Regulation of muscle force in the absence of actin-myosin-based cross-bridge interaction

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Leonard TR, Herzog W. Regulation of muscle force in the absence of actin-myosin-based cross-bridge interaction. Am J Physiol Cell Physiol 299: C14–C20, 2010. First published March 31, 2010; doi:10.1152/ajpcell.00049.2010.—For the past half century, the sliding filament-based cross-bridge theory has been the paradigm of choice for muscle contraction and force production for the past half century (15–17, 19, 20). According to this theory, contraction occurs through the interaction of myosin-based cross bridges that attach cyclically to actin and tend to pull actin past the myosin filaments toward the center of sarcomeres (15, 17, 19). This produces muscle contraction and force. When a muscle is stretched, sarcomeres become longer and actin-myosin filament overlap decreases (Fig. 1), thus decreasing the number of possible cross-bridge interactions and active force while passive forces increase (8, 15, 16, 18, 31). At sarcomere lengths where actin-myosin filament overlap ceases to exist, only passive forces are possible, and these are thought to be essentially invariant at a given muscle or sarcomere length when due account is given to transient viscous effects (Fig. 2). However, recent pilot results suggest that passive forces in isolated myofibrils might be modulated substantially by active stretching (26). In myofibrils, passive forces are known to primarily originate from the structural protein titin (9, 14, 30, 35), the largest protein currently known in the natural world, and thus, we hypothesized that titin, in addition to actin-myosin, might be a strong regulator of force in actively stretched muscles. Therefore, the purpose of this study was to investigate the forces produced in myofibrils that were stretched to lengths too great to allow for actin-myosin interactions and to elucidate the role of titin in producing these forces. Although titin has been associated with force regulation through phosphorylation (37) and calcium binding (25), these effects were assumed much too small to explain our pilot results (26).

METHODS AND MATERIALS

Sample preparation. Strips of rabbit psoas muscle were taken from euthanized animals using Dumont 3 forceps and tied to wooden sticks to preserve the in situ sarcomere length. These strips of muscle were then placed in a rigor-glycerol solution with protease inhibitors (Complete, Roche Diagnostics, Montreal, QB, Canada) and stored at −20°C for 10 to 14 days (32). For experimentation, strips of muscle were placed in a +4°C rigor solution, homogenized, and placed in the experimental chamber (20°C). Solutions used are published elsewhere (22, 34).

Ethics approval was granted from the institutional Animal Ethics Committee.

Testing protocol. Fifty-nine single myofibrils (with an aggregate total of 312 sarcomeres) from rabbit psoas were tested in these experiments and divided into eight testing groups. Myofibrils with three to eight sarcomeres in series were used (mean of 5.3) in these experiments because of the high magnification of the microscope (>100 oil objective with a ×2.5 Optovar) and the large magnitude of the stretches employed.

Group 1 myofibrils (n = 12) were nonactivated and stretched in a solution containing ATP. Group 2 myofibrils (n = 12) were activated in a calcium + ATP solution and then stretched. Group 3 myofibrils (n = 6) were treated with a mild trypsin solution (for titin deletion) (7, 10, 11) and then kept nonactivated and stretched in a solution containing ATP. Group 4 samples (n = 8) were also treated with trypsin but were then placed in the activating calcium + ATP solution and then stretched. Group 5 myofibrils (n = 10) were placed in an activating (calcium + ATP) solution with 20 mM 2,3 butanedione monoxime (BDM), a cross-bridge inhibitor (34), and then stretched. Group 6 (n = 5) myofibrils were nonactivated and lengthened to 3.4 μm and then activated in a calcium + ATP solution and then further lengthened. Group 7 myofibrils (n = 2) were nonactivated and lengthened to ~5 μm, then activated and further lengthened to a mean sarcomere length of ~6 μm. Group 8 myofibrils (n = 4) were activated at optimal length (2.2 μm), lengthened to a mean sarcomere length of ~5 μm, and then deactivated by placement in a relaxing solution.

Tests for all myofibrils were performed starting at sarcomere lengths between 2.0 and 2.4 μm (except group 6) and then lengthened at a speed of 0.1 μm per sarcomere per second (which is ≈5% of the initial sarcomere length per second).

