The Contribution of Titin to Striated Muscle
Shortening

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by
Joseph M. Schuster

Kerry S. McDonald, Ph.D, Thesis Advisor

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled:

The Contribution of Titin to Striated Muscle Shortening

presented by Joseph Schuster,

a candidate for the degree of master of science in physiology

and hereby certify that, in their opinion, it is worthy of acceptance.

Kerry McDonald PhD

Michael Rovetto PhD

Marybeth Brown PhD

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Abstract

Striated muscle shortens under various loads (i.e., performs work), which allows movement of the skeleton and pumping of blood throughout the circulatory system. The amount of work that a muscle can do is determined by the speed at which it shortens against a given load. One sarcomeric protein hypothesized to assist muscle shortening speed is the giant protein titin. Titin is a 3-4 MDa protein that spans the sarcomere from Z line to M line and is thought to both bind actin in the thin filament and proteins of the thick filament. Titin contains extensible regions that are thought to provide passive restoring forces, with the size of these extensible regions being the primary difference between titin isoforms. The larger the extensible regions, the lower the passive force for a given increase in muscle length. The extension of titin, and consequent increase in passive force, may assist in muscle shortening by bearing some of the load normally carried by cross-bridges. To test this hypothesis that titin assists shortening in an isoform dependent manner three muscle types were studied: slow-twitch skeletal muscle fibers (with the largest titin isoform), fast-twitch skeletal muscle fibers (intermediate-sized titin isoform), and cardiac myocytes (smallest titin isoform). Single permeabilized skeletal muscle fibers or cardiac myocyte preparations were attached between a force transducer and a position motor and changes in muscle length were monitored during shortening against varied loads during submaximal Ca^{2+} activations. Force-velocity relationships were obtained before and after a mild trypsin treatment that has been shown to

cleave titin with no apparent effects on other sarcomeric proteins. Although cleavage of titin did not affect isometric force or loaded shortening velocity in any of the three types of striated muscle, titin cleavage did alter the pattern of force re-development by markedly reducing the extent of a transient overshoot in isometric force that typically occurs after muscle re-stretch in all three muscle types. These results imply that titin strain has a negligible effect in determining striated muscle loaded shortening velocity but does appear to modulate the number of force-generating cross-bridges following muscle stretch.

Introduction

Over the past fifty years scientists have made great progress in understanding the mechanism of striated muscle contraction. Their studies have led to many important discoveries including the sliding filament model of contraction (31-35, 37). According to this model myosin molecules cyclically interact with actin to propel the thin filament toward the middle of the sarcomere. This idea was substantiated by the discovery of the crystal structure of myosin II, which described the actin-binding and nucleotide-binding motifs as well as a putative conformational change all of which coincide with mechano-chemical transduction. (56).

A major function of striated muscle is to move a load, which allows movement of the skeleton and pumping of blood throughout the circulatory system. The structural anatomy of striated muscle allows it to perform this vital function. Both skeletal muscle and cardiac muscle are comprised of individual muscle cells that have a highly organized array of cytoskeletal proteins. The array of proteins gives rise to the functional unit of striated muscle known as a sarcomere. The sarcomere is bordered longitudinally by Z-disk proteins, and its body consists of three myofilament systems: the thin filament, the thick filament and titin. The thin filament contains strands of filamentous actin and the troponin-tropomyosin regulatory protein complex. Within the troponin-tropomyosin

complex are four proteins: troponin I (inhibitory), troponin C (calcium binding), troponin T (tropomyosin binding) and tropomyosin. The thick filament contains the motor protein myosin and structural proteins including myosin-binding protein C. The titin system consists of one large protein, titin, and titin's associated proteins including T-cap and S100A1 (Figure 1). For striated muscle to contract, myosin must bind actin in an ATP-dependent manner and form cross-bridges. The initiation of cross-bridge formation relies on the presence of calcium ions. Calcium is released from the sarcoplasmic reticulum and binds troponin C, which induces a conformational change in troponin C that causes the movement of the troponin I/troponin T complex. The movement of troponin T helps pull tropomyosin (which is associated with troponin T) into the actin groove, eventually leading to the exposure of myosin-binding sites on actin. Once myosin-binding sites are exposed, nearby myosin heads originating from the thick filament bind actin and begin the cross-bridge cycle.
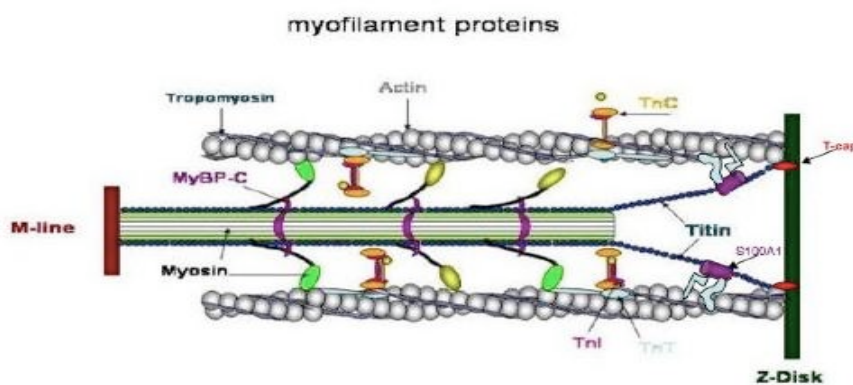

myofilament proteins

Figure 1. Schematic of sarcomeric myofilament proteins: TnC=Troponin C, TnI= Troponin I, MyBP-C= myosin binding protein-C, T-cap= titin cap,

Actin and myosin must be in close proximity to interact, thereby producing force. Power production in striated muscle is determined by the number of force-generating cross-bridges present and the speed at which the muscle shortens against a load (which is determined by the number of cross-bridge cycles per second). Thus, speed of muscle shortening has two key determining factors, the number of force-generating heads present and the rate of cross-bridge cycling, which is determined by the ATP hydrolysis rate of myosin.

In addition to its active force-generating properties striated muscle also possesses the ability to generate passive force (also referred to as restoring force). Passive force exerts its effects as cytosolic calcium concentration is decreasing and passive force actually exceeds active force. During relaxation, passive forces help realign thin and thick filaments in preparation for another round of active cross-bridge cycling. For instance, when striated muscle cells shorten, the cell's width increases, and thus, the thick and thin filaments move apart, which reduces the probability of cross-bridge interaction with actin. Passive force assists the return of actin and myosin to an optimal alignment for attachment during the subsequent activation. The two main elements that provide striated muscle with passive force are titin and extracellular collagen.

In order to better understand the active and passive properties of striated muscle, it is necessary to take a thorough look at the functional and structural properties of muscle proteins, in particular the myofilament proteins which

includes actin, troponin subunits, tropomyosin, myosin, myosin binding protein-C, and titin.

Actin

The major component of the thin filament is actin; it exists in muscle as a filamentous strand (f-actin) of individual globular actin subunits (g-actin). The g-actin subunits polymerize spontaneously within muscle cells to form f-actin. Two strands of f-actin interact to form the alpha helical structure of the thin filament. The actin helix contains various sub-domains (1-4) for protein interaction (64), among which sub-domain 1 is of great importance because it is thought to be the site for actin-myosin interaction (52). The alpha helical structure of actin is anchored to the Z-line of the sarcomere to provide support to the sarcomere and the basis for muscle shortening. The actin helix contains a groove for protein interaction, in which lie some major regulatory proteins, including a ropelike tropomyosin structure and associated troponins. Tropomyosin and the troponins act as regulators of cross-bridge binding in a calcium dependent manner. Actin and myosin interact to form the force producing cross-bridge. Actin, which is anchored to the Z line, can be translated toward the middle of the sarcomere by actively cycling myosin molecules, which underlies muscle fiber shortening. Actin is also a site for interaction with many other sarcomeric proteins such as S100A1 (a calcium binding homodimer protein) and titin. Actin is a versatile sarcomeric protein that provides the basis for muscle shortening, major structural support for muscle fibers and sites for protein-protein interactions (10, 20, 52).

