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ORIGINAL PAPER

Impact of tropomyosin isoform composition on fast skeletal muscle thin filament regulation and force development

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Abstract Tropomyosin (Tm) plays a central role in the regulation of muscle contraction and is present in three main isoforms in skeletal and cardiac muscles. In the present work we studied the functional role of α- and βTm on force development by modifying the isoform composition of rabbit psoas skeletal muscle myofibrils and of regulated thin filaments for in vitro motility measurements. Skeletal myofibril regulatory proteins were extracted (78 %) and replaced (98 %) with Tm isoforms as homogenous ααTm or ββTm dimers and the functional effects were measured. Maximal Ca^{2+} activated force was the same in ααTm versus ββTm myofibrils, but ββTm myofibrils showed a marked slowing of relaxation and an impairment of regulation under resting conditions compared to ααTm and controls. ββTm myofibrils also showed a significantly shorter slack sarcomere length and a marked increase in resting tension. Both these mechanical features were almost completely abolished by 10 mM 2,3-butanedione 2-monoxime, suggesting the presence of a significant degree of Ca^{2+}-independent cross-bridge formation in ββTm myofibrils. Finally, in motility assay experiments in the absence of Ca^{2+} (pCa 9.0), complete regulation of thin filaments required greater ββTm versus ααTm concentrations, while at full activation (pCa 5.0) no effect was observed on maximal thin filament motility speed. We infer from these observations that high contents of ββTm in skeletal muscle result in partial Ca^{2+}-independent activation of thin filaments at rest, and longer-lasting and less complete tension relaxation following Ca^{2+} removal.

Keywords Tropomyosin · Myofilament protein isoforms · Skeletal muscle · Myofibril · Myofilament regulatory proteins · Myopathies

Introduction

Tropomyosin (Tm) is a dimeric α chained coiled-coil actin-binding protein which plays a pivotal role in the regulation of striated muscle contraction, acting as the “gatekeeper” of thin filament Ca^{2+} activation (Gordon et al. 2000; Holmes and Lehman 2008; Lehman et al. 2009; Nevzorov and Levitsky 2011). In the sarcomere, Tm dimers are associated in a head–tail manner and the resulting axial continuity of Tm strands on thin filaments allows cooperative azimuthal movements between switched-on/off positions under the influence of Ca^{2+} binding to troponin (Tn) and strongly bound cross-bridge formation.

In mammalian striated muscle, there are three major highly homologous Tm isoforms which are product of different genes: αTm (TPM1), βTm (TPM2) and γTm (TPM3) (Perry 2001; Gunning et al. 2008). The expression of isoforms varies with species, muscle type and many other, largely unknown, developmental, hormonal and environmental factors. In mammalian fast skeletal muscles, αTm and βTm are present in variable proportions from 9:1 to 1:1 (Bronson and Schachat 1982; Perry 2001). In the past few years, many studies using solution (Boussouf et al. 2007), cell (Lu et al. 2010) and transgenic (TG) models (Jagatheesan et al. 2009) have investigated the potential functional role of Tm isoforms. This has been mainly in terms of the Ca^{2+} sensitivity of the contractile apparatus

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and of cross-bridge access to thin filaments. These studies are complicated by the recent observation that homodimeric and heterodimeric isoforms of Tm are mixed in muscle and their ratios can also affect the overall Tm function in the Ca^{2+} regulation process (Janco et al. 2013). When α and β isoforms are expressed in an adult skeletal muscle, the preferred form is the heterodimer αβ (Lehrer et al. 1989; Hvidt and Lehrer 1992) with αα homodimers being formed only when the α:β ratio is greater than 1. The formation of ββ homodimer is usually not favoured, probably due to the instability of the resulting Tm molecule at physiological temperature, especially when associated with the adult isoforms of Tn (Lehrer 1975). The physiological significance of the scarce presence of ββTm homodimer in adult skeletal muscle has therefore been questioned. Interestingly, the ββTm isoform is the predominant form in fetal life (Amphlett et al. 1976) and is increased considerably in the presence of mutations of the TPM2 gene associated with skeletal muscle myopathies (e.g., nemaline myopathy or distal arthrogryposis; Nilsson and Tajsharghi 2008). Based on these recent findings, it has been hypothesized that the increase in TPM2 expression and the change in dimeric species of Tm could introduce a critical perturbation of the thin filament Ca^{2+} regulation process contributing to the pathogenesis of myopathies (Tajsharghi et al. 2012).

The goal of this current study was to investigate the functional consequences of the enrichment of the ββTm content in skeletal muscle myofibrils to get mechanistic insight into the general role of Tm isoforms in regulation. Native Tm from rabbit psoas myofibrils was exchanged with recombinant Tm as ααTm versus ββTm dimers, using the method we previously developed to remove and reconstitute striated muscle myofibrils with exogenous regulatory proteins (Scellini et al. 2010; Janco et al. 2012; Nixon et al. 2013). Replacement of regulatory proteins in single myofibrils offers a number of advantages, as compared to more conventional muscle preparations (She et al. 2000). The smaller diffusion distances allow a more complete and homogeneous exchange of proteins in a much shorter time (Piroddi et al. 2003). Furthermore, fast solution switching methods (Tesi et al. 1999) can be used to abruptly change the concentration of Ca^{2+} and investigate isometric force development and millisecond-time-scale activation and relaxation kinetics. This approach improves the resolution of previous studies performed in vitro (Clemmens et al. 2005) or in skinned fibres from transgenic (TG) mouse models (Pieples et al. 2002; Jagatheesan et al. 2009) and gelsolin treated/reconstituted systems (Fujita et al. 2002, 2004). In addition, in vitro motility assays of actin sliding were performed to determine the regulation state of ααTm versus ββTm-dimer reconstituted thin filaments. The results of this work showed that increased contents of ββTm in thin filaments has no or only small effects on maximal active force development of myofibrils or on the speed of reconstituted thin filament motility. On the other hand, ββTm compromises regulation at pCa 9.0, suggesting that high levels of ββTm in sarcomeres may result in an altered switch-off mechanism and partial Ca^{2+}-independent activation. These results have been previously presented in a preliminary form (Scellini et al. 2011).

