The increase in non-cross-bridge forces after stretch of activated striated muscle is related to titin isoforms

Anabelle S. Cornachione,1* Felipe Leite,2* Maria Angela Bagni,3 and Dilson E. Rassier2

1Department of Pathology, Universidade de São Paulo, São Paulo, Brazil; 2Department of Kinesiology and Physical Education, McGill McGill University, Montreal, Quebec, Canada; and 3Dipartimento di Medicina Sperimentale e Clinica, Scienze Fisiologiche, University of Florence, Florence, Italy

Submitted 2 June 2015; accepted in final form 18 September 2015

Cornachione AS, Leite F, Bagni MA, Rassier DE. The increase in non-cross-bridge forces after stretch of activated striated muscle is related to titin isoforms. Am J Physiol Cell Physiol 310: C19–C26, 2016. First published September 24, 2015; doi:10.1152/ajpcell.00156.2015.—Skeletal muscles present a non-cross-bridge increase in sarcomere stiffness and tension on Ca2⁺ activation, referred to as static stiffness and static tension, respectively. It has been hypothesized that this increase in tension is caused by Ca2⁺-dependent changes in the properties of titin molecules. To verify this hypothesis, we investigated the static tension in muscles containing different titin isoforms. Permeabilized myofibrils were isolated from the psoas, soleus, and heart ventricle from the rabbit, and tested in pCa 9.0 and pCa 4.5, before and after extraction of troponin C, thin filaments, and treatment with the actomyosin inhibitor blebbistatin. The myofibrils were tested with stretches of different amplitudes in sarcomere lengths varying between 1.93 and 3.37 μm for the psoas, 2.68 and 4.21 μm for the soleus, and 1.51 and 2.86 μm for the ventricle. Using gel electrophoresis, we confirmed that the three muscles tested have different titin isoforms. The static tension was present in psoas and soleus myofibrils, but not in ventricle myofibrils, and higher in psoas myofibrils than in soleus myofibrils. These results suggest that the increase in the static tension is directly associated with Ca2⁺-dependent change in titin properties and not associated with changes in titin-actin interactions.

There is evidence showing that the residual force enhancement is caused by length nonuniformities among half-sarcomeres and a Ca2⁺-induced stiffening of titin (36, 51, 55). If the residual force enhancement is caused by stiffening of titin, it would be directly associated with the static tension.

Surprisingly, the static tension observed in skeletal muscles has not been investigated in cardiac muscles in which titin has several physiological roles. Changes in titin stiffness are associated with several cardiomyopathies, and modulations of cardiac stiffness through titin have important implications. Titin affects the sarcomere length (SL)-dependent Ca2⁺ sensitivity of force production by the heart (8, 14), an important feature of the Frank-Starling mechanism of the heart. Titin degradation decreases Ca2⁺-activated maximal force of cardiac myocyte (7) and rat cardiac trabeculae (15) at SLs longer than the slack length. Finally, titin degradation depresses the SL-dependent increase in Ca2⁺ sensitivity of rat cardiac myocytes (7). Different muscles express different isoforms of titin (17, 48), with large variations observed in the PEVK region of the molecule. Slow skeletal muscles express a long titin isoform N2A, and fast skeletal muscles express a shorter N2A isoform, whereas cardiac muscles express the isoforms N2B and N2BA. As a result, the mechanical properties and passive forces exerted by these muscles are distinctively different, with implications for their physiological roles and length ranges (16, 48). If the passive forces and static tension are regulated by a Ca2⁺-dependent change in titin properties, it would be expected that these muscles would present different responses to stretch under activated and nonactivated conditions.