Myosin

Another important sarcomeric protein is myosin, which functions as the motor for striated muscle shortening, mediated by its interaction with actin and ATP. In the presence of ATP myosin molecules can ratchet down the thin filament causing relative sliding of thick and thin filaments. Myosin is the major protein component of the thick filament. Each myosin molecule is composed of 2 heavy and 4 light chains. The heavy chains form the major structural component and nucleotide-binding component of the myosin molecule. While the myosin light chains appear to play a regulatory role in striated muscle, regulatory light chain (MLC2) phosphorylation is not necessary for striated muscle contraction as it is for smooth muscle contraction. In striated muscle, phosphorylation of regulatory light chain increases both force and force development rates especially during sub-maximal calcium activations such as those present in cardiac muscle during systole (51)**.**

The head and neck region of each heavy chain (referred as the S1 region) contains an actin binding domain, an ATP binding domain, and myosin light chain binding regions (61, 62). As the muscle shortens, myosin molecules move along the alpha helical actin filament propelled by the energy of ATP hydrolysis. This ATP hydrolysis is performed by the S1 region of the myosin molecule. Each cross-bridge cycle is thought to be coupled to one molecule of ATP although the number of cross-bridge cycles per ATP hydrolyzed is still being debated (4, 7, 48, 66).

Each cross-bridge cycle involves some basic steps which can be grouped into three general phases: unbound, bound non-force generating, and bound force generating cross bridges. The steps in the cross-bridge cycle are:

      1. myosin without bound ATP and tightly bound to actin

      2. myosin/ATP complex unbound to actin,

      3. myosin/ADP+Pi weakly bound cross-bridges

      4. myosin/ADP (Pi release) force generating cross-bridges

      5. Myosin/ADP dissociation

Each step in the cross-bridge cycle is governed by its own rate constant. The identity of the rate-limiting step in cross-bridge kinetics is widely debated. Some propose that the rate-limiting step is inorganic phosphate release (3, 73), while others claim it is prior to P_i release at the transition from weakly bound to strongly bound cross-bridges (k_4)(49). The structure of the actin-myosin cross-bridge complex has been resolved by x-ray crystallography and other methods (61, 62). The visualization of cross-bridge protein complex has led to the confirmation of many of the earlier mechano-transduction hypotheses but questions still remain about some fundamental aspects of the structural and functional states in the cross-bridge cycle such as the rate limiting step during different types of contractions (eg., isometric and isotonic) and how many cycles are possible per ATP hydrolyzed (31, 33-38).

Tropomyosin

Tropomyosin (Tm) is a component of the thin filament that mainly functions in regulating strongly bound cross-bridge formation. Tropomyosin exists in the sarcomere as a dimer in a coiled coil alpha helix configuration. Individual tropomyosin dimers overlap one another down the entire length of the thin filament. Tropomyosin lies in the groove of the actin alpha helix occupying subdomain 1 of actin, which prevents actin-myosin head interactions. In the presence of calcium and the troponin complex, tropomyosin moves out of the actin helix groove and reveals myosin-binding regions of actin, which results in subsequent sarcomere shortening (70). The overlapping configuration of tropomyosin dimers in the thin filament may play a role in the cooperative activation of the thin filament by formation of neighboring cross-bridges. Some propose that tropomyosin exists in three states in striated muscle: blocked, closed and open. The transition between states relies on the presence of calcium bound to troponin C and on the presence of weakly bound cross-bridges. Once calcium enters the sarcomere, it binds troponin C, starting a cascade of conformational changes in the troponin/tropomyosin complex. This cascade forces tropomyosin to shift its position and move into the closed state, allowing myosin and actin to form strongly bound cross-bridges. Strongly bound non-force generating cross-bridges cause the transition of tropomyosin into the open conformation. Once in the open conformation, strongly bound force-generating cross-bridges can form.

Troponins

The troponin (Tn) molecules are a group of three small protein subunits so named TnI (inhibitory), TnT (tropomyosin) and TnC (calcium) that are involved in the regulation of cross-bridge binding through their interactions with calcium and tropomyosin. TnT is a 30kD protein that tethers the Tn complex to tropomyosin. Tropomyosin movement out of the actin groove and subsequent thin filament activation depends on TnT interaction with tropomyosin. Some propose that a portion of TnT is involved in the cooperative activation of tropomyosin (40). TnC is a small calcium-binding protein belonging to the EF-hand family. Once calcium is bound, a conformational change in TnC eventually causes the Tm-Tn complex to uncover myosin-binding sites on actin, allowing cross-bridges to form. TnI, the inhibitory protein of the Tn complex, interacts with Tm, TnT, TnC and actin. TnI is responsible for inactivation of the thin filament when calcium is removed from the myoplasm. TnI also contains threonine residues that are sensitive to phosphorylation by protein kinase A (PKA) in response to β-adrenergic stimulation. Several studies have found that phosphorylation of TnI by PKA yields decreased calcium sensitivity of force, which is thought to speed the rate of relaxation in order to assist ventricular filling during times of increased heart rate associated with β-adrenergic stimulation (28, 39, 65, 69).

Myosin-binding protein C

Myosin-binding protein C (MyBP-C) is a regulatory protein located in the sarcomere of striated muscle. MyBP-C interacts with the S2 segment of the

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myosin molecule (11, 63). The S2 segment of myosin is a hinge region that is thought to influence the range of motion of the myosin head (the S2 segment is also thought to interact with titin). MyBP-C forms a ring around the thick filament with radiating projections resembling helicopter blades, which hold myosin heads in a particular conformation (44). MyBP-C is thought to regulate the rate of relaxation in striated muscle through PKA-mediated phosphorylation (23, 27, 42). Some hypothesize that MyBP-C phosphorylation increases myosin ATPase rate and force production by cross-bridges (22, 68). When MyBP-C is phosphorylated, it is thought to release its constraint on myosin S2, which allows greater flexibility and azimuthal range of myosin heads. This greater range of motion of myosin heads would likely increase the probability of cross-bridge formation and/or change the kinetics of cross-bridges cycling.

Titin

The giant sarcomeric protein titin is 3-4 MDa and spans the sarcomere from Z-line to M-line. Titin was first discovered in 1979 by Wang et. al. (71), who initially thought that titin was a collection of polypeptides that formed one large protein. Although titin's physical identification eluded researchers for many years, probably due to its susceptibility to proteolytic cleavage, many scientists, including Earnest Starling and A.F. Huxley, posited its existence (33, 57, 58). Starling and Huxley modeled their theories on the premise that something within striated muscle was regulating passive properties. This is now known to be the role of titin, which is one peptide, encoded by a single gene, TTN (1). Titin is

believed to bind actin in the thin filament and the proteins of the thick filament (16, 55, 74). Titin contains extensible and non-extensible regions. The non-extensible regions provide a scaffold for associated proteins and serve as anchors for titin to the Z- and M-lines within the sarcomere (16, 18, 43). The extensible regions span the I-band segment of the sarcomere and have been shown to play a role in the passive properties of striated muscle (15, 16, 21, 25, 43). Titin plays a pivotal role in the Frank-Starling mechanism of the heart by regulating the extent of ventricular filling. Titin regulates passive stretch of the ventricles during filling and resulting recoil following filling (19, 58). The extensible regions of titin contain both a PEVK-rich region (rich in proline, glutamate, valine and lysine) and tandem immunoglobulin (Ig) repeats (30). These two regions form the molecular spring region in cardiac and skeletal titin. Straightening of the Ig domains and extension of the PEVK region during muscle stretch creates a passive force, which realigns the thick and thin filaments and helps the sarcomere back to its original length after active shortening or after a passive load is removed (6, 12, 18, 24, 45, 53). Extension is the uncoiling movement of titin's elastic regions (as opposed to unfolding, which is changing of the protein's tertiary structure). Cardiac muscle contains an additional spring element, N2B, which further contributes to passive tension development.

