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The Mechanical Properties of Titin within a Sarcomere?

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Titin is a structural protein in muscle that spans the half sarcomere from z-band to M-line. Although there are selected studies on titin's mechanical properties from tests on isolated molecules or titin fragments, little is known about its behavior within the structural confines of a sarcomere. Here, we tested the hypothesis that titin properties might be reflected well in single myofibrils. Therefore, the purpose of this study was to measure the passive mechanical properties of isolated single myofibrils and evaluate whether these properties reflect the basic mechanical properties of the titin molecule. Single myofibrils from rabbit psoas were prepared for measurement of passive stretch-shortening cycles at lengths where passive titin forces become important. Three repeat stretch-shortening cycles with magnitudes between 1.0-3.0μm/sarcomere were performed at a speed of 0.1μm/s·sarcomere and repeated after a ten minute rest at zero force. These tests were performed in a relaxation solution (passive) and an activation solution (active) where cross-bridge attachment was inhibited with butanedione monoxime. Myofibrils behaved viscoelastically producing an increased efficiency with repeat stretch-shortening cycles, but a decreased efficiency with increasing stretch magnitudes. Furthermore, we observed a first distinct inflection point in the force-elongation curve at an average sarcomere length of 3.5μm that was associated with an average force of 68±5nN/mm^{-1}. This inflection point was thought to reflect Ig domain unfolding and was missing after a ten minute rest at zero force, suggesting a lack of spontaneous Ig domain refolding. These passive myofibrillar properties are consistent with those observed in isolated titin molecules, suggesting that the mechanics of titin are well preserved in isolated myofibrils, and thus, can be studied readily in myofibrils, rather than in the extremely difficult and labile single titin preparations.

Introduction

Titin (also known as connectin) is a giant structural protein in muscle. It spans a half sarcomere from the z-band to the M-line (Fig. 1) and has been associated with passive force production in cardiac and skeletal muscles. It has spring like properties in its extensible I-band domain dominated by the immunoglobulin (Ig) segments (both proximal and distal), and the PEVK region, named so for its predominance in proline (P), glutamate (E), valine (V) and lysine (K) residues. Since its discovery in the mid-1970s[1,2], titin has emerged as an important stabilizer of sarcomeres[3,4], a producer of passive force[5,6], a regulator of active force[7,8], and has been associated with a variety of signaling, structural, and mechanical properties[9,10,11,12].

Titin is considered the third sarcomeric protein[11], and knowing its mechanical properties is essential for explaining passive characteristics of muscles[11,12,13], force regulation during stretch[7,8], sarcomere stability[3,4], and residual force enhancement in skeletal muscles[8,14,15]. However, measuring titins mechanical properties is difficult because the isolated protein is highly unstable, and measuring its properties requires specialized equipment. Nevertheless, Kellermayer

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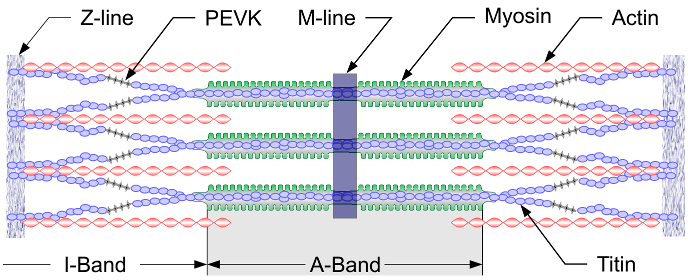

Figure 1:
Schematic illustration of a sarcomere with the sarcomeric filaments titin, actin, and myosin. Titin is anchored in the z-lines of the sarcomere and extends to the M-band in the middle of the sarcomere, thereby spanning a half sarcomere. Because of its spring-like properties in the I-band region, titin is perfectly positioned to stabilize the myosin filaments in the centre of the sarcomere, provide stability to sarcomeres (especially when stretched to long lengths), and to act as a force modulator when sarcomeres are stretched.

