In situ titin properties at long lengths when Ig domain folding/unfolding is prevented

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Titin, also known as connectin, is the largest known protein and was discovered in the mid-1970s and consists of Immunoglobulin (Ig) domains that flank N2A and PEVK segments. At long lengths and high force these Ig domains unfold. There is growing evidence that titin may change its stiffness by attaching its proximal portion to actin upon activation. If so folding/unfolding of Ig domains may become physiologically relevant. This is of interest because Ig domain folding/unfolding is associated with a great loss of energy, but preventing such folding/unfolding has been shown to result in a virtually elastic behaviour with no energy loss in isolated titin molecules. Therefore, the purpose of this study was to test titin properties in situ within sarcomeres at lengths where Ig domain unfolding has occurred while preventing folding/unfolding of Ig domains during small stretch shortening cycles. **Keywords:** muscle; titin; sarcomere.

Introduction

Titin, also known as connectin, is the largest known protein and was discovered in the mid-1970s[1,2]. In striated muscles, titin spans the half sarcomere, from Z-band to M-line (Figure 1). It acts as a molecular spring in the region between its Z-band and thick filament attachments, the I-band region[3], thereby stabilizing the myosin filaments in the centre of sarcomeres[4], providing passive force to muscle[5], and allowing for force transmission between sarcomeres.

The spring-like elements of titin in the I-band region of skeletal muscle consist of two Immunoglobulin (Ig) domains that flank the N2A and PEVK segments (Figure 1). Upon muscle stretching, the randomly oriented Ig domains are aligned first, followed by stretching of the PEVK segment, and finally the unfolding of Ig domains which has been thought to only occur at para-physiological muscle lengths[6,7].

Aside from its acknowledged role in passive force production, titin has recently been implicated in regulating active forces by changing its stiffness, and therefore its force when muscles are actively stretched[8−13]. It is known that such changes in titin stiffness occur when, upon activation and calcium influx into the sarcoplasm, calcium ions bind to specific sites on titin, thereby changing the unfolding characteristics of titin, or specifically, the PEVK[8,9], and Ig domains[14]. Furthermore, there is accumulating evidence that titin may change its stiffness by attaching its proximal portion (proximal Ig domain, N2A region, and possibly some of its PEVK domain) to actin upon activation, thereby leaving the distal Ig domain as the only remaining spring element in activated muscle[12,13,15]. If so, the folding/unfolding properties of the Ig domain would become physiologically relevant, as such folding/unfolding would occur during active (but not passive) muscle stretching within the physiological range of muscle excursions. This is of
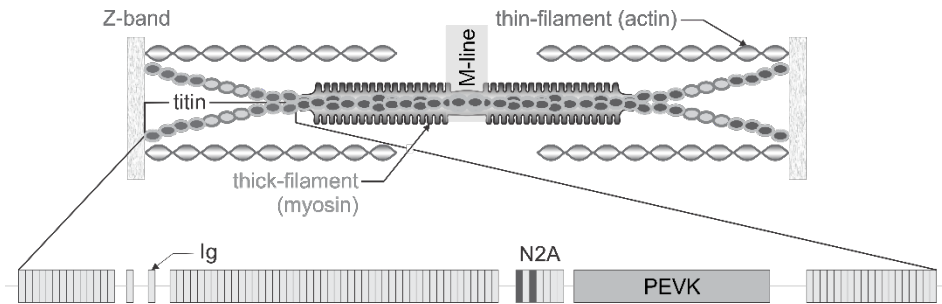
Figure 1:

Schematics of a sarcomere with the contractile filaments, actin and myosin, and the structural protein titin. Titin acts as a spring in the region between its attachments into the Z-band and the myosin filament, a region that is composed of two tandem immunoglobulin (Ig) domains, the N2A, and the PEVK segment. Each of these individual segments has spring like properties of different stiffness, thereby producing a complex mechanical structure of serially arranged springs that come into play at distinct sarcomere lengths (and thus, distinct passive forces).

particular interest since Ig domain folding/unfolding is associated with a great loss of energy[6,7,16], but preventing such folding/unfolding has been shown to result in a virtually elastic behaviour with no energy loss in isolated titin molecules. However, titin properties have been shown to differ substantially between isolated titin preparations and titin in its physiological in situ environment within a sarcomere[7].

