Research Article

Titin-Isoform Dependence of Titin-Actin Interaction and Its Regulation by S100A1/Ca^{2+} in Skinned Myocardium

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Titin, also known as connectin, is a large filamentous protein that greatly contributes to passive myocardial stiffness. In vitro evidence suggests that one of titin's spring elements, the PEVK, interacts with actin and that this adds a viscous component to passive stiffness. Differential splicing of titin gives rise to the stiff N2B and more compliant N2BA isoforms. Here we studied the titin-isoform dependence of titin-actin interaction and studied the bovine left atrium (BLA) that expresses mainly N2BA titin, and the bovine left ventricle (BLV) that expresses a mixture of both N2B and N2BA isoforms. For comparison we also studied mouse left ventricular (MLV) myocardium which expresses predominately N2B titin. Using the actin-severing protein gelsolin, we obtained evidence that titin-actin interaction contributes significantly to passive myocardial stiffness in all tissue types, but most in MLV, least in BLA, and an intermediate level in BLV. We also studied whether titin-actin interaction is regulated by S100A1/calcium and found that calcium alone or S100A1 alone did not alter passive stiffness, but that combined they significantly lowered stiffness. We propose that titin-actin interaction is a "viscous break" that is on during diastole and off during systole.

1. Introduction

Passive tension development in the sarcomere plays a critical role in diastolic function. The giant protein titin/connectin spans the half-sarcomere from Z-disk to M-line [1] and is responsible for the development of passive tension within the sarcomere [2]. Titin-based passive tension constitutes a large fraction of the myocardial passive tension; the other main contributor is the extracellular matrix [2, 3]. Titin contains an extensible *I*-band region that comprises three distinct regions: serially-linked immunoglobulin (Ig)-like domains, the N2B element, and the PEVK region (which primarily contains proline (P), glutamate (E), valine (V), and lysine (K) residues) [4]. These three spring-like elements develop force when extended and, along with collagen, determine myocardial passive stiffness [5].

Isoform splicing is a long-term mechanism that alters titin-based myocardial passive stiffness [6–8]. Cardiac titin is present as two isoforms: the small (3.0 MDa) N2B titin and larger N2BA titin isoform (~3.3 MDa) [4, 6]. Differential expression of titin's *I*-band region results in N2BA titin containing a larger PEVK domain than in N2B titin, in addition to a variable number of additional Ig domains [4, 6, 9]. At a given extension, the cardiac N2BA isoform develops less force than the N2B isoform, which can be explained by its longer end-to-end length. This longer length results in a lower fractional extension of the elastic elements in the *I*-band, which leads to lower force development [10, 11]. Consistent with this, mouse knockout models, in which the spring region of titin has been shortened, develop higher passive force [12, 13].

Adding to the complexity of the sarcomeric response to external stretch is the viscous interaction between cardiac PEVK and actin [14–17]. In vitro motility assays [14–16, 18], single molecule studies [19], myocyte mechanics [14], electron microscopy [20], and single myofibril recoil

experiments [21] have provided evidence for a dynamic interaction between the PEVK and F-actin that retards filament sliding. As the sarcomeres change length during active contraction (systole) and passive stretch (diastole), titin's *I*-band region and actin are displaced with respect to one another. F-actin contains negatively-charged regions on its outer surface [22], and the ionic strength dependence of the PEVK-actin interaction suggests that electrostatic interactions play a role in determining the magnitude of the "viscous drag" force of this interaction [14]. In vitro evidence suggests that the PEVK-actin interaction might be regulated by S100A1 [14], a soluble calcium-binding protein present in the myocardium [23], with the interaction reduced in the presence of S100A1 and physiological calcium levels. Thus, isoform splicing represents a long-term response to changing demands on the cardiovascular system, and Ca^{2+}/S100A1 modulated PEVK-actin association might represent a short-term mechanism for changing passive stiffness.

The presence of titin-actin interaction has mainly been investigated in N2B expressing rodent myocardium [14, 15]. However, large mammals, including human, typically express higher levels of N2BA cardiac titin [8] and here we investigated titin-actin interaction in bovine left atrium (BLA) which expresses predominately N2BA titin, and bovine left ventricle (BLV) which coexpresses both N2B and N2BA titin [24, 25]. We compared results to those from the N2B dominant mouse left ventricle (MLV). We used skinned myocardium and studied passive stiffness before and after actin extraction using the actin severing protein gelsolin. We also focused on the possible regulation of actin-titin interaction by S100A1 and calcium, a mechanism of regulation for which support exists from in vitro binding and in vitro motility studies [14], but which has not been tested in a sarcomeric context. The sarcomere has many geometric constraints and contains countless proteins that are absent in the in vitro assays, and these might mask or accentuate properties measured in vitro. The findings of our studies support that titin-actin interaction is universally present in the different titin isoforms, and that the level scales with the stiffness of the isoform. We also found support for regulation of titin-actin interaction by S100A1 and calcium in skinned myocardium. The data indicate that during the cardiac cycle, titin-actin interaction fluctuates, and is strong during diastole when the calcium level is low but considerably weaker during systole when calcium is high.

2. Materials and Method

2.1. Muscles. Hearts were rapidly excised from ~3 mo old C57/BL6, male mice. The left ventricle (LV) was opened and immediately placed in a modified Krebs solution [composition in mM: NaCl 122, KCl 5, NaHCO₃ 25, MgCl₂ 1.08, NaH₂PO₄ 1.3, CaCl₂ 0.2, 2, 3–6 butanedione monoxime (BDM) 30, glucose 11.2, insulin 5 U/L, gassed with 95% O₂, 5% CO₂, pH 7.4]. Bovine hearts (animals >18 months old and weighing >1100 lbs) were obtained from local slaughterhouses. The hearts were excised within ~15 minutes following death. The left atrium (LA) and left ventricle were cut into small pieces and placed into oxygenated Krebs solution. Small wall muscle strips (length: 1.5–3.0 mm; diameter ~0.3 mm) were dissected using a high-resolution binocular scope. Extreme care was taken to dissect the strips in the direction of the fibers and to avoid stretching during dissection. Visual inspection under the dissection microscope revealed that some areas of the BLA contained relatively high levels of collagen and these areas were avoided in this study. Muscle strips were skinned in relaxing solution (BES 40 mM, EGTA 10 mM, MgCl₂ 6.56 mM, ATP 5.88 mM, DTT 1 mM, K-propionate 46.35 mM, creatine phosphate 15 mM, pH 7.0) (chemicals from Sigma-Aldrich, MO, USA) with 1% Triton-X-100, overnight at ~4°C. The muscle strips were then washed and used for mechanical experiments. To prevent degradation, all solutions contained protease inhibitors (PMSF: 0.5 mM; leupeptin: 0.04 mM, and E64: 0.01 mM). All animal experiments were approved by the University of Arizona Institutional Animal Care and Use Committee and followed the U.S. National Institutes of Health "Using Animals in Intramural Research" guidelines for animal use.

