 3A). Individual sarcomere lengths were measured at the end of stretch in a subset of three active and three passive experiments to ensure that filament overlap was lost in all sarcomeres. Although sarcomere length non-uniformities were present, all measured sarcomeres were beyond filament overlap at the end of stretch (Fig. 3B) (average sarcomere length 6.0 μm). The shortest sarcomeres measured in active and passive myofibrils stretched to an average sarcomere length of 6.0 μm were 4.15 μm and 4.07 μm respectively.

Calcium activated myofibrils produced more force during stretch compared to passive, cross-bridge inhibited (BDM), and TnC-depleted myofibrils at all measured sarcomere lengths (2.5-6.0 μm) ($p < 0.01$) (Fig. 4). Therefore, calcium activated myofibrils produced more force both within the region of filament overlap (< 4.0 μm) and beyond the region of thick-thin filament overlap (~4.0-6.0 μm) in mouse psoas myofibrils that were either not activated, or were activated but force production was prevented, either by depletion of a regulatory protein (TnC) or by inhibiting strong cross-bridge binding (BDM).

BDM treated and TnC-depleted myofibrils produced significantly less force than calcium activated myofibrils at all sarcomere lengths ($p < 0.01$). However, they produced more force than the passively stretched myofibrils across all sarcomere lengths exceeding 3.5μm ($p < 0.05$) (Fig. 4). For sarcomere lengths beyond myofilament overlap, the force increase in BDM treated and TnC-depleted myofibrils averaged approximately 15% of the total increase observed in the

normal (non-inhibited) myofibrils. BDM treated and TnC-depleted myofibrils did not differ in force at any sarcomere lengths.

Simulations using a three-filament model (Schappacher-Tilp et al., *submitted* 2014) assumed that titin's N2A region binds to the thin filament leaving only the PEVK region and the distal Ig domains as free spring elements (Fig.5). Forces predicted in actively stretched sarcomeres with N2A thin-filament binding exceeded passive force predictions. Forces predicted for actively stretched sarcomeres without N2A thin-filament binding were deficient in titin-based force enhancement. There was a small increase in predicted force between actively (without N2A thin-filament binding) and passively stretched myofibrils (~12%). Predicted force-elongation curves of actively stretched sarcomeres without N2A thin-filament binding closely resembled TnC-depleted and BDM treated experimental observations.

DISCUSSION

The first aim of this study was to determine whether titin-based force is increased during active compared to passive stretch of mouse skeletal muscle myofibrils. Previous experiments in rabbit psoas myofibrils showed that at lengths beyond filament overlap, titin-based force is much greater in active compared to passive stretching. The increase in titin-based force from these studies could not be explained with any known mechanism of sarcomere force modulation (Leonard and Herzog, 2010). The results from the present study demonstrate that titin-based force is also greater during active compared to passive stretch in mouse psoas myofibrils. This modulation of titin force allows the sarcomere to maintain its force-generating capability during active stretch to lengths beyond filament overlap and provides a protective mechanism within the sarcomere by which active stretch is limited.

In actively stretched mouse psoas myofibrils, forces exceeded the force observed in passively stretched myofibrils matched at all sarcomere lengths. One would expect that at sarcomere lengths within filament overlap (< 4.0 μm), calcium activated myofibrils produce more force than non-activated myofibrils because of the active forces produced by cross-bridge interactions between the contractile filaments actin and myosin. However, cross-bridge forces cannot be used to explain the increase of force production in calcium activated myofibrils that are stretched beyond filament overlap (> 4.0 μm) (Leonard and Herzog, 2010). At these lengths,

cross-bridges cannot form and titin is virtually the exclusive contributor to myofibril force (Herzog et al., 2012b).

