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Calcium-dependent inhibition of in vitro thin-filament motility by native titin

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Abstract **Titin (also known as connectin) is a giant filamentous protein that spans the distance between the Z- and M-lines of the vertebrate muscle sarcomere and plays a fundamental role in the generation of passive tension. Titin has been shown to bind strongly to myosin, making it tightly associated to the thick filament in the sarcomere. Recent observations have suggested the possibility that titin also interacts with actin, implying further functions of titin in muscle contraction. We show — using in vitro motility and binding assays — that native titin interacts with both filamentous actin and reconstituted thin filaments. The interaction results in the inhibition of the filaments' in vitro motility. Furthermore, the titin-thin filament interaction occurs in a calcium-dependent manner: increased calcium results in enhanced binding of thin filaments to titin and greater suppression of in vitro motility.**

Key words: Muscle contraction; Motility (in vitro); Titin; Calcium regulation; F-actin; Thin filament

1. Introduction

Titin (also known as connectin) is a 3-million-dalton protein — the largest protein known to date — that constitutes about 10% of the total muscle protein [1,2,4]. Titin's role in muscle function has so far been thought to be mostly mechanic: being anchored to the Z-line and to the thick filaments of the A-band (via its strong myosin-binding property) it generates passive tension when the sarcomere is stretched [3,5,6,13]. In invertebrate muscles [14–16] and in the non-muscle cytoplasm [17], however, where such structural constraints are different or absent, titin's function remains elusive. Recent studies have shown that, beyond its strong affinity for myosin, titin also binds several other muscle proteins [7,8,9,10]. Of a particular interest is its binding to actin, because this interaction could potentially provide clues to titin's elusive functions. Indeed, the thin filament of the cardiac muscle I-band has been proposed to be a complex containing titin [18], non-muscle (cellular) titin has been shown to co-localize with the actin-rich stress fibers [19], and a strong association has been demonstrated between F-actin and cloned cardiac titin fragments [11] leading to the inhibition of actomyosin in vitro motility [12]. Interaction between native titin and F-actin or the structurally and functionally more complex thin filament, however, has not been demonstrated. We tested the possibility of interaction between native titin and F-actin/thin filament using in vitro heavy meromyosin (HMM)-supported motility assays and in vitro titin-F-actin/thin filament binding assays. Our results indicate that titin can interact with both unregulated actin filaments and reconstituted thin filaments, which results in the inhibition of the filaments' in vitro motility. The titin-thin filament association was found to be enhanced upon increasing the free calcium concentration.

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2. Materials and methods

2.1. Preparation of proteins

Actin, myosin and heavy meromyosin (HMM) were purified according to established methods [20–22]. F-Actin was fluorescently labelled with molar excess of tetramethyl-rhodamine-isothiocyanate-phalloidin (Molecular Probes, Eugene, OR). HMM was actin-affinity purified prior to use in the in vitro assay to remove irreversible rigor heads [22]. Titin was prepared from rabbit back muscle (longissimus dorsi) essentially according to the method of Soteriou et al. [7]. The final chromatography stage of the procedure resulted in fractions containing a mixture of T1 and T2 titin, followed by fractions containing T2 only [23]. For the in vitro motility and binding assays we used the first fraction devoid of T1. Purity of titin was determined by SDS-polyacrylamide gel electrophoresis using 2.35–12% gels [24].

2.2. Reconstitution of thin filaments

Thin filaments were reconstituted from purified actin and tropomyosin–troponin (Tm–Tn) complex. Tm–Tn complex was prepared essentially according to Sata et al. [25]. In order to obtain homogenous reconstitution of the thin filament we carried out the reconstitution by incubating F-actin (1 mg/ml) and Tm–Tn complex (2 mg/ml) at 40°C for 10 min followed by slow cooling (0.2°C/min) to 10°C [25]. Alternatively, we mixed Tm-Tn complex with G-actin followed by polymerization of actin [26]. The two different methods yielded identical results. pCa was calculated by the computer program of Fabiato [27] and adjusted by adding $CaCl_2$.