Passive tension was measured with a silicon semiconductor strain gauge (model A*E*–801, SensoNor, Horten, Norway). Muscle length was controlled by a high-speed motor (Model 308B, Aurora Scientific Inc., Richmond Hill, Ontario, Canada). Preparations were attached to the motor arm and the force transducer via aluminum clips. Using a small prism, the two perpendicular diameters of the muscle were measured and the cross-sectional area was calculated, assuming the cross-section is elliptical in shape. This area was used to calculate the force per unit area (in mN/mm²), allowing us to compare results from different muscles. Experiments were performed at room temperature (20–22°C). We determined titin's contribution to total passive tension using its fractional contribution in MLV, BLV, and BLA as previously determined (this previous work used the exact same experimental conditions as the present work); see [3]. We also determined the passive stiffness from the local slope of the titin-based tension-SL relation.

Sarcomere length (SL) was measured with laser-diffraction [26, 27]. The diffraction pattern was collected with a bright-field objective, a telescope lens was focused on the back focal plane of the objective, and the diffraction pattern was projected, after compression with a cylindrical lens, onto a photodiode array. The first order diffraction peak position was obtained using a digital spot-position detector board installed in an IBM AT computer. This signal was converted to sarcomere length using a calibration curve that was established with diffraction peaks of a 25 μm grating that was present in the chamber. For further details see [26, 27].

Maximum active force was measured at an SL of 2.0 μm, using an activating solution with pCa 4.5 (pCa = −log [Ca2+]). Passive force was measured while the muscle was in relaxing solution and stretching the preparation with a constant velocity from its slack length to a predetermined amplitude followed by a release to the slack length at the same velocity. Most experiments were done utilizing a stretch/release velocity of 0.1 length/sec with the length referenced to the slack sarcomere length (~1.9 μm), except

for those in Figure 6 that were done at both 0.1 and 2.0 length/sec. Stretch–release cycles were followed by ~20-min rest periods at the slack length.

To eliminate titin-actin interaction in the *I*-band region of the sarcomere, we used a calcium-independent gelsolin fragment (amino acids 1–406; dissolved in 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 1.0 mM EGTA, 1.0 mM Mg-acetate, 20 mM K-propionate, 1 mM DTT, and 20 μg/mL leupeptin, pH 7.0 at 21–23°C), as described previously [28]. Skinned muscle strips were mechanically characterized and the chamber solution was then replaced by gelsolin (3 μg/μL) in relaxing solution, as described previously [29, 30], and incubated at room temperature for 4 hours, followed by extensive washing with relaxing solution and a mechanical characterization identical to the one carried out before actin extraction. To determine the degree of thin filament extraction, the maximal active tension (pCa 4.0) at a starting SL of 2.0 μm was measured before and after actin extraction.

To test the effect of low level of calcium or S100A1, a separate group of skinned muscles were incubated in a relaxing solution (pCa 9.0), a calcium (pCa 6.8) solution (solution composition calculated by the program of Fabiato and Fabiato [31]), and either a relaxing solution that contains S100A1(1 μg/μL; S100A1 from Sigma) or a solution that contains both calcium (pCa 6.8) and S100A1. The skinned muscles were incubated in these solutions and were then stretch/released at 0.1 length/sec, allowed to rest 20 minutes, and then stretch/released at 2.0 length/sec.

2.2. Statistics. Data are presented as mean ± SE. Significant differences were determined using a *t*-test or where appropriate ANOVA. Post hoc comparisons were made using Tukey HSD. Probability values < .05 were taken as significant.

3. Results

We focused on the contribution of titin-actin interaction to passive stiffness and its modulation via S100A1 and calcium. We utilized myocardial wall strips from MLV, as a representative of a myocardium that expresses predominantly N2B titin, BLA, as a representative of an N2BA dominant sample, and BLV, as a representative of myocardium that coexpresses both N2BA and N2B titin isoforms [11]. The N2B : N2BA expression ratio in the BLV tissues was determined with agarose gels [32] to be 1.2 ± 0.2; the inset of Figure 1(b) shows an example of a gel with BLV and BLA samples. We measured passive tension during stretch of the skinned myocardium, before and after extracting actin with gelsolin, in order to deduce whether actin-titin interaction contributes to passive tension. To determine the degree of actin extraction, we measured maximal active tension before and after gelsolin treatment.

Gelsolin treatment resulted in a nearly 95% loss in active tension (Table 1), suggesting that the majority of the thin filaments was extracted in all tissues, consistent with previous work [28]. Although the remaining few percent of active tension could have been eliminated by extending the gelsolin
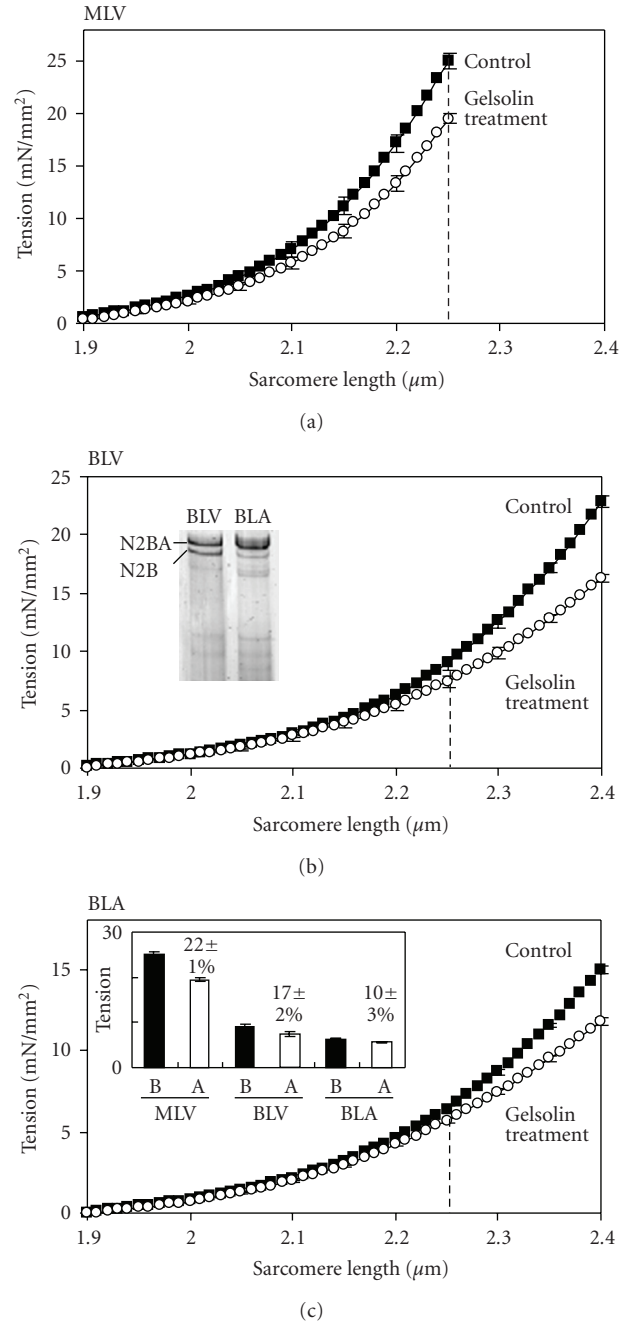