Methods

Myofibril isolation and Tm–Tn replacement procedure

Single myofibrils or thin bundles of myofibrils were prepared from rabbit fast skeletal muscle by homogenization of glycerinated psoas muscle, as previously described (Tesi et al. 1999, 2000). Rabbits were killed with pentobarbital (120 mg/kg) administered through the marginal ear vein. All procedures performed were conducted in accordance with the official regulations of the European Community Council on Use of Laboratory Animals (Directive 86/609/EEC) and protocols were approved by the Ethical Committee for Animal Experiments of the University of Florence. All solutions were kept around 0 °C and contained 0.5 mM DTT and a cocktail of protease inhibitors including 10 μM leupeptin, 5 μM pepstatin A, 200 μM phenylmethylsulphonylfluoride, 10 μM E64 and 500 μM NaN_3. Endogenous skeletal Tm and Tn were extracted and replaced with recombinant homodimer αα or ββTm isoforms and rabbit fast skeletal Tn as previously described (Scellini et al. 2010; Siththanandan et al. 2009). Briefly, myofibrils were washed by centrifugation (five–seven times) in a mildly alkaline/low ionic strength solution (2 mM Tris–HCl, pH 8.0) to remove native Tm and Tn. Extracted myofibrils were then washed in 200 mM ionic strength rigor solution (100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 50 mM Tris–HCl, pH 7.0) and reconstituted with exogenous Tm (5 μM) and Tn (2 μM) in a two steps protocol (0 °C, 2 h incubation per step). Tn, extracted and purified from rabbit fast skeletal muscle, was kindly provided by Dr. E. Homsher (UCLA University, Los Angeles, USA). The recombinant homodimer Tms were made as described below. Reconstituted myofibrils were washed and stored in 200 mM ionic strength rigor solution at 4 °C, and used within 3 days. At each stage of the protocol, samples were retained from both supernatant and pellet fractions for electrophoresis assays. The extent of the Tm–Tn extraction and replacement was determined using 15 % SDS-PAGE gels, stained with Coomassie brilliant blue R-250 (Sigma) to reveal the resolved protein bands. To determine the amount and the relative distribution of the various isoform of Tm, Coomassie stained gels were scanned and the gel images were analyzed using UN-SCAN-IT gel 6.0 software (Silk Scientific, Inc., UT, USA). Usually, peaks referring to βTm, TnT and αTm bands were clearly defined and the degree of Tm extraction and reconstitution was assessed determining the ratio of Tm–actin band intensities for each lane. Data obtained from myofibril preparations not permitting accurate gel analysis of replacement were discarded.

Expression and purification of recombinant N-terminal modified ααTm and ββTm

The full-length cDNAs encoded for α and βTm were obtained from mouse heart total RNA by RT-PCR (mouse and rabbit Tm are 100 % identical). These PCR products were further subcloned in reading frame into pET-24a(+) expression vector (Novagen). To restore full function of α and βTm by mimicking the N-terminal acetyl group, an additional nine nucleotides encoding three amino acids (Met-Ala-Ser) were inserted at the 5′-end of each corresponding cDNA by site-directed mutagenesis. These expression constructs were transformed separately into the *E. coli* strain BL21(DE3) for the production of recombinant Tm proteins. These Tm proteins were purified as previously described (Smillie 1982). The function of the N-terminal modified α or βTm was analyzed by actin binding assays as described before (Monteiro et al. 1994). N-terminal modification of Tm has been shown to mimic the N-terminal acetylation of the native molecule, restoring actin binding, head–tail polymerization and the capacity to inhibit actomyosin ATPase without interfering with functional properties of in vitro (Monteiro et al. 1994; Landis et al. 1999) and myofibril (Siththanandan et al. 2009) systems.

Apparatus for mechanical measurements and rapid solution changes in myofibrils

The system used to record force from single myofibrils and for rapid solution changes has been described earlier (Colomo et al. 1997, 1998; Tesi et al. 2000). Briefly, myofibrils selected for use were mounted horizontally between two glass micro-tools: a calibrated cantilevered force probe and a length-control motor.

The length of attached myofibrils was initially set 10–20 % above slack length (Tesi et al. 1999); initial sarcomere length (sl) was measured by calibrated visualization with a camera and monitor. Isometric force was measured from the deflection of the shadow of the force probe projected on a split photodiode (Cecchi et al. 1993). Myofibrils were activated and relaxed by rapid translation between two continuous streams of relaxing (pCa 9.0) and activating (pCa 4.5) solutions flowing by gravity from a double-barrelled glass pipette placed at right angles to, and within 1 mm of, the preparation. Solution changes after the start of the paired-pipette movement (driven by a stepper-motor-controlled system) occurred with a time-constant of 2–4 ms and were complete within 10 ms (Colomo et al. 1998; Tesi et al. 2000). Experiments were performed at 15 °C in a thermostatically controlled myofibril observation chamber and microscope enclosure.

