



# Are titin properties reflected in single myofibrils?

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## ABSTRACT

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. Single myofibrils from rabbit psoas were prepared for measurement of passive stretch-shortening cycles at lengths where passive titin forces occur. Three repeat stretch-shortening cycles with magnitudes between 1.0 and 3.0  $\mu\text{m}$ /sarcomere were performed at a speed of 0.1  $\mu\text{m/s}$  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 2,3 butanedionemonoamine. 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 3.5  $\mu\text{m}$  that was associated with an average force of  $68 \pm 5$  nN/mm. This inflection point was thought to reflect the onset of 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 observed here 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.

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## 1. Introduction

Titin is a giant structural protein in muscle (Maruyama, 1976; Wang et al., 1979). 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 (Granzier and Labeit, 2007). It has spring-like properties in its extensible I-band domain dominated by the Ig segments and the PEVK region, named so for its predominance in proline (P), glutamate (E), valine (V) and lysine (K) residues. Titin's elasticity has been explored in isolated molecules and recombinant fragments of titin using laser trapping and atomic force microscopy to reveal its properties as an entropic spring (Kellermayer et al., 1997; Rief et al., 1997; Tskhovrebova et al., 1997). Its properties can be modeled as serially linked wormlike-chains with different persistence and contour lengths for the PEVK region and the Ig segments (Granzier and Labeit, 2007). At low forces and small sarcomere lengths, elongation of titin is thought to occur by alignment of the tandem Ig domains initially. With increasing sarcomere lengths, the PEVK region is thought to be stretched, and finally, at long sarcomere lengths and high passive

forces, the Ig domains are expected to unfold (Granzier and Labeit, 2007). Since its discovery in the mid-1970s (Maruyama, 1976; Wang et al., 1979), titin has emerged as an important stabilizer of sarcomeres (Horowitz et al., 1986; Horowitz and Podolsky, 1987), a producer of passive force (Granzier et al., 1997; Granzier and Irving, 1995), a regulator of active force (Leonard et al., 2010; Leonard and Herzog, 2010a), and has been associated with a variety of signaling, structural, and mechanical properties (Cazorla et al., 1999; Fukuda et al., 2005; Granzier and Labeit, 2007; Joumaa et al., 2008).

Titin is the third most abundant sarcomeric protein (Granzier and Labeit, 2007), and knowing its mechanical properties is essential for explaining passive characteristics of muscles (Granzier and Labeit, 2007; Joumaa et al., 2007; Joumaa et al., 2008), force regulation during active stretch (Leonard et al., 2010; Leonard and Herzog, 2010a), sarcomere stability (Horowitz et al., 1986; Horowitz and Podolsky, 1987), and residual force enhancement in skeletal muscles (Herzog and Leonard, 2002; Leonard et al., 2010; Leonard and Herzog, 2010a, 2010b). However, measuring titin's mechanical properties is difficult because the isolated protein is unstable and requires specialized equipment. Nevertheless, it has been possible to isolate titin and measure its passive force–elongation properties using a laser trap approach (Kellermayer et al., 1997). This was done by attaching beads to the “ends” of titin molecules and capturing one bead in a laser trap (for force measurement) and the other end

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to a micro-pipette for producing length changes. Kellermayer et al. (1997) found that titin was virtually elastic below 20 pN, and highly viscoelastic above 20 pN. Their interpretation of this result was that titin behaved as a nonlinear entropic spring which starts to unfold at approximately 20 pN. A variable fraction of the molecule (5%–40%) was found to remain permanently unfolded, thereby providing a range of essentially elastic behavior that changed as a function of the history of titin stretching. 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 (Kellermayer et al., 1997).