FIGURE 1: Measurements of total passive tension, before and after gelsolin treatment. (a) mouse left ventricle (MLV), (b) bovine left ventricle (BLV), (c) bovine left atrium (BLA) before gelsolin extraction (control, black) and after gelsolin treatment (white). Gelsolin treatment decreases passive tension. Inset of (b): example of titin gel (SDS agarose) with BLV and BLA samples. Inset of (c): tension at SL = 2.25 μm before and after gelsolin treatment with % tension reduction. MLV is more greatly affected by thin filament extraction than BLA, with BLV at an intermediate level. Error bars are SE (for clarity plotted only every 0.05 μm).

treatment duration, because this might recruit titin from near the Z-disc [30], which is normally inextensible, we limited the gelsolin treatment duration such that a very

TABLE 1: Measurement of maximal active tension (pCa 4.5) before and after gelsolin treatment. Percent reduction not different between groups. (SL 2.0 μm; n = 6 per group; data are mean $\pm$ SE; ***P < .001.

Muscle	Dominant Isoform	Active tension before gelsonlin treatment (mN/mm^2)	Active tension after gelsonlin treatment (mN/mm^2)	Percent tension reduction (%)
MLV	N2B	36.7 $\pm$ 0.9	2.25 $\pm$ 0.26***	93.8 $\pm$ 0.8
BLV	Coexpressing	41.8 $\pm$ 1.5	2.24 $\pm$ 0.16***	94.6 $\pm$ 0.3
BLA	N2BA	33.2 $\pm$ 0.5	2.31 $\pm$ 0.24***	93.0 $\pm$ 0.7

small amount of active tension remained. Thus by comparing passive stiffness of different tissue types and utilizing gelsolin as a tool to extract actin, we investigated the contribution of titin-actin interaction to passive tension in N2B expressing myocardium (MLV), N2BA expressing myocardium (BLA), and N2B and N2BA coexpressing myocardium (BLV).

### 3.1. Changes in Passive Tension by Thin Filament Extraction.
Because titin-actin interaction is thought to provide a viscous force, the passive tension measured during stretch is expected to be increased by this interaction. We examined passive tension during stretch to sarcomere length 2.25 μm for MLV or 2.4 μm for BLV and BLA. MLV tissues were only stretched to sarcomere lengths (SL) of 2.25 μm so that the tissues would not encounter damage [3]. BLA and BLV tissues were stretched to SL of 2.4 μm, which for these tissues is in the reversible length regime [3]. Between tissue types, N2B expressing MLV was found to be stiffer than N2B/N2BA coexpressing BLV, which was stiffer than N2BA expressing BLA (Figure 1, control curves), reproducing earlier findings [3]. Following thin filament extraction, passive tension was reduced and the magnitude was small at short lengths and grew as SL increased (Figure 1). The effect was most pronounced in MLV, intermediate in BLV, and lowest in BLA. Comparing data at an SL of 2.25 μm, showed that the magnitude of the reduction was 22, 17, and 10%, respectively (Figure 1(c), inset).

Results in Figure 1 represent total passive tension. Previously, we determined titin's and collagen's fractional contribution to total passive tension of MLV, BLV, and BLA (using the exact same experimental conditions as in the present work) and we used those results [3] to calculate from the total measured tension (Figure 1), the titin-based passive tension. Results are shown in Figure 2, with findings at an SL of 2.25 μm shown in the inset of Figure 2(c). The percent titin-based passive tension reduction at the full SL range is shown in Figure 3(a). In all tissue types, titin-based passive tension increases with SL, and at all SLs the reduction is largest in MLV, less in BLV, and lowest in BLA with values at 2.25 μm of 44, 20, and 12%, respectively. We also determined the passive stiffness from the local slope of the tension-SL relation and calculated the stiffness reduction due to actin extraction. Results shown in Figure 3(b) reveal that in all three tissue types, actin extraction has a large effect on titin-based stiffness, and that the reduction is again largest in N2B expressing MLV and smallest in N2BA expressing BLA with BLV being intermediate.

3.2. Hysteresis in Thin Filament Extracted Tissue.
It is well known that passive tension displays hysteresis (i.e., passive tension is higher during stretch than during release) and because titin-actin interaction appears to contribute to passive tension during stretch we wished to examine whether titin-actin interaction contributes to hysteresis. In the three muscle types, we imposed stretch-releases cycles before and after thin filament extraction and determined hysteresis from the area of the force-length loop of stretches to different SLs (an example of the protocol is shown in Figure 4(a)). In all tissue types, hysteresis increased with SL and, interestingly, gelsolin treatment significantly decreased hysteresis at all lengths. At an SL of 2.25 μm (Figure 4(b)), the decrease was the same in MLV and BLA (33 and 35%, resp.) and larger in BLV (52%). It is important to highlight that hysteresis is not abolished by actin extraction, showing that titin-actin interaction is only partially responsible for hysteresis and that there must be other sources of hysteresis as well.