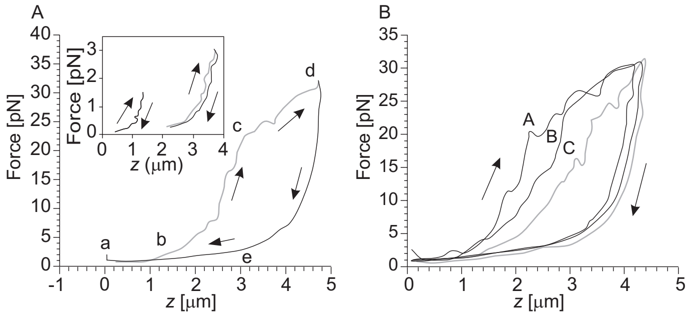

Figure 2:
Force-elongation curves for isolated titin molecules. (A) Titin behaves as an essentially elastic spring over short extension distances (inset), but has a large hysteresis when pulled beyond the point where Ig domains are thought to unfold (point c). (B) Repeat force-elongation curves of a titin molecule illustrating the loss of loading energy for repeat cycles (A first cycle; B second cycle; C fifth cycle) while the unloading energy remains less affected by repeat stretch-shortening cycles (from Kellermayer et al.[16]: by permission).

et al. were able to isolate titin and measure its passive force-elongation properties using a laser trap approach[16]. They found that titin had a virtually elastic response below approximately 20pN[1] and a highly viscoelastic response above 20pN. They also observed that energy loss in passive stretch-shortening cycles decreased with repeat stretch cycles, but increased with increasing stretch magnitudes, and further observed a distinct inflection point in the force-elongation curve which they associated with the start of unfolding of the Ig domain elements[16] (see Fig. 2).

Although mechanical experiments of fractional parts of recombinantly produced titin segments have been performed successfully[17,18,19], and have provided crucial insights into the workings of this molecular spring, full length mechanical experiments of titin are rare[16]. Even with full length testing of titin, there are a variety of limitations. These include the uncertainties of the exact location of fixation of the protein for mechanical measurement, the possibility of measuring properties of multiple rather than single titins, and the difficulty of relating isolated titin properties to its function in the sarcomere, fibre and muscle[16]. Therefore, the purpose of this study was to measure the passive mechanical properties of isolated single myofibrils and evaluate whether these properties reflect the basic mechanical properties of the titin molecule. If so, experiments at the myofibrillar level might be used, as a much simpler alternative to isolated protein tests in assessing the mechanical properties of titin in its native and structurally correct environment in different muscles and under different mechanical loading conditions.

Methods

Preparation

Rabbit psoas myofibrils were used for testing. Myofibrils were harvested from rabbit psoas, chemically and mechanically isolated as described in our previous works[7,8,12,13], and prepared for mechanical testing using micro-electronically machined silicon nitride levers (stiffness 68 pN/nm) for force measurement at one end of the myofibril (resolution <0.5 nN), and a glass needle attached to a motor for producing sub-nanometer step sizes at the other end[7,8,12,13] (Fig. 3).

Testing

Myofibrils (n=28) were passively stretched (see relaxation solution in Joumaa et al, 2008[12]) from a nominal initial average sarcomere length of 2.5-2.7μm by 1.0, 2.0, 2.5, and 3.0μm at a speed of 0.1 μm/s·sarcomere and then released at the same speed to the original length. Three consecutive stretch-shortening cycles were performed without