In this study, we evaluated the relation between the non-cross-bridge, static tension and titin using myofibrils isolated from the psoas, soleus, and heart ventricles, which contain different titin isoforms.1 We used a new system developed in our laboratory that allows testing the myofibrils with a high spatial and time resolution (27), necessary for detecting small changes in passive forces when they are present. We observed that the changes in the static tension correlates with titin isoforms, and that cardiac muscles do not show an increase in non-cross-bridge force, suggesting that Ca2⁺ does not regulate titin forces in cardiac muscles. The results strengthen a mechanism in which Ca2⁺ binding to titin regulates force production in skeletal muscles, but not in cardiac muscles. Such mechanism may have important physiological roles for humans. During eccentric contractions commonly used in daily activities, an increase in titin stiffness may represent an important...
mechanism for the prevention of stretch-induced injury in skeletal muscles.

METHODS

Myofibril preparation. Small muscle bundles of the rabbit psoas, soleus, and heart ventricle muscles were dissected, tied to wood sticks, and chemically permeabilized following standard procedures used in our laboratory (10, 50, 56). Muscles were incubated in rigor solution (pH = 7.0) for ~4 h, after which they were transferred to a rigor-glycerol (50:50) solution for 15 h. The samples were subsequently placed in a fresh rigor-glycerol (50:50) solution with the addition of a cocktail of protease inhibitors (Roche Diagnostics) and stored in a freezer (~20°C) for at least 7 days. The protocol was approved by the McGill University Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care.

On the day of the experiments, small pieces of the samples were homogenized following standard procedures (10, 50, 56), which resulted in a solution containing isolated myofibrils. The myofibrils were transferred to the experimental setup, which contained a system for detection of atomic force cantilever (AFC) displacements (27). With this system, a laser is shined on and reflects from the AFC, which acts as a force transducer. When an attached myofibril is shortened due to activation, it causes AFC deflection, which is detected and recorded using a newly developed optical system with time resolution in the order of milliseconds (27). Since the stiffness of the atomic force cantilevers (K) was known, and we measured the amount of cantilever displacement (Δd), the force (F) could be calculated as F = K·Δd.

Isolated myofibrils were chosen for mechanical testing, based on striation pattern and number of sarcomeres in series (between 8 and 30). Using micromanipulators, the myofibrils were attached between the AFC and the micro-needle, which was used for length changes during the experiments induced with a computer-controlled piezo motor. A multichannel fluidic system connected to a double-barreled pipette (50, 56) was used for fast activation and deactivation of the myofibrils. During the experiments, the position of the double-barreled pipette was rapidly switching to change the solutions surrounding the myofibrils (pCa 4.5 and pCa 9.0, see below).

Myofibril solutions. The rigor solution (pH 7.0) used to store the myofibrils was composed of (in mM) 50 Tris, 100 NaCl, 2 KCl, 2 MgCl₂, and 10 EGTA. The relaxing solution used for muscle dissection (pH 7.0) was composed of (in mM) 100 KCl, 2 EGTA, 20 imidazole, 4 ATP, and 7 MgCl₂. The experimental solutions used during the experiments (pH 7.0) was composed of (in mM) 20 imidazole, 14.5 creatine phosphate, 7 EGTA, 4 MgATP, 1 free Mg²⁺, free Ca²⁺ ranging from 1 mM (pCa²⁺ 9.0) to 32 μM (pCa²⁺ 4.5), and KCl to adjust the ionic strength to 180 mM. The final concentrations of each metal-ligand complex were calculated using a computer program based on previous studies (13, 37).

The active form (+/−) of blebbistatin (Sigma-Aldrich) was dissolved in dimethylformamide to reach a concentration of 20 mM and was stored at ~20°C before use. In the day of the experiment, 1 μl of blebbistatin was diluted in 1 ml of activating (pCa²⁺ 4.5) or relaxing (pCa²⁺ 9.0) solutions to reach a final concentration of 20 μM. Previous study performed in our laboratory with permeabilized skeletal muscle fibers tested both the active (+/−) and inactive (+/+) isomers of blebbistatin and showed that the active isomer inhibits force production while the inactive isomer does not affect significantly the contractile properties of the rabbit psoas (11). Throughout the experiments, the microscope room was maintained dark, and a red filter (650 nm) was placed on the light source of the microscope, because blebbistatin loses its effectiveness when exposed to wavelengths between 365 and 490 nm (57). All experiments were performed at 15°C.