It is well-known that sarcomeres in muscles (Llewellyn et al., 2008), fibers (Page and Huxley, 1963) and myofibrils (Panchangam and Herzog, 2011; Panchangam and Herzog, 2012) are non-uniform in lengths. Despite this non-uniformity, all individual sarcomeres measured in selected experiments were stretched beyond actin-myosin filament overlap (4.0 μm) in our active and passive stretch tests. Therefore, we feel confident that most sarcomeres and half-sarcomeres could not produce any active, cross-bridge based force once the final stretch length was reached. If some sarcomeres or half-sarcomeres still had overlap between actin and myosin filaments, this non-uniformity could not explain the four times higher forces observed in actively stretched myofibrils at the final length (6.0 μm/sarcomere) compared to the passively stretched myofibrils, and it definitely could not explain the four times higher forces compared to the active forces obtained when the average sarcomere length was optimal (active stretch, 2.5 μm/sarcomere; Fig. 4). The reason for this assertion is that a myofibril, by definition, consists of serially arranged sarcomeres. From a mechanical point of view, that means that each half-sarcomere and sarcomere must transmit exactly the same force as all other (half-) sarcomeres, and this force transmission is limited by the weakest half-sarcomere. Thus, independent of whether a single or half-sarcomere maintains some remnant myofilament overlap, it could not explain the results observed here. A completely different mechanism than myofilament overlap or sarcomere length non-uniformity must be at work.

In the presence of calcium, titin-based stiffness increases (DuVall et al., 2013; Labeit et al., 2003). This calcium initiated increase in titin-based stiffness was a likely contributor to the enhanced titin force observed during active stretch of myofibrils. Therefore, with confirmation that titin-based force was enhanced during active stretch in the mouse, subsequent experiments sought to quantify the contribution of calcium to titin's enhanced state. Experiments using BDM and TnC-depletion were conducted to potentially identify the mechanisms underlying the observed increase in titin-based stiffness in actively stretched myofibrils. TnC-depleted myofibrils can be calcium activated but produce no active force as calcium binding to the regulatory TnC protein is not possible, thus the binding site of the cross-bridge on actin remains inaccessible to cross-bridges (Moss et al., 1985). Similarly, BDM treated myofibrils can be

calcium activated, and allow for cross-bridge binding to actin in the weakly bound state, but BDM prevents phosphate release from the nucleotide pocket of the cross-bridge, thereby inhibiting strong cross-bridge binding (Tesi et al., 2002). At sarcomere lengths beyond myofilament overlap (4.0-6.0 μm), calcium activation in BDM treated and TnC-depleted myofibrils increased titin force by approximately 15% of the total increase observed in actively (but non-force inhibited) compared to passively stretched myofibrils. Therefore, it appears that although calcium activation provides increased stiffness to titin, and thus increased force upon sarcomere stretching, this effect is minimal. The full amount of increased titin-based force, which we will refer to as "titin-based force enhancement" requires active force production and strong cross-bridge binding to actin.

Interestingly, the myofibril forces in BDM treated and TnC-depleted myofibrils were the same when sarcomeres were stretched beyond filament overlap. In TnC-depleted myofibrils, there is thought to be no cross-bridge attachment to actin, and titin-enhancement is small, as shown above. In BDM treated myofibrils, weak cross-bridge binding is thought to occur, and small configurational changes in the regulatory proteins troponin and tropomyosin would be expected, but even these events do not produce titin force beyond that observed in the TnC-depleted myofibrils. Thus, weak cross-bridge binding and some configurational changes in the regulatory proteins do not seem sufficient to allow for the full titin-based force enhancement observed in intact, non-inhibited actively stretched myofibrils.

If calcium activation alone is not sufficient to explain the increase in titin stiffness during active stretch, an additional mechanism by which titin-based force is enhanced during active stretch must be at work. Of the various known mechanisms by which titin-based stiffness can be rapidly and reversibly adjusted, we are currently unaware of any mechanism by which titin stiffness is increased by up to 400%. Therefore, we are of the impression that the titin spring must become shorter in actively stretched sarcomeres to explain our findings. It was recently hypothesized that titin-based force may be enhanced when cycling cross-bridges both translate and rotate the thin filaments, winding titin upon the thin filament during activation (Nishikawa et al., 2012). While logistically discreet, this hypothesis conceptually aligns with our assumption that the available spring length of titin must decrease in actively stretched sarcomeres. Shortening of the titin spring could occur by any number of mechanisms. However, the simplest

way to explain titin-based force enhancement is by assuming that titin binds to the thin filament when cross-bridges bind strongly to the thin filament. Thin-filament-titin interactions are well documented in cardiac muscle (Kellermayer and Granzier, 1996), however, much less is known concerning the interaction between skeletal muscle titin and the thin filament. We acknowledge the vast possibilities by which titin-thin filament interactions could increase the stiffness of titin and the precise location and characteristics of this mechanism will require further investigations.

