**Length Dependence of Tension Generation in Rat Skinned Cardiac Muscle**

**Role of Titin in the Frank-Starling Mechanism of the Heart**

Norio Fukuda, PhD; Daisuke Sasaki, MS; Shin’ichi Ishiwata, PhD; Satoshi Kurihara, MD, PhD

**Background**—At the basis of the Frank-Starling mechanism is the intrinsic ability of cardiac muscle to produce active tension in response to stretch. Titin, a giant filamentous molecule involved in passive tension development, is intimately associated with the thick filament in the sarcomere. Titin may therefore contribute to active tension development by modulating the thick filament structure when the muscle is elongated.

**Methods and Results**—Rat skinned right ventricular trabeculae were used. Passive tension at a sarcomere length (SL) of 2.0 to 2.4 μm was decreased after treatment of the preparation with trypsin (0.25 μg/mL) for 13 minutes in the relaxed state at 20°C. This mild trypsin treatment degraded titin without affecting other major contractile proteins. The sarcomere structure was little affected by brief contractions in the trypsin-treated preparations. When SL was adjusted to the slack SL (1.9 μm), active tension was unaffected by trypsin under partial (pCa 5.55) and maximal (pCa 4.8) activation. At longer SLs, however, active tension was significantly (P<0.01) decreased after trypsin treatment at either pCa. The increase in active tension on reduction of interfilament lattice spacing, produced by dextran T-500 (molecular weight ≈500 000), was not influenced by trypsin (SL 1.9 μm). In trypsin-treated preparations, the increase in active tension as a function of muscle diameter was nearly the same for lengthening and osmotic compression at the slack SL.

**Conclusions**—The length-dependent activation in cardiac muscle, an underlying mechanism of the Frank-Starling law of the heart, is at the myofilament level, predominantly modulated by titin and interfilament lattice spacing changes.

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**Key Words:** myocardial contraction ■ myocardium ■ contractility

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Isometric tension in isolated preparations of mammalian ventricular muscle increases dramatically when the muscle is stretched over the range of sarcomere length (SL: from ≈1.8 to ≈2.3 μm), which is observed in the intact heart.¹⁻³ This region of the length-tension relationship (the ascending limb) provides a useful model of the increase in myocardial performance that occurs as a result of increased diastolic filling of the heart.¹⁻³ Because length-dependent activation is seen in skinned cardiac muscle preparations from which the membrane system has been chemically removed, the steep variation of tension generation is due, at least in part, to variations in the activation of the contractile system with length.¹⁻³ The basis of this relationship in skinned muscle preparations is still not fully understood.

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Recent articles,⁴⁻⁶ however, indicate that SL dependence arises largely from a reduction of interfilament lattice spacing rather than from an increase in SL itself. A recent x-ray study with rat skinned ventricular trabeculae confirmed that the lattice spacing decreases as a function of SL, thereby maintaining a nearly constant lattice volume (SL from ≈1.9 to ≈2.4 μm).⁷ A possible consequence of the decreased lattice spacing is an increase in the probability of myosin attachment to the thin filament.⁶ We also found that SL dependence occurs during activation induced by lowering the ATP concentration in the absence of Ca²⁺.⁸ This finding does not support the proposal that the binding of Ca²⁺ to troponin C is the central player in SL-dependent activation⁹ but suggests that structural changes within myofilaments during stretch are the basis of SL-dependent activation.

The giant muscle protein titin (also known as connectin), which spans from the Z-line to the M-line, is responsible for the elasticity of relaxed striated muscle, as well as acting as the molecular scaffold for thick filament formation.¹⁰ The extensible I-band region of cardiac titin has a complex sequence with 3 distinct extensible segments: (1) a PEVK segment, (2) serially linked immunoglobulin-like domains that make up a proximal and a distal immunoglobulin segment, and (3) a unique sequence that is part of the N2B (or...
N2BA) element. Rat ventricular muscle predominately expresses N2B, having higher stiffness than either bovine or porcine ventricular muscle. In the A-band, titin is composed primarily of super repeats of immunoglobulin- and fibronectin-like domains that interact with myosin at the rod portion of this molecule and C-protein. Therefore, it is possible that the extension of titin imposes passive strain on the thick filament and that this strain modifies the actomyosin interaction by changing the position of the myosin heads.

In the current study, we studied the effect of degradation of the elastic segment of titin, by limited tryptic proteolysis, on the length-dependent activation in rat skinned ventricular trabeculae. A preliminary report has been published in abstract form.