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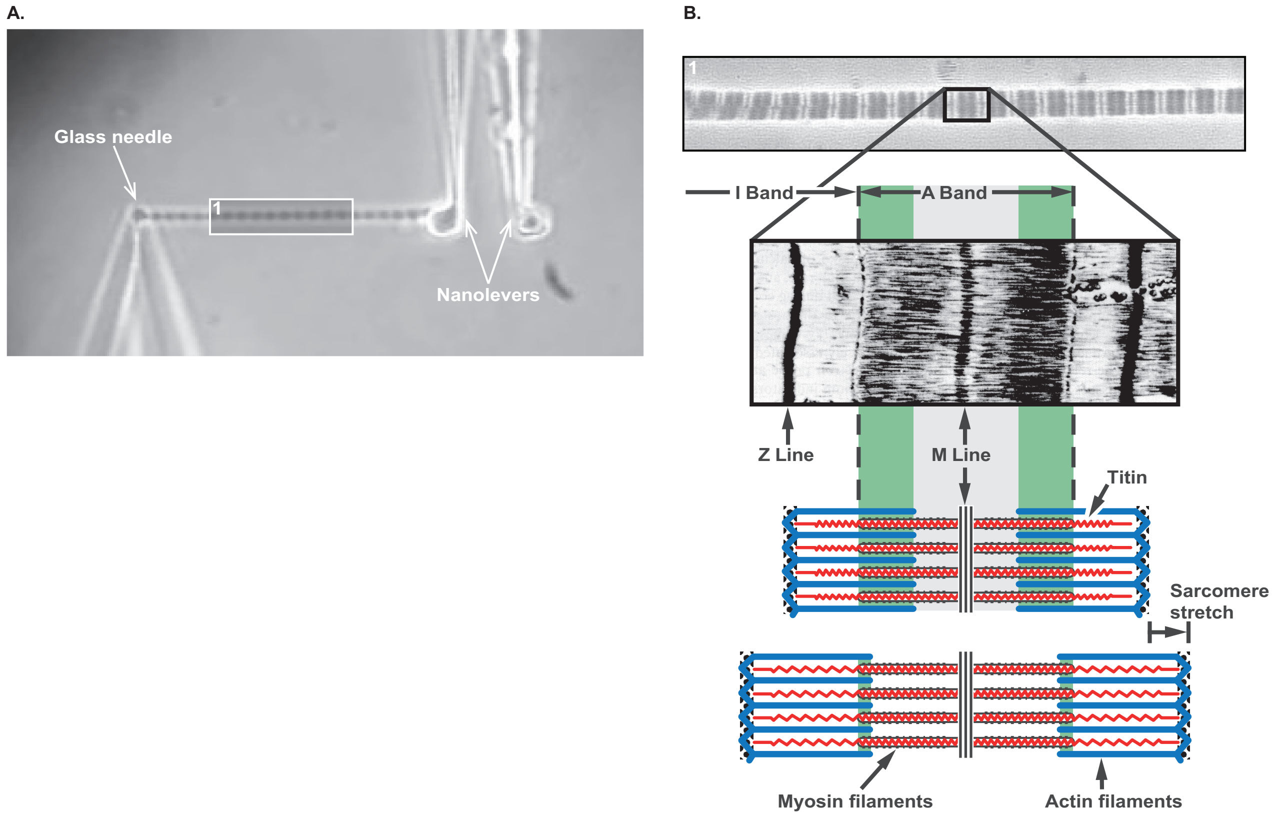

Figure 3:
A. Photo micrograph of a myofibril attached to a glass needle (for imposing controlled displacements) at one end and to a pair of nanolevers (for force measurements) at the other end (left panel). B. Close up view of a myofibril (right panel top), and a single sarcomere (right panel middle), and schematic illustration of the three myofilament structure of a sarcomere (right panel bottom).

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rest between cycles, followed by a ten minute rest and repeat of the original stretch-shortening protocol. For selected myofibrils (n=8), these same stretch-shortening cycles (performed at a speed of 0.1μm/s·sarcomere) were also performed using an activating solution plus a cross-bridge inhibitor (butanedione monoxime, BDM; see activation solution with BDM in (Leonard and Herzog, 2010[8])) to test the passive properties of titin in an activation medium with a saturated calcium concentration ($pCa^{2+} = 3.5$).