We previously proposed that a binding site for titin on the thin filament may be exposed during the movement of regulatory proteins with the influx of calcium (Herzog et al., 2012a; Herzog et al., 2012c; Leonard and Herzog, 2010). However, the results from the present study suggest that movement of regulatory proteins is not sufficient to initiate titin-based force enhancement. This is because there was no difference in force between TnC-depleted myofibrils (no movement of regulatory proteins) and BDM treated myofibrils (presumed movement of regulatory proteins). Therefore, we now speculate that with the influx of calcium and the initiation of cross-bridge cycling, one (or many) site(s) on the thin filament are exposed, allowing titin-thin filament binding to decrease the available spring length of titin in an activated sarcomere (Fig. 6). This hypothesis is particularly attractive as it provides an energetically efficient and reversible mechanism by which titin-based force could be modulated during activation. Experimental support for titin-thin filament interactions is provided by experiments in rabbit longissimus dorsi muscle, which demonstrate calcium-dependent changes in titin-actin interactions. In these studies an unidentified segment of titin demonstrated binding to actin filaments in a calcium-dependent manner (Kellermayer and Granzier, 1996). While this segment of titin has not been identified, these results provide support for the idea that titin may bind to the thin filament during calcium-activation.

Recent speculation that the N2A segment of titin may be the site of thin filament-titin binding (Nishikawa et al., 2012) provides the basis for a focused investigation. The N2A segment of titin has a mechanical role in passive skeletal muscle, resisting stretch at long sarcomere lengths (Granzier and Labeit, 2004) and is also a main player in numerous skeletal muscle signaling events (Huebsch et al., 2005). Binding at the N2A segment of titin would be mechanically advantageous as it would prevent the proximal Ig domain from extending upon stretch (Fig. 6) and allow the high-force PEVK region to be recruited immediately upon stretch

(Nishikawa et al., 2012). The hypothesis that N2A titin is the site of titin-thin filament binding is based on studies in the *mdm* mouse model, which is characterized by a deletion in the N2A region of titin (Garvey et al., 2002). During active unloading of whole mouse soleus muscles, the titin spring is shorter and stiffer than in passive soleus muscles while no change in length or stiffness is observed in soleus muscles of *mdm* mice during active unloading (Monroy et al., 2012). These results suggest that the mechanism by which titin achieves an enhanced state of stiffness during calcium activation is altered in *mdm* titin. Further support for N2A as the site of titin-thin filament binding is provided by reports that suggest a fundamental difference in titin-actin interactions between skeletal muscle that expresses N2A titin and cardiac muscle that expresses N2B (Yamasaki et al., 2001).

To investigate this hypothesis further, we modeled the proposed N2A titin-thin filament interaction to determine whether this mechanism could explain titin-based force enhancement. We assumed that titin's N2A region binds to the thin filament leaving only the PEVK region and the distal Ig domains as free spring elements. While the predicted absolute values of stress based on the rabbit psoas model differ from the experimental results from mouse, we can study the impact of N2A-thin filament binding on force predictions conceptually (Fig. 5). Forces predicted during actively stretched sarcomeres with N2A thin-filament binding show a substantial force gain at long sarcomere lengths vastly exceeding purely passive forces and forces predicted for activation without N2A binding. With N2A-thin filament binding, titin-based force predicted by the model was doubled in actively stretched sarcomeres. Experimental results demonstrate 3-4 times greater titin-based force in actively stretched mouse psoas myofibrils and approximately 3 times greater in rabbit psoas myofibrils (Leonard and Herzog, 2010) which exceeds the force predicted by the model. By shifting the binding site on titin towards the M-line, the force gain would be substantially higher; suggesting that the binding site on titin would be located at or distal to the N2A segment. When simulations were performed in the absence of N2A-thin filament binding, the model predictions of titin-based force coincided closely with cross-bridge inhibited (TnC-depleted and BDM) sarcomeres, which were deficient in titin-based force enhancement (Fig. 4). The predicted force of actively stretched myofibrils without titin binding still exceeded passive forces, presumably due the binding of calcium to the PEVK and Ig domains (DuVall et al., 2013; Joumaa et al., 2008; Labeit et al., 2003; Ting et al., 2012), but only to a small extent. Overall, these simulations conceptually reflect the experimental results and

proposed mechanism that titin is bound to the thin filament during activation. To reproduce the absolute values of the experimental results one might need to fit the parameters for the mouse model. Nevertheless, the conceptual premise of the results observed in mouse myofibrils supports titin binding to the thin filament at or distal to the N2A segment.