2.3. In vitro motility assay

The in vitro motility assay was carried out essentially according to Kron et al. [22]. Fluorescent actin filaments were visualized by using a Nikon Diaphot 300 inverted epifluorescence microscope equipped with rhodamine interference filter set (Nikon) and a 64×, 1.4 NA oil-immersion objective (Nikon), and using a microchannel-plate intensified CCD camera (ICCD-100F, VideoScope International Ltd., Sterling, VA). The detected images were recorded on a Hi8mm VCR (SONY EV-S7000), and subsequently digitized by an LG-3 frame grabber board (Scion Corporation, Frederick, MD) in an Apple Power Macintosh 6100/60 computer using image analysis software (Scion Image Version 1.57c, based on NIH Image, Wayne Rasband, NIH, Bethesda). Experiments were carried out at 30°C, adjusted by using a temperature-regulated copper microscope stage and a copper coil around the objective in which water was circulated by a heating/cooling thermostat (VWR Scientific, Seattle, WA). The flow-through microchamber was identical to the one used by Kellermayer et al. [28] with an internal volume of ~10 μl. First, 100 μl of HMM–titin mixture in 0.6 M KCl-Assay Buffer (AB, 25 mM imidazole-HCl, 25 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, pH 7.4) was pipetted through the microchamber and incubated at room temperature for 1 min. HMM concentration was varied between 40–100 μg/ml, and titin concentration between 0.29–427 μg/ml. Molar ratios were calculated assuming a molecular weight of 360 kDa for HMM

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[21] and 2000 kDa for T2 [2]. Titin was omitted from the control experiment. Non-specific binding sites were blocked by washing in 100 μl of 0.5 mg/ml bovine serum albumin (BSA) in AB buffer. Fluorescent actin filaments were then added, at a concentration of 70 ng/ml, and allowed to bind to the HMM–titin-coated surface for 1 min. The flow-cell was then washed with 100 μl of AB buffer supplemented with 6 mg/ml D-glucose, 40 μg/ml catalase, 200 μg/ml glucose oxidase, and 100 mM β-mercaptoethanol to reduce photobleaching. Filament movement was initiated by the infusion of 1 mM ATP. The velocity of the actin filaments was measured on digitized video sequences (usually 30 frames) by user-developed macro programs. Velocity was calculated by automatically following the centroid position of a selected filament as a function of time. For spatial calibration we used a 10-μm optical grating (VWR Scientific, Seattle, WA). Results were plotted either as histograms (Fig. 1b) or as mean velocities with standard error of the mean for all filaments (including immobile) in 5–10 microscope fields of view (Fig. 3a and b).

2.4. *In vitro microscopic binding assay*

The interaction between titin and actin/thin filaments was studied in an in vitro microscopic binding assay. In the assay, the amount of fluorescent actin/thin filaments bound to a titin-coated nitrocellulose surface was assessed by measuring the total filament length per field of view. Measuring the total filament length instead of counting the number of filaments provided a better estimate of the titin-bound actin/thin filament because mean filament length increased with in-