Analysis

Peak force, loading energy (area under the force elongation curve in the stretch phase), unloading energy (area under the force-elongation curve in the shortening phase), hysteresis or loss of energy (difference between the loading and unloading energies), change in stiffness (defined as the inflection point of the force-elongation curve; that is where the second derivative of this curve became zero), shortest sarcomere length at the inflection point and average force at the inflection point were all evaluated for each myofibril. All values are given as means and corresponding standard deviations. Statistical differences for all outcome measures between the tests performed passively (relaxation solution) and actively with cross-bridge inhibition (activation solution with BDM) were performed using the non-parametric Mann-Whitney signed-rank test[20]. Changes in outcome measures between the first, second and third stretch-shortening cycle, and from the first set of three cycles and the repeat set after ten minutes, were performed using non-parametric Kruskal-Wallis repeated measures testing[20]. All analyses were performed using a level of significance of 0.05.

Results

Change in stiffness of force-elongation curve

A distinct change in stiffness of the force-elongation curves was observed in 8 of the 28 tested myofibrils (Fig. 4). The smallest sarcomere length where this was observed was 3.5μm while the average force at this inflection point was 68 nN ($\pm$5 nN) when normalized to 1.0μm of myofibril cross-sectional area.

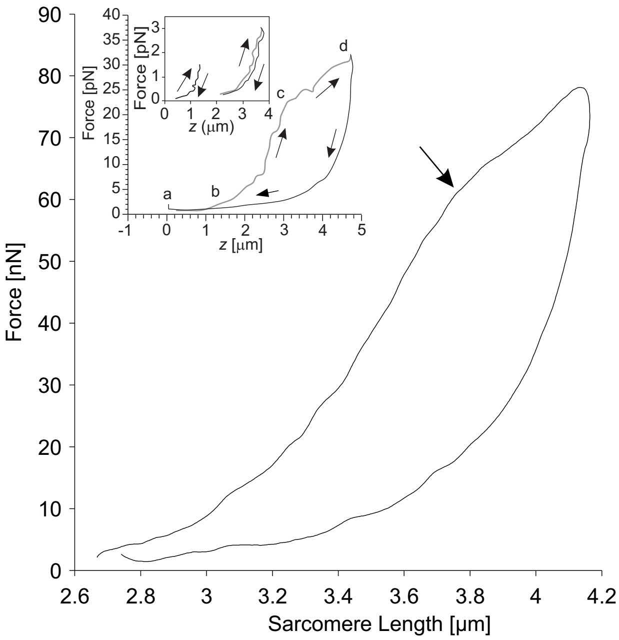

Figure 4:
Passive force-elongation curve of a single myofibril showing a characteristic inflection point (arrow) as has been found in tests using isolated titin molecules (inset point c). This inflection point is thought to reveal the length at which Ig domain unfolding starts to occur in the sarcomeres of a myofibril. Note the similarity in shape of the single molecule and single myofibril curves. Sarcomere length is the mean length of all sarcomeres in the myofibril.

Loss of energy (hysteresis)

Energy loss decreased significantly ($p<0.05$) from the first to the second, and from the second to the third repeat stretch-shortening cycle (Table 1 and Fig. 5). This decrease was primarily associated with a significant ($p<0.05$) decrease in loading energy, while the unloading energies for repeat cycles also decreased significantly ($p<0.05$) but to a much lesser degree (Fig. 5). The energy loss for the first cycle of the repeat testing set (10 minutes following the first set) was significantly smaller ($p<0.05$) than the first cycle of the initial set, but was similar (not significantly different) to the third cycle of the original set (Fig. 6). Finally, efficiency decreased with the magnitude of stretch ($p<0.05$) from the smallest (1.0μm) to the greatest stretch magnitude (3.0μm) for all corresponding cycles (1st, 2nd and 3rd in Table 1).