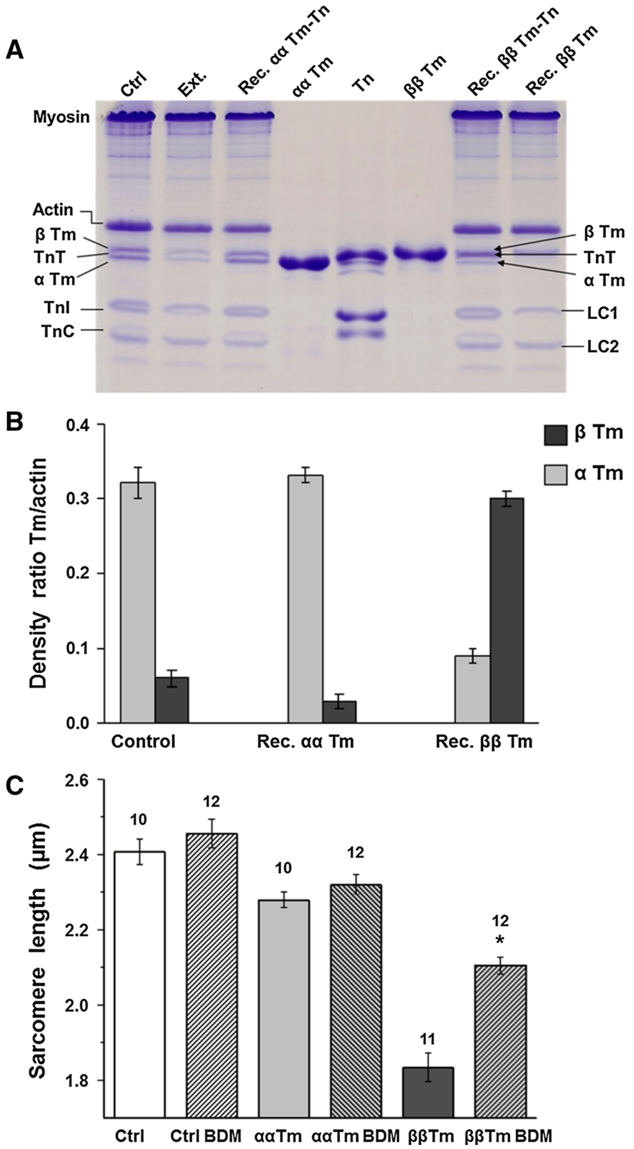
Maximal isometric force was measured and normalized by cross-sectional area of the preparation (P_0); Ca^{2+} activation rate (k_{ACT}) and the rate of tension redevelopment (k_{TR}) following the imposition of a release–restretch protocol (about 30 % myofibril length; Brenner 1988) were estimated from single-exponential fits as previously described (Tesi et al. 2000). Relaxation rate for the slow phase (slow k_{REL}) was calculated from the slope of the regression line fitted to the tension trace normalized to the entire amplitude of the tension relaxation transient. The relaxation rate for the fast phase (fast k_{REL}) was measured from a single-exponential decay fitted to the data. For fitting, transition from the slow to rapid phase was determined subjectively from individual traces. The duration of the slow phase was measured from tension traces from the onset of solution change at the myofibril to the intercept of the regression line with the fitted exponential. Resting tension development (RT) of myofibrils was measured at pCa 9.0 by imposing 30 % releases of initial length. Analysis of variance was used to compare between myofibril groups after Tm–Tn replacement. Student's unpaired *t* tests were used to assess significance.

Data acquisition

Force and length signals were continuously monitored throughout the experiment using commercial software and programs modified for our use (National Instruments®, LabVIEW®). The same signals were also recorded during experimental protocols and later used for data analysis. Data measurements were made directly with a homemade LabVIEW Analysis program converting the analogue signals to numeric values and commercial software (Origin®, SigmaPlot®).

Solutions

All activating and relaxing solutions were calculated as described previously (Tesi et al. 2000) at pH 7.0. The solutions contained: 10 mM total EGTA, 5 mM Mg-ATP, 1 mM free Mg^{2+}, 10 mM MOPS, propionate and sulphate to adjust the final solution to an ionic strength of 200 mM and a monovalent cation concentration of 155 mM. Although continuous solution flow minimizes alterations in the concentration of Mg-ATP and its hydrolysis products in



◀**Fig. 1** Protein isoform composition and slack sarcomere length of rabbit psoas myofibrils reconstituted with Tn and ααTm or ββTm versus control. **a** Representative Coomassie-stained 15 % SDS-PAGE gel of Tm–Tn extraction and replacement in rabbit psoas myofibrils before (*lane 1* Ctrl) and after (*lane 2* Ext) removal of endogenous Tm–Tn complex and following reconstitution (*lane 3* and 7 Rec) with exogenous ααTm–Tn (*lane 3*) or ββTm–Tn (*lane 7*). Fast skeletal Tn complex (*lane 5*), ααTm (*lane* 4), ββTm (*lane* 6), myofibrils extracted and reconstituted only with ββTm (*lane* 8 Rec ββTm). **b** Relative amount of αTm and βTm in control, reconstituted ααTm–Tn and reconstituted ββTm–Tn myofibrils averaged from five SDS-PAGE gels. Tm band intensities (α or β, respectively) were expressed relative to actin band. All values are given as mean ± s.e.m. *Control* αTm 0.32 ± 0.02, βTm 0.06 ± 0.01, *reconstituted ααTm* αTm 0.33 ± 0.01, βTm 0.03 ± 0.01, *reconstituted ββTm* αTm 0.09 ± 0.01, βTm 0.30 ± 0.01. **c** Mean slack sarcomere length (sl) of control (Ctrl), ααTm and ββTm reconstituted myofibrils measured in relaxing solution before and after addition of 10 mM BDM. The value of sl in ββTm myofibrils is significantly shorter compared to ααTm and Ctrl myofibrils. BDM affects sl of ββTm myofibrils while it has no effect on Ctrl and ααTm reconstituted myofibrils. All values (μm) are given as mean ± s.e.m.: Ctrl 2.41 ± 0.03, Ctrl + BDM 2.46 ± 0.04; ααTm 2.28 ± 0.02, ααTm + BDM 2.32 ± 0.03; ββTm 1.83 ± 0.04, ββTm + BDM 2.11 ± 0.02. *$P < 0.05$ versus ββTm

the myofibrillar space, the measurements were made in the presence of creatine phosphate (10 mM) and creatine kinase (200 units/ml) to prevent any ADP gradients. Contaminant [Pi] (around 170 μM in standard solutions) was reduced to less than 5 μM (Pi-free solutions) by a Pi-scavenging enzyme system (purine-nucleoside-phosphorylase with substrate 7-methylguanosine; Tesi et al. 2000, 2002a). Ca–EGTA:EGTA ratio was set to obtain a fully relaxing solution of nominal pCa 9.0 and a maximally activating solution of pCa 4.5 (Brandt et al. 1972). In a few experiments, 10 mM 2,3-butanedione 2-monoxime (BDM) was added to relaxing solution. Nucleoside phosphorylase (‘bacterial’), 7-methylguanosine, ATP, BDM, DTT, leupeptin, E64, phenyl-methylsulphonylfluoride, NaN_3 and pepstatin A were purchased from Sigma, creatine phosphate and creatine kinase from Roche Diagnostics.