The contribution of titin to passive force is determined by the size, extension and unfolding of I-band regions. The progression of straightening, extension, and unfolding have been extensively studied using atomic force

microscopy (AFM) and immunoelectron microscopy (IEM). These techniques

have shown titin behaves as two worm-like chains in series (17). The worm-like

chain (WLC) model is described by the equation ($FL_p/k_bT = z/L_c + 1/4(1-z/L_c)^2 - ¼$),

where a polymer chain is a bendable continuum in which thermally excited

motion evokes the contraction of the chain (reduction of its end-to-end distance)

and increases the chain's conformational entropy (45, 47, 56). A chain's

conformational entropy is the energy stabilizing the protein in its unfolded state.

L_p is the persistence length, which is the stiffness of a bendable chain. L_c is the

contour length or the end-to-end length of the chain when stretched with an

infinite amount of force. It is the theoretical length of a chain because no force is

infinite physiologically. During single molecule AFM experiments, both the Ig and

PEVK regions extend and unfold as predicted by the WLC model (45, 62).

However, under physiological conditions it is entropically unfavorable for the Ig

regions to unfold and refold during each muscle contraction-relaxation cycle. In

contrast, the PEVK regions extend and recoil during contraction/relaxation. It is

important to define the terms lengthening and recoiling in this context.

Lengthening is not the same as unfolding. Lengthening consists of partial

uncoiling of regions of the titin molecule but not complete protein unfolding.

Recoiling is the return of titin's PEVK or N2B to their shapes prior to lengthening.

IEM, AFM and WLC modeling have uncovered the progression of titin extension

and lengthening within the physiologic sarcomere length range. Following initial

stretch, Ig regions straighten, producing a small amount of passive force which

initiates PEVK extension. In cardiac muscle the N2B region lengthens as

predicted by the WLC model following PEVK straightening. The energetically

favorable PEVK recoiling after extension contributes to the bulk of titin's passive

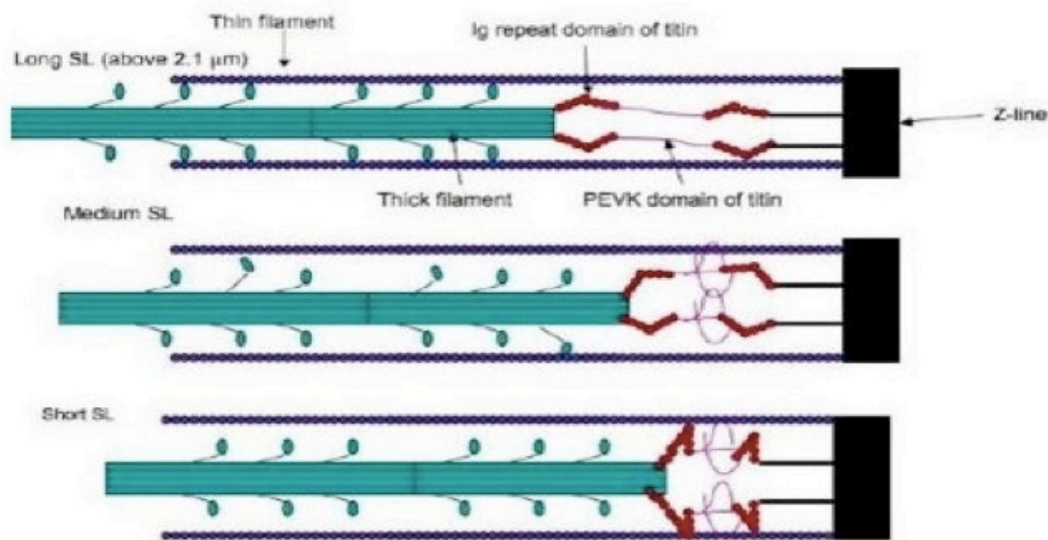force production in striated muscle (Figure 2).

Figure 2. Titin's contribution to passive force development. At short sarcomere length both Ig repeat and PEVK domains begin coiled. Following initial muscle stretch Ig repeats begin to extend. Further sarcomere lengthening results in the uncoiling of the PEVK domain.

The WLC model provides a quantitative assessment of flexible proteins such

as titin. Since WLC is a mathematical model it does not fully explain the

properties of titin, which is why I have chosen the further investigate the

properties of titin.

Genetics of Titin

Striated muscle is able to genetically vary the size of titin through

differential splicing of the titin gene (TTN). The size of the extensible regions is

the major determinant in the size of the titin isoform present and a large

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determinant in the amount of passive tension produced in a given muscle type. The ability of muscle to modulate passive tension plays a role in the diastolic properties of the ventricle. As the sarcomere is stretched, PEVK begins to extend. Once the PEVK region begins to extend passive force begins to rise. By genetic modulation the length of the PEVK region, passive forces will vary (45, 62). For example, slow-twitch muscle (such as soleus) genes code for the largest titin isoform, which has the longest PEVK region. The long PEVK region is able to extend to a longer length and therefore produces less passive tension at a given sarcomere length than a shorter PEVK, like the one in fast-twitch skeletal muscle and cardiac muscle. Titin's passive properties also are variable through PKA phosphorylation of I-band residues. In 2005 Fukuda et. al. showed that phosphorylation of titin's I-band residues causes a decrease in passive tension in N2B and N2BA titin isoforms (13). This is significant because during beta-adrenergic stimulation, reduced ventricular stiffness would be advantageous to allow for more rapid ventricular filling (Figure 3) (16).
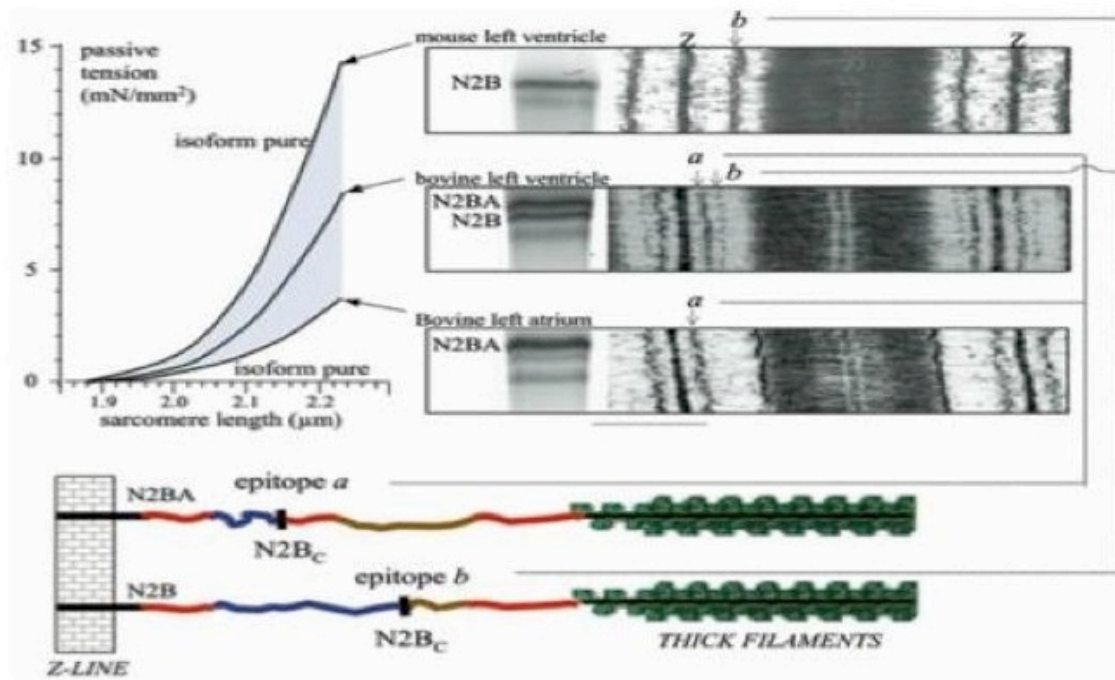
Figure 3. Role of different titin isoforms on passive tension development in striated muscle. As the N2B region increases in length the passive tension produced by titin decreases. Epitope a marks the site the N2Bc antibody binding in N2BA titin, epitope b marks the site of the N2Bc antibody binding site in N2B titin. Marking of the epitopes allows researchers to show full extension of titin isoforms at a given level of passive tension. Reprinted with permission from the Journal of Physiology (17).