### 3.3. Modulation of Titin-Actin Interaction via S100A1 and Ca^{2+}.
While titin-actin interactions might be useful in providing resistance to stretch during diastole, during systole, these interactions could hinder ejection when thin filaments rapidly slide past the extensible region of titin. Thus it is worth examining whether titin-actin interaction is regulated. Based on in vitro studies, we had previously proposed that the Ca^{2+}-binding protein S100A1 regulates actin-titin interaction [3]. Here we investigated the possibility of modulation of passive stiffness via S100A1 and Ca^{2+}. We used a pCa of 6.8 because this is just below the level that causes activation [33]. We focused on MLV because titin-actin interactions are most pronounced in this tissue type, making it easier to detect regulation, if indeed it exists. The example experiment of Figure 5 shows that Ca^{2+} alone or S100A1 alone did not change tension during stretch (Figure 5(a)) but that when combined (pCa 6.8 plus S100A1) passive tension was reduced (Figure 5(b)). We performed experiments at two stretch speeds (0.1 and 2.0 length/sec) and their summarized results are shown in Figure 6. In the presence of Ca^{2+} alone or S100A1 alone, passive tension at both speeds was slightly increased compared to that measured in Ca^{2+}free solution (Figure 6, upper curves). In the presence of both S100A1 and Ca^{2+}, a significant decrease in passive stiffness was observed, with up to 20% decrease at the fastest speed (Figure 6, bottom two curves).
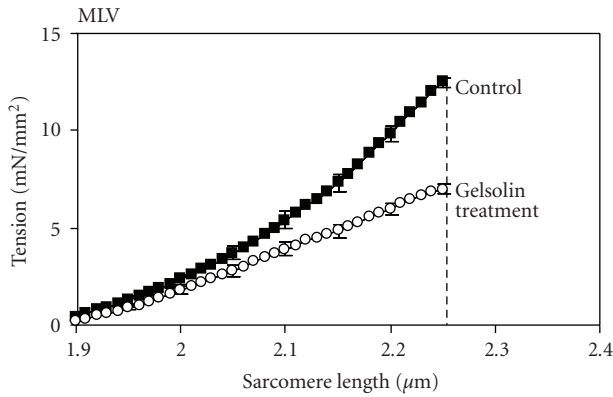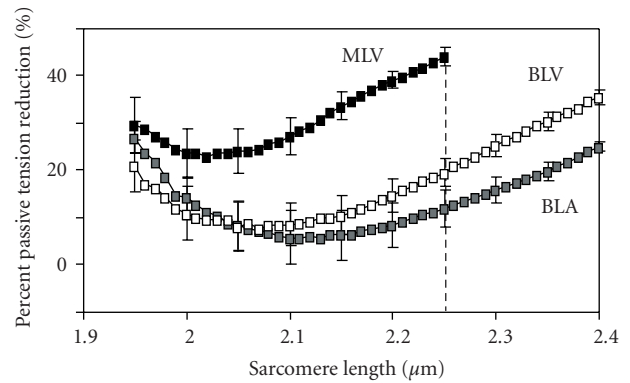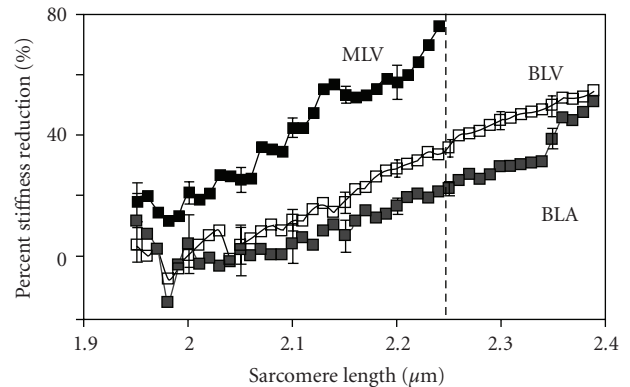
(a)
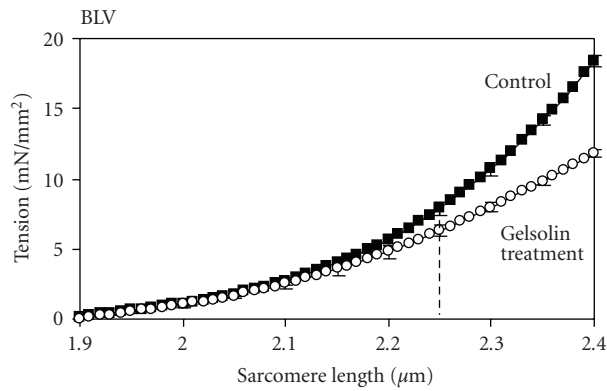
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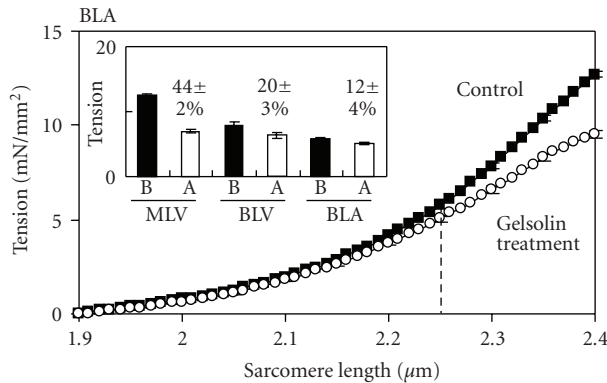
(c)

Figure 2: Measurements of titin-based passive tension, before and after gelsolin treatment. (a)–(c) In MLV, BLV, and BLA titin-based passive tension is reduced following gelsolin treatment. At a SL of 2.25 μm. Decrease in titin-based passive tension is largest in MLV, smallest in BLA, with BLV being intermediate. Data are based on Figure 1; see text for details. Error bars are SE (for clarity plotted only every 0.05 μm).

(a)

(b)

Figure 3: Reduction in titin-based tension (a) and titin-based stiffness (b). Data are based on measurements shown in Figure 2 (tension) and from the slope of the tension-SL curve shown in Figure 2 (stiffness). The reductions in tension and stiffness are SL dependent, in all tissue types, and are highest in MLV, lowest in BLA and intermediate in BLV. Error bars are SE (for clarity plotted only every 0.05 μm).