Methods

Experimental Procedure

The heart was removed from male Wistar rats (250 to 300 g) anesthetized with sodium pentobarbital (50 mg/kg IP). Cylindershaped, thin trabecular muscles (diameter, 100 to 150 μm) were dissected from the right ventricle in oxygenated Tyrode’s solution without Ca2+ at 30°C. The preparations were skinned in 1% (vol/vol) Triton X-100 in the relaxing solution (in mmol/L: Mg-ATP 4, MOPS 10, EGTA 10, free Mg2+, and ionic strength 180 [pH 7.0]) for 60 minutes at ~2°C. The ionic strength was adjusted with KCl. The preparations were stored at ~2°C. Then SL was increased in the relaxing solution containing 50% (vol/vol) Triton X-100™ and 2 mmol/L leupeptin for 1 week or less.

Isometric tension was measured in solutions containing 4 mmol/L Mg-ATP, 10 mmol/L MOPS, 1 mmol/L free Mg2+, various concentrations of free Ca2+ (adjusted with Ca[10 mmol/L EGTA]), 0.1 mmol/L P1,P5-di(adenosine-5')pentaphosphate, 15 mmol/L creatine phosphate, 15 U/mL creatine phosphokinase, and 180 mmol/L ionic strength (adjusted with KCl).

Protocols

SL was measured by laser diffraction. Passive tension was measured at SL 2.0, 2.1, 2.2, 2.3, and 2.4 μm by slowly increasing SL (at ~100 nm/s per sarcomere) from the slack SL (1.9 μm, no passive tension) in the relaxing solution. SL was then readjusted to 1.9 μm for trypsin treatment (see below). Thereafter, passive tension was measured at the above SLs.

Ca2+-activated tension was measured according to a previously described procedure. The preparation was bathed in the low-EGTA (1 mmol/L) relaxing solution for ~15 seconds just before contraction to minimize the buffering effect of EGTA (the low-EGTA relaxing solution was used only for this purpose). Contraction was stopped by transferring the preparation to the relaxing solution containing 10 mmol/L EGTA. SL was decreased in the relaxing solution from 1.9 μm, and active tension was measured at SLs of 1.9, 2.0, 2.1, 2.2, 2.3, and 2.4 μm at the partial- (pCa 5.55) or full- (pCa 4.8) activation condition. Then, SL was readjusted to 1.9 μm for trypsin treatment; the active tension level was also checked before treatment. We used only those data in which the tension was >95% of that measured at the beginning of the experiment. After trypsin treatment, we measured active tension at the above SLs. Active tension was characterized as the total tension produced minus passive tension. Passive and active tension development were reproducible without trypsin treatment.

The control pCa-tension relationship was first obtained at SL 1.9 μm and then at 2.3 μm before trypsin treatment. Then SL was readjusted to 1.9 μm for trypsin treatment. After trypsin treatment, the pCa-tension relationship was obtained at the 2 SLs. The muscle diameter was measured in the relaxing solution on a microscope at a magnification of ×225. All experiments were performed at 20±0.2°C.

Trypsin Treatment

Leupeptin was removed from the skinned trabeculae by immersing the preparations in the relaxing solution, which was stirred for 10 minutes at 20°C. This washing procedure was repeated 7 times. Trypsin (Sigma, type I, ~11 000 N-Benzoyl-L-arginine ethyl ester [BAEE] units per milligram protein) was made up fresh in the leupeptin-free relaxing solution at 0.25 μg/mL. The muscle preparation was immersed in this solution for 13 minutes at 20°C, and the reaction was stopped by immersing the preparation in the relaxing solution containing 2 mmol/L leupeptin for 10 minutes.

Confocal Microscopy

Skinned preparations were stained with 3.3 μmol/L Alexa488 phalloidin (Molecular Probes) in the relaxing solution containing 20 mmol/L 2,3-butanedione monoxime for 7 hours at 4°C. Both ends of the preparation were fixed on the glass slide with double-sided adhesive tape and covered with a coverslip to make a solution-exchangeable flow cell. The preparation was activated under the microscope (IX70, Olympus Co) equipped with a real-time confocal scanning unit (CSU10, Yokogawa Electric Co). The confocal fluorescence image of the preparation was recorded on a videotape recorder. SL was calculated from the profile of fluorescence intensity by averaging the lengths of 25 sarcomeres. NIH Image software was used. Each solution contained 1 mmol/L dithiothreitol.