Methods

Preparation: Myofibrils were prepared for mechanical testing as described previously[17,18]. Briefly, small pieces of muscle were harvested from rabbit psoas, and were isolated to obtain short myofibrils. Isolated myofibrils were then immersed into a bath on top of an inverted microscope in a rigor solution (see solutions below). After ten minutes, the rigor solution was replaced with a low calcium relaxation solution (see solutions below) that prevented active force production. Myofibrils in suspension were then washed away, leaving those attached to the bottom cover glass. Myofibrils of appropriate length (typically 6-12 sarcomeres in series) with a distinct striation pattern were selected for mechanical testing by attaching them at one end to a silicon nitride lever for force measurements (stiffness of 68pN/nm, force resolution of 0.5nN) and at the other end to a rigid glass needle attached to a motor for controlled, sub-nanometre step size, length changes (Figure 2).

The image of the attached myofibril was projected onto a high density photo diode array (Schafter/Kirschoff, Hamburg, Germany, resolution of 7nm) for identification of the A- and I-bands, Z-bands, and the calculation of sarcomere lengths from Z-band to Z-band or between the centroids of adjacent A-bands if Z-bands were not clearly visible.

Solutions: The rigor solution (pH 7.4) was composed of (in mM): 50 Tris, 100 NaC1, 2 KC1, 2 MgC12, and 10 EGTA. Protease inhibitors were added to the final solution, in the following concentrations (in pM): 10 leupeptin, 5 pepstatin A, 0.2 PMSF, 0.5 N, and 0.5 DlT. The relaxing solution (pH = 7.0; pCa2+ = 8) was composed of (in mM): 10 MOPS, 64.4 K+ proprionate, 5.23 Mg proprionate, 9.45 Na2S04, 10 EGTA, 7 ATP, 10 creatine phosphate.

Protocol: Myofibrils (n=9) were stretched passively from an average sarcomere lengths of about 3.0m to an average sarcomere length of about 5.5m at a speed of 0.1sarcomere lengths/s, and then held at that length for 120s for stress relaxation to occur and passive force to reach a near steady-state (Figure 3). Immediately following the 120s, hold, myofibrils were subjected to ten shortening-stretch cycles of approximately 0.5m/sarcomere magnitude and then released to the original lengths of about 3.0m/sarcomere.