In vitro motility assay

Rabbit actin and myosin heavy meromyosin (HMM) were purified from rabbit fast skeletal muscle as previously described (Clemmens et al. 2005). Tn subunits and αα or ββTm were recombinant proteins (see above). Unregulated (actin) and regulated (actin + Tm + Tn) motility was measured at 30 °C in flow cells with surfaces containing skeletal HMM. Construction of flow cells and measurement of filament sliding speed and moving filaments were as previously described (Clemmens and Regnier 2004; Clemmens et al. 2005). The concentrations of Tn and Tm used in motility buffer to reconstitute thin filaments that stopped movement varied with the Tm isoform used. Motility buffer (mM): 25 imidazole, 2 Mg-ATP, 1 EGTA, 1 free Mg^{2+}, 50 ionic strength (KCl) pH 7.4. Filament motility data were collected at pCa 9.0 and pCa 5.0 in the presence of antioxidant agents (0.018 mg/ml catalase, 0.1 mg/ml glucose oxidase, 3 mg/ml D-glucose, 40 mM DTT) to minimize photobleaching and photo-oxidative protein damage. Speed or moving fraction versus pCa data were fitted to a four-parameter Hill equation using non-linear regression weighted for SD of individual points (Origin®).

Results

Gel electrophoretic studies of Tm replacement in rabbit psoas myofibrils

Two different reconstituted (Rec) myofibril groups were obtained from control (Ctrl) rabbit psoas myofibrils by

Tm–Tn replacement procedure: ααTm (Rec ααTm–Tn) and ββTm (Rec ββTm–Tn) myofibrils. Samples of all myofibril preparations used for mechanical measurements were retained for electrophoretic analysis, as well as fractions of Tm–Tn extracted myofibrils (Ext). Figure 1a shows a representative 15 % Tris–HCl SDS-PAGE gel after Coomassie blue staining of skeletal rabbit psoas myofibril samples kept at each step of the Tm–Tn extraction–reconstitution protocol. For each lane, the amount of protein loaded (4–10 μg) is different due to protein loss during the preparation. Data in the literature regarding the quantitative α and β isoform ratio in fast skeletal muscle are controversial, but it is generally assumed that the α isoform predominates at a ratio of about 4:1 to βTm (Cummins and Perry 1973; Salviati et al. 1982; Perry 2001). The degree of Tm extraction or replacement in each lane was assessed from the intensity profile of scanned gels and determining the ratio of Tm (αTm or βTm) to actin band intensities in the control, extracted and reconstituted samples. After extraction, the (α + β)Tm/actin ratio (lane Ext) is 0.09 ± 0.01 compared to 0.38 ± 0.02 in the controls (lane Ctrl) and 0.35 ± 0.01 and 0.39 ± 0.01 in the reconstituted αα and ββTm myofibrils (lanes Rec), representing about 78 % ± 3 mean value of (α + β)Tm removal and 98 % ± 6 mean value of (α + β)Tm replacement (mean ± s.e.m.; $n = 5$). Extraction–replacement of fast skeletal Tn was 100 %. Figure 1b shows mean data from five gels regarding the relative amount of α and βTm in control myofibrils and in myofibrils reconstituted with ααTm or ββTm. It can be seen that extraction–replacement protocol strongly affects the α:β Tm isoform ratio, shifting from a prevalence of α over β in control myofibrils (0.84:0.16) to a correspondent prevalence of β over α in ββTm reconstituted myofibrils (0.23:0.77). As expected, enrichment in αTm isoform in ααTm-reconstituted myofibrils is only slight (0.92:0.08). ββTm replacement in rabbit psoas myofibrils ensures the presence of significant amounts of ββTm homodimers in sarcomeres. Potential formation of new αβTm heterodimers by chain exchange during the replacement protocol should be negligible as temperature is maintained around zero throughout the whole procedure (Hvidt and Lehrer 1992).

Impact of the modification of α:β Tm ratio on active force development of myofibrils after extraction–replacement of Tm–Tn

Single or thin bundles of rabbit psoas myofibrils were mounted in the isometric force recording apparatus (15 °C) and Ca^{2+}-activated by fast solution switching from relaxing (pCa 9.0) to fully activating (pCa 4.5) solution. Relaxation from maximal isometric force was then induced by switching myofibrils back to pCa 9.0 solution. Figure 2a shows recordings of a representative activation–relaxation cycle of myofibrils both native (Ctrl, upper trace) and after endogenous Tm–Tn extraction and reconstitution with αα (middle trace) or ββTm (bottom trace). In each contraction, we measured maximal steady-state tension (P_0 i.e., force at pCa 4.5 normalized over cross-sectional area) and both the rate of force activation upon Ca^{2+} switch from pCa 9.0 to pCa 4.5 (k_{ACT}) and the rate of tension redevelopment upon a 30 % rapid release–restretch length manoeuvre imposed on maximal activated steady force (k_{TR}; Brenner 1988). As previously observed, k_{ACT} and k_{TR} of myofibrils activated by rapid solution switching had similar values, indicating that the activation mechanisms including Ca^{2+} binding to TnC and subsequent thin filament switch-on do not limit the apparent rate of force generation (Colomo et al. 1998).