Not only does titin play an important role in passive tension, it also may assist in active muscle contraction. Cazorla et. al. (6) have proposed a model in which titin exerts a radial force that pulls actin and myosin closer together as the sarcomere length increases. Because titin is attached obliquely to the Z-line and the thick filament, in the sarcomere it is able to produce radial forces strong enough to overcome the electrostatic forces repelling actin and myosin molecules. The radial force will increase the number of force-generating cross-bridges by cross-bridge induced cooperative activation of the thin filament. More myosin heads may then able to bind actin due to the presence of titin's radial force; this would increase force production during a given calcium activation.

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Studies by Fukuda et. al. have shown that titin's contribution to calcium sensitivity may be isoform dependent and that the smallest titin isoforms have the greatest effect on calcium sensitivity (14). Titin's contribution to calcium sensitivity may be due to changes in interfilament lattice spacing or influences on cross-bridge mobility (14). There is good evidence that titin's contributions to lattice spacing is a major facilitator of active tension development, because addition of dextran after titin degradation restores lattice spacing to original state and increases calcium sensitivity of force in a proportionate manner.

Titin also may influence muscle shortening velocities. A possibility is that as the muscle shortens, titin exerts a recoiling force, which would assist, and thereby speed shortening. Titin acts as a spring in striated muscle as titin is stretched it builds potential energy, and then as the muscle begins to shorten titin begins to recoil and return to its original shape. The recoiling force of titin could reduce the opposing forces acting on cross-bridges. Reduction in the load borne by cross-bridges would allow them to cycle faster and thereby increase overall shortening speed of the muscle cell. This hypothesis suggests smaller titin isoforms would create greater recoiling forces, and, therefore, titin's contribution to shortening velocity would be greatest in striated muscle with the shortest isoform. For example a fast-twitch muscle myocyte would have a larger titin-based shortening velocity than a slow-twitch muscle myocyte, due simply to the shorter titin isoform present. In addition, experiments by Yamasaki et. al. also have shown that addition of PEVK slows actin velocities on heavy mero-myosin

(HMM) (74). This influence has been shown to be calcium dependent and also may rely on the ability of S100A1 to bind titin. S100A, an EF-hand calcium binding protein, was shown to bind the PEVK domain of titin. S100A1/Ca competes with actin for PEVK binding sites. This competition reduces the PEVK-actin interactions within the muscle freeing more actin to bind with myosin.

Other Titin Functions

Titin's functions appear not to be limited to only passive and active force development. Titin also plays a role in cellular signaling and assembly of the sarcomere during development, when the large titin molecule serves as a scaffold for assembly of the thick and thin filaments. Titin contributes to muscle cell signaling by serving as a substrate for many cellular tyrosine kinases as well as having kinase activity itself. Titin kinase has been localized to the M-line region of the titin molecule. Studies by Peng et. al. have shown that a knockout of this region results in a reduction of muscle contractility and the development of cardiac hypertrophy in mice (59). The pathways associated with titin kinase range from the progression of necrosis during muscular dystrophies through titin-cap protein to influencing the balance of muscular atrophy through a MURF-2 mediated pathway (59). It also should be noted that titin mutations are seen in a variety of muscular diseases from limb girdle muscular dystrophy to types of cardiomyopathies (2, 16).

Although there is evidence that titin contributes to muscle shortening during very lightly loaded contractions, there are no known studies that directly

address its role during loaded contraction, which is how striated muscle contracts in vivo. This thesis addresses the role of titin during loaded contractions. The hypothesis tested was that titin contributes to loaded shortening by bearing some of the load on the cross-bridge, thus allowing faster loaded shortening. This hypothesis was addressed by examining the idea that loaded shortening and power output in muscle myocytes would be faster after titin degradation.

Materials and Methods

Experimental animals

All procedures involving animal use were performed according to the Animal Care and Use Committee of the University of Missouri-Columbia. Male Sprague-Dawley rats (~6 wk of age) were obtained from Harlan (Madison, WI), housed in groups of two or three individuals, and provided with access to food and water ad libidum. Young rats were used in order to ensure the α-myosin heavy chain (MyHC) isoform was conserved for all the studies. Several studies indicate that myosin heavy chain isoforms change from α-MyHC to β-MyHC with age (9, 26).

Solutions

The composition of relaxing and activating solutions were as follows (in mmol/L, obtained from Sigma, St. Louis): 1 free Mg^{2+}, 7 EGTA, 4 Mg-ATP, 20 imidazole, and 14.5 creatine phosphate (pH 7.0); calcium concentrations were between $10^{-4.5}$ (for maximal Ca^{2+}- activating solution) and 10^{-9} M (for relaxing solution).

Trypsin (obtained from Sigma, St. Louis) stock 2.5 mg/ml was stored in 1M HCl at -20^0 C. The stock was diluted to concentrations of 250 ng/ml for skeletal muscle protocols and 25 ng/ml f or myocyte protocols in relaxing solution. Trypsin solution was made fresh each experimental day.

Protease inhibitor solution (in mmol/L) contained Pefabloc (Sigma), 0.25 leupeptin (CalBiochem), and 0.5 PMSF (Sigma).

Myocardial and skeletal muscle preparations

Skinned cardiac myocytes were obtained by mechanical disruption of hearts from Sprague-Dawley rats as described by McDonald et. al. (41). Briefly, rats were anesthetized by inhalation of isoflorane (20% v/v in olive oil), and hearts were excised and rapidly placed in ice-cold relaxing solution. The ventricles were dissected away from the atria, cut into to 2-3 mm pieces, and further disrupted for 5s in a Waring blender. The resulting suspension of cells was centrifuged for 105 s at 165 g, after which the supernatant was discarded. The myocytes were skinned by suspending the cell pellet for 5 min in 0.3% Triton

X-100 (Pierce Chemical) in cold relaxing solution. The skinned cells were washed twice with cold relaxing solution, suspended in 10-15 ml of relaxing solution and kept on ice during the day of the experiment. For skeletal muscle experiments psoas and soleus were dissected from the Sprague-Dawley rats as well. Once the heart was placed in cold relaxing solution the skeletal muscles were dissected out and immediately placed in cold relaxing solution. The muscles were separated into bundles of approximately 50 fibers and tied to a capillary tube. The muscle bundles were placed in glycerol skinning solution (1:1 v/v in relaxing solution) for 24 h and stored at -20^0 C until needed for up to one month. For each experimental protocol, single skinned myocytes were teased out of a bundle, then attached to the apparatus.

Experimental apparatus

The experimental apparatus for physiological measurements of cardiac and skeletal myocytes was similar to the one previously described (41). Briefly, cardiac or skeletal myocytes preparations were attached between a force transducer and a torque motor by gently placing the ends of the myocyte into stainless steel 25 gauge troughs. The ends of the myocyte were secured by overlapping a 0.5 mm-long piece of 3-0 myofilament nylon suture (Ethicon) onto each end of the myocyte and then tying the suture to the troughs with two loops of 10-0 monofilament suture (Ethicon). The attachment procedure was performed under a stereomicroscope (approximately X100 magnification) using finely

shaped forceps. The myocytes appeared to have comparable sarcomere integrity.