Although mechanical experiments of fractional parts of recombinantly produced titin segments have been performed successfully (e.g. (Duvall, 2010; Kulke et al., 2001; Williams et al., 2003)), and have provided crucial insights into the workings of this molecular spring, full length mechanical experiments of titin are rare (Kellermayer et al., 1997), and are associated with several 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, fiber and muscle (Kellermayer et al., 1997). 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. Most importantly, we wanted to

compare the energy loss of myofibrils for repeat stretch-shortening cycles and for stretches of different magnitudes, and wanted to identify the inflection point in the force–elongation curve, relate it to the molecular forces observed for titin at this transition, and identify the sarcomere lengths at which inflection occurs. These parameters were also measured by Kellermayer et al. (1997), and thus, direct comparison with single protein data is possible. Furthermore, it has been suggested that titin might become stiffer with activation because of calcium binding (Labeit et al., 2003; Joumaa et al., 2008), but recent evidence does not support these results (Leonard and Herzog, 2010a; Herzog et al., 2012). In order to quantify the effect of calcium activation on passive myofibrillar properties, we repeated selected stretch-shortening experiments in an activation solution but in the presence of a cross-bridge inhibitor.

If results of single titin and myofibril experiments are similar, experiments at the myofibrillar level might be used, as a simpler alternative to isolated protein tests in assessing the mechanical properties of titin. Myofibrillar testing has the additional advantages that titin can be evaluated in its native environment, and that its properties can be assessed in different muscles and under different mechanical loading conditions.

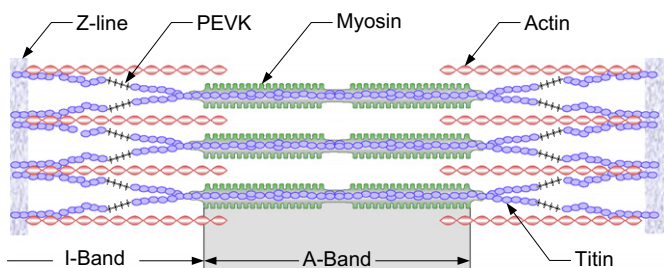
## 2. Methods

### 2.1. Preparation

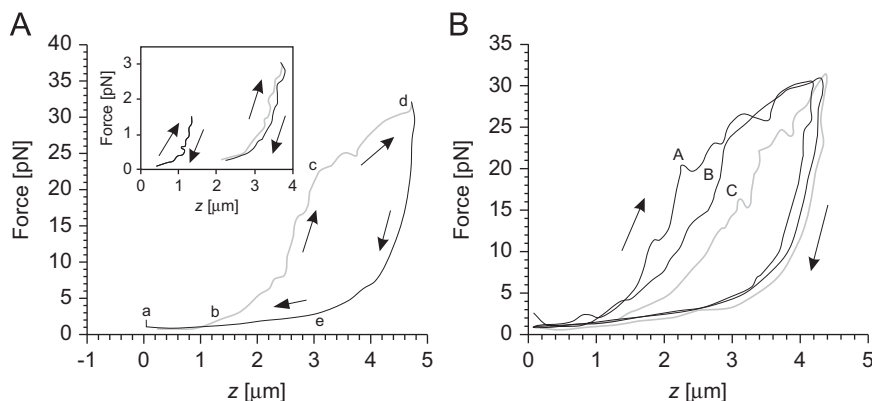
Myofibrils were harvested from rabbit psoas, chemically and mechanically isolated as described previously (Joumaa et al., 2007; Leonard et al., 2010; Leonard and Herzog, 2010a; Leonard and Herzog, 2010a; Leonard and Herzog, 2010a), and prepared for mechanical testing using micro-electronically machined 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 (Fig. 3) (Joumaa et al., 2007, 2008; Leonard and Herzog, 2010a).