Gel Electrophoresis

Skinned preparations either before or after trypsin treatment were dissolved in lysis solution, heated for 3 minutes at 90°C, and electrophoresed on a 2.5% to 12% acrylamide gradient (or 10% acrylamide) gels. The gels were stained with Coomassie Brilliant Blue. The lanes were scanned densitometrically, and peak areas were integrated geometrically (AE-6910CX, ATTO Co).

Data and Statistical Analyses

Significant differences were tested by the paired Student’s t test. The unpaired t test was used for Figure 3 as well as for the analysis of internal shortening with laser diffraction. For protein content analysis, Tukey’s multiple comparison was used. In all cases, statistical significance was verified at P<0.05. The pCa-tension relationship was fitted to the Hill equation. Correlations between active tension and muscle diameter were evaluated by testing the correlation of these parameters. All data were expressed as mean±SEM, with n representing the number of muscles.

Results

Titin Degradation by Trypsin

Titin consisted of a doublet before trypsin treatment, T1 and T2, on SDS–polyacrylamide gel electrophoresis (Figure 1, left). T2 is generally considered a degradation product of T1 (intact titin). After trypsin treatment, T1 was degraded and a new band appeared on the gels below T1 (T3). Major thick and thin filament–based proteins other than titin were unaffected (Figure 1).

Passive tension was exponentially increased with an increase in SL to a degree similar to that reported by Kentish et al using rat skinned ventricular trabeculae (Figure 2 top). After trypsin treatment, passive tension was significantly (n=10, P<0.05) decreased (SL 2.0 to 2.4 μm). A significant level of passive tension was, however, present in trypsin-treated preparations, particularly at long SLs. Trypsin treatment did not influence muscle diameter at SL 1.9 μm (130.1±7.9 and 131.9±7.7 μm, n=10, P>0.05, before and after trypsin, respectively). Highly correlated linear regression lines were obtained before and after trypsin treatment between (1-relative diameter) and SL (Figure 2 bottom). Both regression lines were very close to the volume constant line.
Reproducibility of Ca^{2+}-Activated Tension

The reproducibility of active tension development was checked (Figure 3). Brief contractions, sufficient for the estimation of peak active tension, were repeated 10 times at SL 1.9 or 2.4 μm (~20 seconds and 5 to 7 seconds for SL 1.9 and 2.4 μm, respectively) at pCa 4.8. Then, a sustained contraction (1 minute) was induced, followed by a brief contraction. At SL 1.9 μm, we observed no differences in relative tension and time to half-maximal tension ($t_{1/2}$) between untreated and trypsin-treated preparations before, during, and after a sustained contraction. At SL 2.4 μm, the degree of tension reduction with repetitive brief contractions was almost the same in untreated and trypsin-treated preparations. During sustained contraction, however, tension was slightly (n=5, $P<0.05$) decreased in trypsin-treated preparations. In the subsequent brief contraction, tension was markedly depressed and $t_{1/2}$ was prolonged, indicating that the sarcomere structure had been disorganized during the preceding sustained contraction. We concluded that in trypsin-treated preparations, brief contractions could be induced at least 10 times with minimal damage to the sarcomere structure.

Effect of Trypsin on SL-Tension Relationship

Active tension was dramatically increased with SL before trypsin treatment, and the slope was steeper at partial activation than at full activation (Figure 4 left). Trypsin treatment did not influence active tension at SL 1.9 μm. Active tension was, however, significantly ($P<0.01$) decreased after trypsin treatment at SLs of 2.0 μm and longer under both activating conditions. We performed another series of experiments, maintaining SL at 2.4 μm (Figure 4 right). Active tension was markedly decreased after trypsin treatment. The degree of tension reduction was similar to that obtained when preparations were treated with trypsin at SL 1.9 μm: 93.2±8.4 and 64.2±8.1 kN/m² (n=6, $P<0.01$), respectively, at pCa 4.8 before and after trypsin and 75.0±5.1 and 51.3±7.1 kN/m² (n=6, $P<0.01$), respectively, at pCa 5.55 before and after trypsin.
We measured average SL in the central region of the muscle during contraction (pCa 4.8) by laser diffraction. SLs set at 2.0, 2.1, 2.2, 2.3, and 2.4 μm in the relaxing condition (increased stepwise from 2.0 μm), respectively, were decreased to a similar degree in untreated and trypsin-treated preparations: 1.94±0.02 vs 1.93±0.02, 2.01±0.02 vs 2.00±0.02, 2.11±0.02 vs 2.10±0.03, 2.20±0.03 vs 2.19±0.02, and 2.29±0.02 vs 2.28±0.04 μm (n=4, P>0.1 for each comparison). The observed degree of internal shortening (ie, 3% to 5%) was consistent with that reported previously in rat skinned ventricular trabeculae. 5 If the degree of internal shortening is small enough, it can be concluded that titin-based passive tension is present during contraction in the preparations not treated with trypsin (see Figure 2 top).