Measurements of active and passive myofibril forces. Once the myofibrils were attached between the AFC and the micro-needle, they were adjusted to average SLs of 2.4 μm for the psoas, 2.6 μm for the soleus muscles, and 1.8 μm for the ventricle. Under high magnification, the contrast between the dark bands of myosin (A bands) and the light bands of actin (I bands) provided a dark-light intensity pattern, representing the striation pattern produced by the sarcomeres, which allowed measurements of SL during the experiments (43, 50). The SL was measured with a video camera simultaneously with a linear photodiode array (SK4096ZPD, Schafter + Kirchhoff) connected to the right side port of the microscope. The myofibrils were activated (pCa 4.5) to produce a fixed-end isometric contraction for ~15–20 s. After 3-min rest, the myofibril was activated again at 2.8, 3.0, or 2.0 μm (psoas, soleus, and ventricle, respectively). Finally, the myofibrils were set at SLs of 2.6, 2.8, and 1.8 μm (psoas, soleus, and ventricle, respectively), activated, and stretched (amplitude 0.2 μm) after maximal force was stabilized during activation. Following, a similar stretch was repeated at pCa 9.0, similar to the stretch performed at activation conditions.

Finally, the myofibrils were tested for passive force development. They were stretched passively (pCa 9.0) in consecutive steps of 0.2 μm per sarcomere at a speed of 10 μm/s. The stretch was initiated in SL of 2.93–1.95 μm (psoas muscle), 2.68–2.70 μm (soleus muscle), or 1.51–1.55 μm (ventricle muscle) (see Supplemental Movie S1 in supplementary material for one typical experiment; supplemental material for this article is available online at the journal website).

After the first set of mechanical testing was completed, the fibers were treated for extraction of troponin C (TnC), according to a standard protocol (11, 18, 38, 46). Briefly, fibers were set at average SLs of 1.8 μm (ventricle muscle) or 2.8 μm (soleus and psoas muscle) and incubated for 90 min in rigor-EDTA solution (pH 8.5) composed of 5 mM EDTA and rigor solution at 8°C. The timing for the extraction procedure differed slightly from previous studies, but it followed procedures used previously in our laboratory (11). The force decreased to ~15–20% of the initial isometric force and did not decrease further after 90 min. The myofibrils were then treated with FX45 gelsolin fragment for extraction of actin filaments. Briefly, the myofibrils were bathed in solution (pH 7.0) containing 20 mM 2,3-butanedione monoxime, activation solution (pCa 4.5), and 2 g gelsolin (Sigma-Aldrich) at 8°C for 20 min and subsequently washed with relaxing solution for 5 min. Finally, the myofibrils were treated for 3 h with 50 μM myosin-mutant actin (50% by incubating them during 30 min in relaxing solution containing blebbistatin. After these treatments, the entire mechanical protocol, as explained above, was repeated with all myofibrils.

Throughout the experiments, homogeneity of SL was accessed to detect potential damage in the myofibrils. There was a nonuniformity among SLs that was formed during myofibril activation, but almost none during passive stretches, either before or after thin-filament removal, in pCa 4.5 and pCa 9.0. When nonuniformity of SLs formed during activation did not recover into a regular striation pattern before the next activation, the experiment was stopped, and the myofibril was discarded from further analysis.

Titin isoforms. Samples from the ventricle, soleus, and psoas muscles collected from the same animals used in the mechanical experiments were evaluated for titin isoforms using SDS-agarose electrophoresis, as previously described (17, 48). Briefly, muscle tissue samples were pulverized using a pestle and mortar. The yield was solubilized (40:1 vol/wt) in equal volume of urea (8 M urea, 2 M thiourea, 3% SDS, 75 mM DTT, 50 mM Tris-HCl, 0.03% bromophenol blue, pH 6.8) and glycerol (50% glycerol and complete protease inhibitors cocktail, Roche) buffer at 60°C. Agarose gels (1%) were run at 15 mA per gel for 3 h and 20 min at 40°C (Hoefer SE 600, Amersham Pharmacia Biotech, Piscataway, NJ). The gels were stained using Coomassie brilliant blue and then scanned.