Analysis: The mean force relaxation (1SD) for the two minute stress relaxation period was evaluated as the percentage decrease of force from the peak force at the end of stretch, to the (nearly) steady-state force averaged across the last five seconds of the 2 minute holding period (Figure 3). The mean decrease in force (1SD) for the ten stretch-shortening cycles following the stress relaxation period was calculated
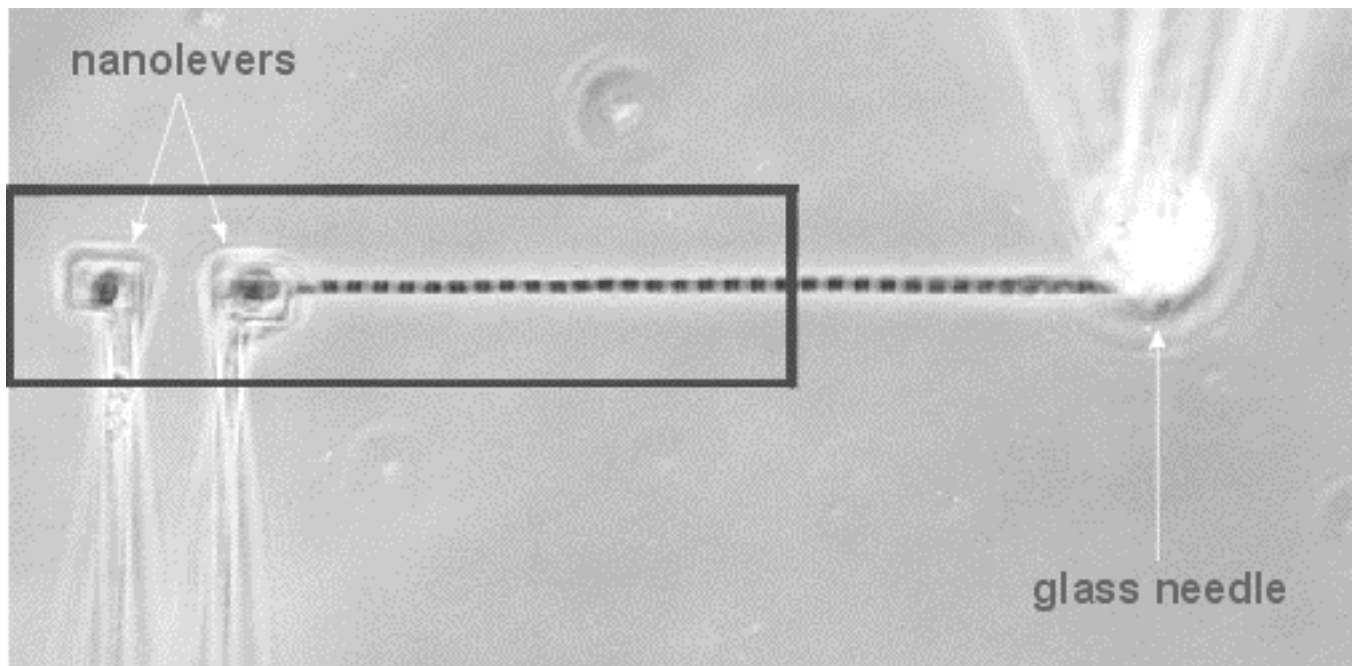
Figure 2:
Experimental setup showing a myofibril comprised of about 30 serially arranged sarcomeres. The myofibril is attached at the left end to one nano-lever of a pair for force measurements and at the right end to a glass needle that is connected to a motor that can impose computer controlled length changes with a resolution of less than 1nm.

as the percentage decrease of the peak force from the first to the tenth stretch-shortening cycle expressed as the average decrease per cycle (Figure 3). Finally, the mean hysteresis (1SD) (energy loss) for each of the ten stretch-shortening cycles following the relaxation period was calculated as the percentage energy loss relative to the energy during the stretch across all ten cycles and all nine myofibrils.

Results

Holding the myofibrils for two minutes at the final stretched length of about 5.5m/sarcomere resulted in a force relaxation averaging 35"%" (6"%"), and resulted in near steady-state forces by the end of the hold, indicating that Ig domain unfolding had been essentially complete. The following ten stretch-shortening cycles resulted in an average decrease in force of 0.7"%" (0.9"%") per cycle, thereby indicating an essentially elastic behavior in terms of force retention. There was a consistent average loss of energy (hysteresis) for the ten stretch-shortening cycles across all myofibrils of 13"%" (3"%") (Figure 4).