Furthermore, we checked SL changes in the trypsin-treated preparation by using a confocal microscope. Figure 5 suggests that in the center of muscle, the resting SL of 2.33±0.01 μm was shortened to 2.25±0.07 μm during contraction (pCa 4.8) and returned to 2.32±0.01 μm on deactivation (internal shortening, ∼3.5%). Figure 5 also suggests that major deterioration does not occur in the sarcomere structure before, during, or after contraction after trypsin treatment.

Effect of Trypsin on the Increase in Active Tension by Osmotic Compression

It is well established that dextran T-500 osmotically decreases interfilament lattice spacing in skinned cardiac muscle7 and consequently augments tension development. 5,6 When the muscle diameter was reduced by increasing the concentration of dextran T-500 (up to 6% wt/vol; Amersham Pharmacia Biotech), active tension was augmented, and significantly correlated linear regression lines were obtained between muscle diameter and active tension under partial and maximal activation conditions (Figure 6, SL 1.9 μm). The slope of the linear regression line was steeper under partial activation. The slope of the linear regression line under the partial or maximal activation condition was unaffected by trypsin treatment.

Effect of Trypsin on Ca²⁺ Sensitivity

Increasing SL from 1.9 to 2.3 μm caused a decrease in muscle diameter from 121.8±6.9 to 107.2±6.6 μm (−12% reduction, n=5, P<0.01) and shifted the pCa-tension relationship to the left (Figure 8 top). 6 Dextran T-500 (6% wt/vol) reduced the diameter from 126.3±9.2 to 109.8±6.9 μm (−13% reduction, n=4, P<0.01) at SL 1.9 μm and shifted the pCa curve leftward to a similar degree as that obtained by lengthening (Figure 8 bottom). 6 It was found that trypsin treatment did not affect the increase in Ca²⁺ sensitivity, as reflected by the shift of the midpoint of the pCa curve, by both lengthening (0.184±0.005 and 0.178±0.006 pCa units, P>0.05, before and after trypsin, respectively) and osmotic compression (0.163±0.013 and 0.162±0.017 pCa units, P>0.05, before and after trypsin, respectively).

The Hill coefficient values 6 for untreated and trypsin-treated preparations were 6.25±0.59 and 6.47±0.55 (P>0.05), respectively, at SL 1.9 μm and 5.97±0.56 and 5.74±0.15 (P>0.05), respectively, at SL 2.3 μm. Those for untreated and trypsin-treated preparations were 6.60±0.45 and 6.81±0.39 (P>0.05), respectively, without dextran T-500 and 5.58±0.44 and 5.40±0.29 (P>0.05), respectively, with dextran T-500. Trypsin treatment did not affect the cooperative activation of the thin filament as reflected by the Hill coefficient.

SL Change vs Osmotic Compression in Trypsin-Treated Preparations

Figure 7 compares the diameter–active tension relationships converted from the SL–active tension relationships (Figure 4 left) with those obtained by osmotic compression (Figure 6) for trypsin-treated preparations. At each pCa, the slope of the linear regression line obtained by SL change nearly overlapped that obtained by osmotic compression.
by 2 factors, 1 of which is related to titin extension and the other to a reduction of interfilament lattice spacing (Figures 4, 6, and 7). Consistent with earlier reports,4–6 Ca\(^{2+}\) sensitivity was predominantly modulated by changes in interfilament lattice spacing but was unaffected by trypsin treatment (Figure 8). We now discuss these results, focusing on the role of titin in the regulation of the formation of force-generating cross-bridges.

Helms et al\(^{16}\) found that the anti-titin antibody 9D10, which labels the PEVK segment of titin, failed to label the sarcomere after trypsin treatment in rat skinned cardiac cells. The authors concluded that passive tension was lowered after trypsin treatment due to degradation of the PEVK segment. In rat skinned ventricular trabeculae, titin and collagen are the most significant contributors to passive tension, with titin dominating at the shorter end of the working range of SL and collagen at longer SLs.\(^{20}\) We therefore consider that the passive tension insensitive to trypsin (Figure 2 top) is predominantly attributable to collagen. It has also been pointed out that collagen could cause shrinkage of the lattice with increasing SL when the plasma membrane is absent.\(^{7}\) Therefore, the lattice spacing may tend to remain unchanged on titin degradation in collagen-rich fibers, such as in rat ventricular trabeculae (Figure 2 bottom).