Data analysis. The static tension was measured as described previously (4, 11). Briefly, three force records were taken for each measure: 1) the response of the myofibrils to stretches in pCa 4.5; 2) the response of the myofibrils to stretches in pCa 9.0; and 3) the
response of myofibrils to isometric contractions. The isometric response at the corresponding final length and the stretch response in pCa 9.0 were subtracted from the response to the stretch in pCa 4.5, to provide a value for the static tension after the stretch. During isometric contractions developed at the plateau of the force-SL relation, the rate of force development ($K_{act}$) was calculated by fitting a double-exponential equation ($F = \{a \times \left[1 - \exp(-K_{act} \times t) - \exp(-l \times t)\right] + b\}$) to the data, and the rate of relaxation ($K_{rel}$) was calculated by fitting a single-exponential equation ($F = \{a \times \exp(-K_{rel} \times (t - c)) + b\}$) to the data (44, 56). For both equations, $F$ is force; $t$ is time; $K_{act}$ is the rate constant for force development; $l$ is the second rate constant; $K_{rel}$ is the rate constant for force decrease during relaxation; and $a$, $b$, and $c$ are constants.

The isometric forces and the static tension values in the three groups of myofibrils (psoas, soleus, ventricles) were compared using a one-way ANOVA. The forces produced by each of the muscle types during the consecutive stretches before and after blebbistatin treatment were compared with a two-way ANOVA (condition, SL) for repeated measures. When significant changes were observed, post hoc analyses were performed with Newman-Keuls tests ($P < 0.05$). All results are shown as means ± SE.

RESULTS

Figure 1 shows an isometric contraction produced by a myofibril isolated from the soleus activated at a SL of 2.4 μm, before and after depletion of titin and thin filaments, and treatment with blebbistatin. Before treatment, the force developed rapidly on myofibril activation, reaching a plateau and stabilizing for as long as activation persisted. Note that the signal-to-noise ratio in our myofibril system is very high, which allows us to evaluate precisely small changes in myofibril force. After treatment for depletion of TnC and thin filaments and treatment with blebbistatin, there was virtually no force produced by the myofibril when it was exposed to pCa 4.5. There were some detectable changes in the baseline signal toward the end of the activation period, but not sufficient to produce significant levels of force. Mean (±SE) values for the isometric forces produced by the three preparations used in this study before and after blebbistatin are shown in Table 1 and confirm the results presented in Fig. 1.

The force produced during activation is well within the range observed in other studies performed at similar temperatures (24, 45, 49, 50, 56, 65). The force produced by psoas myofibrils was higher than the force produced by soleus, and both were higher than that produced by ventricle myofibrils. Furthermore, the rates of activation and relaxation observed in myofibrils are similar to those observed in other studies (44, 45, 56, 58, 59, 64, 65) and show that the psoas myofibrils are faster than the soleus and ventricles myofibrils, as expected and reported previously. The rates of activation and relaxation are also characteristic of fast and slow muscle fibers, as reported in many studies in the past (for a review see Ref. 47).

Figure 2 shows force traces recorded during experiments in which myofibrils isolated from the psoas, soleus, and ventricle were stretched at pCa 4.5 after maximal force was reached. It also shows isometric contractions produced at the corresponding final myofibril lengths, and traces recorded when myofibrils were stretched at pCa 9.0 and pCa 4.5 after blebbistatin treatment. Before blebbistatin treatment, the myofibrils presented a typical behavior observed when skeletal and cardiac muscles are stretched during activation; a fast increase in force was observed during stretches. In the soleus and psoas, the increase in force was followed by an exponential decay until it stabilized at levels that were higher than those produced previous to the stretch, and higher than the force produced during isometric contractions at corresponding lengths. This increase in force is referred to as residual force enhancement (51). In the ventricle myofibrils, the force decayed abruptly after the stretch, to then develop to a new steady state. Importantly, the ventricle myofibrils did not produce a residual force enhancement. The force produced when the