### 2.2. Testing

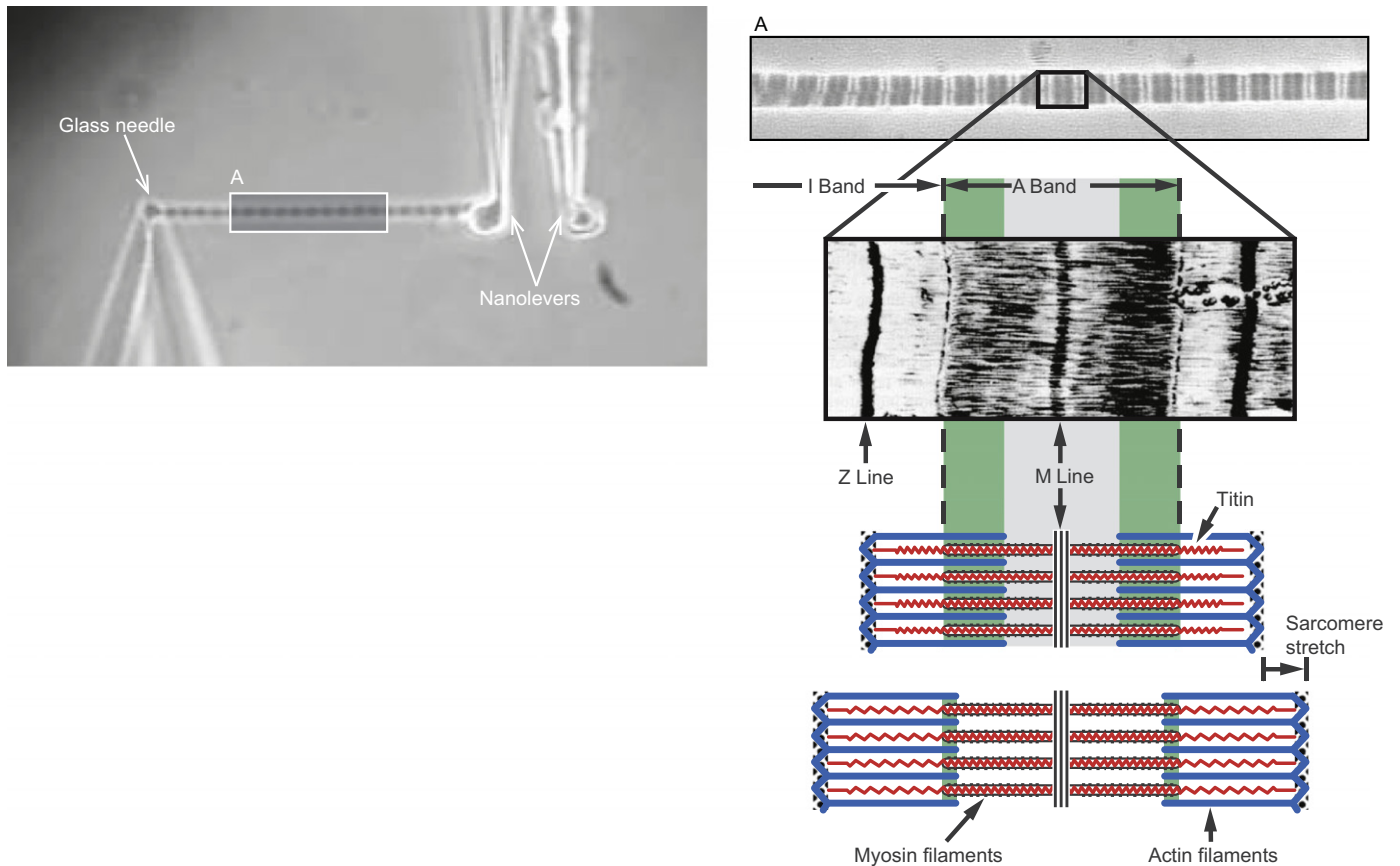
Myofibrils ( $n=28$ ) were passively stretched (see relaxation solution in Joumaa et al. (2008)) from a nominal initial average sarcomere length of 2.5–2.7  $\mu\text{m}$  by 1.0, 2.0, 2.5, and 3.0  $\mu\text{m}$  at a speed of 0.1  $\mu\text{m/s}$  · sarcomere and then released at the same speed to the original length. Three consecutive stretch-shortening cycles were performed without rest, followed by a ten minute rest and repeat of the original stretch-shortening protocol. For selected myofibrils ( $n=8$ ), these same stretch-shortening cycles were also performed using an activating solution plus a cross-bridge inhibitor (2,3 butanedionemoxime), BDM; see activation solution with BDM in Leonard and Herzog (2010a) to test the passive properties of titin in an activation medium with a saturated calcium concentration ( $\text{pCa}^{2+}=3.5$ ).



