Titin (Visco-) Elasticity in Skeletal Muscle Myofibrils

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Abstract: Titin is the third most abundant protein in sarcomeres and fulfills a number of mechanical and signaling functions. Specifically, titin is responsible for most of the passive forces in sarcomeres and the passive visco-elastic behaviour of myofibrils and muscles. It has been suggested, based on mechanical testing of isolated titin molecules, that titin is an essentially elastic spring if Ig domain un/refolding is prevented either by working at short titin lengths, prior to any unfolding of Ig domains, or at long sarcomere (and titin) lengths when Ig domain un/refolding is effectively prevented. However, these properties of titin, and by extension of muscles, have not been tested with titin in its natural structural environment within a sarcomere. The purpose of this study was to gain insight into the Ig domain un/refolding kinetics and test the idea that titin could behave essentially elastically at any sarcomere length by preventing Ig domain un/refolding during passive stretch-shortening cycles. Although not completely successful, we demonstrate here that titin's visco-elastic properties appear to depend on the Ig domain un/refolding kinetics and that indeed, titin (and thus myofibrils) can become virtually elastic when Ig domain un/refolding is prevented.

Keywords: skeletal muscle, titin, actin, myosin, stretch-shortening cycles, passive properties, stiffness, elastic, visco-elastic, energy loss, hysteresis, muscle properties, cross-bridge theory, sliding filament theory, sarcomere, sarcomere mechanics.

1 Introduction

When a muscle is stretched to a certain threshold length (which differs between muscles), it starts to exhibit passive forces that increase non-linearly with increasing muscle lengths [1-3]. These passive forces are caused primarily by collagen

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fibrils embedded into the muscle's connective tissues and by the structural protein titin, the third most abundant protein in sarcomeres [4-6]. While the connective tissues and associated collagen fibrils are (visco-) elastic structures with predictable properties, titin's properties are not nearly as predictable. Titin contributes to force regulation in actively stretched muscle by changing its stiffness through binding of calcium at specific sites [7-9] and is thought to change its free spring length by binding or interacting with actin [10-14]. During passive stretching, the various domains of titin are successively engaged [15] and give rise to the changing passive properties observed in myofibrils [16].



Figure 1: Schematic drawing of a sarcomere with the contractile proteins actin and myosin, and the structural protein titin. Titin spans the half-sarcomere from the M-line in the middle of the sarcomere to the Z-line at the ends of the sarcomere. It is fixed to myosin and is inextensible in the A-Band region, but acts as a molecular spring in the I-Band region. Its primary spring elements are the proximal and distal Ig domains (schematically indicated by the blue circles close to the Z-line and the A-Band, respectively) and the PEVK region separating the two Ig domains (adapted from [45], with permission).

Titin (also called connectin) is a sarcomeric protein that spans the half sarcomere from the M-line to the Z-line [5, 6, 17] (Figure 1). It produces most of the passive force within the contractile unit, the sarcomere [18, 19], provides stability to the thick (myosin) filament in the centre of the sarcomere [20, 21], contributes to the residual force enhancement observed in muscles following active stretching [8, 22, 23], is responsible for force regulation in actively stretched muscles [24, 25], and provides stability for serially arranged sarcomeres on the descending limb of the force-length relationship [26, 27]. Titin acts like a spring in its extensible I-Band region. At short sarcomere lengths and small passive forces, the immunoglobulin (Ig) domains of titin are thought to align. With increasing sarcomere lengths, the



Figure 2: Force-elongation curves for isolated titin molecules stretched using a laser tweezer approach [31]. Titin shows an inflection point (c) at the point where Ig domain unfolding is supposed to occur, and shows a great hysteresis when stretched and shortened beyond point (c). Below length (c), titin behaves essentially elastic (inset – short length). Similarly, titin also behaves virtually elastic at long length (inset – long length), but only if stretch-shortening occurs without appreciable unfolding and refolding of Ig domains (adapted from Kellermayer et al. 1997, with permission).

so-called PEVK region, named so for its abundance in proline (P), glutamate (E), valine (V), and lysine (K) residues, is thought to become stretched, while at very long lengths, the Ig domains start to unfold [15].