Before mechanical measurements were obtained, the experimental apparatus was mounted on the stage of an inverted microscope (model IX-70; Olympus Instruments), which rested on a pneumatic anti-vibration table with a cutoff frequency of ~1Hz. Force measurements were made using a capacitance-gauge transducer (model 403; sensitivity, 20 mV/mg (plus X 10 amplifier for myocyte preparations); resonant frequency, 600 Hz; Aurora Scientific; Aurora, Ontario, Canada). Length changes during mechanical measurements were introduced at one end of the preparation using a DC torque motor (model 308; Aurora Scientific) driven by voltage commands from a personal computer via a 12-bit digital-to-analog converter (AT-MIO-16E-1; National Instruments, Austin TX). Force and length signals were digitized at 1 kHz using a 12-bit analog-to-digital converter. Each was displayed and stored on a personal computer using custom software based on LabView for Windows (National Instruments). Images of the myocytes were monitored while relaxed, using an IonOptix SarcLen system (IonOptix, Milton, MA)

Force-velocity and power-load measurements

The protocol for obtaining force-velocity and power-load measurements has been described in detail by McDonald (41, 54), and all measurements were

made at 13 ± 1^0 C. The attached myocyte was first transferred to maximal Ca^{2+}-activating solution (pCa 4.5) and allowed to obtain steady-state isometric force after which a series of sub-isometric force clamps were applied to determine isotonic shortening velocities. The isotonic force was maintained using a servo system for 150-250 ms while length changes during this time were monitored. After the force clamp, the myocyte was slackened to near zero force to estimate the relative load sustained during isotonic shortening, after which the myocyte was returned to its initial length.

Titin cleavage by trypsin treatment

To cleave titin the skeletal muscle myocyte or cardiac myocyte preparation were treated with 250ng/ml or 25ng/ml trypsin in relaxing solution, respectively. The myocyte was incubated in trypsin solution for 6 min, after which it was switched to a pCa 9.0 solution containing protease inhibitors [leupeptin (CalBiochem) 40 μM, PMSF (Sigma) 0.5mM and 0.1mM Pefabloc (Sigma)] for 6 min in order to stop enzymatic degradation of contractile proteins. The extent of titin degradation was determined by visualization of silver stained SDS-AGE (agarose gel electrophoresis).

SDS-agarose gel electrophoresis

Agarose gel electrophoresis protocol has been described in detail by Warren et. al (72). Briefly, after mechanical measurements, skeletal muscle myocytes or cardiac myocytes preparations were frozen in sample buffer

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(containing 8M urea, 2M thoiurea, 3% SDS w/v, 75mM DTT, 0.03% bromophenol blue and 0.005 Tris) at -80°C until gel was run.

The 1% agarose resolving gel was prepared as follows; first a 12% acrylamide plug was poured between two Hoefer mini-gel plates using 1.5mm spacers. The acrylamide plug was poured ~1 inch from the bottom of the plate. The plug was allowed to polymerize 1 hr. The plates were then placed into an oven at 65°C for 30 min along with a 15 ml syringe and 10 well gel comb. During this time the 1% agarose resolving gel was prepared by mixing 0.4g of Sea Kem Gold agarose (obtained from Sigma St. Louis), 12 ml of 100% glycerol, 8mL 5X running buffer and 20 mL distilled water. This solution was weighed and placed in a microwave oven for 2 min to dissolve the agarose. Once the powder was dissolved a heated syringe was used to draw up the agarose. The gel plates were removed from the oven and injected with the agarose solution care being taken to avoid air bubbles in the gel. The plates were allowed to cool to room temperature for 10 min then placed in 4°C cool room for 1 hr. Once the gel was sufficiently cool, and solidified, 2X-running buffer was added to the gel apparatus and the samples were loaded on to the gel. The gel was run for 2 hr.

Following electrophoresis the gel was fixed in 50% methanol, 12% glacial acetic acid, and 5% glycerol w/v for 1 hr and dried at 37°C overnight. The next day the gel was washed 3 times using de-ionized water then washed in potassium ferrocyanide for 5 min, and subsequently washed 3 more times in de-

ionized water. Following the last wash the gels were stained by adding to a solution of 50g sodium carbonate, 2g ammonium nitrate, 2g silver nitrate, 10g tungstosilicic acid and 6.7 mL 37% formaldehyde in 1 L water. This silver stain solution was used to detect titin bands. After titin's bands appeared the gel was washed in 1% v/v glacial acetic acid to quench the silver staining process. The gel was then dried overnight between two sheets of Mylar.

STATISTICAL METHODS

The data, force, velocity and k_{tr} were compiled and are shown as means and standard deviations. A paired t-test was used to determine the difference in k_{tr}, shortening velocity, power output, relative titin degradation and peak power output values. Force-velocity curves were fitted using the Hill equation (29). Power-load curves were made by multiplying force by velocity at each load on the force velocity-curve (50). Microsoft Excel was used to generate these values as well as the correlation coefficients. A p value of 0.05 was chosen as the limit for statistical significance.

Results

Effects of trypsin treatment on myofibrilar protein content and force generation

Treatment of skinned skeletal myocyte preparations with 0.25 mg/ml trypsin for 6 min resulted in ~35% titin cleavage (Figure 4) as determined by SDS-PAGE and silver staining. Although titin was degraded by addition of trypsin to the preparations, the contractile apparatus (including actin and myosin) appeared unaffected (Figure 5 and Table 1).

Figure 4. Myocyte treatment with 0.25ng/ml trypsin causes 35.38 +/- 3.86% (n=13) titin degradation. Lane 1 shows slow-twitch skeletal muscle fibers following trypsin treatment, lane 2 shows a fast-twitch skeletal muscle fiber after trypsin treatment, lane 3 shows a control fast-twitch skeletal muscle fiber myocyte, and lane 4 shows control slow-twitch skeletal muscle fiber (control= no trypsin treatment).

Table 1 Maximal tension for cardiac myocytes, slow-twitch skeletal muscle fibers and fast-twitch skeletal muscle fibers

Fast-twitch pre- trypsin (kN/m^2)	Fast-twitch post-trypsin (kN/m^2)	Slow-twitch pre-trypsin (kN/m^2)	Slow-twitch post-trypsin (kN/m^2)	Cardiac pre- trypsin (kN/m^2)	Cardiac post- trypsin (kN/m^2)	p>0.1 for all muscle types
36.7	37.84	26.93	27.18	241.01	234.05	**Tension**
15.15	14.90	14.77	14.18	77.28	74.04	**Std dev**

Helmes et. Al. also have shown that this degree of trypsin treatment does not affect contractile proteins, but does significantly degrade the titin molecule (24). Titin degradation did not compromise the capabilities of the myocyte or myocyte preparations to generate force, because maximal Ca^{2+} activated force development did not change after trypsin treatment (see Table 1). SDS-PAGE analysis suggested that MyHC content was unaffected by trypsin treatment (Figure 5). Interestingly, there was smearing of the MyHC band in several lanes that were trypsin treated, which may be due to some trypsin induced degradation, however this was not a consistent finding and did not always occur (for example, see lane 8 in Figure 5). The smearing was more likely due to unequal amounts of loaded protein content between lanes.
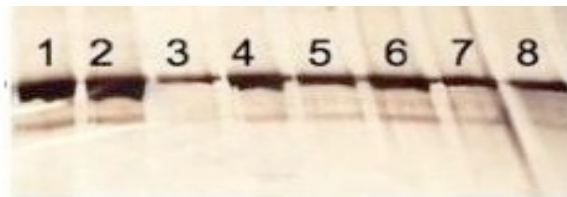

Figure 5. Trypsin treatment of cardiac myocytes does not appear to cleave myosin heavy chain as determined by SDS-PAGE analysis. Lane 1- control myocytes, lane 2- trypsin treated myocytes, lane 3- control myocytes, lane 4- trypsin treated myocytes, lane 5- control myocytes, lane 6- trypsin treated myocytes, lane 7- control myocytes, lane 8- trypsin treated myocytes.