**Fig. 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 anchor the myosin filaments in the center of the sarcomere, provide stability to sarcomeres (especially when stretched to long lengths), and to act as a force modulator when sarcomeres are stretched.



**Fig. 2.** Force–elongation curves for isolated titin molecules. (A) Titin behaves as an essentially elastic spring over short extension distances (inset) and in regions that are permanently unfolded, 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 single titin molecule illustrating the loss of loading energy for repeat cycles (A – second cycle; B – third cycle; C – fifth cycle) while the unloading energy remains less affected by repeat stretch-shortening cycles (from Kellermayer et al. (1997); by permission).



**Fig. 3.** 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). Close up view of a myofibril (right panel top), and a single sarcomere (right panel middle), and schematic illustration of the three myofibril structure of a sarcomere (right panel bottom).

Although BDM affects the active properties by inhibiting cross-bridge force production, it does not affect the passive properties of myofibrils (Leonard and Herzog, 2010a). All force data were collected at 30 Hz, thereby providing a minimum of 600 individual data points for the shortest stretch-shortening cycles.

### 2.3. 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), efficiency (percentage of work obtained in the shortening phase compared to the work obtained in the stretching phase), change in stiffness (defined as the inflection point of the force–elongation curve; that is where the second derivative of this curve becomes zero), shortest sarcomere length at the inflection point, and average force at the inflection point were quantified. All values are given as means  $\pm$  1 SD. Statistical differences for all outcome measures between the tests performed passively and actively with cross-bridge inhibition were performed using the non-parametric Mann–Whitney signed-rank test. Changes in outcome measures between repeat stretch-shortening cycles, and from the first to the repeat set of cycles after ten minutes, were performed using non-parametric Kruskal–Wallis repeated measures testing (Hinkle et al., 1979). All analyses were performed using a level of significance of 0.05.

## 3. Results

### 3.1. Inflection point

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 3.5  $\mu$ m (with a range from 3.5 to 4.5  $\mu$ m). The average force at this inflection point was 68 nN ( $\pm$  5 nN) when normalized to a cylindrical myofibril of 1.0  $\mu$ m diameter.

### 3.2. Loss of energy

Energy loss is the difference in loading and unloading energy within a single cycle and this difference decreased significantly for repeat cycles (Table 1 and Fig. 5). Energy loss was primarily associated with a significant decrease in loading energy, while the unloading energies for repeat cycles decreased to a much lesser degree (Fig. 5). The energy loss for the first cycle of the repeat testing set (10 min following the first set) was significantly smaller than the first cycle of the initial set, but was similar to the third cycle of the original set (Fig. 6). Finally, efficiency decreased significantly with the magnitude of stretch from the smallest (1.0  $\mu$ m) to the greatest stretch magnitude (3.0  $\mu$ m) for all corresponding cycles (1st, 2nd and 3rd in Table 1).

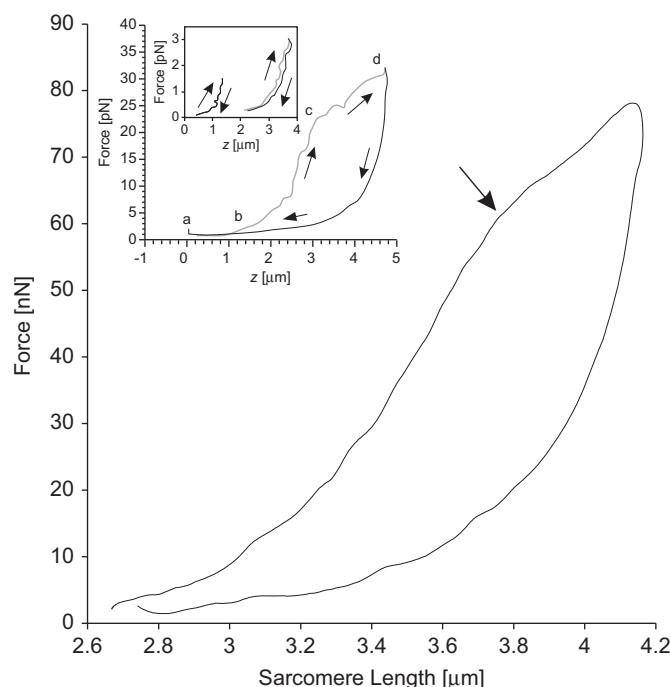
### 3.3. Active vs. passive stretch-shortening tests