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Table 1:
Mean (±1SD) percent efficiency of the three repeat stretch cycles of a test set as a function of the stretch magnitude per sarcomere of passive myofibrils. Note that efficiency increases with increasing number of repeat stretch cycles and decreases with increasing stretch magnitudes (as observed for single titin molecules).

Stretch magnitude (per sarcomere)	1st stretch	2nd stretch	3rd stretch
1.0 μm	50% (6%)	66% (9%)	72% (5%)
2.0 μm	49% (5%)	63% (7%)	65% (6%)
2.5 μm	38% (8%)	52% (12%)	55% (11%)
3.0 μm	36% (8%)	45% (8%)	48% (12%)

Active vs. passive stretch-shortening tests

There were no differences in any of the outcome measures for the tests performed using the relaxation solution (passive tests) and the activation solution (active tests) with the added cross-bridge inhibitor (results not shown).

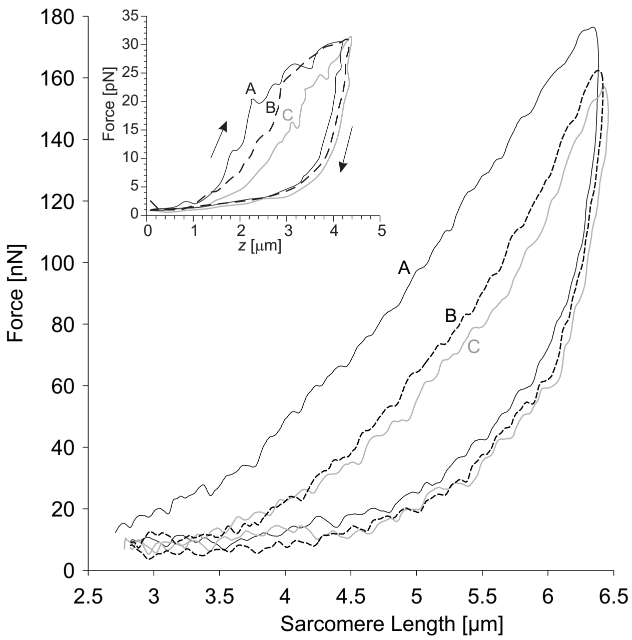

Figure 5:
Force-elongation curves of three repeat stretch-shortening cycles of a single myofibril illustrating the loss of loading energy, the decrease in the hysteresis and the relatively unchanged unloading energies from cycle 1 to cycle 3. Note the similarity of the single myofibril curves with the curves obtained from a single titin molecule (inset).

Discussion

Changes in stiffness

We observed a change in stiffness (inflection point) of the myofibril force-elongation curves similar to that seen in the isolated titin tests[16]. However, this observation was only made for a sub-set of the myofibrils. We think there might be two reasons for this inconsistent observation: first, the attachment of myofibrils and its handling prior to testing often involved considerable stretching of the sarcomeres. This stretching in the preparatory phase preceding the actual tests might be responsible for unfolding of some of the Ig domains (which we think are the cause for the change in myofibril stiffness see below), and if these Ig domains do not refold within a short period of time (which we think they do not see below), the change in stiffness could only be observed in myofibrils that were prepared without significant stretching of the sarcomeres prior to testing. Second, we estimate that 1.0μm of cross sectional area of a muscle contains approximately 2700 titin molecules[6,21]. Since sarcomere lengths in a myofibril (like in a muscle) are non-uniform[22], one would expect titin Ig domain unfolding to occur at different myofibril lengths for the individual sarcomeres, and this might obscure the clear change in stiffness observed in isolated titin preparations[16].