With the enhanced force of titin confirmed in the mouse skeletal muscle, future experiments can utilize the *mdm* model to further investigate the role of N2A titin in titin-based force enhancement. In a preliminary investigation, we isolated and stretched calcium activated and non-activated *mdm* myofibrils using the same experimental protocol described below to determine whether the *mdm* deletion in N2A titin affects titin-based force enhancement. Isolated *mdm* myofibrils did not differ in diameter or structure and thus were expected to contain the same quantity of contractile material and force-producing capability as in wild-type myofibrils. Despite our expectations, when activated at the plateau of the force-length relationship, contraction force was decreased in *mdm* compared to wild-type myofibrils activated at corresponding sarcomere lengths. During active stretch, titin-based force enhancement was present in *mdm* (Fig. 7) but to a much lesser extent than in actively stretched wild-type myofibrils (Fig 4), suggesting that titin-based force enhancement is affected by the *mdm* deletion. These pilot results are in accordance with the proposed mechanism that N2A titin binds to the thin filament to achieve enhanced force during active stretch; however, additional experiments and considerations are required in the interpretation of these results. Whether N2A is the site of titin that binds to the thin filament during calcium activation will require a more focused investigation. Nevertheless, this preliminary data is a strong indication that N2A titin is a binding site or otherwise involved in the mechanism of titin-based force enhancement.

When considering an interaction between titin and the thin filament in explaining enhanced titin-based force during active stretch, it is important to consider that with the exception of calcium stiffening of titin, titin-based force enhancement was abolished when strong cross-bridge cycling was inhibited. This result suggests that titin's enhanced state is at least in part dependent on the presence of cross-bridge cycling and active force. Previous results from rabbit psoas myofibrils showed that titin-based force enhancement was decreased when the myofibril was activated at long sarcomere lengths, with less than optimal filament overlap (Leonard and Herzog, 2010). If titin-thin filament interactions are partially responsible for the

enhancement of titin-based force, these results would suggest that the interaction occurs with the onset of cross-bridge cycling, and thus, at sarcomere lengths where initial activation occurs. This indicates that titin's contribution to the force produced during active stretch spans the full length of stretch. While the present study could not disseminate any contribution of titin to active force within the region of overlap, the question of whether titin contributes to the force generated within the region of overlap during active stretch is an important topic for future studies.

When considering the property of titin-based force enhancement within the context of an intact muscle fiber it is important to consider the various additional structures which provide resistive forces to protect the sarcomeres (and titin) from potentially hazardous elongation. Within working muscle fiber lengths, titin and collagen are the main contributors to passive force (Granzier and Irving, 1995). Individual sarcomeres are also surrounded by a network of intermediate filaments which resist sarcomere stretch to lengths that cause structural damage (>4.5 μm) (Wang et al., 1993). At these long sarcomere lengths, the intermediate filament network acts as a force-bearing structure, contributing about one quarter of the total passive force (Wang et al., 1993). In the absence of resistive forces provided by collagen and intermediate filaments, which are integral in providing protection against stretch-induced sarcomere injury, our results refer only to titin-based mechanics in the sarcomere. As residual force enhancement has been observed at the single sarcomere level (Leonard et al., 2010; Rassier, 2012), these experiments were designed to specifically address titin-based force enhancement in the actively stretched intact sarcomeres. While intermediate filaments, collagen and additional structures also contribute to the resistive force limiting sarcomere stretch within a muscle fiber, our results indicate that titin-based force enhancement is a fundamental mechanical property of the sarcomeres themselves, which provides an inherent resistance to actively stretching sarcomeres.

In summary, we found that titin-based force is increased in actively stretched mouse psoas myofibrils at lengths beyond filament overlap. With studies from rabbit and mouse demonstrating this property, we propose that enhanced titin-based force beyond the effects of calcium is an inherent property of actively stretched skeletal muscle. Calcium activation alone could only explain 15% of the increase in titin-based force beyond filament overlap; therefore we propose that with the onset of cross-bridge cycling and active force, titin binds to the thin filament and decreases its available spring length resulting in a drastic increase in its spring

stiffness. This mechanism would provide the sarcomere with an additional source of force production during active stretch, which could potentially provide an explaination for how skeletal muscles achieve enhanced force following active stretch. While additional experiments are needed to elucidate the mechanism by which titin-based force enhancement occurs, our results suggest that it is initiated when a site on titin (at or distal to the N2A segment) binds to the thin filament with the onset of cross-bridge cycling. With past and present studies demonstrating a role for titin in active muscle, our understanding of force production should begin to expand the contribution of titin in actively stretched skeletal muscle.