### Table 1. Contractile properties of myofibrils measured during mechanical experiments

<table>
<thead>
<tr>
<th></th>
<th>Ventricle</th>
<th>Soleus</th>
<th>Psoas</th>
</tr>
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<tbody>
<tr>
<td>Isometric force, nN/μm²</td>
<td>70 ± 6.1</td>
<td>90 ± 7.5</td>
<td>130 ± 14.4</td>
</tr>
<tr>
<td>$K_{act}$, s⁻¹</td>
<td>1.1 ± 0.05</td>
<td>2 ± 0.2</td>
<td>7 ± 1.7</td>
</tr>
<tr>
<td>$K_{rel}$, s⁻¹</td>
<td>1.8 ± 0.4</td>
<td>2 ± 0.3</td>
<td>18 ± 5.6</td>
</tr>
<tr>
<td>Residual force enhancement, %</td>
<td>0.1 ± 0.03</td>
<td>5.0 ± 0.5*</td>
<td>10.4 ± 1.4*</td>
</tr>
<tr>
<td>Static tension, %</td>
<td>0.04 ± 0.02</td>
<td>8.1 ± 0.3*</td>
<td>13.8 ± 1.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Isometric forces, $K_{act}$ (rate of force development) and $K_{rel}$ (rate of relaxation) of myofibrils measured at sarcomere lengths of 1.8 μm (ventricle), 2.4 μm (psoas), and 2.6 μm (soleus) are shown. Residual force enhancement and static tension were measured at final sarcomere lengths of 2.0 μm (ventricle), 2.8 μm (psoas), and 3.0 μm (soleus). *Significant increase in force after stretch compared with isometric conditions ($P < 0.05$).
myofibrils were stretched in pCa 9.0 follows the same pattern as that observed previously (56).

Figure 2 also shows that the force was increased in response to the stretch performed in pCa 4.5 after blebbistatin treatment, as it has been shown before (11, 37). The increase in force during the stretches produced at pCa 4.5 compared with pCa 9.0, but not in the ventricle myofibrils. The increase is more substantial in the psoas myofibrils. The results shown in Fig. 3 were confirmed when all myofibrils tested in this study were grouped to construct a passive force-SL relation (Fig. 4). Note that the range of SLs is different for the muscles, due to their different titin isoforms (Fig. 4, top) (48).

There was an upward shift in the curves produced by the psoas and soleus myofibrils in pCa 4.5 compared with pCa 9.0, but not in the curve produced by the ventricle myofibrils. The increase in force was higher (8 nN/µm²) at a SL of 3.37 µm in the psoas myofibrils, and 5 nN/µm² at a SL of 3.86 µm for the soleus myofibrils, a range that is similar to previous studies investigating the static tension (4, 41).

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Figure 2 also shows that the force was increased in response to the stretch performed in pCa 4.5 after blebbistatin treatment, as it has been shown before (11, 37). The relative increase in force after stretch is different in the three muscles: it increased beyond the force developed during stretches produced at pCa 9.0 in the psoas and soleus muscles, but not in the ventricle muscle. The increase observed in the skeletal muscles is characteristic of the static tension previously observed in skeletal muscle fibers (3, 4, 11, 42). The results were confirmed statistically, showing that the static tension is dependent on muscle type. It increased the force by 4 ± 0.2 nN/µm² in the ventricle myofibrils, 8.1 ± 0.3 nN/µm² in the soleus myofibrils, and 13.8 ± 1.2 nN/µm² in the psoas myofibrils; all of these values are significantly different (P < 0.05) (see also Table 1).

Figure 3 shows results of experiments in which myofibrils were stretched in discrete steps of 0.2 µm along a large range of SLs. The stretches were performed in pCa 9.0 and pCa 4.5 after treatment with rigor-EDTA, gelosin, and blebbistatin. Note that the peak forces during the stretches and the passive forces after stretch were increased in the presence of Ca²⁺ in the psoas and soleus, but not in the ventricle myofibrils. The increase is more substantial in the psoas myofibrils. The results shown in Fig. 3 were confirmed when all myofibrils tested in this study were grouped to construct a passive force-SL relation (Fig. 4). Note that the range of SLs is different for the muscles, due to their different titin isoforms (Fig. 4, top) (48).