There were no differences in any of the outcome measures for the tests performed passively and those performed actively in the presence of BDM (results not shown).

## 4. Discussion

### 4.1. Changes in stiffness

We observed a change in stiffness (inflection point) of the myofibril force–elongation curves similar to that seen in isolated titin, which has been attributed to the unfolding of Ig domains of titin (Kellermayer et al., 1997). However, this observation was only made for a sub-set of the myofibrils. There are two primary reasons



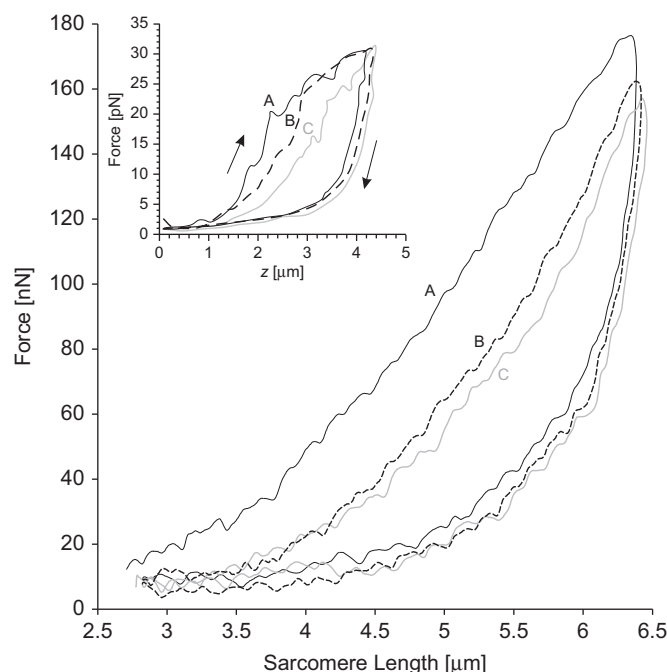
**Fig. 4.** Passive force–elongation curve of a single myofibril showing a characteristic inflection point (arrow) as has been found in tests using isolated titin molecules (inset point c). This inflection point is thought to reveal the length at which Ig domain unfolding starts to occur in the sarcomeres of a myofibril. Note the similarity in shape of the single molecule and single myofibril curves. Note further that in this example the inflection point was found at an average sarcomere length of approximately 3.7  $\mu\text{m}$ , while we found inflection points at average sarcomere lengths as short as 3.5  $\mu\text{m}$ . This variation of sarcomere length at which the inflection point was observed is consistent with the findings made here and by Kellermayer et al. (1997) that unfolded Ig domains may not readily refold, and some may remain unfolded following stretch, thereby showing inflection points (i.e. the onset of “new” Ig domain unfolding) at different sarcomere lengths.