Relaxation of force, which as previously observed in myofibrils took place in two phases (Tesi et al. 2002b), was characterized by measuring the duration and the rate of the slow phase (slow k_{REL}) and the rate of the fast exponential phase (fast k_{REL}). Mean data are reported in Table 1.

As previously observed (Siththanandan et al. 2009; Scellini et al. 2010), the protocol of extraction–replacement of Tm–Tn led in all myofibril batches to a decrease of maximal isometric tension that here was about 35 %. However, P_0 measured after the extraction–replacement protocol was not lower than usually reported for control rabbit psoas myofibril batches (de Tombe et al. 2007; Kreutziger et al. 2008). No difference in P_0 was observed between ααTm and ββTm replaced myofibrils (Fig. 2b). In ααTm, k_{ACT} (Fig. 2c) and k_{TR} (Fig. 2d) were also preserved compared to controls. Interestingly, both these rates were significantly decreased in ββTm replaced myofibrils versus control and ααTm replaced myofibrils (for data and significance of the effects see Table 1).

Interestingly, relaxation of force was markedly prolonged in ββTm-reconstituted myofibrils, as shown in Fig. 3a where representative traces of ββTm and ααTm reconstituted myofibrils are shown on an expanded time scale. The comparison of mean relaxation parameters of ββTm versus ααTm replaced myofibrils (Table 1) showed a significant increase in the duration of the slow phase of relaxation (about 20 % $P < 0.01$) and a significant decrease in fast k_{REL} (about 35 % $P < 0.01$) with no effect on slow k_{REL} in the presence of an increased sarcomeric content of the ββTm isoform. Replacement with ααTm had a much smaller impact on the rate of fast relaxation, likely due to non-specific "run-down"-like effects of the extraction–replacement treatment, as previously observed (Nixon et al. 2013).

The effects on relaxation kinetics found in ββTm myofibrils are the same as previously observed when control myofibrils were partially relaxed to Ca^{2+}-

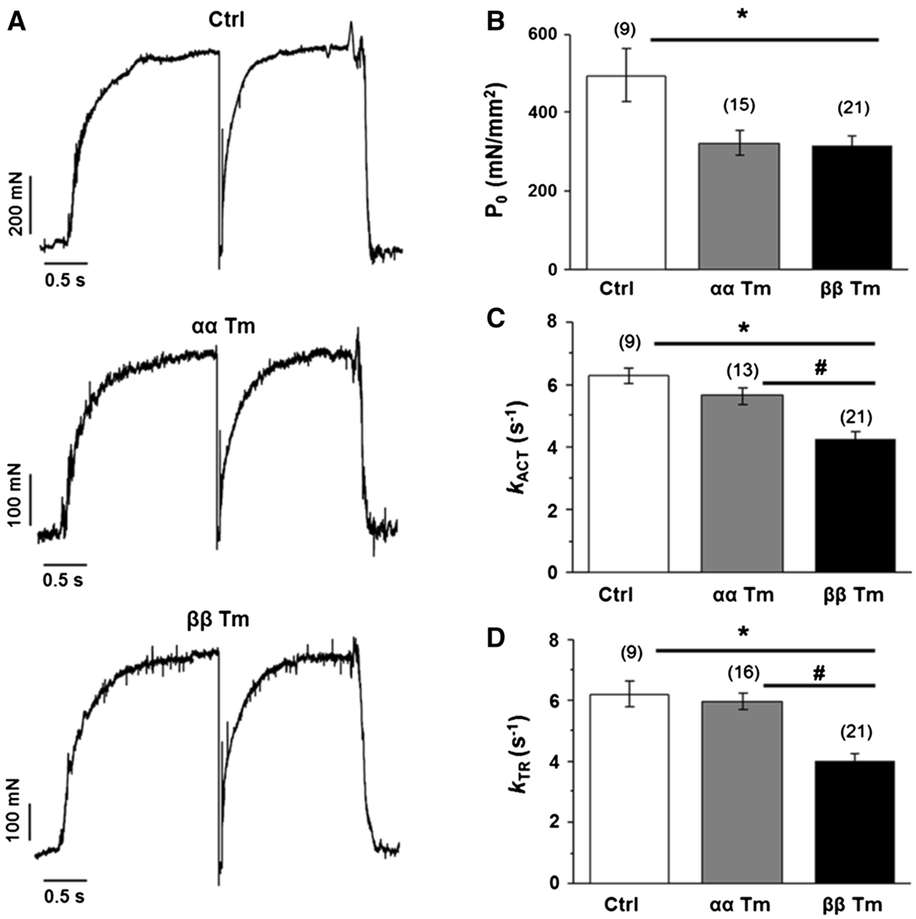


Fig. 2 Isometric active tension and kinetics of force generation in control and ααTm and ββTm reconstituted skeletal myofibrils. **a** Representative traces of rabbit psoas myofibrils maximally activated and fully relaxed by fast solution switching (15 °C) before (Ctrl) or after Tm–Tn extraction replacement with ααTm or ββTm. The rate of tension generation (k_{ACT}) was measured from the kinetics of force development following fast Ca^{2+} activation. Fast length changes of about 30 % (release–restretch protocol; length traces not shown) were applied to myofibrils under conditions of steady tension generation to measure the rate constant of tension redevelopment k_{TR}. *Ctrl* sl 2.77 μm, P_0 573 mN/mm², k_{ACT} 6.1 s^{-1}, k_{TR} 7.2 s^{-1}, *ααTm* sl 2.41 μm, P_0 365 mN/mm², k_{ACT} 5.5 s^{-1}, k_{TR} 4.8 s^{-1}, *ββTm* sl 2.5 μm, P_0 474 mN/mm², k_{ACT} 4.6 s^{-1}, k_{TR} 5.3 s^{-1}. **b** Histograms of mean values of maximal active tension P_0. **c** Kinetics of force development k_{ACT}. **d** Kinetics of force redevelopment k_{TR} of untreated (Ctrl, *white*), ααTm (*gray*) and ββTm (*black*) myofibrils. P_0 values were significantly reduced for ααTm and ββTm reconstituted myofibrils compared to Ctrl. In ββTm-reconstituted myofibrils, k_{ACT} and k_{TR} values were significantly decreased vs both control and ααTm reconstituted myofibrils. *Bars above columns* are s.e.m., number of myofibrils in *parenthesis*; values and statistical significance in Table 1 ($^{*,\,\#}$ $P < 0.05$)