The smallest mean sarcomere length at which the change in stiffness in the first force-elongation cycle occurred was 3.5μm. For subsequent cycles, the inflection point occurred at increasingly longer sarcomere lengths and slightly increasing myofibril forces (Fig. 7). This result suggests that (i) unfolding of the Ig domains occurs at approximately 3.5μm in rabbit psoas muscle, and (ii) that Ig domain refolding is not complete in the shortening phase, thereby shifting the start of unfolding of Ig domains to greater average sarcomere lengths

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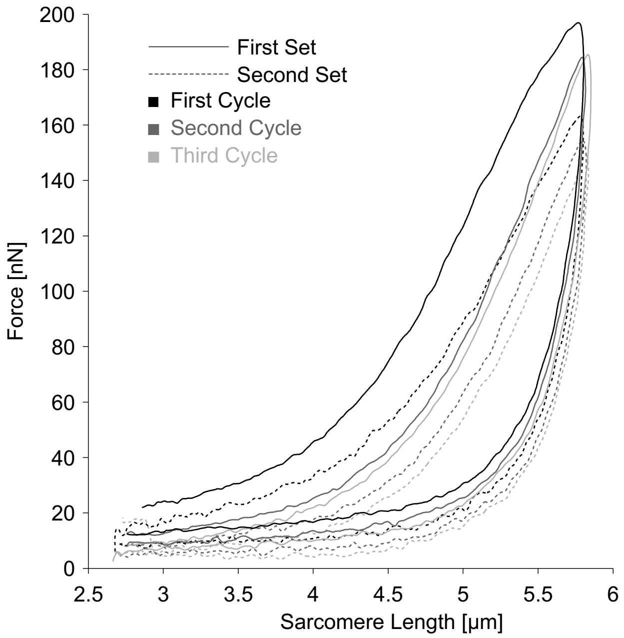

Figure 6:
Force-elongation curves for three repeat stretch-shortening cycles of two sets of cycles separated by ten minutes of rest at an average sarcomere length of approximately 2.5μm. Note that the first cycle of the second set has a substantially lower loading energy than the first cycle of the first set suggesting that refolding of the Ig domains was not completed in the ten minute rest between sets. Similarly, the loading energies of the second and third cycles of the second set are significantly smaller than the corresponding cycles of the first set.

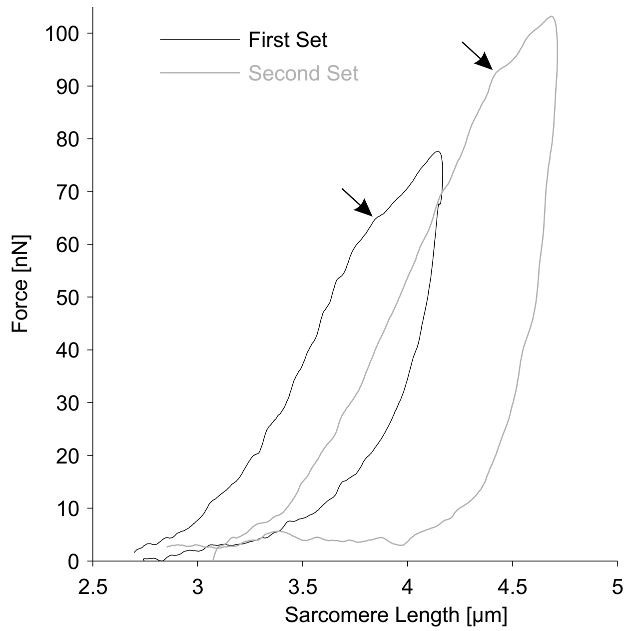

Figure 7:
Force-elongation curves for two first stretch-shortening cycles that were separated by ten minutes. The inflection point (where Ig domain unfolding is supposed to start - arrows) is shifted to greater sarcomere lengths and increased force for the second cycle suggesting that Ig domain refolding did not occur in the ten minute rest between trials, and that Ig domains have different unfolding forces.

in repeat stretch-shortening cycles. This result is in agreement with observation by Kellermayer who found that Ig domain unfolding occurred at increasing titin lengths for repeat stretch-shortening cycles (Fig. 2), and that Ig domain unfolding is incomplete if stretch-shortening cycles occur without a break[16]. The increase in Ig domain unfolding force with repeat stretch cycles (Fig. 7) suggests that unfolding strengths across Ig domains differ, thereby suggesting structural differences across Ig domains.