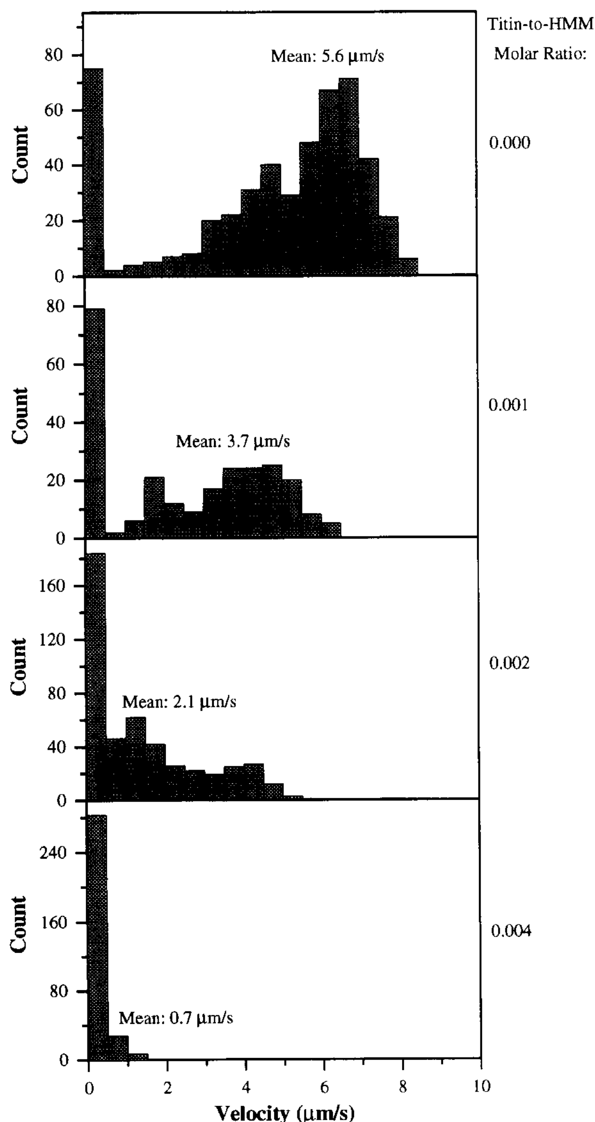

Fig. 1. Effect of titin on in vitro actin-filament motility. With increasing titin-to-HMM molar ratio the velocity of the mobile actin-filaments decreased and the number of immobile filaments increased. The actual titin-to-HMM ratios are indicated on the right side of the figure.