Exploring the passive mechanical properties of titin has been difficult, as isolation of this giant protein has proven tricky, and the individual domains of titin have vastly differing properties [28, 29]. However, Kellermayer et al. [30, 31] were able to isolate single titin molecules and test their mechanical properties using a laser trap approach. By attaching a bead to one end of titin that could then be trapped using a laser, and by fixing the other end of titin rigidly, they performed stretch-shortening experiments of controlled magnitudes, thereby exploring the properties of titin when different regions became recruited during stretch. Kellermayer et al. [30, 31] observed that single titin molecules behaved essentially elastic below a stretch force of approximately 25pN but highly viscoelastic above 25pN (Figure 2). However, if the stretch-shortening cycles were repeated, the hysteresis of

the force-elongation/shortening curves became smaller, and if stretch-shortening cycles were restricted to long lengths (and high passive forces) where Ig domain unfolding and refolding was essentially abolished, titin was shown to exhibit virtually elastic properties with little or no hysteresis [30]. They argued that if titin was completely unfolded, and refolding of Ig domains was prevented during cyclic stretch-shortening cycles, titin would act as an elastic spring, while it would behave viscoelastic if un/refolding of Ig domains was allowed. Since large hystereses in stretch-shortening cycles are associated with great losses of energy, it would be of distinct advantage if such losses were minimized in working muscles.

Kellermayer et al. [31] found that titin forces need to be very small (<2.5pN) for effective Ig domain refolding, and we demonstrated that even in the absence of measurable force, titin refolding remained incomplete even after as long as ten minutes in single myofibrils of rabbits when held at average sarcomere lengths of 2.6μ m [16]. These results suggest that stretching of titin "easily" unfolds Ig domains, while shortening does not readily allow for refolding [32], thus, in repeat stretch-shortening cycles titin becomes increasingly more elastic and loss of energy is minimized. Therefore, we hypothesized that titin is a (visco-) elastic spring that can change its domain of "virtually" elastic behaviour to any sarcomere lengths of repeated use if Ig domain un/refolding is prevented.

The purpose of this study was to test if titin behaves essentially elastic at long lengths where effective un/refolding of Ig domains is prevented, thereby testing Kellermayer's ideas for the first time with actin in its proper structural environment. Furthermore we wanted to test if the rate of refolding of Ig domains, in the absence of force, is sarcomere length dependent. All experiments were performed using isolated myofibrils from rabbit psoas muscle, which allows for accurate determination of the mechanical properties of titin in its native structural arrangement [16, 32].

2 Methods

Preparation: Myofibrils were prepared for mechanical testing as outlined previously by our group [8, 16, 24, 33]. Briefly, myofibrils were obtained from psoas muscles of New Zealand White rabbits, and were chemically and mechanically isolated to form single fibrils with serially arranged sarcomeres. Isolated myofibrils were then immersed into a bath on top of an inverted microscope in a "rigor" solution (see solutions below). After ten minutes of stabilization, the rigor solution was replaced with a low calcium relaxation solution (see solutions below) that prevented any active actin-myosin based-cross bridge forces [8]. 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) that

showed distinct striation patterns were then 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 spatial resolution of 7nm) and at the other end to a rigid glass needle attached to a motor for controlled, sub-nanometre step size, length changes (Figure 3). Three distinct experiments were performed as outlined below.



Figure 3: Myofibril set up for mechanical testing. A myofibril with approximately 30 sarcomeres is attached at its right end to a rigid glass needle, which in turn is attached to a motor with less than nm step resolution for imposing computer controlled stretch-shortening cycles. The left end of the myofibril is attached to one of a pair of nano-levers for force measurements. Upon force production the myofibril will pull the attached lever away from its reference lever, and knowing the stiffness of the lever (68pN/nm in this case), instantaneous forces can be determined with a resolution of less than 0.5nN. For reference, myofibrils are approximately 0.7-1.0 μ m in diameter and sarcomeres shown here have an average length of approximately 2.2 μ m.