4. Discussion

We found in this work that thin filament extraction, with the actin-severing protein gelsolin, reduces passive tension during stretch of skinned myocardium. These findings support previous studies that suggest that passive tension measured during stretch in cardiac muscle is not solely determined by titin's intrinsic elasticity, but also includes contributions from a transient (or viscous) force that is due to titin–actin interaction [14, 15]. In vitro binding and motility studies have shown that amongst the three spring elements of cardiac titin, the tandem Ig segments and N2B element do not bind actin, but that the PEVK region does interact with titin [14, 15, 18]. Important evidence for functional significance of titin-actin interaction was obtained in mechanical experiments with mouse cardiac myocytes in which a recombinant protein fragment consisting of the PEVK region of the N2B titin isoform was added to skinned myocytes, and passive tension during stretch was lowered due to a competition with endogenous titin molecules for binding sites on the thin filament [14]. Our present work on mouse skinned myocardium in which we found that thin filament extraction also lowers passive tension during stretch, similarly to what had been found earlier in myocytes with exogenous PEVK, further supports the notion that titin-actin interaction contributes to passive tension
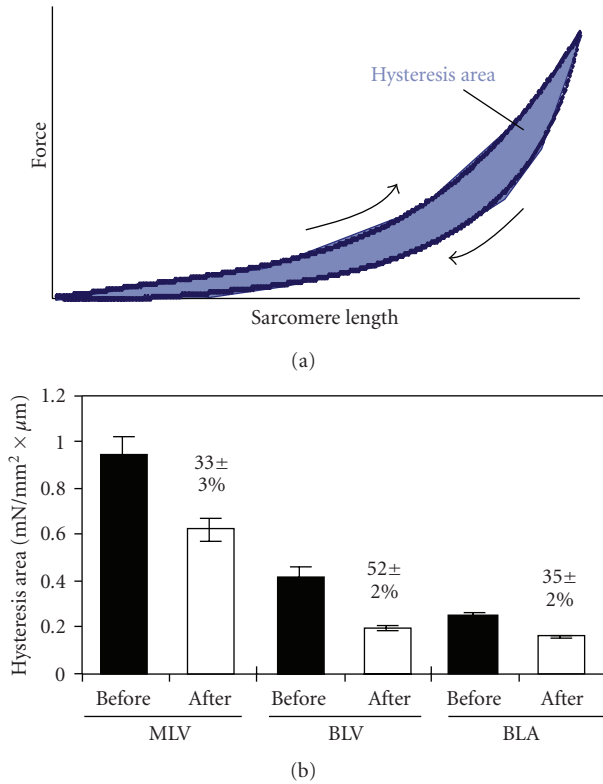
(a)

(b)

FIGURE 4: Hysteresis before and after gelsolin treatment. (a) Protocol. Skinned muscles were stretched to a given length followed by a release back to slack and hysteresis was calculated from the area of the resultant force-sarcomere length curve. Measurements were made before and after gelsolin treatment. (b) Comparison of hysteresis at SL 2.25 μm with % hysteresis reduction. All tissues showed an increase in hysteresis with SL and a reduction in hysteresis following actin extraction with gelsolin. Error bars are SE. $^*P < .01$.
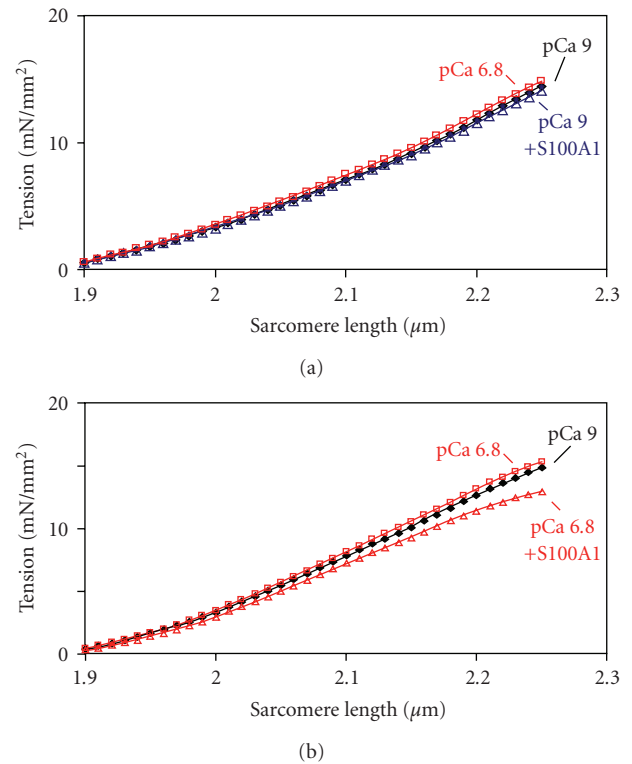

(a)

(b)

FIGURE 5: Example experiment addressing effect of calcium and S100A1 on tension in skinned muscle during stretch. (a) Skinned MLV muscle was stretched (0.1 length/sec) in pCa 9.0 (black), pCa 6.8 (red), and pCa 9.0 plus S100A1 solution (blue). Calcium (pCa 6.8) or S100A1 alone did not affect tension during stretch. (b) Skinned MLV muscle was stretched (0.1 length/sec) in pCa 9.0 (black), pCa 6.8 (red squares), and pCa 6.8 plus S100A1 solution (red triangles). Only when calcium (pCa 6.8) and S100A1 are both present is passive tension reduced.

during stretch. Furthermore, our finding that thin filament extraction lowers passive tension in bovine myocardium as well as mouse myocardium (Figures 1–3) suggests that N2BA cardiac titin also interacts with actin and that PEVK-titin interaction is universally represented in different cardiac isoforms.

In vitro binding studies have shown that the binding between F-actin and the PEVK is ionic strength dependent [14, 18, 19], suggesting that the interaction is at least in part electrostatic. F-actin contains a large patch of negatively charged residues on its exposed surface [22], and several actin-binding proteins are known to bind to this region via basic charge clusters [34–36]. The PEVK region of N2B cardiac titin is comprised of basic (pI 9-10) ~28-residue PEVK repeats [6, 37]. At physiological pH, the cardiac PEVK therefore carries a net positive charge, which likely facilitates its interaction with the negatively charged actin filament. Proline-rich regions (PRRs) often assume extended conformations, such as the polyproline II (PPII)-helix that has been predicted for PEVK fragments [38]. Due to the conformational restrictions that proline residues impose on

the polypeptide backbone, PRRs experience a small reduction of entropy upon ligand binding [39]. This property makes them energetically favorable sites for protein-protein interactions, and there are numerous examples of PRRs that perform binding functions in vivo [39]. The binding of actin by the N2B PEVK may therefore be both electrostatically and thermodynamically favorable.