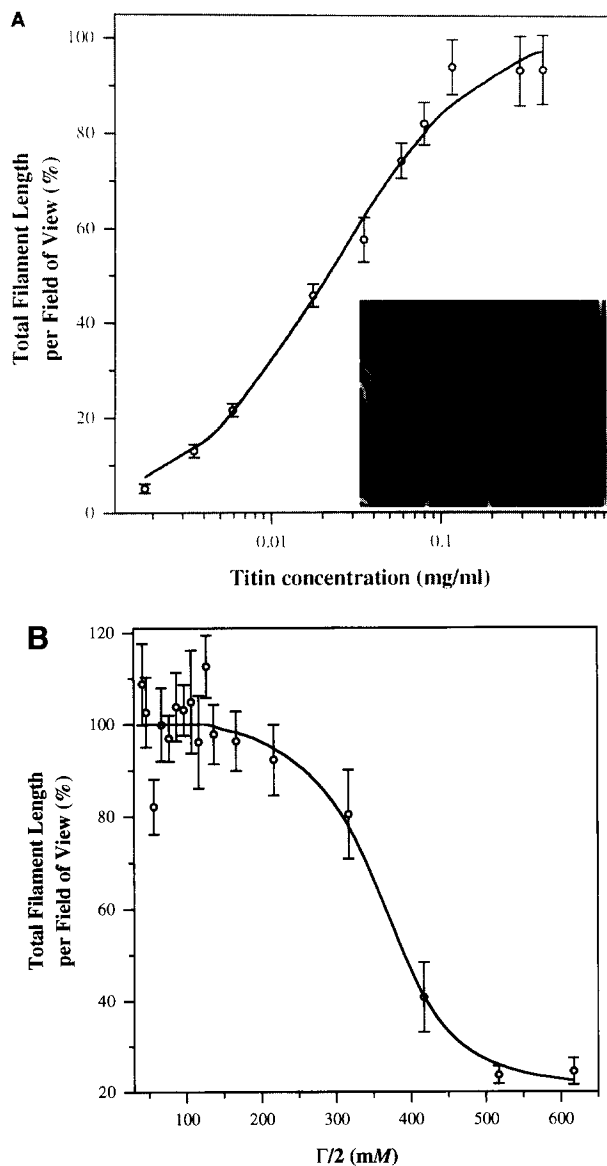

Fig. 2. In vitro F-actin–titin binding assay. (a) Actin-filament binding to increasing densities of titin. Titin density on the nitrocellulose surface was adjusted by varying the solution titin concentration in the range of 1.2–400 μg/ml. The extent of filament binding was measured as the mean total actin-filament length per field of view versus titin concentration in solution. Total filament length increased with increasing titin concentration. Above 400 μg/ml titin concentration, the nitrocellulose surface apparently became saturated. Data were fit with the hyperbolic function $f(x)=ax/(b+x)$, where a is the maximal total filament length at saturating titin concentrations and b is the titin concentration at 50% saturation. The inset shows a representative fluorescence microscopic image of actin filaments bound to a surface coated with titin. Scale bar=10 μm. (b) Actin-filament binding to the titin-coated nitrocellulose surface as a function of ionic strength (Γ/2). The nitrocellulose surface was coated with titin at a solution concentration of 400 μg/ml. Data were fit with the logistic function $f(x)=(a-d)/[1+(x/c)^b]+d$, where a and d are the maximal and minimal total filament lengths, respectively, b is the slope parameter, and c is the ionic strength at the half-maximal value of total filament length.

creasing titin concentration, indicating that a single filament may bind to several titin molecules. Titin, diluted in 0.6 M KCl-AB buffer was added to the microchamber and allowed to bind to the nitrocellulose surface for one minute. Non-specific binding sites were then blocked with 0.5 mg/ml BSA in AB buffer. Fluorescent actin/thin filaments, at a concentration of 70 ng/ml were then added and allowed to bind to the titin-coated surface for one minute. Unbound filaments were washed away with AB buffer supplemented with the oxygen-scavenger enzyme system (see above). Filament length was measured by either manually tracing the filaments using the freehand tool of the image analysis software or by using a user-developed macro program that automatically traced filaments and measured their length in a sequence of digitized images. Ionic strength was adjusted by washing in solution containing progressively increasing concentrations of KCl. Ionic strength was calculated using the computer program of Fabiato [27]. At an ionic strength above ~150 mM, filaments began to partially dissociate from the titin-coated surface. This resulted in actin filaments with segments wiggling in solution plus segments remaining immobile. Dissociated, wiggling segments were excluded from the length measurement by frame averaging (8–16 frames) and thresholding.

2.5. Capillary binding assay

Thin filament-titin capillary binding assay was carried according to Li et al. [12]. Nitrocellulose-coated glass capillaries (Drummond Scientific Co., Broomall, PA; internal volume 87 μl) were filled with titin (100 μg/ml) and incubated at room temperature for 1 min. Non-specific binding sites were blocked with BSA (0.5 mg/ml in AB buffer). Thin filaments (unlabeled) were then added, with an actin concentration of 100 μg/ml, and the pCa adjusted to either 5.5 or 6.5, and the ionic strength to either 42 mM or 150 mM. After one minute, the unbound thin filaments were washed out with 900 μl buffer (identical to the one in which the thin filaments were diluted), and the contents of the capillary were solubilized and analyzed for protein composition by electrophoresis using a 2.6–12% polyacrylamide gel [24]. For protein detection, ammoniacal silver staining was used [24]. Titin was omitted from the negative control. Gel scanning was carried out using a UC1260 color scanner (UMAX Data System Inc., Fremont, CA) in the transparency mode at 600 dpi resolution.

3. Results and discussion

3.1. Interaction between native titin and F-actin

The effect of titin on in vitro motility was studied by following the movement of fluorescent actin filaments on a lawn composed of a mixture of HMM and native titin. To exclude the effect of the known actin-binding titin segment (near the Z-line) [29] we used the T2 (also known as β-connectin) portion of the molecule from which the near-Z-line segment is absent [2]. The addition of titin in the in vitro motility assay resulted in a decrease in velocity of the moving filaments and in an increase in the number of immobile filaments (Fig. 1). The decrease in velocity increased with increasing molar ratio of native titin to HMM, similarly to previous experiments with genetically expressed titin fragments [12].