The image of the attached myofibrils 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-lines, and the calculation of sarcomere lengths from z-line to z-line or between the centroids of adjacent A-bands if z-lines 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 DIT. The relaxing solution (pH = 7.0; pCa²⁺ = 8) was composed of (in mM): 10 MOPS, 64.4 K+ proprionate, 5.23 M \sim pr \sim opr+ion ate, 9.45 Na2S04, 10 EGTA, 7 ATP, 10 creatine phosphate. Note specifically that the

low calcium concentration of the relaxing solution did not permit any actin-myosin based active force production during the experiments [8, 24, 34].

Protocol: Experiment 1: Myofibrils (n=8) were passively stretched [8] from a nominal initial average sarcomere length of about 2.6 μ m by nominal amounts of 2.0, 2.5, and 3.0 μ m/sarcomere at a speed of 0.1 μ m/s/sarcomere [16] and then released at the same speed to the starting length. Three consecutive stretch-shortening cycles were performed without rest, followed by one or two subsequent sets of three stretch-shortening cycles separated by a ten minute rest at an average sarcomere length of 2.6 μ m.

Experiment 2: Using separate myofibrils (n=8), the testing protocol described in the previous paragraph was repeated with the exception that myofibrils were rested for ten minutes between sets at an average sarcomere length of 1.8 μ m (instead of 2.6 μ m used in the first protocol). Note that neither resting length was associated with a measurable passive force.

Experiment 3: Finally, another set of myofibrils (n=5) was stretched passively from an average sarcomere length of 2.6 μ m by a nominal amount of 2.0 μ m/sarcomere at a speed of 0.1 μ m/s/sarcomere. When the final stretch length was reached, myofibrils were subjected to ten stretch-shortening cycles of nominal magnitude 0.5, 1.0, or 1.5 μ m per sarcomere, and then, following the last of these cycles, released to the starting length. In contrast to the first two experiments where at least partial re-folding of Ig domains was expected during myofibril shortening, in these last set of experiments, Ig domain re-folding was not expected to occur because of the length of titin during the repeat stretch-shortening cycles and the significant amount of force [30-32].

Analysis: The primary outcome measures in this study were the peak forces obtained in the stretch shortening cycles, the loading energies of all stretch cycles, the hysteresis for the three repeat stretch-shortening cycles of experiments 1 and 2, and the peak forces, loading energies, and hysteresis for the ten stretch-shortening cycles of 0.5μ m amplitude in experiment 3. Loading energies were calculated as the integral of the force-elongation curves during the loading phase. Unloading energies were calculated as the integral of the force-elongation curves during the unloading phase. Hystereses were calculated as the loading minus the corresponding unloading energy in a stretch-shortening cycle.

The primary statistical analyses that were performed included comparisons of the anticipated decrease in peak forces, loading energies, and hysteresis for the three repeat stretch-shortening cycles, the recovery of peak force and hysteresis after the ten minute rest at average sarcomere lengths of 2.6μ m (experiment 1) and 1.8μ m (experiment 2), and the decrease in peak force, loading energies, and hysteresis in

the ten repeat shortening-stretch cycles of 0.5μ m amplitude at the long sarcomere lengths of experiment 3. All values are given as means ±1SE, and changes in outcome measures between repeat stretch-shortening cycles (either with or without break) were assessed using non-parametric Friedman's repeated measures testing [35] with α =0.05.

3 Results

Experiment 1:

Peak forces during the stretch shortening cycles were reduced significantly (p<0.05) by 16% and 11% for cycles 1, 2, and insignificantly for cycle 3 (3%), respectively of set 2 compared to the corresponding cycles in set 1 (Figure 4A). Furthermore, the peak forces of the 1st cycle of the second set were similar (109±26nN) to the peak forces of the 3rd cycle of the first set (112+26nN), indicating that there was no significant force recovery in the 10 minute rest period between sets, when myofibrils were rested at an average sarcomere length of 2.6 μ m (Figure 4B).

Similarly to the peak forces, the loading energies were reduced significantly (p< 0.05) by 30% and 13% for cycles 1, 2, but not for cycle 3 (0%), respectively of set 2 compared to the corresponding cycles in set 1 (Figure 4A). Also, the loading energies of the 1st cycle in the 2nd set were similar (86±25nN· μ m) to the 3rd cycle in the first set (78±18nN· μ m), indicating that there was no substantial recovery of loading energy in the 10 minute rest period between sets, when myofibrils were rested at an average sarcomere length of 2.6 μ m (Figure 4B).