Discussion

The purpose of this study was to test titins properties in situ within sarcomeres at lengths where Ig domain unfolding has occurred, while simultaneously preventing folding/unfolding of Ig domains for small stretch-shortening cycles. We hypothesized that titin properties would be virtually elastic for such conditions, as has been found in isolated titin preparations tested using laser trapping techniques[6]. In order to prevent folding of Ig domains, testing was performed at very long sarcomere lengths (¿4.5m) thereby ensuring that refolding of Ig domains was highly unlikely[6,7]. In order to prevent unfolding of Ig domains during the ten stretch-shortening cycles, we let myofibrils stress-relax at long lengths (approximately 5.5m/sarcomere) for two minutes, resulting in a force loss averaging 35"%" and reaching near-steady-state conditions ensuring that Ig domain unfolding for the subsequent stretch-shortening cycles was minimal. Since force decrease during the stretch-shortening cycles following stress relaxation averaged less than a percent per cycle, we feel confident that Ig domain unfolding played no (or only a minor) role in our results. In previous
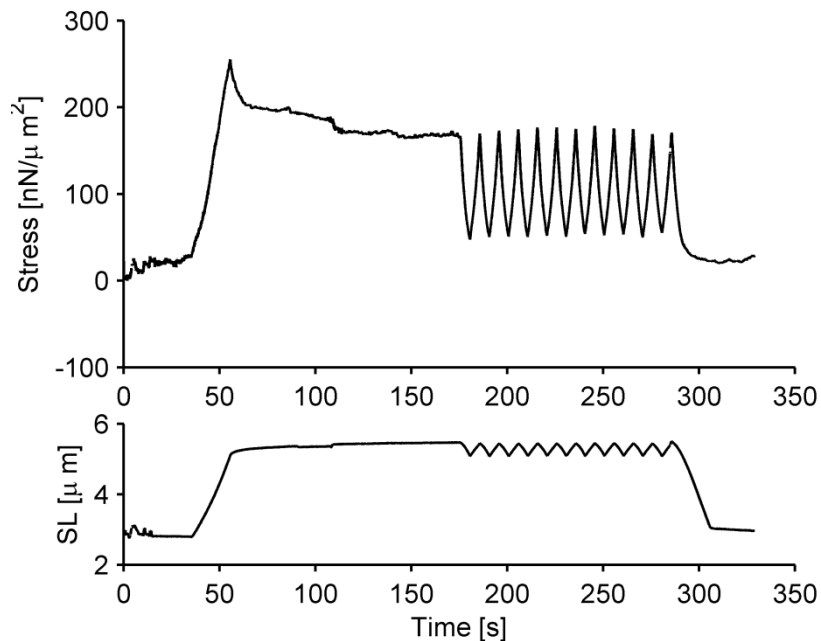
Figure 3:

Stress (force per cross-sectional area) vs. time and sarcomere length (SL) vs. time trace for a representative myofibril that was stretched form an average sarcomere length of about 3.0m to an average sarcomere length of about 5.5m, held for two minutes at the final length, and then subjected to ten stretch-shortening cycles of about 0.5m/sarcomere. Note that the maximum and minimum forces during the ten stretch-shortening cycles remained virtually the same, indicating an essentially elastic behaviour of the myofibril for these conditions.

work, we performed similar experiments using ten stretch-shortening cycles at long sarcomere lengths, but in those experiments no stress relaxation preceded the stretch-shortening cycles (Figure 4B)[7]. This resulted in a relatively great decrease in peak forces in the cycling period. We interpreted these results as being affected by Ig domain unfolding, which resulted in substantial force loss throughout the stretch-shortening cycles and hystereses decreasing from the first (42"%") to the last cycle (7"%") averaging 18"%" (12"%")[7]. Here, we ensured that Ig domain folding/unfolding was minimized, possibly completely prevented, and, as a consequence, peak forces during the stretch-shortening cycles remained virtually constant for many tests (e.g. Figure 4A). However, this did not result in the anticipated elastic behavior of titin within the myofibrils as had been observed for isolated single titin preparations, but resulted in a consistent hysteresis averaging 13"%" that remained essentially constant across the ten stretch-shortening cycles[6,19]. Of course, there is always the possibility that the isolated titin results by Kellermayer et al. (1997) are not correct. However