MATERIALS AND METHODS

Sample Preparations

A myofibril is the smallest structural unit of muscle that maintains the natural architecture of the contractile apparatus (Yang et al., 1998). In a single myofibril, extracellular connective tissues are absent and all force measured must be generated by the proteins comprising sarcomeres in series. Therefore, myofibril experiments are ideal for the investigation of the contribution of titin to force production in sarcomeres as described previously (Leonard and Herzog, 2010).

Experiment setup

All tests were conducted using an inverted microscope (Zeiss Axiovert 200M) equipped with a ×100 oil immersion objective (numerical aperture 1.3) with a ×2.5 Optovar (Leonard and Herzog, 2010) under ×100 oil immersion.

Myofibril forces were determined using custom-built nanofabricated silicon nitride cantilevers with two arms and a stiffness of 21 or 68 pN/nm (Fig. 2). Myofibrils were glued (Dow Corning 3145) to one of the lever arms at one end and wrapped around a stiff glass needle at the other end. Displacement of one cantilever arm attached to the myofibril relative to the second reference arm was measured using a custom MATLAB program. Force was calculated from the measured displacement and the known lever stiffness and expressed in units of stress ($nN/\mu m^2$) by normalizing the measured force to the cross-sectional area of each myofibril.

Materials

Strips of mouse psoas muscle were extracted from euthanized animals and tied to wooden strips to preserve the *in situ* length. The muscle strips were placed in a rigor-glycerol solution (-20°C, pH 7.0) with protease inhibitors (Complete®, Roche Diagnostics, Montreal, QB, Canada) and stored at -20°C for 12-17 days (Leonard and Herzog, 2010). For experiments, muscle strips were homogenized in a rigor solution at 4°C and tested at 20°C (Leonard and Herzog, 2010). Activation of myofibrils was achieved by direct application of calcium ions in an activation solution (pCa 3.5) (Leonard and Herzog, 2010). Ethics approval was granted by the Life and Environmental Sciences Animal Ethics Committee of the University of Calgary.

Experimental Protocol

Single myofibrils with four to twelve sarcomeres in series were mounted between a needle for controlled length changes and a cantilever to measure force (Fig. 3). Myofibril lengths were adjusted to an initial average sarcomere length of 2.5 μm. Previous experiments demonstrated a force-dependent component to titin-based enhanced force (Leonard and Herzog, 2010). Therefore, to maximize the magnitude of titin-based force enhancement in the present study, myofibrils were activated slightly above the plateau region and then stretched. The thin filament length in mouse psoas is ~1.11 μm (Bang et al., 2009; Burkholder and Lieber, 2001) and myofilament overlap in mouse psoas would be expected to be lost at sarcomere lengths of approximately 3.95 μm (Herzog et al., 1992). Therefore, when the average sarcomere lengths within a myofibril exceed 4.0 μm, we can safely assume that some sarcomeres and half-sarcomeres are pulled beyond myofilament overlap, and thus their forces, which are strictly in series with the rest of the myofibril, have to match those produced in the remaining sarcomeres. Individual sarcomere lengths were measured at the end of stretch to confirm the loss of filament overlap.

Average sarcomere lengths were determined by dividing the total length of the myofibril by the number of sarcomeres. All myofibrils were stretched from an average sarcomere length of approximately 2.5 μm to lengths of approximately 6.0 μm, thereby achieving lengths that far exceeded filament overlap. Stretching was applied at a speed of 0.1 μm per sarcomere per second. After stretch, myofibrils were held isometrically until a steady-state force was reached.

The inclusion criterion for successful myofibril experiments were determined and strictly adhered to. A myofibril experiment was considered successful if (1) all sarcomeres lengthened and (2) the force increased with stretch for the entire duration of stretch. In actively stretched myofibril experiments, in addition to the two criteria stated above, all sarcomeres had to visibly shorten upon activation for the experiment to be considered viable. All experiments which did not exhibit the established criterion were excluded from statistical analyses.

Experimental Groups

Group 1 – passive: myofibrils (n = 10) were passively stretched in a non-activating (pCa = 8.0) solution containing ATP. This group demonstrates the mechanical properties of the passive titin spring.