The energy loss (hysteresis) expressed relative to the corresponding loading energies were the same for the first and second sets and averaged 60, 53, and 48% for cycles 1, 2, and 3, respectively.

Experiment 2:

In contrast to experiment 1, the peak forces during the stretch-shortening cycles were not reduced between the first and second set of stretch-shortening cycles in experiment 2 (Figure 5A). In fact, peak forces in the second set tended to be slightly (but not significantly) higher by 8%, 7%, and 7% respectively for cycles 1, 2, and 3 compared to the corresponding peak forces in the first set. Consistent with these results, the peak forces in the 1st cycle of the second set were significantly higher (p<0.05) ($107\pm11nN$) than the peak forces in the 3rd cycle of the first set ($92\pm11nN$), indicating a substantial recovery of force when myofibrils were rested for ten minutes at an average sarcomere length of 1.8μ m (Figure 5B).

Although the loading energies were slightly reduced in the second set by 11%, 4%, and 4% for cycles 1, 2, and 3, respectively, only the values for the first cycle were statistically different (Figure 5A). In agreement with the peak forces,



Figure 4: Exemplar stretch-shortening cycles of two myofibrils (one for figure A and another one for figure B) stretched from an initial average sarcomere length of about 2.6 μ m. (A) Stretch 1 is the first stretch-shortening cycle of set 1, while stretch 2 is the first stretch-shortening cycle of set 2 which occurred after a ten minute rest period with the myofibril relaxed at an average sarcomere length of 2.6μ m. Note that the peak force and loading energy is substantially decreased in stretch 2 compared to stretch 1, indicating incomplete recovery, and thus incomplete refolding of Ig domains in the ten minute rest period. (B) Stretch 1 is the third stretch-shortening cycle of set 1, while stretch 2 is the first stretch-shortening cycle of set 2 which occurred after a ten minute break with the myofibril relaxed at an average sarcomere length of 2.6μ m. Note the similarity in loading energy and peak force between the first stretch of set 2 and the last stretch of set 1, suggesting that there was little (if any) recovery of passive force in the ten minute rest period at a sarcomere length of 2.6 μ m. indicating little or no refolding of Ig domains in the break. Forces on the left axes are the measured myofibril forces (nN) while forces on the right axes represent an estimate of the forces in a single titin molecule (pN) assuming 2700 titin molecules per um².

the loading energy of the 1st cycle in the second set was significantly (p<0.05) higher (79±8nN· μ m) than the loading energies of the 3rd cycle in the first set (51±6nN· μ m), thereby indicating a great recovery of loading energy in the ten minute rest period when myofibrils were held at an average sarcomere length of 1.8 μ m (Figure 5B).

Energy losses (hysteresis) relative to the loading energies were the same for the



Figure 5: Exemplar stretch-shortening cycles of two myofibrils (one for figure A and another one for figure B) stretched from an initial average sarcomere length of about 2.6 μ m. (A) Stretch 1 is the first stretch-shortening cycle of set 1, while stretch 2 is the first stretch-shortening cycle of set 2 which occurred after a ten minute rest period with the myofibril relaxed at an average sarcomere length of 1.8μ m. Note that the peak force and loading energy are essentially the same, indicating complete recovery, and thus complete refolding of Ig domains in the ten minute rest period. (B) Stretch 1 is the third stretch-shortening cycle of set 1, while stretch 2 is the first stretch-shortening cycle of set 2 which occurred after a ten minute break with the myofibril relaxed at an average sarcomere length of 1.8μ m. Note the substantial increase in loading energy and peak force in the first stretch of set 2 (purple) compared to the last stretch of set 1 (yellow), suggesting a big recovery of passive force in the ten minute rest period at a sarcomere length of 1.8μ m, indicating substantial refolding of Ig domains in the rest period. Forces on the left axes are the measured myofibril forces (nN) while forces on the right axes represent an estimate of the forces in a single titin molecule (pN) assuming 2700 titin molecules per um².

corresponding stretch-shortening cycles in the first and second set and averaged 68, 49, and 45% for cycles 1, 2, and 3, respectively, values similar to those obtained in experiment 1.