**Table 1**

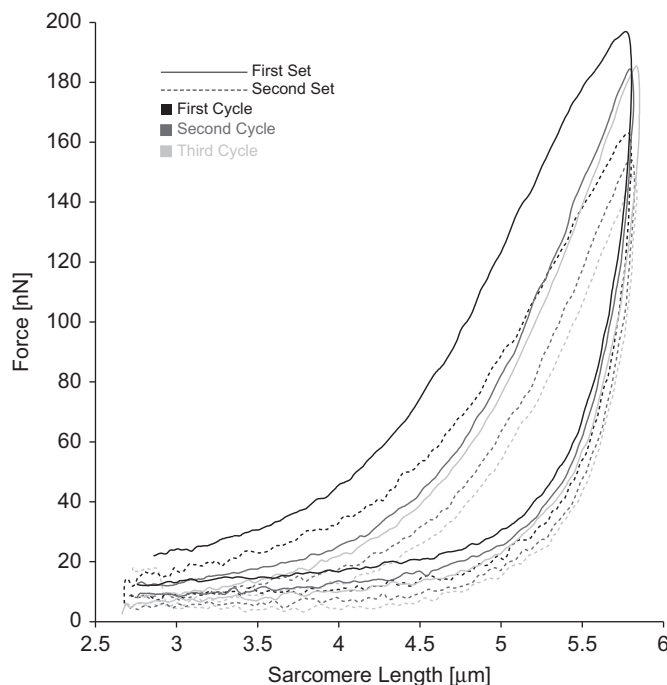
Mean ( $\pm 1$  SD) percent efficiency of the three repeat stretch cycles of a test set as a function of the stretch magnitude per sarcomere of passive myofibrils. Note that efficiency increases with increasing number of repeat stretch cycles and decreases with increasing stretch magnitudes (as observed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

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

for this inconsistent observation: first, the attachment of myofibrils and its handling prior to testing often produces considerable stretching of the sarcomeres. This stretching 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), and if these Ig domains do not refold within a short period of time (in accordance with the model of Kellermayer et al. 1997), the change in stiffness can only be observed in myofibrils that were prepared without significant stretching of the sarcomeres prior to testing. Second, since sarcomere lengths in a myofibril are non-uniform (Herzog et al., 2010), one would expect 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 (Kellermayer et al., 1997).



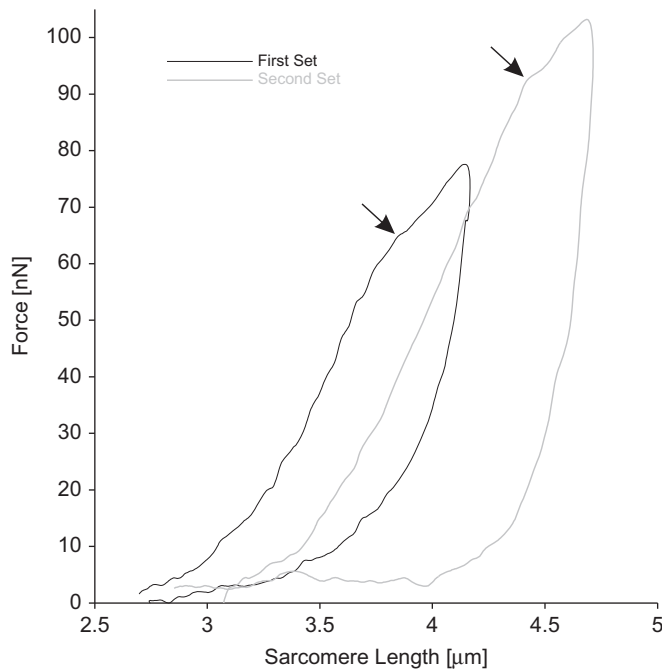
**Fig. 5.** Force–elongation curves of three repeat stretch-shortening cycles of a single myofibril illustrating the loss of loading energy, the decrease in the hysteresis and the relatively unchanged unloading energies from cycle 1 to cycle 3. Note the similarity of the single myofibril curves with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



**Fig. 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 2.5  $\mu\text{m}$ . 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.

The smallest mean sarcomere length at which a change in stiffness of the force–elongation curve was observed was 3.5  $\mu\text{m}$ . For subsequent cycles, the inflection point occurred at increasingly longer sarcomere lengths and slightly increasing myofibril





**Fig. 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.

forces (Fig. 7). This result suggests that unfolding of the Ig domains occurs at approximately  $3.5\ \mu\text{m}$  in rabbit psoas, and 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 in repeat stretch–shortening cycles. This finding agrees with observation by Kellermayer et al. (1997) who found that Ig domain unfolding occurred at increasing titin lengths for repeat stretch–shortening cycles (their Fig. 2), and that Ig domain unfolding is incomplete if stretch–shortening cycles occur without a break. The increase in Ig domain unfolding force with repeat stretch cycles (Fig. 7) suggests that unfolding strengths across Ig domains differ, as reported by Li et al. (2002) and Rief et al. (1998).