Kellermayer et al. observed that Ig domain unfolding occurred at a titin force of approximately 20-25pN[16] (Fig. 2). In a myofibrillar cross-sectional area of 1.0μm, one would expect approximately 2700 titin molecules (assuming a lattice spacing of 42nm between thick filaments[21] and 6 titin molecules per half thick filament[11]. Multiplying the total number of titin molecules in a myofibril by the force of first Ig domain unfolding (20-25pN) provides an estimate of the myofibril force at which to expect a force-elongation inflection point; that is about 54-68nN. The upper estimates of this theoretical range agreed well with the mean myofibril force (normalized to a 1.0μm cross-sectional area) where we measured the inflection point in our myofibril experiments (68$\pm$5nN). This good agreement between the isolated titin and single myofibril results suggests that the inflection point observed in the myofibril experiments indeed corresponds to the point at which titin Ig domain unfolding starts to occur. The average sarcomere length of rabbit psoas with the hip fully extended is 2.6μm (unpublished results). Therefore, we conclude that Ig domain unfolding likely does not occur during physiological use of the rabbit psoas muscle.

Loss of energy

The loss of loading energy, but the relative steady values for the unloading energy with repeat stretch-shortening cycles, is also in good agreement

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with observations on single titin molecules[16] (Fig. 2). The lack of recovery of the loading energy for repeat stretch cycles, and indeed for repeat sets of stretch cycles after a ten minute break (Fig. 6), supports the observation that Ig domain unfolding is incomplete within repeat stretch cycles and after ten minutes of rest. We should emphasize here that although our myofibrils were rested at a mean sarcomere length of 2.5-2.7μm where passive forces are zero, and thus no strain on titin is expected, this length is at the very end of that encountered in the normally functioning rabbit psoas (maximal in vivo sarcomere length of about 2.6μm), thus refolding of Ig domains might have been prevented here because of the relatively long resting sarcomere lengths, despite a zero passive force. Future experiments on the kinetics of Ig domain refolding will need to focus on resting sarcomeres for different periods of time at different sarcomere lengths, as sarcomere length might have a greater effect than passive force on titin refolding when titin is within its mid-range within a sarcomere.

Active vs. passive stretch-shortening tests

Titin is known to have binding sites for calcium, and it has been argued that calcium activation of muscles affects the mechanical properties of titin by increasing its stiffness[8,12,23] and/or by modulating its interaction with other sarcomeric proteins, such as actin[8,24,25,26,27]. Based on this evidence, we expected that there would be differences in the passive forces measured in a low calcium relaxation solution and a high (saturating) calcium activation solution in which "active" cross-bridge based forces were chemically inhibited by BDM[12]. However, no such differences were observed, which is in contrast to previous reports[12,23] but is consistent with some of our most recent work[8]. In contrast to the work by others[12,23], our work here was performed at very long sarcomere length. Possibly, calcium activation has an effect on titin at short but not at long sarcomere lengths, or the relatively small effects observed at the short sarcomere lengths cannot be resolved at long sarcomere lengths when passive myofibril forces are in excess of 20 times greater than the observed differences. This puzzle will need further attention in the future, by measuring the effects of calcium activation at short sarcomere lengths.

Conclusions

The results of this study suggest that the properties of isolated titin molecules are well reflected in whole myofibril testing. Titin properties appear to be well preserved when titin operates within the structural boundaries of a sarcomere. This result is exciting insofar as passive myofibril testing is rather simple compared to the complex isolation, stabilization and mechanical testing of single titin proteins. Not only is a myofibril approach much easier technically, it also offers the advantage that titin can be studied in its native environment and that titins properties can be directly related to sarcomere forces and lengths, and thus can be extrapolated to myofibril, fibre and muscle properties.

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