Experiment 3:

The loss in peak force across the ten repeat stretch-shortening cycles at long sar-



Figure 6: Force-sarcomere length (A) and force-time relationships (B) for an exemplar myofibril in experiment 3. The myofibril was stretched from an initial length of approximately 2.7μ m/sarcomere to a final length of about 4.2μ m/sarcomere followed by ten repeat shortening-stretch cycles of small magnitude (note the last cycle is only half the magnitude of the previous cycles). There is a small loss of peak force in the ten repeat stretch-shortening cycles indicating visco-elastic creep. Note the virtually elastic behaviour for the ten repeat stretch-shortening cycles at long sarcomere lengths where (some) Ig domains are unfolded. The left inset shows the first of the ten stretch-shortening cycles, the right inset the tenth one. In the left inset, the shortening (yellow) is matched by the subsequent stretch (blue) curve for the low force values (50-90nN) but then the stretch curve (blue) falls off to longer length. This behaviour suggests that there was no effective refolding of Ig domains, but that towards the end of the stretch, additional Ig domains became unfolded, causing the slight decrease in peak force and increased length. In the right inset (tenth full cycle), the red (shortening) and blue (stretching) curves essentially overlap, thereby displaying an essentially elastic response, suggesting that effective unfolding and refolding of Ig domains was prevented. This is in stark contrast to the big hysteresis (and energy loss), when the myofibril is returned to its original $(2.7\mu m)$ length. Forces on the left axes are the measured myofibril forces (nN) while forces on the right axes represent an estimate of the forces in a single titin molecule (pN) assuming 2700 titin molecules per um^2 .

comere lengths was 11% for nominal magnitudes of 0.5μ m (Figure 6) and was 11% and 5% for single observations at stretch-shortening magnitudes of 1.0 and 1.5μ m, respectively. In experiments 1 and 2, losses in peak force were 7% and 8%, respectively for the three repeat cycles. The corresponding three cycle loss in experiment 3 (stretch-shortening at long sarcomere lengths where refolding of Ig domains was prevented) was similar: 8%, 8%, and 2%, respectively for cycle amplitudes of 0.5, 1.0, and 1.5 μ m, respectively.

Most importantly, however, the hystereses observed in experiments 3 were much smaller than in experiments 1 and 2 and ranged from $5nN\cdot\mu m$ (for cycle 1) to $1nN\cdot\mu m$ (for cycle 10) with an average value of $3nN\cdot\mu m$, compared to 72 $nN\cdot\mu m$ (cycle 1, experiment 1) to $37nN\cdot\mu m$ (cycle 3, experiment 1), and 61 $nN\cdot\mu m$ to $23nN\cdot\mu m$, for cycle 1 and 3, respectively of experiment 2. However, the absolute hysteresis might not be good for comparison, since the stretch-shortening amplitude affects these values. However, by expressing the hysteresis (energy loss) in terms of the loading energy provides an indication of the energy loss in the different experiments.

The energy loss (hysteresis) relative to the loading energies averaged 14%, 14% and 27% for stretch-shortening amplitudes of 0.5, 1.0 and 1.5μ m, respectively, with the highest value of 34% (1st cycle for 1.5μ m amplitude) and the smallest value of 8% (cycles 7-10 for 0.5μ m amplitude). These values are all much smaller than the corresponding values given above for experiments 1 (48-60%) and 2 (45-68%), indicating a much more elastic (less energy loss) response. The smallest energy loss values of 8% are similar to those obtained for tendinous materials [36].