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Fig. 2. Four superimposed contractions produced by myofibrils isolated from the psoas (A), soleus (B), and ventricle (C) muscles. Black traces: isometric contractions and passive stretches performed in pCa 4.5 and 9.0, respectively. Red traces: stretches produced after full development in pCa 4.5. Blue traces: stretches applied in pCa 4.5 after myofibrils were treated with rigor-EDTA, gelosin, and BB. Note the high signal-to-noise ratio in the force traces, which allows the detection of small force differences during the experiments. During the stretches in pCa 4.5, the force increased substantially, and after the stretch the force decreased to attain a steady state that was higher than the force obtained during isometric contractions at a similar length. During the stretches in pCa 4.5 after the myofibrils were treated with rigor-EDTA, gelosin, and BB, the force was higher than that produced during stretches in pCa 9.0 for the psoas and soleus muscles, but not for the ventricle muscle.

Fig. 3. Consecutive stretches performed with myofibrils isolated from the psoas (A), soleus (B), and ventricle (C) muscles in pCa 4.5 after treatment with rigor-EDTA, gelosin, and BB, and pCa 9.0. An increase in Ca²⁺ concentration caused an increase in forces produced by the myofibrils during and after stretches of psoas and soleus myofibrils, but not in ventricle myofibrils.
titin isoforms to adjust sarcomere stiffness. N2B isoform is expressed with a springlike segment called N2B, whereas N2BA presents two springlike domains, N2B and N2A, N2A is also found in skeletal muscles: slow twitch skeletal muscle expresses a longer N2A isoform, whereas fast twitch muscle expresses a shorter N2A titin (40). The number of springlike elements and its size defines the extensibility of titin. The shorter isoforms of titin start to produce passive forces at shorter SLs; in fact the passive tension is strongly determined by the number and length of spring elements in titin.

Although it is accepted that titin is the main factor responsible for passive forces in striated muscles, its regulation by Ca\(^{2+}\), and consequently muscle activation, is still a matter of debate. It has been suggested that titin is responsible for the static tension by changing its mechanical properties on muscle activation (1, 3, 4, 11, 54). Such hypothesis is based on studies showing Ca\(^{2+}\) may directly regulate titin function. Early studies have suggested that Ca\(^{2+}\) ions bind to titin filaments (61, 62), and that the binding sites are restricted to the area spanning from the N2A segment to the M-line. This PEVK segment has a strong negative net charge at physiological pH (pI of 5.0) (23), and thus it is amenable for positively charged ions (63). Subsequently, Labeit et al. (26) observed that Ca\(^{2+}\) binding to the PEVK region of isolated titin molecules caused a decrease in its persistence length, which is associated with an increase in stiffness, and potentially an increase in passive force production. After testing several constructs, the authors also showed that the minimal titin fragment that responded to Ca\(^{2+}\) ions contained a central E-rich domain with glutamates flanked by PEVK repeats. Since different skeletal muscle titin isoforms contain a different number of PEVK repeats and E-rich motifs (5), their result is consistent with the idea that Ca\(^{2+}\) affects the conformation of the PEVK segment. Since the glutamate-rich (E-rich) motif is essential for titin response to Ca\(^{2+}\), and it is present in the N2BA cardiac isoform and in skeletal muscle isoforms, this finding has general implications for muscle regulation.