Effects of titin degradation on skinned skeletal and cardiac

myocyte contractile properties

As mentioned above there were no differences in force-generating capacities between skinned myocyte/fiber preparations before and after trypsin treatment. The relationship between titin degradation and loaded shortening/power output was examined in fast-twitch skeletal muscle fibers, slow-twitch skeletal muscle fibers, and rat skinned cardiac myocytes. Figure 6 shows the force-velocity and power-load curves for fast-twitch skeletal muscle fibers, slow-twitch muscle fibers, and cardiac myocytes. During these experiments force velocity curves were obtained prior to titin degradation by measuring velocity of myocyte/fiber shortening at varied loads (ranging from 5%-90% of isometric). Next, titin was degraded by mild trypsin treatment and a second force velocity relationship was obtained. Titin degradation did not significantly affect the loaded shortening velocities or power output of any of the three muscle cell types (Figures 6 and 7).
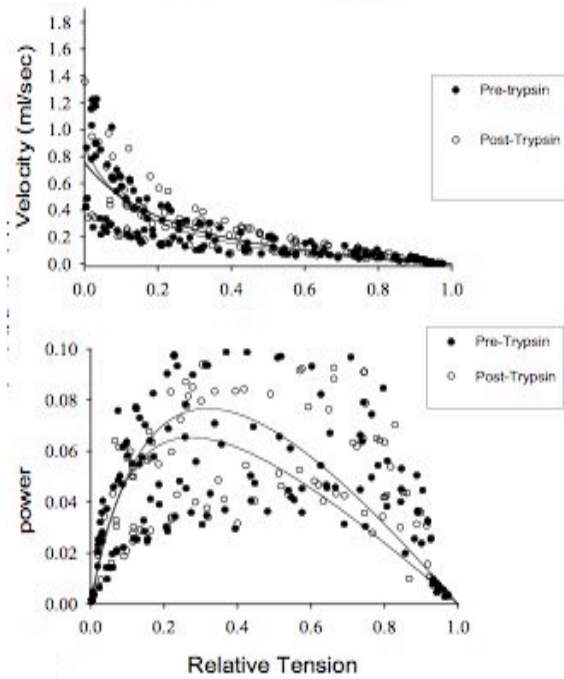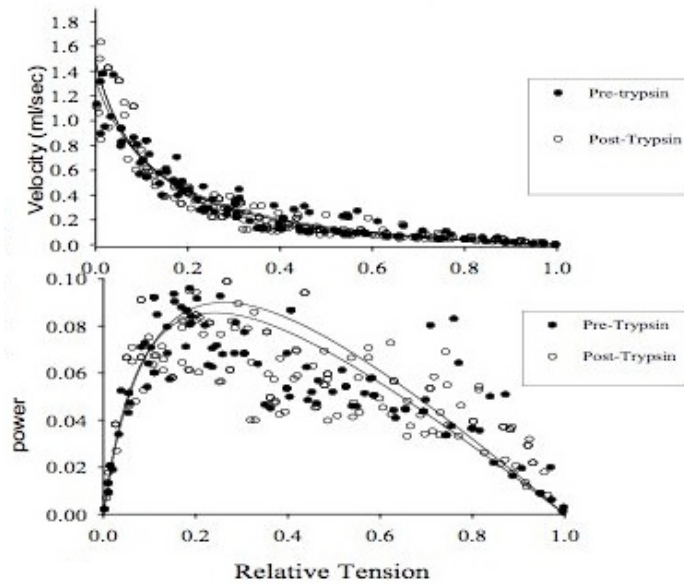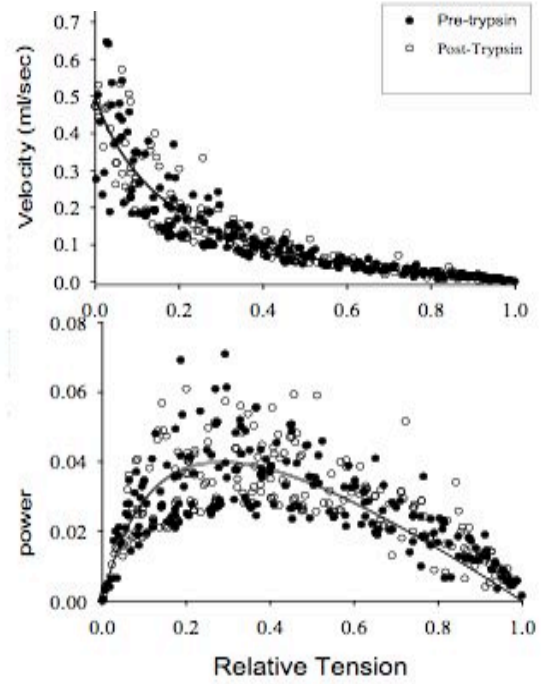
Figure 6. Partial titin degradation by trypsin did not change loaded shortening velocity or power output in fast-twitch skeletal muscle fibers (A), slow-twitch skeletal muscle fibers (B), or cardiac myocytes (C).
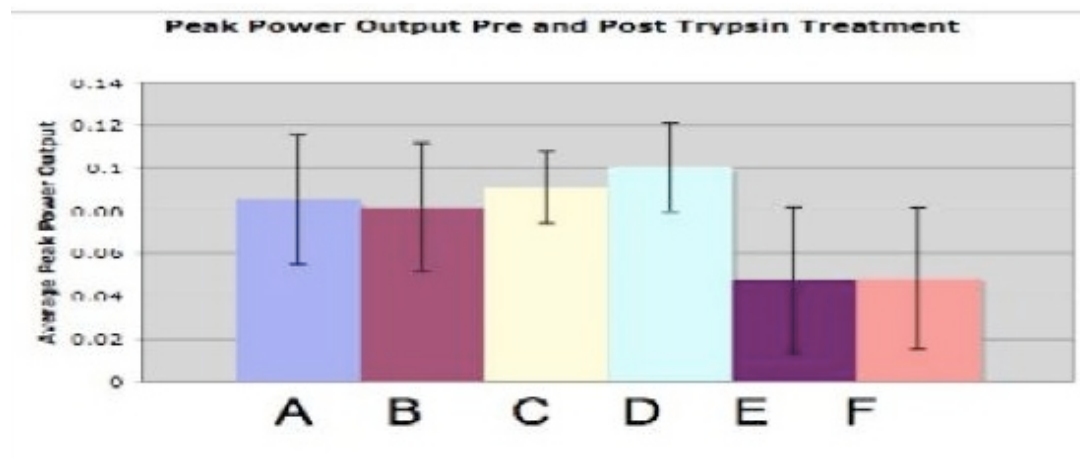
Figure7. There was no significant difference in peak power output in fast-twitch skeletal muscle fibers (n=7), slow-twitch skeletal muscle fibers (n=7) or cardiac myocytes (n=7) after trypsin treatment: A) cardiac myocytes pre-trypsin treatment peak power (0.085+/-0.03, B) cardiac myocytes post-trypsin treatment power (0.081+/-0.028), C) fast-twitch skeletal muscle fibers pre-trypsin peak power (0.091+/- 0.012), D) fast-twitch skeletal muscle fibers post-trypsin peak power (0.1 +/- 0.02), E) slow-twitch skeletal muscle fibers pre-trypsin peak power (0.047 +/- 0.03), F) slow-twitch skeletal muscle fibers post-trypsin peak power (0.048 +/- 0.033), peak power= P/P_0-ML/sec.