Group 2 – active: myofibrils (n = 7) were activated in a calcium-ATP (pCa 3.5) activation solution and then stretched. Active, actin-myosin-based cross-bridge forces cease to exist at sarcomere lengths beyond myofilament overlap (i.e., ~4.0 µm) (Gordon et al., 1966; Huxley, 1957; Leonard and Herzog, 2010).

Group 3 – TnC depleted: myofibrils (n = 8) were incubated in a low ionic strength rigor-EDTA solution (pH 7.8) for 10 minutes to inhibit cross-bridge formation by depletion of Troponin C (TnC) (Joumaa et al., 2008) and then stretched in calcium-ATP activation solution. The TnC-depletion protocol used in this study has been described previously and its effectiveness in eliminating TnC from actin, thereby preventing any cross-bridge based forces, has been verified using gel electrophoresis and force measurements, respectively (Joumaa et al., 2008).

Group 4 – BDM: myofibrils (n = 8) were stretched in a calcium-ATP activation solution with 20nM butanedione monoxime (BDM), an inhibitor of strong cross-bridge binding to actin (Tesi et al., 2002). All solutions used in this study have been previously published (Joumaa et al., 2008; Leonard and Herzog, 2010).

Modeling

The proposed N2A titin-thin filament interaction was simulated to determine whether it could explain titin-based force enhancement in previous (Leonard and Herzog, 2010) and present

observations. A detailed description of the mathematical model is provided elsewhere (Schappacher-Tilp et al., *submitted* 2014). Briefly, the simulations consider active force production based on a five-state cross-bridge model (Piazzesi and Lombardi, 1995; Rayment et al., 1993) and passive force production based on elongation of titin. In the case of actin-titin binding upon activation, we assume that titin's N2A region binds to the virtually rigid thin filament thereby reducing titin's free spring length. The binding between actin and titin does not generate force but directly influences passive force generation. The total force exerted by a sarcomere is given by the sum of cross-bridge based active force and variable titin-based passive force. The latter is calculated by Monte Carlo simulations performed for 200 titin strands, and normalized to the passive forces in a sarcomere of 1μm² cross sectional area. In order to eliminate any bias toward the titin-thin filament binding model, we refrained from fitting the experimental data, but rather used well known model parameters from the literature based on rabbit psoas titin (Linke et al., 1998; Linke et al., 2002; Prado et al., 2005). The model predictions were scaled to experimental mouse psoas values and expressed as predicted stress ($nN/\mu m^2$) by average sarcomere length (μm)

Statistical Analysis

A Kruskal Wallis non-parametric ANOVA was performed and followed-up with a Mann-Whitney U group-wise comparison to determine how the force produced during stretch differed between myofibril groups. Significance was determined at 0.05 and used for all analyses.

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486 **DISCLOSURES**

487 No conflicts of interest, financial or otherwise, are declared by the authors.

488 **AUTHOR CONTRIBUTIONS**

489 Krysta Powers - data collection, analysis, paper preparation; Gudrun Schappacher-Tilp-
490 modeling; Azim Jinha - analysis, programming, editing; Tim Leonard – conception,
491 experimental design, editing; Kiisa Nishikawa - conceptual development, interpretation and
492 editing; Walter Herzog - experimental design, conceptual development, interpretation and final
493 editing.

494

FIGURE CAPTIONS

Fig 1. Schematic representation of a sarcomere including thick, thin and titin filaments. The thick and thin filaments are the main contractile proteins. A-band titin runs the length of the thick filament and is mainly structural. I-band titin is attached to the thin filament at the Z-disc and contains titin's extensible spring segments. The proximal Ig, N2A, PEVK and distal Ig domains of titin can extend during sarcomere stretch. Extension of the PEVK domain is the main source of titin-based force.

Fig. 2. Experimental set-up. A single myofibril is attached to a glass needle used to manipulate the length of the myofibril, and a cantilever used to measure the force produced by the myofibril.

Fig. 3. Force and sarcomere length traces. Force traces (A) in $nN/\mu m^2$ and individual sarcomere lengths (B) from exemplary non-activated (blue) and activated (red) myofibrils. The initiation of activation and duration of stretch are denoted by the vertical dotted lines. The force (A) of activated myofibrils (red) remains higher than the force of the non-activated myofibrils (blue) for the duration of stretch and following force relaxation to a steady-state. Individual sarcomeres lengths (B) are non-uniform, however all measured sarcomeres are stretched beyond filament overlap (gray area) during the implemented stretch.