Tellingly, we observed that the static stiffness is correlated with different isoforms of titin in myofibrils isolated from the soleus, psoas, and ventricle muscles. These results agree with recent indirect observations by Nocella et al. (42), who showed that that the ratio between the static tension and the stretch amplitude (static stiffness) is approximately five times greater in extensor digitorum longus than in soleus intact fibers, which contain different titin isoforms. Furthermore, the rate of development of the static stiffness on stimulation is slightly faster in extensor digitorum longus than in soleus fibers, in agreement with the different time course of the intracellular Ca\(^{2+}\) transients in these muscles (6). These results were confirmed in the present study, since the static tension was higher in psoas muscle myofibrils than in soleus muscle myofibrils. The finding that cardiac myofibrils do not show a static tension is novel; since cardiac muscles are not stretched while contracting in regular activities, a Ca\(^{2+}\)-dependent increase in titin stiffness may not play any physiological role in cardiac contractions.

Although investigators have concentrated mostly on the PEVK domain of titin, there is one study using molecular dynamics simulation, suggesting that binding of Ca\(^{2+}\) ions could regulate the Ig domains of titin molecules, also involved in passive forces (34). However, an experimental study performed by Watanabe et al. (67) using atomic force microscopy...
investigated the potential effect of Ca$^{2+}$ on differentially spliced (I65-70) and constitutive (I91-98) regions from Ig domains of titin. The authors observed that the average domain unfolding force in I91-98 and the persistence length of the unfolded I91-98 chain were not different when experiments were conducted in pCa 9.0 and pCa 3.0, suggesting that Ca$^{2+}$ does not change the properties of titin through an increased stiffness of Ig domains.

Alternative mechanism. Another mechanism by which Ca$^{2+}$ could increase the titin role on force production is by facilitating the binding of titin with actin, increasing the overall sarcomere stiffness. Given the proximity between titin and actin filament in the I band of the sarcomeres (21, 22, 31, 66) and the malleability of the PEVK domain of titin, that may transition between different conformational states (35) and bind F-actin (25, 33, 39, 68), this hypothesis is tempting. In fact, it has been shown that the binding of the PEVK domain of titin to actin can be modulated by S100A1, a member of the S100 family of EF-hand Ca$^{2+}$ binding proteins (68), which is present in high concentrations in striated muscles (20). A study investigating in vitro motility assays for myosin-driven actin motility showed that titin inhibited significantly the sliding of the actin filaments in the presence of Ca$^{2+}$ (21), and this inhibitory effect was enhanced with increased concentrations of Ca$^{2+}$. However, most evidence suggests that Ca$^{2+}$ has an opposite effect on titin-actin binding. Kulke et al. (25) found that the PEVK-induced inhibition of actin filament sliding over myosin was reversed with a high Ca$^{2+}$ concentration. Yamasaki et al. (68) showed that S100A1-PEVK binding alleviates the PEVK-based inhibition of F-actin motility, inhibiting PEVK-actin interaction and providing the sarcomere with a mechanism to free the thin filament from titin before contraction. The findings suggesting that binding of titin or PEVK fragments to actin are inhibited by Ca$^{2+}$ were confirmed by Stuyvers et al. (60) in a cardiac muscle model. The authors demonstrated that the stiffness of the rat cardiac trabeculae, based on titin-actin interactions, increased when Ca$^{2+}$ levels decayed during muscle relaxation, i.e., when Ca$^{2+}$ levels are decreasing, the titin-actin based stiffness is increasing. Our results are in line with these studies, as when skeletal muscle myofibrils were treated for TnC removal, thin-filament removal, and blebbistatin, they still presented a static tension. Thus our results suggest that the increase in forces produced during stretch in the presence of Ca$^{2+}$ is caused entirely by titin, and not by titin-actin interactions.

Residual force enhancement. The persisting increase in force commonly observed after skeletal muscles are stretched was confirmed in psoas and soleus myofibrils (for reviews see Refs. 12, 51, 52) and was absent in ventricle myofibrils. As far as we know, this is the first study to investigate the possibility of such force increase in cardiac muscles. Since cardiac muscles do not operate with imposed stretches and shortenings during activation and isometric contractions, this result is not surprising; a Ca$^{2+}$-induced increase in titin stiffness is not needed for cardiac muscles to operate in physiological conditions. Most interestingly, the absence of residual force enhancement was observed in parallel with an absence of the static tension, conferring further support to the hypothesis that titin activation by Ca$^{2+}$ is responsible for both phenomena, as suggested by previous researchers (11, 41, 51, 52, 54).