Sarcomere length and force measurements. All tests were conducted using an inverted microscope (Zeiss Axiovert 200M) (22) equipped with a ×100 oil immersion objective (numerical aperture 1.3) and a ×2.5 Optovar. Individual sarcomere lengths were measured using an ultra-high-resolution linear diode line scan camera (model SK10680 DJR, Schafer and Kirschoff) with a resolution of 6.7 nm/pixel (22). Sarcomere lengths were calculated from Z line to Z line.

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of adjacent sarcomeres, and only when this was not possible because the striation pattern tended to disappear with excessive stretching, average sarcomere length was determined by dividing the specimen length by the number of sarcomeres. A custom-built piezo-tube motor with a drawn glass pipette was used to manipulate the length of the specimen with nanometer resolution. LabView software (National Instruments, Austin, TX) controlled the motor and data acquisition. Myofibril forces were determined using custom-built nanofabricated silicon nitride cantilevers (6) with a stiffness of 22 pn/nm (for passive and titin-deleted experiments) or 178 pn/nm (for all active experiments). Displacement of one lever attached to the myofibril relative to a reference lever was measured, and forces were calculated from the measured displacement and the known lever stiffness (22). Myofibrils were glued (Dow Corning 3145)(28) to one of the levers and wrapped around the lever to help prevent detachment. Forces were normalized to the cross-sectional area by measuring myofibril diameter (28) and were expressed in units of stress (nN/μm²).

RESULTS

Activated and stretched myofibrils show much greater force within the actin-myosin filament overlap zone (sarcomere lengths <4.0 μm) than nonactivated and stretched myofibrils, as one would expect (Fig. 3A); however, completely unexpected, forces in the activated and stretched myofibrils remain much higher, and increase more rapidly, than those of myofibrils that were lengthened while not activated, even at sarcomere lengths beyond 4.0 μm (Fig. 3A).

To elucidate the possible role of titin in the increased force of actively stretched myofibrils, we repeated the active and

Fig. 2. Representation of the force-length relationship showing active force (red) and passive force (green) contributions with the total force (black dashed). Up to sarcomere lengths of ~3.6 μm, active forces dominate; between 3.6 and 4.0 μm, passive forces dominate; and beyond 4.0 μm, the passive forces become the only source of force for rabbit psoas myofibrils. [From Gordon et al. (8)].
nonactivated stretch experiments following depletion of titin. The absence of titin, forces during activated and nonactivated stretching were essentially zero and did not increase systematically with increasing sarcomere lengths (blue and yellow triangles, Fig. 3B), suggesting that titin is crucial for nonactivated force production, as suggested by others (9, 14, 30, 35) and that titin is essential for the dramatic increase in force observed here beyond myofilament overlap in the activated and stretched myofibrils (Fig. 3A).

Activation of myofibrils has been associated with calcium binding to titin and an associated increase in titin’s spring stiffness and force upon stretch (23, 25). These effects, however, have been thought to be minor but have only been studied within the region of actin-myosin filament overlap. Thus, to elucidate the role of calcium on titin’s force and stiffness regulation in the absence of actin-myosin-based cross-bridge forces, we performed stretch experiments with calcium-activated myofibrils while inhibiting cross-bridge force through BDM (2, 34). Stretching “activated” myofibrils in BDM conditions was successful in abolishing all actin-myosin-based forces (orange squares, Fig. 3B) and produced passive forces that were essentially the same as those produced for purely nonactivated (passive) myofibril stretching (green circles, Fig. 3B). This result suggests that calcium activation, and calcium binding to titin, has a negligible effect on myofibril force regulation and cannot explain the results observed in Fig. 3A. Thus, we hypothesized that actin-myosin-based cross-bridge forces before stretching are essential to produce the observed force increase of actively compared with nonactivated and stretched myofibrils.