The inhibition of actin-filament motility by titin could be caused by the tethering of the filaments, as proposed in the case of titin fragments by Li et al. [12]. To test for this, we carried out an in vitro actin–titin binding assay (Fig. 2). The nitrocellulose-coated surface of the solution chamber used for the in vitro assay was coated with varying concentrations of titin. Then, following the blocking of non-specific binding sites with BSA, fluorescent actin filaments were allowed to bind to the surface. The degree of actin binding to titin was assessed by measuring the total length of all actin filaments per microscopic field of view of the titin-coated surface. Our results showed that F-actin binds to native titin, evidenced by the numerous actin filaments bound to the titin-coated surface (Fig. 2a, inset). The total filament length increased with increasing surface density of titin (Fig. 2a). Since titin binds actin filaments strongly under our experimental conditions, the idea that titin inhibits in vitro actin-filament motility by tethering the filaments is well supported.

In vitro motility assays are generally carried out at ionic strengths (typically 30–50 mM) lower than what is considered to be physiological (~180 mM) [30]. To test whether the demonstrated actin-titin association will occur under ionic conditions resembling physiological, we measured the degree of actin-titin interaction at increased ionic strengths (Fig. 2b). The binding of F-actin to titin was not affected by increasing ionic strength up to ~150 mM. Above ~150 mM, actin filaments started to dissociate from the titin-coated surface: partially attached, wiggling filaments began to appear, indicating that the filaments, originally bound at several sites, are held at a reduced number of attachment points. The actin filaments were found to be tethered strongly at the focal points of attachment; the linkage withstood solution flow, and allowed for actin-filament rotation around the attachment point. Upon further increase of ionic strength, the majority of actin filaments completely dissociated from the titin-coated surface. The ionic strength at the half-maximal value of the total filament length was 361.7 mM (±21.52, S.E.M.). Thus, the actin–titin interaction may have physiological significance because it occurs at ionic strengths considered to be physiological.

3.2. Interaction between native titin and reconstituted thin filaments

In the intact muscle sarcomere, regulatory proteins (troponin, tropomyosin) are associated with actin, forming the thin filament. To study whether the presence of regulatory proteins influences the actin-titin interaction, we replaced pure F-actin with reconstituted thin filaments and carried out calcium(pCa)-dependent in vitro motility assays. In the in vitro thin filament-HMM motility assay, at pCa 4.2 (fully activated state), increasing titin density resulted in the progressive inhibition of motility, as shown by reduced filament velocities (Fig. 3a). Thus, titin also inhibits thin-filament motility. To measure the effect of titin at different levels of activation, thin-filament velocity was measured at varying pCa levels (Fig. 3b). In the absence of titin, motility was present even at high pCa, similarly to the recent observations of Fraser and Marston [31]. The minimal velocities (in the pCa range of 6.5–6.1) for the control and titin experiments were 0.59 (±0.16, S.E.M.) and 0.45 μm/s (±0.18, S.E.M.), respectively. The difference between these velocities is statistically insignificant (Student's t-test, $P<0.05$). Thus, the effect of titin on motility at high pCa was negligible. At low pCa (5.9–5.5), the inhibition of motility became pronounced: the maximal velocities for the control and titin experiments were 3.17 (±0.13, S.E.M.) and 1.51 μm/s (±0.20, S.E.M.), respectively. The difference between the velocities is statistically significant (Student's t-test, $P<0.05$). Inhibition of motility at low pCa (pCa 5.9–5.4) reached ~50%. Thus, the inhibition of thin-filament motility by titin is calcium dependent.