activation levels just above the contractile threshold (Tesi et al. 2002b) or in the presence of truncated TnI subunits unable to fully inhibit acto-myosin interactions in the absence of Ca^{2+} (Narolska et al. 2006). This may indicate the presence in ββTm myofibrils of significant levels of Ca^{2+}-independent tension at rest. The finding that the rate of slow phase relaxation (Table 1) was unchanged by Tm isoforms support the hypothesis that slow k_{REL} is primarily determined by the cross-bridge detachment rate at maximal Ca^{2+} activation and then by the isoform of the myosin heavy chain motor (Poggesi et al. 2005).

Resting properties of myofibrils after extraction–replacement of Tm–Tn

Another consequence of the treatment associated to the extraction–replacement of Tm–Tn (besides the unspecific but significant decrease in P_0 and fast k_{REL}) is the decrease in mean sl of myofibrils mounted on the recording apparatus (Table 1; Fig. 1c). This length is adjusted before activation just above slack length, in order to reduce inhomogeneity in sarcomeres and to avoid movements of the myofibril during solution switching. As shown in

Table 1 Effect of Tm–Tn extraction–replacement with skeletal Tn and ααTm or ββTm on tension generation and relaxation of rabbit psoas myofibrils at 15 °C

Myofibril batches	Tension generation				Relaxation		
					Slow phase		Fast phase
	sl (μm)	P_0 (mN mm^{-2})	k_{ACT}(s^{-1})	k_{TR}(s^{-1})	Duration (ms)	k_{REL} (s^{-1})	k_{REL} (s^{-1})
Control	2.65 ± 0.03 (9)	494 ± 67 (9)	6.19 ± 0.41 (9)	6.27 ± 0.24 (9)	65 ± 6 (9)	2.13 ± 0.28 (9)	59 ± 6 (9)
ααTm	2.48 ± 0.02* (16)	323 ± 31* (15)	5.96 ± 0.26 (13)	5.63 ± 0.28 (16)	68 ± 3 (12)	2.16 ± 0.19 (11)	42 ± 5* (12)
ββTm	2.33 ± 0.04* (19)	313 ± 26* (21)	4.00 ± 0.23*# (21)	4.26 ± 0.22*# (21)	80 ± 3*# (19)	2.14 ± 0.23 (19)	27 ± 2*# (19)

Each group of data are from different myofibril batches. All values are given as mean ± s.e.m.; values in parentheses are the myofibril numbers

Control solution treated myofibrils not subjected to extraction–reconstitution protocol, *ααTm* ααTm–Tn reconstituted myofibrils, *ββTm* ββTm–Tn reconstituted myofibrils, *sl* slack sarcomere length of myofibrils mounted for force recording, P_0 maximum isometric tension, k_{ACT} rate constant for force development following maximal Ca^{2+}-activation, k_{TR} rate constant for force redevelopment following release–restretch of maximally activated myofibrils, k_{REL} rate constant of tension relaxation for slow and fast relaxation phases

* $P < 0.05$ (Student's *t* test) versus control myofibrils, # $P < 0.05$ versus ααTm myofibrils

Table 1 and Fig. 1c, ββTm myofibrils had a large decrease in mean sl that is significant also when compared to ααTm-enriched myofibrils (about 6 %).

The large decrease in mean sl of ββTm myofibrils mounted for force recording at pCa 9.0 suggests that the enrichment in ββTm is associated with an incomplete switched-off state of thin filaments in the absence of Ca^{2+}. To check this hypothesis, mean slack sl of control, ααTm and ββTm enriched myofibrils was measured in free myofibrils in the experimental chamber at pCa 9.0. As shown in Fig. 1c, slack sl of ββTm myofibrils was significantly shorter (1.83 ± 0.04 μm) compared to ααTm (2.28 ± 0.02 μm) and control (2.41 ± 0.03 μm). The addition of 10 mM BDM, an inhibitor of strong cross-bridge formation (McKillop et al. 1994; Regnier et al. 1995) did not significantly affect sl of control or ααTm myofibrils while it had a strong effect on ββTm myofibrils, increasing sl by about 15 % (2.11 ± 0.02; $P < 0.05$). This result suggests the presence of a significant level of Ca^{2+}-independent activation of force generating acto-myosin interactions in myofibrils replaced with ββTm. This feature is much less evident in the ααTm-replaced myofibrils.

To better investigate passive properties of myofibrils extracted and replaced for Tm–Tn, the steady-state sl–RT relationship was determined in the three myofibril groups by stretching them from slack to increasing sls and measuring the force-drop to zero following the imposition of a large and sudden release (about 30 % l_0). sl and RT were measured in conditions minimizing stress relaxation (Belus et al. 2010), i.e., about 10 min after the sl change. Details of the experimental protocol applied to myofibrils are shown in Fig. 4a (bottom trace), together with tension traces for a representative myofibril per each group. The average sl–passive tension relationships of control, ααTm and ββTm (Fig. 4b) are evidence that at rest (pCa 9.0), stiffness is significantly higher in ββTm and the relationship is significantly left-shifted compared to ααTm and non-exchanged controls. Modifications observed in ααTm myofibrils are much smaller and probably due to non-specific effects of the replacement treatment. Interestingly, ββTm reconstituted myofibrils also show an important recovery of force following the release of force to zero (Fig. 4a, third trace from top), suggesting the presence of actively cycling cross-bridges in the absence of Ca^{2+}. As shown in Fig. 4c (and Fig. 4a fourth trace from top), this feature as well as the difference in sl–RT relation between ββTm, ααTm and control myofibrils is almost completely abolished by inhibiting actively cycling cross-bridge formation by 10 mM BDM. The presence of a large and significant amount of Ca^{2+}-independent tension only in ββTm enriched myofibrils compared to ααTm and controls suggests that the finding is not merely due to the replacement method and supports the hypothesis that ββTm is