The working sarcomere excursion for rabbit psoas muscles ranges from  $2.0$  to  $2.6\ \mu\text{m}$  (unpublished observations). Therefore, Ig domain unfolding occurs outside the physiological range of this muscle, and Ig domain alignment and extension of the PEVK region are likely the only mechanisms of titin extension in the intact rabbit psoas. Although passive forces within the physiological range of rabbit psoas are likely small, experiments with titin depletion, using a mild trypsin solution, showed that about 90%–95% of all passive forces are attributable to titin in rabbit psoas myofibrils, and that active force transmission and sarcomere integrity is lost when titin is depleted, rendering the myofibrils useless (Leonard and Herzog, 2010a).

Kellermayer et al. (1997) scaled up their single titin results to an entire rabbit psoas fiber, and estimated that Ig domain unfolding should occur at an average sarcomere length of about  $3.0\ \mu\text{m}$  (their Fig. 5 – open circles). When estimating the theoretical sarcomere lengths of Ig domain unfolding, they made some non-trivial assumptions. Nevertheless, if we assume that their sarcomere length estimate is accurate, then our values overestimate the actual sarcomere length of first Ig domain unfolding. This discrepancy may be reconciled if some Ig domains were already unfolded prior to our experiments. On the other hand, Kellermayer's extrapolation from a single molecule to a whole

fiber might have underestimated the actual sarcomere length of first Ig domain unfolding. Only an experiment in which Ig domain unfolding is directly observed can resolve this puzzle. We are working on doing precisely such experiments using quantum dot labeled antibodies that demarcate the ends of the proximal and distal Ig domain segments in rabbit psoas myofibrils.

Kellermayer et al. (1997) also observed that Ig domain unfolding occurred at a titin force of  $20$ – $25\ \text{pN}$  (their Fig. 2). In a cylindrical myofibril with a diameter of  $1.0\ \mu\text{m}$ , one would expect approximately 2700 titin molecules (assuming a lattice spacing of  $42\ \text{nm}$  between thick filaments (Herzog, 1999) and 6 titin molecules per half thick filament (Granzier and Labeit, 2007)). Multiplying the number of titin molecules by the force of first Ig domain unfolding ( $20$ – $25\ \text{pN}$ ) provides an estimate of the myofibril force at which to expect a force–elongation inflection point; that is about  $54$ – $68\ \text{nN}$ . The upper estimate of this theoretical range agrees well with the force we measured at the inflection point in our myofibril experiments ( $68 \pm 5\ \text{nN}$ ). This result suggests that the inflection point observed in the myofibril experiments indeed corresponds to the point at which titin Ig domain unfolding starts to occur.

#### 4.2. Loss of energy

The loss of loading energy, and the relative steady values for the unloading energy with repeat stretch–shortening cycles, agrees with the observations on single titin molecules (Kellermayer et al., 1997). 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), suggests that Ig domain unfolding is incomplete within repeat stretch cycles and after ten minutes of rest. Our myofibrils were rested at a mean sarcomere length of  $2.5$ – $2.7\ \mu\text{m}$  where passive forces were zero. Nevertheless, this length is at the very end of the normal range of motion, thus refolding of Ig domains might have been prevented because of the relatively long resting sarcomere lengths. Future experiments should focus on the conditions required for effective Ig domain refolding.