Fig. 4. A comparison of myofibril force-length curves from experimental conditions. Force normalized to myofibril cross-sectional area ($nN/\mu m^2$) ± s.e.m. as a function of average sarcomere length (μm). Myofibrils were stretched from an average initial sarcomere length of 2.5 μm to an average final sarcomere length of 6.0 μm. The loss of filament overlap is designated by the dotted line. Myofibrils were stretched in a Ca^{2+} -rich activation solution (active, diamonds), in activation solution with BDM, a cross-bridge inhibitor (BDM, squares), in activation solution following chemical depletion of troponin-C (TnC-X, triangles), and in a low Ca^{2+} relaxing solution (passive, circles). Activated myofibrils generated significantly more force than BDM, TnC-X, and Passive myofibrils at all sarcomere lengths both within and beyond the region of filament overlap ($p < 0.01$). BDM and TnC-X myofibrils generated significantly more force than passive myofibrils at average sarcomere lengths of 3.5 - 6.0 μm ($p < 0.01$).

Fig. 5. Predicted force-sarcomere length relationships from simulated data. The predicted titin-based force (grayed region) of actively stretched skeletal sarcomeres with N2A thin-

filament binding (black diamonds) exceeds predicted titin-based forces of actively stretched sarcomeres without N2A thin-filament binding (gray diamonds) and predicted forces of passively stretched sarcomeres (open circles). Predicted force of actively stretched sarcomeres in the absence of N2A thin-filament binding is nearly diminished, with a small increase in predicted force from passively stretched sarcomeres which can be attributed to calcium stiffening of titin's PEVK and Ig domains.

Fig 6. Proposed mechanism by which titin-based force is enhanced in the half sarcomere. The mechanism proposed occurs at the initiation of cross-bridge cycling when titin reversibly binds to the thin filament at the N2A segment. This binding would significantly reduce the available spring length of the passive titin spring (top panel, *Lp*). Thus, during Ca^{2+} - activation and cross-bridge cycling the active titin spring length (bottom panel, *La*) would be much shorter and stiffer, if the proposed mechanism is correct. If the sarcomere was stretched with an active spring length of *La* rather than *Lp*, the passive force in the top panel half sarcomere would be substantially less than that for the bottom panel half sarcomere even for stretches of identical magnitudes.

Fig. 7. Pilot data comparing force-length curves from active and passively stretched *mdm* myofibrils. Force normalized to myofibril cross-sectional area ($nN/\mu m^2$) as a function of average sarcomere length (μm) for a single active (diamond) and passive (circle) *mdm* myofibril. Myofibrils were stretched from an average initial sarcomere length of 2.5 μm to an average final sarcomere length of 6.0 μm. The loss of filament overlap is designated by the dotted line. The force of activated *mdm* myofibrils (triangle) remains higher than the force of the non-activated *mdm* myofibrils (blue) for the duration of stretch. Titin-based force was increased during active stretch, but to a lesser extent than in actively stretched wild-type myofibrils (Fig. 3).

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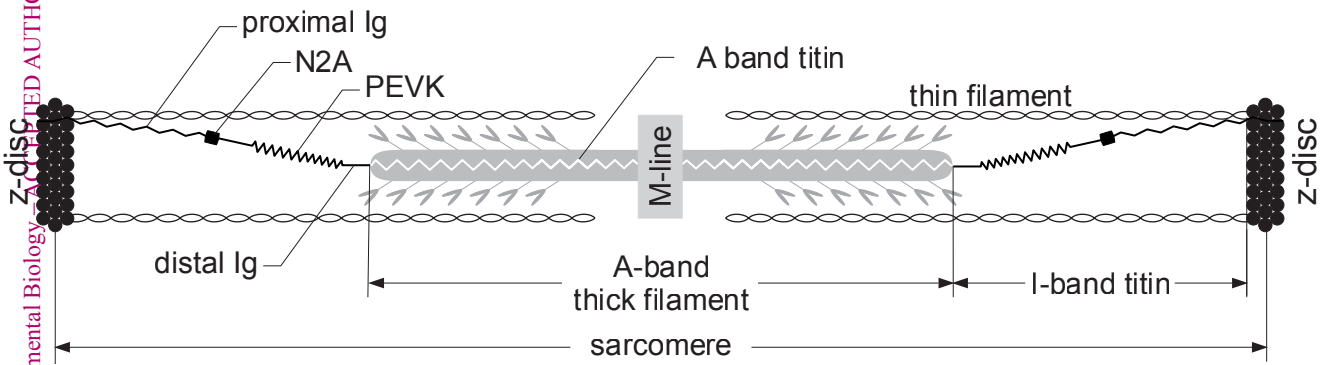