The in vitro studies discussed above were all carried out with a recombinant PEVK fragment that represents the PEVK sequence found in the human N2B cardiac isoform. The high sequence conservation of the N2B PEVK [28] makes it likely that the same binding properties exist in different species. Additionally, the PEVK sequence of the N2B titin isoform is constitutively expressed in different titin isoforms and thus PEVK-titin interaction is expected to occur in N2BA cardiac titin as well [4, 7, 28]. The N2BA isoform contains additional PEVK residues many of which belong to the above discussed PEVK repeat sequence motif. Others belong to a second PEVK motif (absent in the N2B isoform), the so-called E-rich motif which contains a preponderance of glutamate [4, 40]. Nagy et al. [18] studied
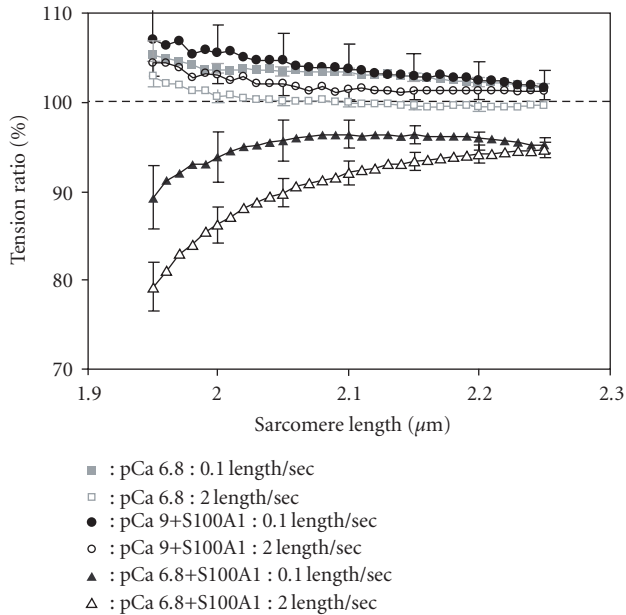
Figure 6: Effect of calcium, S100A1, and calcium plus S100A1 on tension. Skinned MLV was stretched at two velocities (0.1 and 2.0 length/sec; dark and open symbols, resp.) in calcium alone (pCa 6.8; grey), S100A1 alone (circles), or calcium plus S100A1 (triangles). Calcium alone or S100A1 alone slightly increases tension (3–8%). When S100A1 is added to pCa 6.8 solutions, tension is decreased, an effect that is largest at the fastest stretch speed. Tensions are expressed relative to tensions measure in the pCa 9.0 solution (See methods for details). Error bars are SE (for clarity plotted only every $0.05\,\mu$m).

recombinant PEVK proteins with different motif structures and found that the presence of E-rich motifs *enhances* the actin binding property of the PEVK. Thus, although the PEVK of the N2BA isoform has not been studied in vitro, it is expected that PEVK-actin interaction is present in this titin isoform, consistent with our present findings.

We found that the effect of thin filament extraction was highest in N2B expressing MLV and lowest in N2BA expressing BLA. Although this might suggest that the interaction between the PEVK and actin is less in N2BA titin, as discussed above, this is unlikely to be the case (the interaction might actually be enhanced in N2BA titin) and it is worthwhile therefore to explore alternative explanations. A likely explanation is as follows. The interaction between the PEVK region and the actin filament will during sarcomere stretch impede the extension of the PEVK element, and as a result the extension of the tandem Ig and N2B spring elements (which do not interact with actin) will be increased. The degree to which this increased extension affects force will depend on how much it will increase titin's fractional extension (end-to-end length divided by the contour length) [41–43]. Because N2BA titin has a much longer contour length than N2B titin (due to the extra PEVK residues and additional Ig domains), its increase in fractional extension will be less and hence its increase in force will be less as well (titin's force is a function of its fractional extension; see

[41, 44]). Thus, our findings are consistent with a model in which all titin isoforms interact with actin and in which the isoform's contour length in inversely related to the titin-actin interaction induced force increase. The contour length of N2BA titin is longer than that of N2B titin [11], explaining why the passive tension increase of BLA is much lower than that of MLV. It is also important to highlight that although the effect of titin-thin filament interaction at a given SL is lower in bovine tissues than in mouse, the effects are more similar in the two species when they are compared at their end diastolic SL, which in large mammals has been reported to be $\sim$2.4 μm [45], and in rodents $\sim$2.2 μm [46]. Thus, titin-actin interaction is likely to play important physiological roles in species as diverse as mouse and bovine.

We have previously shown a correlation between the level of N2B expression and heart beat frequency [8]. We proposed that the short times available for diastolic filling when the heart beats fast (in the mouse, the filling time is only $\sim$40 msec) necessitates a stiff ventricle, to ensure that early diastolic filling is fast (titin contributes to the early diastolic suction force [47–49]) and that during late diastole, overfilling is prevented [8]. Because the increased stiffness due to titin-actin interaction is most pronounced at longer SLs (Figure 3(b)), titin-actin interaction might function primarily as a determinant of the maximal filling volume. It is also important to highlight that the contribution of titin-actin interaction to passive stiffness increases with stretch speed (due to its viscous nature, see e.g., [19]), and thus as the heart beats more forceful and faster (as occurs during beta-adrenergic stimulation) titin-actin interaction will further increase stiffness at the large volumes and enhance thereby control over the maximal filling volume. Titin also plays a structural role that includes centering of the A-band in the middle of the sarcomere [50]. Any unevenness in active force generation in the two halves of a sarcomere (which might be due to, for example, micro-variation in the timing of calcium release) is expected to result during systole in movement of the A-band away from its central location in the sarcomere. During diastole, passive tension will reset the A-band to its central location. However, if diastolic durations are extremely short, this is hard to accomplish and stiff titin will speed recentering, with a viscous component at longer length preventing overshoot and possible oscillations. Thus, we propose that titin-actin interaction is important for controlling both late diastolic filling and the structural integrity of the sarcomere.

Although titin-actin interaction is likely to be functionally important during diastole, during systole it is not beneficial because it impedes filament sidling. It has been shown in vitro that S100A1 regulates PEVK-actin interaction [14] and, thus, S100A1 might provide a mechanism for switching PEVK-actin interaction off during part of the heart cycle when it is not a desirable feature to have. S100 is a 25-member family of EF hand proteins, with S100A1 as the most abundant member found in the heart [23]. Physiological levels of calcium activate S100A1, by exposing a hydrophobic domain on S100A1 that is a primary site for protein interactions [23]. Immunoelectron microscopy has shown that in the presence of calcium, S100A1 binds

at several sites along titin's extensible region, including the PEVK domain [14]. Furthermore, gel overlay analysis has revealed that S100A1 binds the PEVK region in a calcium-dependent manner, and in vitro motility assays indicate that S100A1-PEVK interaction reduces the force that arises as F-actin slides relative to the PEVK domain [14]. Thus we had previously proposed that S100A1 provides a mechanism to free the thin filament from titin and reduce titin-based tension before active contraction and rapid filament sliding take place [14]. The present work is the first critical test of this proposal. We used a low level of calcium (pCa 6.8), just below the threshold for contraction, and showed that this alone has no clear effect on tension of skinned myocardium during stretch, and neither has S100A1 alone (5 and 6). However, when both are present, a significant reduction in passive tension during stretch is observed, that scales with stretch speed, as expected from being derived from a viscous source. Thus because S100A1 is constant during the cardiac cycle, and calcium fluctuates, titin-actin interaction will vary: it is strong during diastole when the calcium level is low but considerably weaker during systole when calcium is high.