Kellermayer et al. (1997) observed a virtually elastic behavior when titin was not stretched beyond its inflection point. Since the inflection point was observed at an average sarcomere length as short as  $3.5\ \mu\text{m}$ , we would have expected an essentially elastic response for our shortest ( $1.0\ \mu\text{m}$ ) stretch–shortening experiments which had a peak sarcomere length of about  $3.5\ \mu\text{m}$ , but we did not (see Table 1). The reasons for this lack of elastic behavior cannot be explained with the data collected in our work. However, the following possibilities exist: (i) the true inflection point might occur at shorter sarcomere lengths than  $3.5\ \mu\text{m}$ . Kellermayer et al. (1997) suggested a sarcomere length of about  $3.0\ \mu\text{m}$  based on their theoretical estimates. This is perfectly feasible as handling of the myofibrils and installing them in the testing apparatus was associated with some stretching, and might have produced Ig domain unfolding prior to testing; (ii) although titin might behave virtually elastically prior to Ig domain unfolding in isolation, it might behave visco-elastically in situ where titin is known to interact transiently with actin (Kulke et al., 2001; Linke et al., 2002; Yamasaki et al., 2001) and this interaction might produce viscous properties; (iii) the purely elastic properties of single titin might not be observable in an ensemble of titin proteins located in serially arranged sarcomeres of different length and size; (iv) finally, titin might exist in different isoforms within the same muscle. The inflection point might be visible for one of the isoforms while the other isoform might unfold at shorter sarcomere lengths, and so might provide the observed visco-elasticity. These are just some of the possibilities for the lack of an elastic behavior of titin for small “stretch–shortening” cycles of a single myofibril. Undoubtedly, other explanations exist, and this observation deserves attention in future experiments.

#### 4.3. Active vs. passive stretch-shortening tests

Titin has binding sites for calcium, and it has been argued that calcium activation of muscles affects the mechanical properties of titin by increasing its stiffness (Joumaa et al., 2008; Labeit et al., 2003); and/or by modulating its interaction with other sarcomeric proteins, such as actin (Astier et al., 1998; Bianco et al., 2007; Herzog et al., 2012; Leonard and Herzog, 2010a; Linke et al., 2002; Nishikawa et al., 2011; Yamasaki et al., 2001). Based on this evidence, we expected a difference between the passive forces measured in a low calcium relaxation solution and those obtained in a high calcium activation solution in which “active” cross-bridge based forces were chemically inhibited (Leonard and Herzog, 2010b). However, no such differences were observed, which is in contrast to previous reports (Joumaa et al., 2008; Labeit et al., 2003) but is consistent with recent work, where no differences in myofibril stiffness and passive forces were found between actively and passively stretched myofibrils ((Leonard and Herzog, 2010a), Fig. 3B). In contrast to the work by others, (Joumaa et al., 2008; Labeit et al., 2003), our work was performed at long sarcomere lengths (up to 5.5  $\mu\text{m}$ ). Possibly, calcium activation has an effect on titin at short but not at long sarcomere lengths, or the relatively small effects observed at short sarcomere lengths cannot be resolved at long sarcomere lengths when passive forces are in excess of 20 times greater than the differences observed at short sarcomere lengths. Furthermore, the approach chosen here to measure the effects of  $\text{Ca}^{2+}$  activation on titin (high  $\text{Ca}^{2+}$  concentration in the presence of BDM) was different from the approach by Joumaa et al. (2008) who used troponin C depletion, and different from Labeit et al. (2003), who used gelsolin to remove actin from their single fiber preparations. Therefore, the discrepancy in results might be associated with the details of how preparations were treated prior to measuring titin properties in the presence of  $\text{Ca}^{2+}$  activation.

#### 5. Conclusions

The results of this study suggest that the viscoelastic and force properties of isolated titin molecules are well reflected in whole myofibril testing, except possibly the onset of Ig domain unfolding, and the purely elastic behavior of titin prior to reaching the inflection point. The onset of Ig domain unfolding can be observed in myofibril preparations if stretching of myofibrils in the preparatory phase is minimized. The increase in efficiency with repeat stretch-shortening cycles and decreasing stretch magnitude, as well as the observation of the inflection point for some of the myofibrils at a force perfectly predicted by single titin testing, seems 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 easier technically, it also offers the advantage that titin can be studied in its native environment and that titin's properties can be directly related to sarcomere forces and lengths, and thus can be extrapolated to myofibril, fiber and muscle properties.

#### Conflict of interest statement

Please let it be known that all authors have no financial or personal relationship with other people or organizations that would inappropriately influence our work.

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