that seems rather unlikely as their experiments were performed extremely carefully and were confirmed later in independent experiments[19]. It could also be that our results, with a consistent hysteresis, were not correct. However, this is also rather unlikely since the observed hystereses were consistent in magnitude and were observed in all ten trials of all myofibrils. However, if isolated titin properties, as observed by others[6] do not match titin properties observed here in situ, then titin might behave differently in the intact sarcomere compared to how it behaves when tested as an isolated protein. The result that titin peak force during the ten stretch-shortening cycles remained essentially constant suggests that titin behaves elastically within the sarcomere, and that the hysteresis is not caused by the stretching and shortening of the titin molecule, but is associated with titins in situ behavior. Tentatively, we suggest that in the in situ condition, titin binds to another protein upon shortening of the sarcomere and associated force loss, while this bond is broken during stretching giving the stretch phase additional energy compared to the shortening phase. The
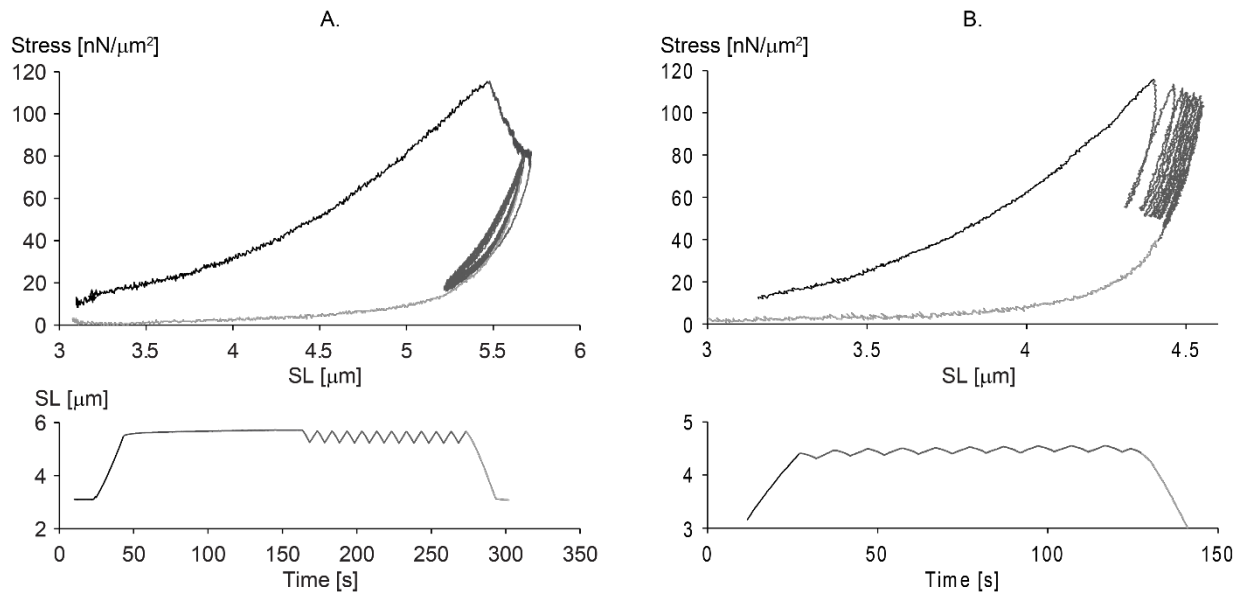
Figure 4:

Stress (force per cross-sectional area) vs. time and sarcomere length (SL) vs. time trace for a representative myofibril that was stretched form an average sarcomere length of about 3.0m to an average sarcomere length of about 5.5m, held for two minutes at the final length, and then subjected to ten stretch-shortening cycles of about 0.5m/sarcomere. Note that the maximum and minimum forces during the ten stretch-shortening cycles remained virtually the same, indicating an essentially elastic behaviour of the myofibril for these conditions.

most likely candidate for such loose binding of titin is actin. Therefore we suggest that in the absence of Ig domain folding/unfolding titin behaves elastically in isolation, but binds/unbinds to another protein (actin) upon shortening/stretching, thereby producing the observed hysteresis of consistent 13"%" magnitude with little variation across cycles and myofibrils (3"%" standard deviation). All experiments were performed passively (i.e. at low calcium concentrations) and at long sarcomere lengths where a great number of Ig domains would have been unfolded[6,7]. Although the sarcomere lengths used here were beyond the physiological limits of the rabbit psoas muscle in vivo[7], Ig domain unfolding as studied here is likely relevant for active muscle contractions, as the proximal part of titin is thought to bind to actin upon muscle activation[13,15], thereby just leaving the distal Ig domain as the single spring element in activated muscles. Thus, studying passive sarcomeres at long lengths might provide insight into titins properties in active muscles within the physiologically relevant working range.

Conclusion

In the absence of Ig domain folding/unfolding, titin properties measured within the structural environment of a sarcomere are different from those of single isolated titin preparations. While Ig domain unfolding likely plays no role at physiological sarcomere lengths in passive muscle, Ig domain unfolding is likely a normal physiological occurrence in active muscle stretching.

Acknowledgements

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