To test this hypothesis, we performed a further set of experiments in which myofibrils were fully activated at different sarcomere lengths (2.4 μm and 3.4 μm/sarcomere) and then lengthened. The active forces for myofibrils activated at 3.4 μm (purple diamonds, Fig. 3A) were about half of those activated at 2.4 μm (red squares, Fig. 3A), and when stretched beyond actin-myosin overlap, the forces remained smaller than those of the myofibrils activated and stretched from sarcomere lengths of 2.4 μm, but were greater than those of the nonactivated and stretched myofibrils (green circles, Fig. 3A). These results suggest that the force regulation observed here at sarcomere lengths beyond actin-myosin filament overlap depends on the active forces before stretching.

To test whether actin-myosin-based cross-bridge forces could be produced at sarcomere lengths >4.0 μm, we stretched nonactivated myofibrils from sarcomere lengths of ~2.2 μm to a mean sarcomere length of approximately 4.5 to 5 μm, then activated them and stretched them further to sarcomere lengths of ~6 μm (Fig. 4). Activating myofibrils at sarcomere lengths >4.0 μm did not alter the force-time curves, indicating that cross-bridge interactions did not occur at these long lengths.

To confirm that the increased passive forces of actively compared with nonactivated and stretched myofibrils were not caused by remnant cross-bridge attachments due to myosin filaments being elongated at their ends as suggested by previous work (36) or torn from the A-band region due to the large stretch imposed in our testing, we stretched fully activated myofibrils from a mean sarcomere length of 2.4 μm to a mean sarcomere length of ~5 μm and then replaced the activating with a relaxing solution (Fig. 5). We observed no effect on force when the solutions were exchanged, thereby confirming that the increase in force in the absence of actin-myosin overlap was not due to “rogue” myosin filaments attaching to actin even at very long sarcomere lengths.
The main result of this study is that forces in the absence of actin-myosin-based cross-bridge forces can be modulated significantly in skeletal muscles, thereby suggesting that there must be significant mechanisms of force production that are not explained by the cross-bridge theory and the traditional expectation of viscoelastic passive forces. The force regulation observed here is significantly greater in magnitude than the maximal actin-myosin-based cross-bridge forces at optimal sarcomere length (Fig. 3A).

Specifically, we show increases in force of up to four times in actively (compared to nonactivated) stretched rabbit psoas myofibrils at sarcomere lengths beyond actin-myosin filament overlap where active, cross-bridge-based forces do not exist (Figs. 4 and 5). When eliminating titin, forces in the activated and nonactivated and stretched myofibrils remain nearly zero even at very long sarcomere lengths, suggesting that titin must be present for this force regulation to take place. Furthermore, since titin is well known to be the primary passive force producer in rabbit psoas myofibrils (3, 13), it is safe to assume that titin plays a crucial role in the force regulation observed here for the first time.

It has been known for some time now that calcium binds to titin upon activation and increases titin’s resistance to stretch (22, 25). However, this calcium-induced stiffening of titin, although well accepted, has been thought to be of small magnitude and thus functionally irrelevant. Nevertheless, to test whether calcium binding to titin might play a role in the dramatic upregulation of force during active elongation, we stretched myofibrils in the activated (activation solution with high-calcium concentration) state but inhibited cross-bridge attachment with BDM (34). In these experiments, the forces measured in the myofibrils were essentially identical to those measured for the purely passive stretches (in relaxing solution), thereby strongly suggesting that calcium activation of titin was not responsible for the increased forces during activated myofibril stretching observed in this study (Fig. 3B; orange squares and green circles, respectively). On the basis of these results, we speculate that either “active (actin-myosin based) force” or cross-bridge attachment to actin is required to produce the

Fig. 4. Individual sarcomere length-time curves and force-time curve for one nonactivated and lengthened myofibril (passive) from the group 7 tests. All individual sarcomere lengths could be measured throughout the test, with all sarcomeres >4.0 μm in length before the introduction of the activating solution; no change in force is observed upon activation (at vertical dashed line), indicating that cross-bridge interactions are not present at these sarcomere lengths. Further lengthening from a mean sarcomere length of 4.5 μm to ~6 μm in a high-calcium activating solution produces force that is essentially the same as that observed in purely passively lengthened myofibrils at the corresponding sarcomere length.