4 Discussion

Titin's I-band regions act as a molecular spring [15], providing most of the passive force in isolated sarcomeres [8, 22] and myofibrils [8, 22, 32, 34]. Titin consists of several spring regions with different properties arranged in series [15, 28-31]: the proximal Ig domain, the N2A and PEVK domains, and the distal Ig domain. While studying isolated titin molecules using a laser tweezer approach, Kellermayer and colleagues [31] discovered that titin acted virtually elastically for stretch-shortening cycles corresponding to short sarcomere lengths and forces below approximately 25pN (Figure 2), and at lengths corresponding to long sarcomeres where Ig domains were assumed to remain permanently unfolded [30, 31]. However, when titin was pulled to lengths beyond Ig domain unfolding, it showed a large hysteresis with a corresponding great loss of energy in the stretch-shortening cycle. These results by Kellermayer et al. [30, 31] suggest that titin can act as a virtually elastic spring, if Ig domain unfolding and refolding is prevented. Such situations could occur at very short sarcomere lengths, prior to any Ig domain unfolding [32], and at

long sarcomere lengths if Ig domain un/refolding is prevented. Since it appears impossible to have all Ig domains unfolded within physiologically relevant sarcomere lengths [16, 31, 32], the elastic region of titin can potentially be shifted to different sarcomere lengths, by pre-unfolding titin Ig segments permanently in cyclic movements over a given range of sarcomere lengths.

However, these theoretical considerations, and experimental findings on isolated titin molecules have never been tested systematically for titin in their native and structurally correct environment of a sarcomere, although detailed analysis of titin's properties at sarcomere lengths below which Ig domain unfolding occurs (3.0- 3.5μ m in rabbit psoas muscles – [16, 31] have been made [32]. Therefore, the purpose of this study was to test under what conditions Ig domain refolding occurs in sarcomeres, and if there is indeed a virtually elastic region of passive sarcomere stretch-shortening cycles when Ig domain refolding is prevented at long sarcomere lengths. Experiments were performed using isolated myofibrils from rabbit psoas muscles which have been shown to have passive forces that originate almost exclusively from titin, and represent titin properties well [16, 32].

We would like to emphasize two major results obtained in this study. The first is that after ten minutes of recovery, peak forces and loading energies did not recover substantially when myofibrils were rested at an average sarcomere length of 2.6µm (Figures 4A and B), but peak forces and loading energies recovered almost completely when myofibrils were rested at an average sarcomere length of 1.8μ m (Figures 5A and B). These findings suggest that in the ten minute recovery between stretch-shortening sets, unfolded Ig domains were able to refold at sarcomere lengths of 1.8μ m but not at sarcomere lengths of 2.6μ m. There is no passive force in psoas myofibrils at sarcomere lengths of 2.6μ m or less, thus forces in titin would be zero too. This result suggests, that in contrast to isolated experiments in titin [31], and previous work in rabbit psoas myofibrils [32] spontaneous refolding of Ig domains does not necessarily occur when forces in titin become low (less than about 2-3pN [30, 31], but requires sarcomere lengths, and thus titin lengths, to be short as well for effective refolding. The threshold length for Ig domain refolding to occur effectively lies somewhere between 2.6 and $1.8\mu m$ (for rabbit psoas), but cannot be narrowed down at present as only observations from these two resting lengths are available.

When no rest was given between stretch-shortening cycles, peak forces, loading energies, and hysteresis decreased from cycles 1-3 in experiments 1 and 2, indicating that Ig domains remained permanently unfolded after the first cycle, thereby providing a more elastic response with increasing cycle number. This property has been observed in a previous study on isolated myofibrils [16, 32] and is consistent with observations on isolated titin molecules [30, 31].

The second result to be emphasized is the virtually elastic behaviour of myofibrils in the last few stretch-shortening cycles of experiment 3, in which effective Ig domain un/refolding was prevented by the experimental protocol design (Figure 6). Experiment 3 was designed to minimize Ig domain refolding for stretch-shortening cycles at long sarcomere lengths where Ig domains were known to have unfolded in the first stretch cycle. Experiments for all three magnitudes of stretch-shortening cycles (0.5, 1.0 and 1.5μ m) at these long sarcomere lengths showed substantially less energy loss (hysteresis) relative to the loading energies, than stretch-shortening cycles in experiments 1 and 2. This finding suggests that titin can act almost elastically at long lengths, if Ig domain refolding is prevented, thereby minimizing energy loss for repeat passive motion. The sarcomere lengths where this virtually elastic behaviour can be elicited can vary, as it merely requires that the Ig domains within the range of the stretch-shortening cycle remain permanently unfolded. This range of sarcomere lengths can vary, as the number of permanently unfolded Ig domains determines at what lengths elastic behaviour is observed.