We next examined whether titin modulates the rate of force development of striated muscle cells. For these experiments we tested whether titin degradation by mild trypsin treatment altered the rate constant of force redevelopment (k_{tr}) following a slack-restretch maneuver. Figure 8 shows force redevelopment traces of a slow-twitch skeletal muscle myocyte preparation before and after mild trypsin treatment. Titin degradation increased the rate of force redevelopment and, interestingly, also decreased the overshoot of force development typically observed after a slack-restretch maneuver (Figures 8 and 9). This effect of titin degradation to attenuate force overshoot was observed in all three muscle cell types examined (Figure 10).
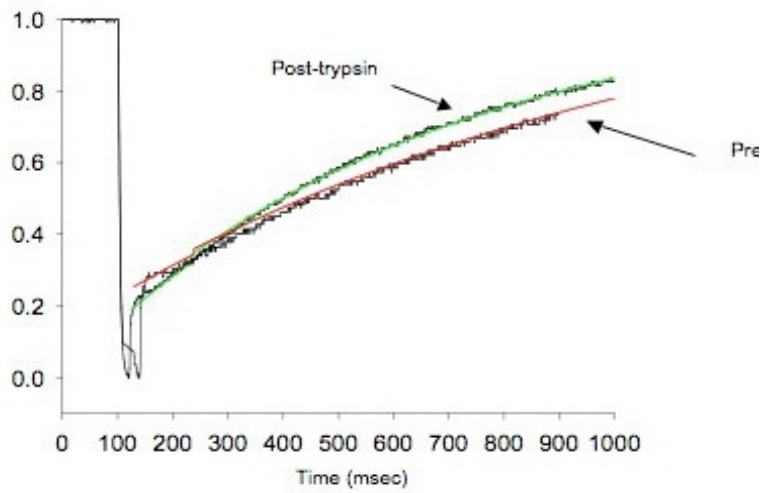
Figure 8. Force redevelopment traces from a slow-twitch skeletal muscle fiber before (red) and after (green) trypsin treatment.
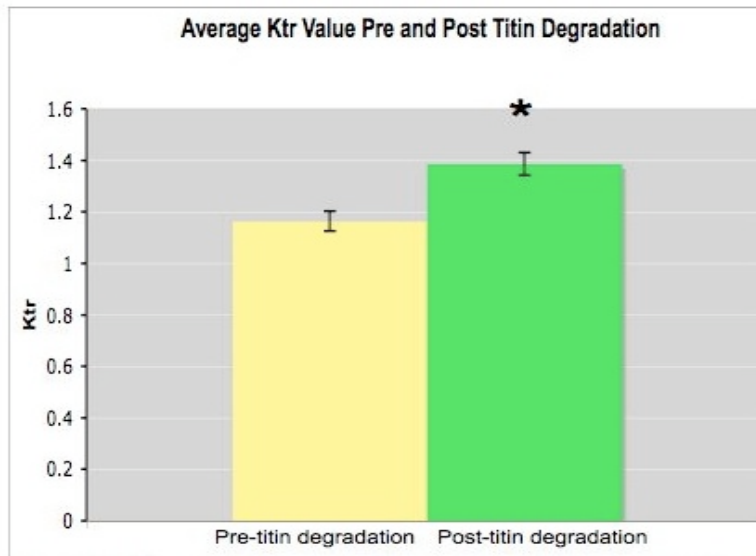

Figure 9. Titin degradation caused a significant increase in k_{tr} (rate constant of force redevelopment) in slow-twitch skeletal muscle fibers (n=6, p<0.001, error bar = standard deviation).
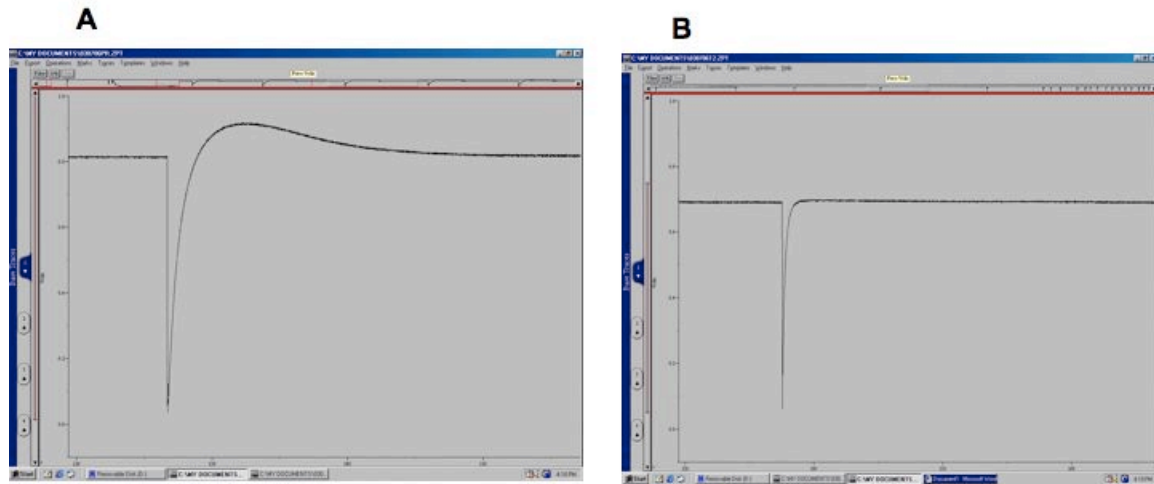
Figure 10: Force redevelopment traces before (A) and after (B) trypsin treatment in a slow-twitch skeletal muscle fiber.

Figure 11: Change in percent overshoot before and after trypsin treatment of skinned cardiac myocyte preparations (A), fast-twitch skeletal muscle fibers (B), and slow-twitch skeletal muscle fibers (C). In cardiac myocytes force overshoot fell from ~8% to ~1% (n=7). In fast-twitch skeletal muscle fibers overshoot fell from ~6% to ~1% (n=6). In slow-twitch skeletal muscle fibers overshoot fell from ~14% to ~1% (n=7) (p<0.001).

Discussion

The primary focus of this study was to determine the contribution of titin to active contraction of striated muscle. Titin did not appear to directl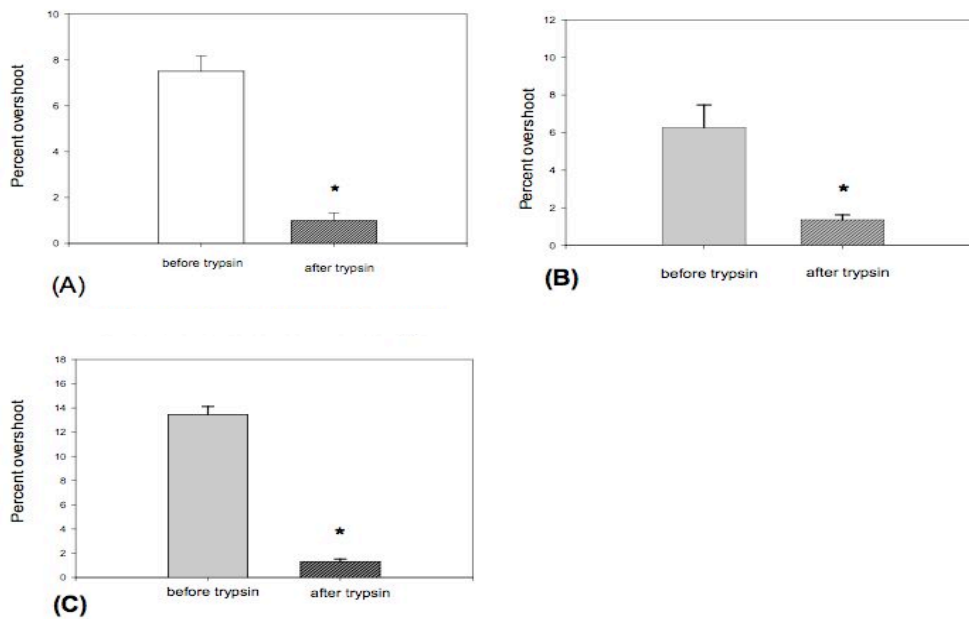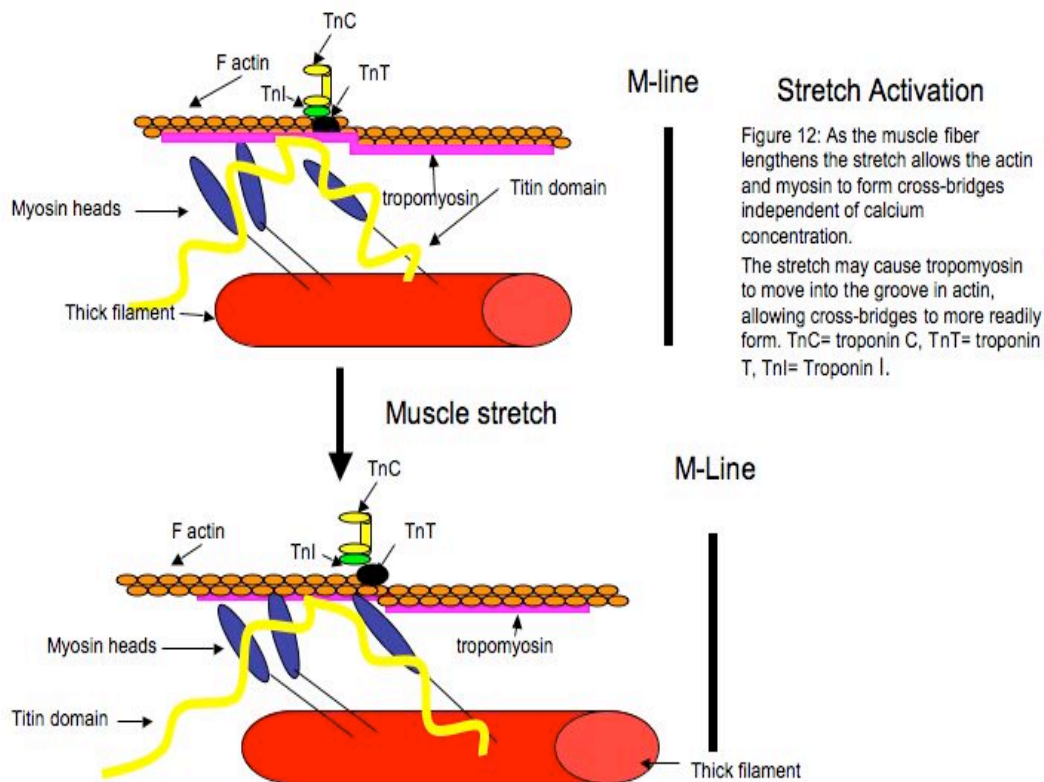y affect the loaded shortening velocity or power output of skeletal muscle myocytes or cardiac myocyte preparations. Degradation of titin, however, did affect the rate of force redevelopment in striated muscle. Mild trypsin treatment of striated muscle myocytes resulted in an increase in the rate of force redevelopment and significantly reduced the overshoot normally observed after a slack-restretch maneuver.