The greater degree of inhibition at low pCa could be indirectly caused by the higher mean filament velocity leading to larger number of contacts between titin and the thin filament per unit time (see model proposed by Li et al. [12]). Alternatively, low pCa may have a direct effect resulting in an in-

creased affinity of the thin filament to titin. To test the effect of velocity on the degree of motility inhibition by titin, we varied filament velocity by an independent method, by changing the temperature in the in vitro motility assay. Increasing temperature results in increasing velocities [32]. Thus, if inhibition of motility by titin is enhanced at increased filament velocities, greater degree of inhibition is expected upon raising the temperature. In the acto-HMM in vitro motility assay, using a titin-to-HMM molar ratio of 0.0015 we found a 74% (±6, S.E.M.) inhibition of motility at 20°C. In contrast, at 30°C, the degree of inhibition was only 41% (±10, S.E.M.). Since the degree of motility inhibition by titin decreased upon raising temperature (hence increasing mean velocity), the idea that the increased inhibition of thin-filament motility at low pCa is caused by the increased mean velocity is not supported. Rather, the calcium dependence of motility inhibition suggests that at low pCa the thin filament's affinity to titin is increased.

To further test whether the thin filament-titin affinity is calcium dependent, we carried out in vitro thin filament-titin binding assays as a function of ionic strength at high (6.5) and low (5.5) pCa. Raising the ionic strength results in the dissociation of actin filaments from titin (see Fig. 2b). Thus, the