Fig. 5. Individual sarcomere length-time curves and force-time curve for an actively lengthened single myofibril composed of 5 sarcomeres in series from the group 8 tests. There are distinct sarcomere length nonuniformities before activation, and upon activation all sarcomeres shorten. The myofibril is then lengthened to a mean sarcomere length of ~5 μm to 6 μm in a high-calcium activating solution produces force that is essentially the same as that observed in purely passively lengthened myofibrils at the corresponding sarcomere length.
increased (non-actin-myosin based) forces observed with activated myofibril stretching.

If active force or cross-bridge attachment was required for this phenomenon to occur, one would expect that the increase in non-actin-myosin-based forces was directly dependent on the magnitude of the cross-bridge-based forces. To test this hypothesis, we performed another set of experiments where stretching of the activated myofibrils was not started at optimal (2.4 μm) sarcomere length, but at a longer (3.4 μm) average sarcomere length where active forces would be reduced to ~40% of those at optimal length (Fig. 3A). Stretching myofibrils with reduced active force also produced forces in the nonoverlap zone (sarcomere lengths >4.0 μm) that were smaller than those obtained for the myofibrils for which active force was greater, but produced forces that were greater than those of nonactivated and stretched myofibrils (Fig. 3A). This result provides strong evidence that the increase in non-actin-myosin-based forces with active stretching is directly linked to active force or cross-bridge attachments to actin.

Before attempting to find explanations for a possible “titin”-based force regulation, we need to make sure that more traditional explanations might not be possible. Probably the most frequently used explanation for force enhancement following muscle stretching is the so-called sarcomere length instability and nonuniformity theory (24, 29). According to this theory, sarcomeres are unstable on the descending part of the force-length relationship, and short sarcomeres remain short upon stretching, while long sarcomeres are pulled beyond overlap where they are rescued by passive forces (12). However, the sarcomere length nonuniformity theory is an unlikely explanation for our results for two reasons: first, if sarcomere length nonuniformity produced the dramatic increase in the non-actin-myosin-based cross-bridge forces, then the highest forces measured in our experiments should never exceed the active forces at optimal sarcomere length, as that would be the limiting force of the short (active force producing) sarcomeres.

However, this result was not found, because the forces of the activated and stretched myofibrils were approximately five times those measured at optimal sarcomere length (Fig. 3A). Even if we assume that the short sarcomeres were stretched, and further assume a maximal stretch force enhancement of 100%, we would still not be near the forces required to explain our measurements.

Second, if sarcomere length nonuniformity was responsible for the observed results, then the long (overstretched) sarcomeres should follow the passive force-extension curve once they are pulled beyond actin-myosin filament overlap. However, our experiments observed that nonactivated and stretched myofibrils failed mechanically at sarcomere lengths of ~6.3 μm and at a force level (263 nN/μm²) that was substantially less than the forces obtained in the activated and stretched myofibrils, thus sarcomere length nonuniformity seems an unlikely explanation for the results observed here.

Nevertheless, to make absolutely sure that sarcomere length nonuniformity could not explain the current results, we measured the individual sarcomere lengths of the stretched myofibrils. Myofibrils are fairly fragile preparations, and one might expect that activation alone might produce a vast increase in sarcomere length nonuniformities, but this was not observed. Furthermore, and in contrast to the expectations of the sarcomere length nonuniformity and instability theory (12), we observed continuous elongation of all sarcomeres when myofibrils were stretched. Sarcomere lengths were not uniform, neither for nonactivated and stretched nor for activated and stretched myofibrils, but on the basis of the structural assumptions underpinning the force-length relationship, we assumed (without verification by a direct observational method like electron microscopy) that all sarcomeres were always pulled beyond actin-myosin filament overlap in all cases where such measurements were made (e.g., Figs. 4, 5, and 6). Furthermore, activation of myofibrils with all sarcomeres at lengths >4.0 μm did not produce an increase in force (Fig. 4), nor did...
deactivation in this same situation produce a decrease in force (Fig. 5), strongly suggesting that there were no actin-myosin-based cross-bridge forces that affected (or caused) the observed increases in force in the activated and stretched myofibrils at sarcomere lengths greater than actin-myosin overlap. From all these observations, we may safely conclude that whatever the cause for the dramatic increase in non-cross-bridge-based forces during active muscle stretching, it cannot be explained with sarcomere instability and the associated development of sarcomere length nonuniformity. Classic studies by others (5, 27) have proposed the transverse cytoskeleton as a regulator of force during stretch. The transverse cytoskeleton produces lateral force transfer between myofibrils in single fibers (and presumably in bundles of myofibrils) but because our preparation is based on a single myofibril, this possibility of force increase during stretch of active myofibrils is precluded.