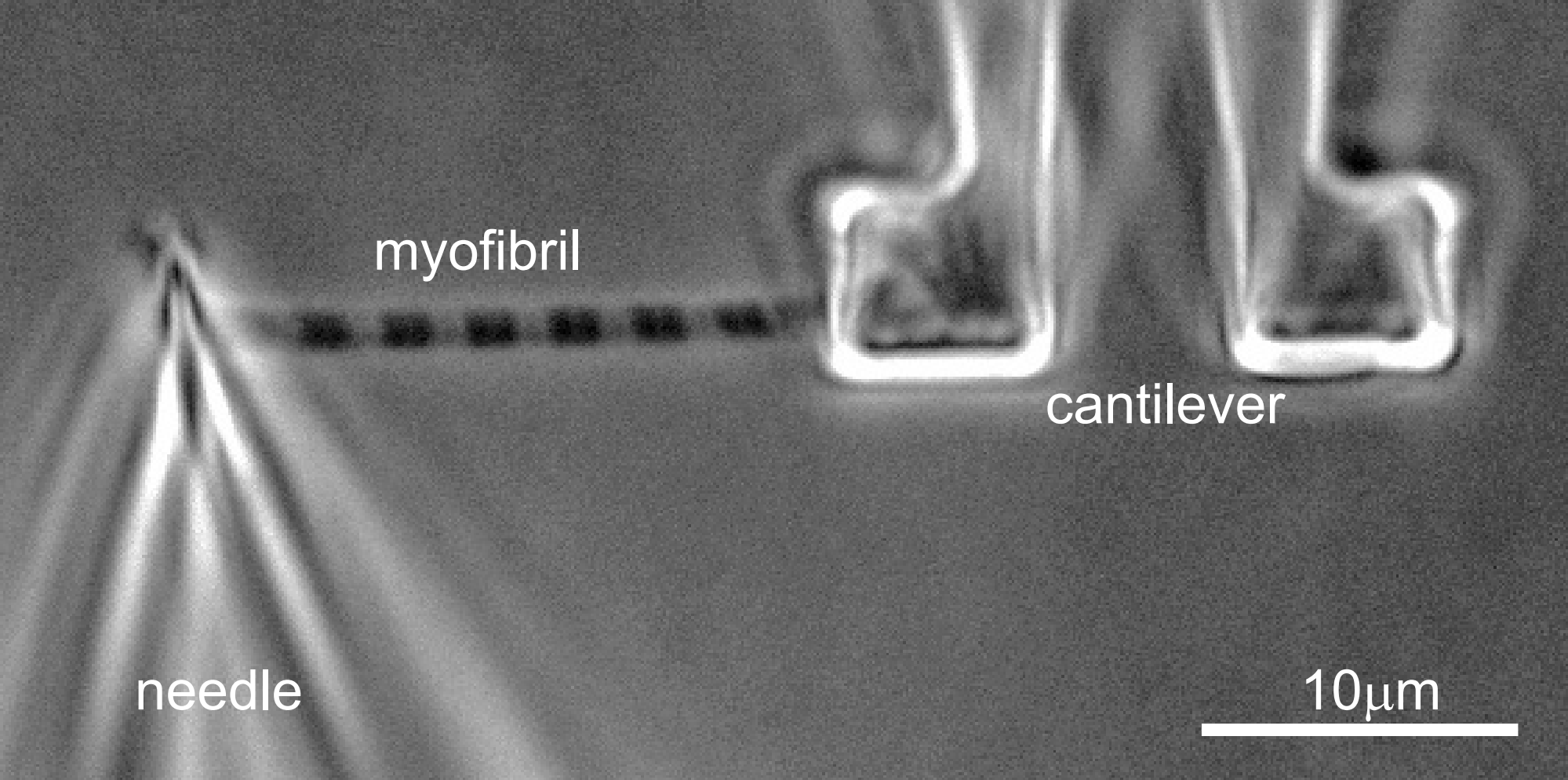
myofibril
cantilever
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10μm