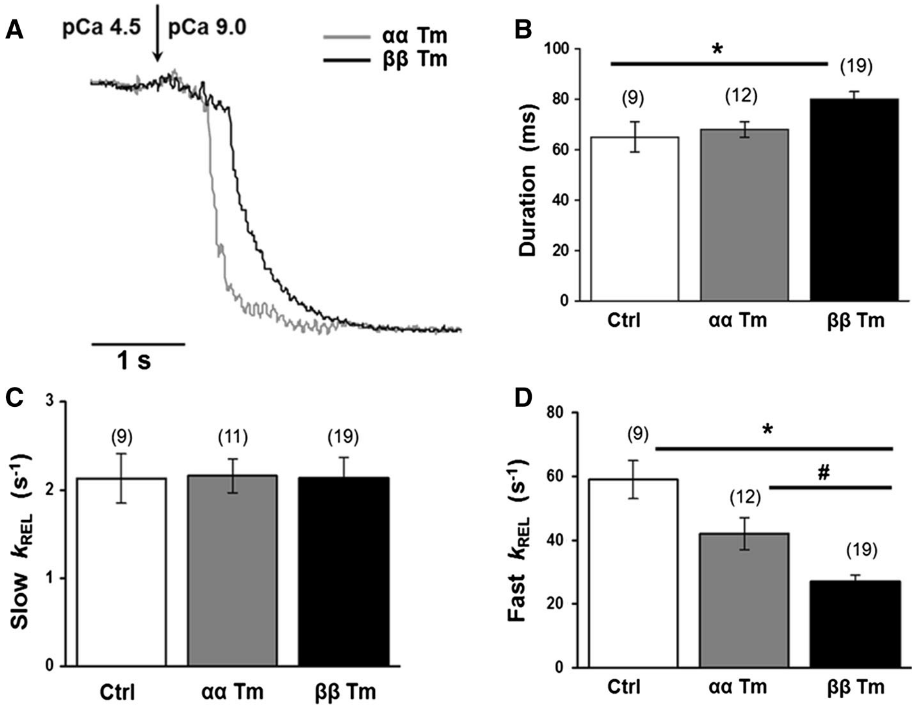


Fig. 3 Biphasic relaxation of force in ααTm and ββTm reconstituted skeletal myofibrils **a** Normalized force relaxation traces of representative ααTm and ββTm reconstituted myofibrils relaxed by switching perfusion back to the relaxing solution. *Gray trace* ααTm myofibril. Duration of slow phase 61 ms, slow k_{REL} 2.40 s^{-1}, fast k_{REL} 59 s^{-1}. *Black trace* ββTm myofibril. Duration of slow phase 88 ms, slow k_{REL} 1.95 s^{-1}, fast k_{REL} 27 s^{-1}. **b** Histograms of mean values of duration of slow phase. **c** Slow k_{REL}. **d** Fast k_{REL} of untreated (Ctrl, *white*), ααTm (*gray*) and ββTm (*black*) myofibrils. Relaxation of ββTm myofibrils was significantly prolonged. *Bars above columns* are s.e.m., number of myofibrils in *parenthesis*, values and statistical significance in Table 1 ($*^{,\,\#}$ $P < 0.05$)

unable to fully inhibit actomyosin interactions in the absence of Ca^{2+}.

ααTm versus ββTm reconstituted thin filament sliding in in vitro motility assays

For motility assays, thin filaments were reconstituted in flow cells on rabbit skeletal HMM coated surfaces, as previously described (Clemmens and Regnier 2004; Clemmens et al. 2005). Motility assay experiments showed that the presence of ββTm or ααTm did not affect reconstituted thin filament motility speed at high Ca^{2+} concentration. As shown in Fig. 5, the speed (A) and the fraction (B) of moving filaments at pCa 5.0 were indistinguishable in ααTm and ββTm filaments and similarly decreased with decreasing Ca^{2+} concentration. In agreement with previous observations in gelsolin-treated cardiac fibres (Lu et al. 2010), ββTm-reconstituted thin filaments showed a trend to a higher Ca^{2+} sensitivity of both the speed and the fraction of moving filaments, but the difference in the present study was not significant. Interestingly, the amount of Tm–Tn needed to reconstitute regulated actin filaments and stop the movement on HMM in resting conditions was different in the presence of ββTm or ααTm ($\geq$2 % moving at pCa 9.0). Achievement of complete regulation of the in vitro systems required greater ββTm (70–75 nM) concentrations compared to αα or native Tm–Tn (30–35 nM). These data further support the hypothesis that high levels of ββTm may result in an altered switch-off mechanism and partial Ca^{2+}-independent activation of reconstituted thin filaments.