In the absence of an obvious traditional explanation for the observed force increases during active stretching beyond actin-myosin filament overlap (such as the cross-bridge or the sarcomere length nonuniformity theory), we must search for other possibilities. Reviewing the experimental evidence, it appears that titin must be present for the observed force increases, but calcium activation of titin does not do the trick. However, it is well known that titin can also change its spring stiffness (and thus resistance to stretch) by changing its free spring length in the I-band region by attaching selectively to actin. Here, we would like to tentatively propose that titin binding to actin might be the cause for the dramatic increase in non-cross-bridge-based forces when muscles are stretched actively compared with when they are stretched in the nonactivated state.

Although the exact molecular details for such a mechanism need careful evaluation, we would like to suggest the following scenario. Upon active force production, titin preferentially attaches to actin, thereby shortening its spring length and increasing its resistance to stretch (Fig. 7). To fully explain our observations, such titin-actin interactions must be modulated either by active force or by the number of attached cross bridges. Force could be the modulator of titin-actin attachment through stretching of the actin filament, thereby exposing “titin attachment sites” on actin, similar to the way stretching of the talin rod enhances binding of vinculin (4). Alternatively, attachment of the cross-bridges to actin, and the associated movements of the regulatory proteins, troponin and tropomyosin, might free up previously covered titin attachment sites on actin. Whatever the detailed mechanisms, the force modulations produced by it must be as great as the actively produced muscle forces through actin-myosin binding by cross bridges.

The idea about titin-actin binding in actively stretched myofibrils can be tested relatively easily. Imagine, for example, that titin is labeled at specific sites with a fluorescent marker so that lengths of individual titin segments can be carefully measured. If we now stretch a nonactivated myofibril, we would expect all segments to elongate on the basis of their constitutive stress-strain properties. However, if a myofibril is activated and stretched by the same amount and produces substantially more force as observed here, we would expect that some titin segments (those bound to actin) would not stretch (or stretch significantly less than in the passive state), while others (those not bound) would stretch more and thereby compensate for the “fixed” attached segments of titin. This then would produce greater stiffness and increased forces during stretching of activated muscle.

The proposed mechanism has the advantage that it is independent of actin-myosin filament overlap and would continue to be in operation at lengths greater than myofilament overlap (i.e., 4.0 μm in rabbit psoas). Therefore, this mechanism might provide powerful protection against stretch-induced muscle injuries, the most common mode of damage of skeletal muscles. Also, such a stretch-induced modulation of force would provide an elegant explanation for the observed stability of sarcomeres on the descending limb of the force-length relationship (21, 33), which has been thought (erroneously) to be unstable for more than half a century (12). Finally, the proposed mechanism might also offer a partial explanation for the so-called “residual force enhancement” of muscles following active stretching (1).

Conclusion. We conclude from the results of this study that there is a powerful mechanism for force regulation in muscles that is independent of actin-myosin-based cross bridges. This force modulation depends crucially on the presence of titin and active force. We tentatively suggest that force (or cross-bridge attachment)-dependent titin-actin interactions cause the dramatic increase in force of activated and stretched compared with nonactivated and stretched muscles.

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DISCLOSURES

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

Fig. 7. Proposed mechanism of force regulation through titin-actin binding. A: passive myofibril lengthening shown with titin spanning the I-band region. No titin-actin interactions occur, so titin is able to extend over its entire free length. B: active force is thought to enhance interaction and binding of titin to actin thereby shortening titin’s natural spring length. Upon stretch, strain in titin’s free spring element is greater for a given absolute increase in sarcomere length, thereby increasing titin’s force contribution. Thus, forces in stretched muscle are regulated not only through actin-myosin-based cross-bridge forces but also through force-dependent interactions of titin with actin.
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