The data collected during these experiments also suggest that titin plays a role in stretch activation properties of muscle. Stretch activation has been defined as a delayed increase in force of calcium-activated muscle triggered by stretch of that muscle (46). This type of activation is particularly important in insect flight muscle as well as vertebrate cardiac muscle. Stretch activated muscle is able to create its own rhythm, known as myogenic rhythm, which helps to support oscillatory work without a neurological rhythm. Stretch of muscle is thought to cause movement of tropomyosin into the actin groove on the thin filament and hence allow actin and myosin to bind and form force-generating cross-bridges. The force redevelopment overshoot seen in Figure 10a has been attributed to stretch activation (5). Campbell had discounted titin's involvement in overshoot, because the overshoot was calcium dependent but titin's properties are thought

to be calcium independent (5). However, our experimental results implicate a direct role of titin because there was a significant reduction in force redevelopment overshoot with ~35% titin cleavage. Titin degradation eliminated overshoot regardless of the level of calcium activation. Titin's involvement in overshoot could be attributed to its positioning within the sarcomere along with its interactions with the thick and thin filaments. Titin's elasticity within the I-band region would most likely respond to stretching of muscle cells by producing a force, which could yield an allosteric effect on the thin filament proteins and result in greater thin filament activation and cross-bridge binding to actin. I propose that as titin is stretched it causes movement in tropomyosin that is translated down the entire thin filament. Titin acts as a series elastic element within the sarcomere, analogous to a tendon of the whole muscle (35). Before tropomyosin can be moved by titin a portion of the force exerted is used to stretch the elastic elements of titin. Titin's participation in the series elastic element allows titin to modulate active muscle tension by the following mechanism: Movement of tropomyosin allows more thick and thin filament interactions and therefore formation of more cross-bridges. The formation of more cross-bridges results in a transient increase in force causing the observed overshoot (depicted in Figure 12). Alternatively the strain on titin could transmit through the cross-bridges to transiently increase force per cross-bridge.

Figure 12: As the muscle fiber lengthens the stretch allows the actin and myosin to form cross-bridges independent of calcium concentration.

The stretch may cause tropomyosin to move into the groove in actin, allowing cross-bridges to more readily form. TnC= troponin C, TnT= troponin T, TnI= Troponin I.

From a physiological standpoint, the experimental results suggest that titin plays a role in isovolumic contraction phase of the cardiac cycle (phase 2) (57, 58). The slack-restretch maneuver performed in these experiments likely mimics, in part, the isovolumic phase of the cardiac cycle, and the data support the idea that titin slows this phase, possibly by increasing cooperativity of force-generating cross-bridges. Greater cooperativity effectively increases the amount of force produced, which manifests as force overshoot (8, 10, 67). This cooperative recruitment of more cross-bridges in parallel (which yields more force) takes a finite amount of time, which tends to slow the measured rate of

force development (and perhaps rate of pressure development during phase 2 of the cardiac cycle (Figure 8).

In addition to the discovery of changes in force redevelopment rates and force overshoot with titin cleavage, it was also shown that 35% titin cleavage did not diminish loaded shortening velocity or power output in cardiac myocytes or in fast-twitch or slow-twitch skeletal muscle fibers. In contrast to this finding, Minejeva et. al. reported that unloaded shortening velocity decreased with titin cleavage (53). The discrepancy between results may be because the putative recoiling force of titin may not be large enough to affect the force produced by the number of active cross-bridges that are needed to support loaded muscle fibers. In other words, the difference may be due to the fact that the current study examined loaded shortening (similar to which occurs in vivo) versus unloaded muscle shortening.

One experimental limitation encountered with this work was the extent of titin degradation. Others have claimed that they can achieve 100% titin degradation with 0.25mg/ml trypsin for 13 min (6, 14, 25). However when this concentration of trypsin was used for this duration there was a significant drop in isometric force production from control levels, and often the myocyte structure was visibly deteriorated. These changes imply that contractile proteins such as myosin or actin are being affected by the trypsin added to the solution. The myocyte preparations that were treated, however, with 0.25-mg/ml trypsin for 6 min did not exhibit any changes in force production and when those myocytes

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were assessed by SDS-PAGE analysis, there appeared to be little to no change in myosin heavy chain content. It certainly is possible that complete titin cleavage would reduce loaded shortening velocity and power output. However, we were unable to completely cleave titin without significant damage to other contractile proteins of skinned myocyte preparations. Titin knockout mice have been attempted by others but these experiments result in lethality, most likely because titin acts as a scaffold for the contractile apparatus during muscle development. Interestingly, Radke et. al. have been able to produce an N2B region knockout mouse line (60). This genotype leads to cardiac atrophy and diastolic dysfunction. The consequences of N2B knockout on myocyte mechanics have not been systematically investigated. Such experiments would be beneficial for testing our idea that titin elasticity plays little to no role in loaded shortening. It also would be interesting to develop a knock-in of the PEVK region to produce a more compliant titin isoform. This would simulate titin cleavage and allow further assessment of our idea that titin elasticity has minimal effects on cardiac myocyte power output capabilities.

Last, once technology allows optical visualization of titin during active muscle shortening, such visualization may help determine the role of titin in myocyte force regulation. For instance, does titin mediate changes in interfilament lattice spacing and does titin elasticity contribute to myocyte shortening dynamics? While this study sheds new light on the role of titin in the

active shortening process in striated muscle, more work is necessary in order to

uncover the biophysical mechanisms by which titin regulates passive and active

properties of striated muscle.

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