The results of Figure 3, as well as those of Figure 5, suggest that brief exposure of the trypsin-treated preparation to Ca\(^{2+}\) does not deteriorate the sarcomere structure. This appears to be contrary to the conclusion of a well-known earlier report.\(^{21}\) That study, however, was conducted with rabbit fast skeletal muscle in the longer SL range (ie,constantly up to 3.0 µm) under sustained contraction for electron microscopic observation.

We found that active tension was decreased at SLs beyond the slack SL after trypsin treatment (Figure 4). Because the degree of internal shortening was nearly the same for untreated and trypsin-treated preparations, it is unlikely that the decrease in active tension was due to excessive shortening of sarcomeres after trypsin treatment. Alternatively, it is possible that the decrease in active tension was due to the effect of trypsin on minor and/or unknown proteins. However, if one assumes that the mechanical as well as regulatory properties of the cardiac muscle contractile system are predominantly determined by the major contractile proteins examined in the current study, as is generally thought, it is reasonable to conclude that passive tension due to titin extension regulates active tension development, because the major proteins other than titin were not degraded (Figure 1) and major deterioration did not occur in the sarcomere structure on contraction (Figures 3 and 5) after trypsin treatment. Similar evidence for an interplay between passive and active tension generation has been obtained in earlier work on the role of minititin, a passive tension generator, in insect indirect flight muscle.\(^{22}\) Active tension and active stiffness were increased with passive tension, and passive stiffness due to weak-binding cross-bridges increased linearly with passive tension.\(^{22}\) Experiments were also performed in skeletal muscle,\(^{23}\) and the authors concluded that the number of force-generating cross-bridges was increased with passive tension.\(^{23}\)

Wakabayashi et al,\(^{24}\) using synchrotron x-rays, found that in frog skeletal muscle, stretching the resting muscle from...
full-overlap to nonoverlap length caused an increase in the myosin periodicity by $\approx 0.6\%$. This result suggests that passive tension due to extension of titin alters the thick filament structure. The authors also noted that stretching of the resting muscle caused loss of the characteristic resting order of myosin heads around the thick filament backbones. It is thus reasonable to assume that a similar mechanism operates at a shorter SL range in cardiac muscle, which has a higher stiffness than skeletal muscle owing to different expressions of titin.

Recent lines of evidence suggest that cross-bridge arrangement can vary in resting muscle, and cross-bridges are distributed in 3 populations: the cross-bridges that are ordered on the thick filament helix and those that are disordered; the cross-bridges that are ordered, which enhances the interaction of myosin heads with thin filaments and consequently increases the number of force-generating cross-bridges.

A recent study showed that the magnitude of the SL-dependent shift of the pCa-tension relationship was diminished by trypsin treatment in rat skinned ventricular cells. This finding is in disagreement with our results (Figure 8).

Because those authors used cells that reportedly have lesser amounts of collagen than do trabecular preparations, the constraint by collagen to maintain the lattice volume may have been lost. This may result in a decrease in Ca$^{2+}$ sensitivity by expansion of interfilament lattice spacing, especially at long SLs. Other factors contributing to the difference in the effect of titin degradation on Ca$^{2+}$ sensitivity are differing ionic conditions and experimental protocols.

More recently, Cazorla et al. presented evidence that titin-based passive tension affects Ca$^{2+}$ sensitivity through changes in the lattice spacing in skinned cardiac muscle. The lattice spacing is under the influence of titin as well as of collagen. Therefore, in preparations that have a low amount of collagen, such as in cardiac cells, titin-based passive tension likely influences the lattice spacing and subsequently, Ca$^{2+}$ sensitivity. Our trabecular preparations, on the other hand, showed a constant volume behavior after titin degradation (Figure 2 bottom), leading us to propose that titin-based passive tension affects the thick filament structures.

It is well known that the SL dependence of tension generation is markedly attenuated in intact and skinned muscle preparations from the failing human heart. It is also known that titin is downregulated in failing human myocardium. The current study suggests that attenuation of the SL-dependent activation in failing myocardium may be due, at least in part, to the downregulation of titin. It would be interesting to investigate the correlation between the expression level of titin and the magnitude of SL-dependent activation in various animal models of heart failure.

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