In summary, our studies support that all cardiac titin isoforms interact with actin and that the degree to which this increases passive stiffness is inversely related to the isoform's contour length. Titin-actin interaction is expected to contribute to the structural integrity of the sarcomere (*A*-band centering) and the control of late diastolic filling. Furthermore, evidence was obtained for regulation of titin-actin interaction by S100A1/Ca^{2+}. We propose that titin-actin interaction functions as "viscous break" that is on during diastole and off during systole.

Acknowledgments

References

[1] D. O. Furst, M. Osborn, R. Nave, and K. Weber, "The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy: a map of ten nonrepetitive epitopes starting at the Z line extends close to the M line," *Journal of Cell Biology*, vol. 106, no. 5, pp. 1563–1572, 1988.

[2] H. L. Granzier and T. C. Irving, "Passive tension in cardiac muscle: contribution of collagen, titin, microtubules, and intermediate filaments," *Biophysical Journal*, vol. 68, no. 3, pp. 1027–1044, 1995.

[3] Y. Wu, O. Cazorla, D. Labeit, S. Labeit, and H. Granzier, "Changes in titin and collagen underlie diastolic stiffness diversity of cardiac muscle," *Journal of Molecular and Cellular Cardiology*, vol. 32, no. 12, pp. 2151–2161, 2000.

[4] S. Labeit and B. Kolmerer, "Titins: giant proteins in charge of muscle ultrastructure and elasticity," *Science*, vol. 270, no. 5234, pp. 293–296, 1995.

[5] M. Helmes, K. Trombitas, T. Centner, et al., "Mechanically driven contour-length adjustment in rat cardiac titin's unique N2B sequence. Titin is an adjustable spring," *Circulation Research*, vol. 84, no. 11, pp. 1339–1352, 1999.

[6] A. Freiburg, K. Trombitas, W. Hell, et al., "Series of exon-skipping events in the elastic spring region of titin as the structural basis for myofibrillar elastic diversity," *Circulation Research*, vol. 86, no. 11, pp. 1114–1121, 2000.

[7] H. Granzier, M. Radke, J. Royal, et al., "Functional genomics of chicken, mouse, and human titin supports splice diversity as an important mechanism for regulating biomechanics of striated muscle," *American Journal of Physiology*, vol. 293, no. 2, pp. R557–R567, 2007.

[8] O. Cazorla, A. Freiburg, M. Helmes, et al., "Differential expression of cardiac titin isoforms and modulation of cellular stiffness," *Circulation Research*, vol. 86, no. 1, pp. 59–67, 2000.

[9] M.-L. Bang, T. Centner, F. Fornoff, et al., "The complete gene sequence of titin, expression of an unusual approximately 700-kDa titin isoform, and its interaction with obscurin identify a novel Z-line to I-band linking system," *Circulation Research*, vol. 89, no. 11, pp. 1065–1072, 2001.

[10] K. Trombitás, A. Freiburg, T. Centner, S. Labeit, and H. Granzier, "Molecular dissection of N2B cardiac titin's extensibility," *Biophysical Journal*, vol. 77, no. 6, pp. 3189–3196, 1999.

[11] K. Trombitaás, A. Redkar, T. Centner, Y. Wu, S. Labeit, and H. Granzier, "Extensibility of isoforms of cardiac titin: variation in contour length of molecular subsegments provides a basis for cellular passive stiffness diversity," *Biophysical Journal*, vol. 79, no. 6, pp. 3226–3234, 2000.

[12] M. H. Radke, J. Peng, Y. Wu, et al., "Targeted deletion of titin N2B region leads to diastolic dysfunction and cardiac atrophy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 9, pp. 3444–3449, 2007.

[13] H. L. Granzier, M. H. Radke, J. Peng, et al., "Truncation of titin's elastic PEVK region leads to cardiomyopathy with diastolic dysfunction," *Circulation Research*, vol. 105, no. 6, pp. 557–564, 2009.

[14] R. Yamasaki, M. Berri, Y. Wu, et al., "Titin-actin interaction in mouse myocardium: passive tension modulation and its regulation by calcium/S100A1," *Biophysical Journal*, vol. 81, no. 4, pp. 2297–2313, 2001.

[15] M. Kulke, S. Fujita-Becker, E. Rostkova, et al., "Interaction betweeen PEVK-titin and actin filaments origin of a viscous force component in cardiac myofibrils," *Circulation Research*, vol. 89, no. 10, pp. 874–881, 2001.

[16] W. A. Linke, M. Kulke, H. Li, et al., "PEVK domain of titin: an entropic spring with actin-binding properties," *Journal of Structural Biology*, vol. 137, no. 1-2, pp. 194–205, 2002.

[17] M. S. Z. Kellermayer and H. L. Granzier, "Calcium-dependent inhibition of in vitro thin-filament motility by native titin," *FEBS Letters*, vol. 380, no. 3, pp. 281–286, 1996.

[18] A. Nagy, P. Cacciafesta, L. Grama, A. Kengyel, A. Málnási-Csizmadia, and M. S. Z. Kellermayer, "Differential actin binding along the PEVK domain of skeletal muscle titin," *Journal of Cell Science*, vol. 117, no. 24, pp. 5781–5789, 2004.

[19] P. Bianco, A. Nagy, A. Kengyel, et al., "Interaction forces between F-actin and titin PEVK domain measured with optical tweezers," *Biophysical Journal*, vol. 93, no. 6, pp. 2102–2109, 2007.

[20] T. Funatsu, E. Kono, H. Higuchi, et al., "Elastic filaments in situ in cardiac muscle: deep-etch replica analysis in combination with selective removal of actin and myosin filaments," *Journal of Cell Biology*, vol. 120, no. 3, pp. 711–724, 1993.

[21] C. A. Opitz, M. Kulke, M. C. Leake, et al., "Damped elastic recoil of the titin spring in myofibrils of human myocardium," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 22, pp. 12688–12693, 2003.

[22] W. Kabsch, H. G. Mannherz, D. Suck, E. F. Pai, and K. C. Holmes, "Atomic structure of the actin:DNase I complex," *Nature*, vol. 347, no. 6288, pp. 37–44, 1990.

[23] R. Donato, "S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles," *International Journal of Biochemistry and Cell Biology*, vol. 33, no. 7, pp. 637–668, 2001.