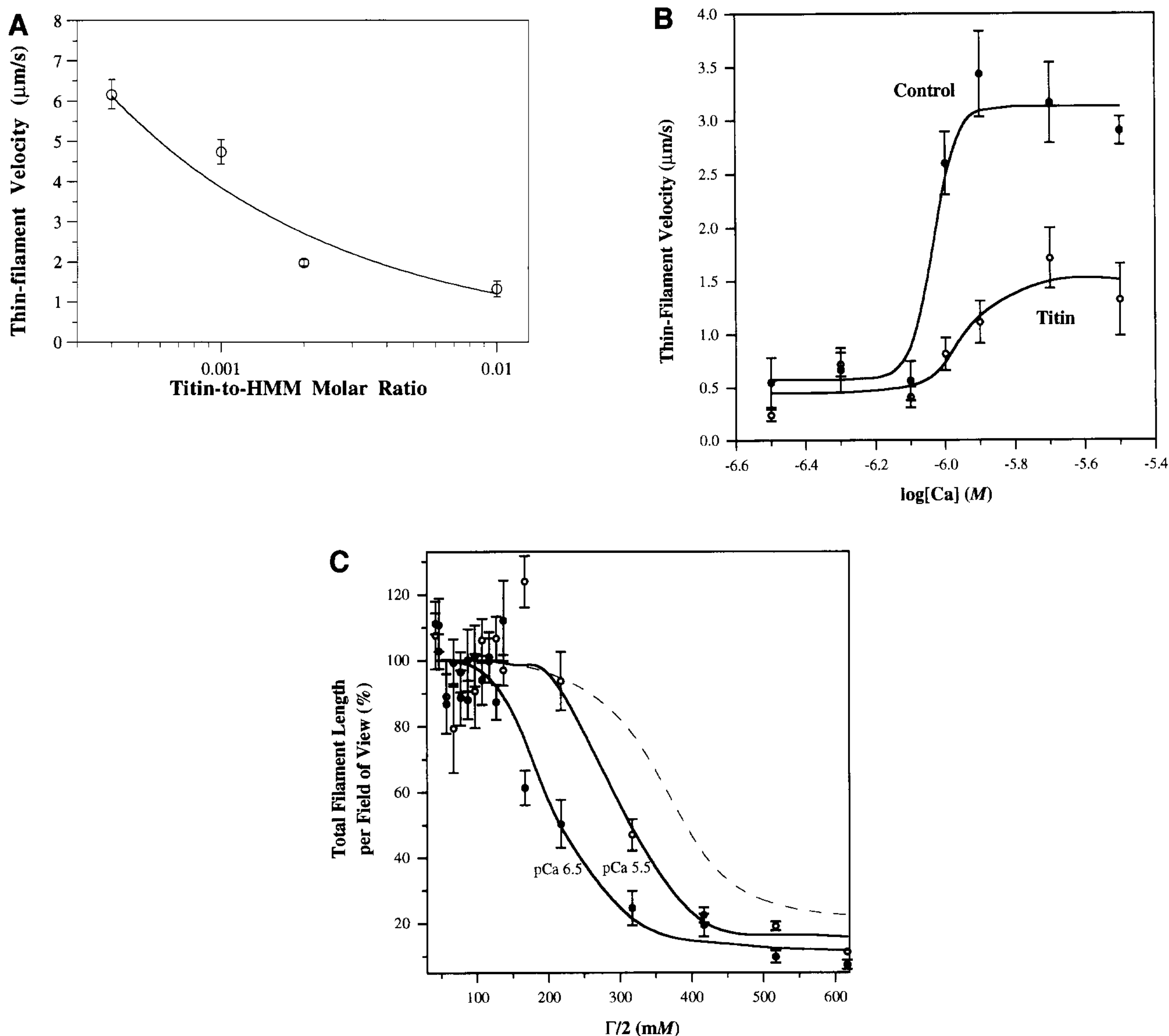

Fig. 3. Interaction between titin and reconstituted thin filaments. (a) Filament velocity as a function of titin-to-HMM molar ratio. The titin-to-HMM ratios used in this experiment were: 0.0004, 0.001, 0.002 and 0.01. Experiments were carried out at activating pCa levels (pCa 4.2, except for the titin-to-HMM ratio of 0.01, which was carried out at pCa 5.5). Filament velocity decreased as the titin-to-HMM molar ratio increased. (b) Velocity–pCa relationship in the absence ('control') and the presence ('titin') of titin. The titin-to-HMM ratio was 0.01. Data were fit with the logistic function used in Fig. 2c. (c) In vitro thin filament-titin binding assay at pCa 5.5 and 6.5 as a function of ionic strength. Degree of binding was measured as the total length of thin filaments bound to a field of view of the titin-coated surface, as described under Fig. 2. Data were fit with the logistic function used in Fig. 2c. Broken line is data (from Fig. 2b) for unregulated actin filaments. (d) SDS-polyacrylamide gel showing the results of the thin filament-titin capillary binding assay. Left-side panel shows the photograph of the gel and the right-side the scan of lanes 7–10. The lanes correspond to the following: (1) rabbit m. longissimus dorsi protein profile, (2) actin, (3) reconstituted thin filament, (4) titin, (5) BSA, (6) negative control: thin filaments in the absence of titin, (7) thin filaments added to titin at Γ/2 of 42 mM and pCa 5.5, (8) at Γ/2 of 42 mM and pCa 6.5, (9) at Γ/2 of 150 mM and pCa 5.5, and (10) at Γ/2 of 150 mM and pCa 6.5. The reduced Tm+TnT to actin ratios in lanes 7 and 8, compared to that of the control (lane 3) and lane 9, are likely to be caused by the reduced affinity of tropomyosin to F-actin at low ionic strength [33].