The reason why the stretch-shortening cycles of experiment 3, even after ten repeat cycles were not perfectly elastic (there remained a tiny hysteresis of about 8%), is likely associated with the time-dependent unfolding of Ig domains [7, 37, 38]. Ig domain unfolding does not only depend on the force acting on titin and its Ig domains, but is also a stochastic process, in which the energy barrier for Ig domain unfolding can be exceeded even in the absence of high external forces. Since our stretch-shortening cycles were performed at a slow speed $(0.1 \mu m/s per$ sarcomere) a stretch-shortening cycle of 0.5μ m/s per sarcomere takes 10s to complete, which is ample time for Ig domains to unfold. We predict however, that if our experiments had been done at a greater speed (let's say 1μ m/s per sarcomere), the hysteresis observed in experiment 3 would have been essentially abolished giving a virtually elastic passive behaviour of the myofibrils. Another way to test the idea of stochastic unfolding of Ig domains in the stretch-shortening experiments of experiment 3 would be to stretch myofibrils to the initial long lengths, but then allow the myofibrils to stress relax to allow for time-dependent unfolding of Ig domains to occur. Starting the ten repeat stretch-shortening cycles after such stress relaxation should give an essentially elastic behaviour of passive myofibrils as all Ig domains would be unfolded (in the ideal experiment) and no refolding could occur as force and sarcomere length are too high for refolding to occur. This last experiment would essentially mimic the approach by Kellermayer and colleagues [30] where they stretched isolated titin molecules to such long lengths that all Ig domains were unfolded. Shortening from such long lengths then revealed an essentially elastic behaviour of titin. Unfortunately, such an experiment cannot be performed with titin in situ, as the stretch required for all Ig domains of titin to

unfold, would go beyond the failure lengths of intact sarcomeres [39]. However, increasing the stretch-shortening speed, and achieving (near) complete Ig domain unfolding with a stress relaxation test at long sarcomere lengths, should allow for testing the changing mechanical properties of titin.

A secondary result of potential importance is the fact that stiffness of titin changes as a function of the history of loading. For example, in figure (6), stiffness of titin for the first stretch cycle from 2.7-4.2 μ m is substantially smaller than the stiffness observed in the ten repeat stretch shortening cycles, as can be seen by the slope of the force-sarcomere length curves. Therefore, it appears that not only titin's viscoelastic properties but also its stiffness at a given (sarcomere-) length can change substantially and affect the instantaneous passive properties of muscle.

In summary, our results suggest that titin is a molecular spring with high viscoelasticity and associated energy loss during passive stretch-shortening cycles when Ig domain unfolding and refolding is allowed to occur. However, at lengths prior to Ig domain unfolding (average sarcomere lengths of about $3.0-3.5\mu$ m for rabbit psoas) [16, 31], or at lengths after Ig domain unfolding has occurred (3.5μ m) but Ig domain refolding is prevented, titin can act as an essentially elastic spring, thereby minimizing energy losses in passive stretch-shortening cycles. Since not all Ig domains will ever be unfolded within feasible sarcomere lengths, it appears that the region of near-elastic behaviour can be adapted to any lengths for repeat stretchshortening cycles if Ig domain refolding is prevented either by the speed of the stretch-shortening cycles or by the length of the sarcomeres.

Titin's properties are known to vary with activation because of calcium binding, phosphorylation and titin-actin interaction in active muscle [13, 14, 26, 40-44]. It will be important to elucidate the detailed behaviour and properties of titin in stretch-shortening cycles while activated. However such experiments are infinitely more difficult to perform than the passive experiments presented here, as one must distinguish between the time dependent cross-bridge forces and titin forces contributing to the total force in activated preparations.

In conclusion, we suggest that titin is a molecular spring whose (visco-) elastic properties and stiffness are governed by the unfolding/refolding kinetics of the Ig domains, and that the elastic behaviour of titin can shift to the required working range for repeat stretch-shortening cycles.

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