[24] N. Fukuda, Y. Wu, G. Farman, T. C. Irving, and H. Granzier, "Titin isoform variance and length dependence of activation in skinned bovine cardiac muscle," *Journal of Physiology*, vol. 553, no. 1, pp. 147–154, 2003.

[25] K. Trombitás, Y. Wu, D. Labeit, S. Labeit, and H. Granzier, "Cardiac titin isoforms are coexpressed in the half-sarcomere and extend independently," *American Journal of Physiology*, vol. 281, no. 4, pp. H1793–H1799, 2001.

[26] H. L. M. Granzier and G. H. Pollack, "The descending of the force-sarcomere length relation of the frog revisited," *Journal of Physiology*, vol. 421, pp. 595–615, 1990.

[27] H. L. M. Granzier and G. H. Pollack, "Effect of active preshortening on isometric and isotonic performance of single frog muscle fibres," *Journal of Physiology*, vol. 415, pp. 299–327, 1989.

[28] C. Hidalgo, B. Hudson, J. Bogomolovas, et al., "PKC phosphorylation of titin's PEVK element: a novel and conserved pathway for modulating myocardial stiffness," *Circulation Research*, vol. 105, no. 7, pp. 631–638, 2009.

[29] H. Granzier, M. Helmes, and K. Trombitás, "Nonuniform elasticity of titin in cardiac myocytes: a study using immunoelectron microscopy and cellular mechanics," *Biophysical Journal*, vol. 70, no. 1, pp. 430–442, 1996.

[30] K. Trombitás and H. Granzier, "Actin removal from cardiac myocytes shows that near Z line titin attaches to actin while under tension," *American Journal of Physiology*, vol. 273, no. 2, part 1, pp. C662–C670, 1997.

[31] A. Fabiato and F. Fabiato, "Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells," *Journal de Physiologie*, vol. 75, no. 5, pp. 463–505, 1979.

[32] S. Lahmers, Y. Wu, D. R. Call, S. Labeit, and H. Granzier, "Developmental control of titin isoform expression and passive stiffness in fetal and neonatal myocardium," *Circulation Research*, vol. 94, no. 4, pp. 505–513, 2004.

[33] O. Cazorla, Y. Wu, T. C. Irving, and H. Granzier, "Titin-based modulation of calcium sensitivity of active tension in mouse skinned cardiac myocytes," *Circulation Research*, vol. 88, no. 10, pp. 1028–1035, 2001.

[34] G. Fulgenzi, L. Graciotti, A. L. Granata, et al., "Location of the binding site of the mannose-specific lectin comitin on F-actin," *Journal of Molecular Biology*, vol. 284, no. 5, pp. 1255–1263, 1998.

[35] E. Friederich, K. Vancompernolle, C. Huet, et al., "An actin-binding site containing a conserved motif of charged amino acid residues is essential for the morphogenic effect of villin," *Cell*, vol. 70, no. 1, pp. 81–92, 1992.

[36] M. Pfuhl, S. J. Winder, and A. Pastore, "Nebulin, a helical actin binding protein," *EMBO Journal*, vol. 13, no. 8, pp. 1782–1789, 1994.

[37] M. Greaser, "Identification of new repeating motifs in titin," *Proteins*, vol. 43, no. 2, pp. 145–149, 2001.

[38] K. Ma, L.-S. Kan, and K. Wang, "Polyproline II helix is a key structural motif of the elastic PEVK segment of titin," *Biochemistry*, vol. 40, no. 12, pp. 3427–3438, 2001.

[39] M. P. Williamson, "The structure and function of proline-rich regions in proteins," *Biochemical Journal*, vol. 297, no. 2, pp. 249–260, 1994.

[40] D. Labeit, K. Watanabe, C. Witt, et al., "Calcium-dependent molecular spring elements in the giant protein titin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 23, pp. 13716–13721, 2003.

[41] M. S. Z. Kellermayer, S. B. Smith, C. Bustamante, and H. L. Granzier, "Complete unfolding of the titin molecule under external force," *Journal of Structural Biology*, vol. 122, no. 1-2, pp. 197–205, 1998.

[42] K. Watanabe, C. Muhle-Goll, M. S. Z. Kellermayer, S. Labeit, and H. Granzier, "Different molecular mechanics displayed by titin's constitutively and differentially expressed tandem Ig segments," *Journal of Structural Biology*, vol. 137, no. 1-2, pp. 248–258, 2002.

[43] K. Watanabe, P. Nair, D. Labeit, et al., "Molecular mechanics of cardiac titin's PEVK and N2B spring elements," *The Journal of Biological Chemistry*, vol. 277, no. 13, pp. 11549–11558, 2002.

[44] H. Granzier, M. Kellermayer, M. Helmes, and K. Trombitás, "Titin elasticity and mechanism of passive force development in rat cardiac myocytes probed by thin-filament extraction," *Biophysical Journal*, vol. 73, no. 4, pp. 2043–2053, 1997.

[45] E. K. Rodriguez, W. C. Hunter, M. J. Royce, M. K. Leppo, A. S. Douglas, and H. F. Weisman, "A method to reconstruct myocardial sarcomere lengths and orientations at transmural sites in beating canine hearts," *American Journal of Physiology*, vol. 263, no. 1, part 2, pp. H293–H306, 1992.

[46] A. F. Grimm, H. L. Lin, and B. R. Grimm, "Left ventricular free wall and intraventricular pressure-sarcomere length distributions," *American Journal of Physiology*, vol. 239, no. 1, pp. H101–H107, 1980.

[47] M. Helmes, K. Trombitás, and H. Granzier, "Titin develops restoring force in rat cardiac myocytes," *Circulation Research*, vol. 79, no. 3, pp. 619–626, 1996.

[48] N. Preetha, W. Yiming, M. Helmes, F. Norio, L. Siegfried, and H. Granzier, "Restoring force development by titin/connectin and assessment of Ig domain unfolding," *Journal of Muscle Research and Cell Motility*, vol. 26, no. 6–8, pp. 307–317, 2005.

[49] S. P. Bell, L. Nyland, M. D. Tischler, M. McNabb, H. Granzier, and M. M. LeWinter, "Alterations in the determinants of diastolic suction during pacing tachycardia," *Circulation Research*, vol. 87, no. 3, pp. 235–240, 2000.

[50] R. Horrowits and R. J. Podolsky, "The positional stability of thick filaments in activated skeletal muscle depends on sarcomere length: evidence for the role of titin filaments," *Journal of Cell Biology*, vol. 105, no. 5, pp. 2217–2223, 1987.