Regulation of the passive force-SL relation. The relations between passive force and SL observed in our experiments, and the range in which SL were tested, are similar to previous studies using isolated myofibrils and fibers from striated muscles (19, 40, 48). Within this range, we observed differences in passive forces between the ventricle, psoas, and soleus myofibrils before increasing the Ca$^{2+}$ concentration (Fig. 4). The force magnitudes are within the range reported previously (40, 48), which provides confidence that the myofibrils were healthy. The difference in the passive force-SL relations is commonly correlated to titin isoforms (25, 29, 30, 32). In skeletal muscle myofibrils, the passive force starts to develop in longer lengths than in cardiac myofibrils. Furthermore, a lower passive force is observed in soleus myofibrils compared with psoas myofibrils. This result strengthens the notion that striated myofibrils can achieve lower or higher stiffness by expressing longer or shorter titin isoforms, respectively.

Most importantly, we observed a small but significant upward shift in the passive force-SL relation in skeletal muscle myofibrils treated with rigor-EDTA, gelsolin, and blebbistatin in pCa 4.5, but not in cardiac myofibrils. A similar result has been observed with permeabilized fibers in studies performed in our laboratory (11) and others (26) after removal of thin filament. Tellingly, the magnitudes of increase in passive forces are very similar across these studies. The passive force was increased both during and after the stretch, which suggests that the effects of Ca$^{2+}$ on titin are observed when the molecular springs are unveiled and remain, for a period of time, associated with the static tension. Since the shifts in the passive force curves also follow the muscle type, the Ca$^{2+}$ dependence of the entire passive force-SL relation seems to be regulated by titin.

The increases in the passive force with high Ca$^{2+}$ concentration observed in this study are in the range presented by most studies available in the literature, using intact amphibian fibers (53), permeabilized mammalian fibers (11, 26), and intact mammalian fibers (41). In one particular study, we investigated the static tension and changes in non-cross-bridge forces in permeabilized fibers that were treated sequentially for TnC depletion, cross-bridge inhibition, and, finally, thin-filament depletion (11). The levels of static force were not changed over all treatments and were similar to the levels that we detected in the present study. The results are strengthened by experiments conducted with amphibian fibers using a wide range of mechanical and chemical interventions to block cross-bridge formation that also reported the presence of the static tension stretch, in levels similar to ours (2, 4, 41, 42).

The conclusion that the static tension and the changes in the passive force-SL relation are associated directly with stiffening of titin and not actin-titin interactions is in contradiction with a study performed with myofibrils. Leonard and Herzog (28) have published results in which myofibrils stretched to up to 6.0 μm (where there is no filament overlap, but actin filaments are conceptually intact) produce increases in force between ~300 and ~350% in activated myofibrils compared with nonactivated myofibrils. In fact, their study reports forces produced by myofibrils stretched to a SL of 6 μm that is approximately seven times higher than the maximal active force produced at 2.0–2.2 μm, a regions corresponding roughly to the plateau of the force-length relation. Increases of such magnitudes are not only substantially different from what...
has been reported by others, but arguably incompatible with the current knowledge on the mechanisms of titin function and muscle contraction. One difference between our studies is the range of SLs investigated. When we tried to stretch myofibrils to SLs longer than ~5 μm in the presence of Ca²⁺, they would be irreparably damaged; if we tried to activate myofibrils on the plateau region of the force-SL relation following activation/stretch at such long SLs, the force would have dropped to 10–20% of maximal force, and the striation appearance of the myofibrils would have been lost.

Summary. In conclusion, our study shows that the presence of a static tension in striated muscles is associated with titin isoforms. Cardiac myofibrils do not show static tension or the residual force enhancement after stretch. Since the two phenomena are directly associated, static tension produced by titin seems to be directly responsible for the residual force enhancement, observed for many years, but still not well understood.

GRANTS
This study was supported by the Natural Sciences and Engineering Research Council of Canada.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


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