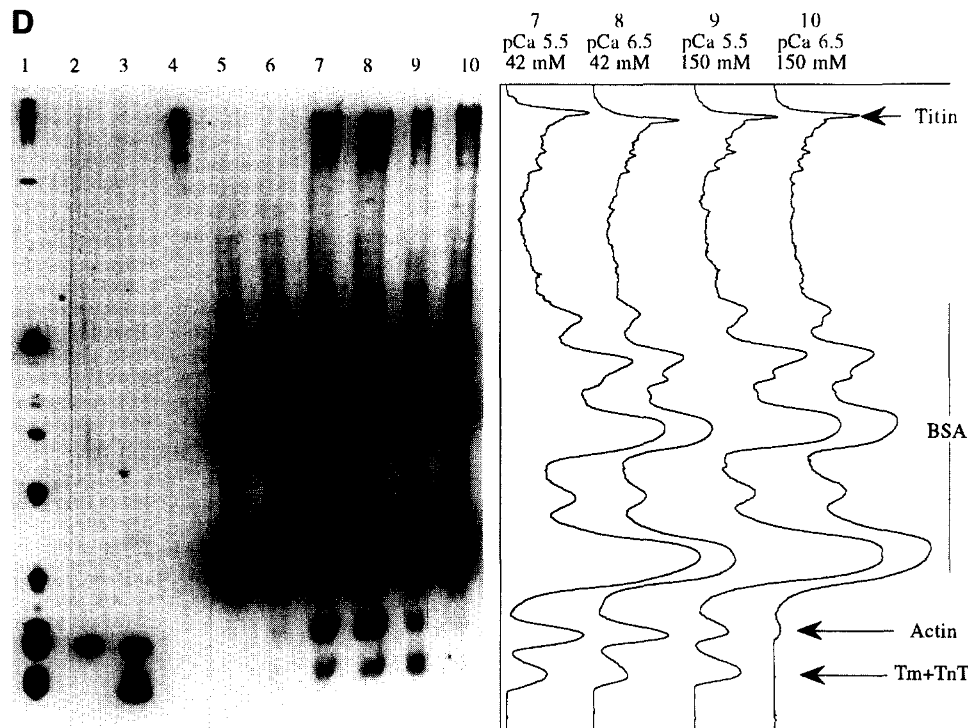

Fig. 3 (*continued*).

ionic-strength dependence of thin filament-titin binding provides means to compare the affinity of thin filaments to titin at different pCa levels. If thin filaments bind titin stronger at low pCa, they are expected to stay attached to the titin-coated surface at higher ionic strengths than in the case of high pCa. Fig. 3c summarizes our observations. At high pCa (6.5), thin filaments began dissociating at an ionic strength as low as ~100 mM. At low pCa (5.5), however, thin filament dissociation began only at ~200 mM ionic strength. The ionic strengths at the half-maximal values of total filament length were 205.2 (±19.9, S.E.M.) and 299.5 mM (±20.2, S.E.M.) for pCa 6.5 and pCa 5.5, respectively. Thus, the in vitro binding assay supports the idea that at low pCa the affinity of thin filaments is greater for titin than at high pCa levels. To confirm the results of the in vitro binding assay by an alternative method, we carried out a thin filament-titin capillary binding assay [12]. Thin filaments were added to titin attached to the nitrocellulose-coated inner surface of a glass capillary. The contents of the capillary were then solubilized and analyzed for protein by SDS-polyacrylamide gel electrophoresis [24]. Binding of thin filaments to titin was analyzed in the cases of pCa 5.5 and 6.5 and ionic strengths of 42 mM (identical to that used in the in vitro motility assay) and 150 mM. The gel analysis (Fig. 3d) showed that, at an ionic strength of 150 mM, larger quantity of thin-filament protein bound to a given amount of titin at pCa 5.5, than at pCa 6.5. The finding provides a qualitative support for the results of the in vitro motility and binding assays, and point to the stronger thin-filament titin interaction at low pCa.

Our observations indicate that – in the experimental environment of the in vitro motility assay – native titin is able to bind both actin and reconstituted thin filaments. This binding results in the inhibition of the motility of actin or thin filaments over HMM. The molecular mechanism of the movement inhibition is likely to be the tethering of actin or thin filaments to the nitrocellulose surface, a possibility suggested previously in the case of cardiac titin fragments [12], and supported by our in vitro binding assays. The possibility of titin tethering the actin or thin filaments to HMM is unlikely since titin has been shown not to bind to HMM [7,12]. The Ca-dependent in vitro motility and binding assays point to an increased affinity of thin filaments to titin at low pCa. Possibly, titin binds to F-actin at sites covered by tropomyosin in the relaxed state (high pCa). These sites might then become exposed as the pCa is lowered, allowing titin to access the actin filament. Our observation that, for a given titin-to-HMM ratio, the reconstituted thin filament slides faster than the unregulated actin filament lends support for this mechanism. An alternative mechanism might be a direct effect of pCa on titin structure, affecting its affinity to the thin filament. Whatever the actual molecular mechanism, the observation that native titin influences in vitro thin filament motility suggests that titin's role may extend beyond the generation of passive tension, to a possible regulatory role in muscle contraction and cell motility. Such a regulatory role might involve the calcium-dependent modulation of filament sliding in the sarcomere.

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