Discussion

In the work we present here, we aimed to understand the functional role of the α and βTm isoforms of fast skeletal muscle by enriching the sarcomere of rabbit psoas myofibrils with their homodimeric forms, together with adult fast skeletal muscle Tn. The results of this work further confirmed the advantages and the limitations of the protocol we use to replace the entire Tm–Tn complex of regulatory proteins. As previously observed (Scellini et al. 2010) the mechanical performance of myofibrils was preserved in spite of a trend of the kinetics of force generation and fast force relaxation to slow down and a significant decrease in maximal isometric force. Myofibrils after the replacement procedure may also show a small loss of regulation, as suggested by the decrease of slack sl and its sensitivity to BDM also observed with the ααTm homodimer. These effects can be explained by some “run-down” of the

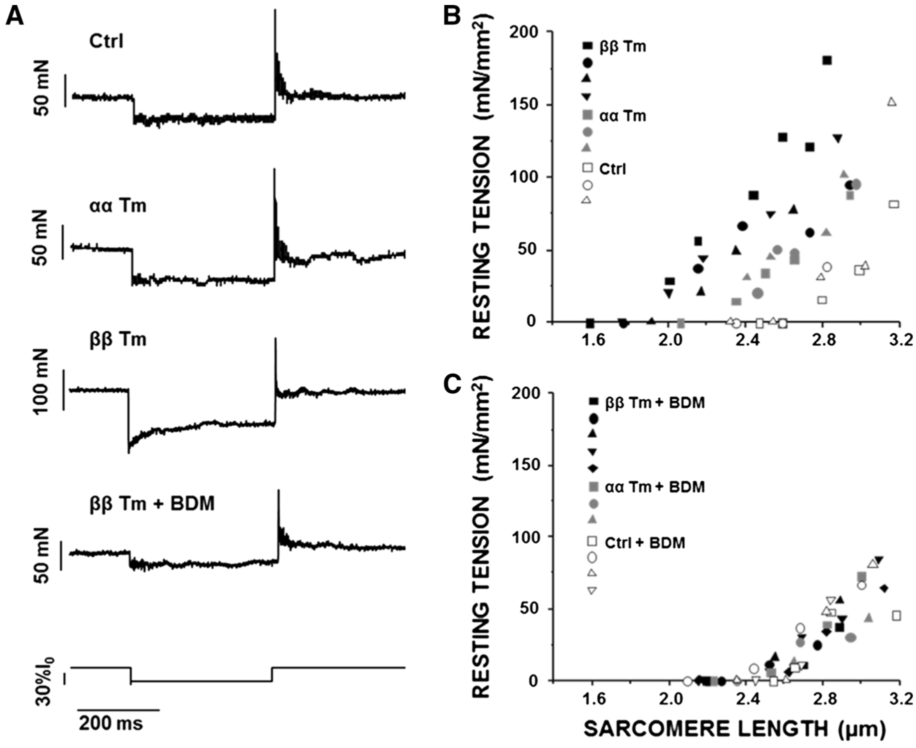


Fig. 4 Ca^{2+}-independent tension in ααTm and ββTm reconstituted myofibrils. **a** Resting tension responses following the imposition of 30 % length step releases to representative myofibrils mounted at slack length in relaxing solution (pCa 9.0). *Control* (*Ctrl*) *myofibril* sl 2.79 μm, RT 31 mN/mm^2, *ααTm myofibril* sl 2.76 μm, RT 52 mN/mm^2, *ββTm myofibrils* sl 2.73 μm, RT 122 mN/mm^2, *ββTm myofibril in the presence of 10 mM BDM* sl 2.77 μm, RT 25 mN/mm^2. *Bottom trace* myofibril length change. **b** Relation between sarcomere length and resting tension in control (*open symbols*), αα (*gray symbols*) and ββTm (*black symbols*) myofibrils. **c** Effect of 10 mM BDM. Data from single experiments

preparation, likely a consequence of the need to use rather un-physiological conditions (pH 8) to extract Tm from its tight association with thin filaments (Siththanandan et al. 2009). For this reason, we carefully designed experiments in order to differentially estimate the effects of the various Tm replacements, above the non-specific effect of the treatment. A further limitation of our method is that we could not fully control the Tm compositions of thin filaments, although we did achieve a significant modification of Tm isoform content in skeletal sarcomeres, from a prevalence of α over β (80:20) in control rabbit psoas muscle to an almost correspondent prevalence of β over α (77:23) in ββTm reconstituted myofibrils. Unfortunately, methods that permit a full control of thin filament composition by gelsolin treatment (Fujita et al. 1996, 2002, 2004; Lu et al. 2010) only work in the myocardium and leave the study of the functional role of Tm in skeletal muscle an unexplored field, at least by direct manipulation of sarcomere protein composition. Specific aim of this work was the investigation of the functional role of βTm, the product of the TPM2 gene that is usually present in low percentages in adult skeletal muscle and almost always in the heterodimeric αβ form (Bronson and Schachat 1982; Schachat et al. 1985; Briggs et al. 1990; for a review see Perry 2001). Interestingly, an increase in the presence of the ββTm is also observed in adult human skeletal muscles in the presence of TPM2 mutations associated with skeletal muscle myopathies (Tajsharghi et al. 2012). As the functional role of ββTm in skeletal muscle is unknown, in case of TPM2 mutations associated with skeletal myopathies, it is impossible to dissect functional effects due to Tm mutations themselves or due to the increased presence of ββTm in sarcomere. For this reason, we focused our study on the effects of ββTm per se, considering this as a test - case for the interpretation of the many studies that over the last 20 years tried to understand the possible differential role of Tm isoforms (mainly βTm) in striated muscle. Most of these studies were performed in cardiac muscle, using gelsolin-treated fibres or TG animals. Lu et al. (2010), using bovine cardiac fibres with cardiac Tn and ββTm reconstituted thin-filaments, reported no change in mechanical properties of contraction at saturated Ca^{2+} with a significant (0.2 pCa units) increase in Ca^{2+} sensitivity and a significant decrease of cooperativity. The preservation of maximal force and the increase in Ca^{2+} sensitivity was also observed in adult TG murine models overexpressing βTm in the heart at organ (Muthuchamy et al. 1995), trabeculae (Palmiter et al. 1996) or single cell (Wolska et al. 1999) levels. The presence of βTm in TG mouse hearts (NTG hearts are pure αTm) was accompanied by a decrease in the kinetics of force relaxation (Muthuchamy et al. 1995; Wolska et al. 1999) and in some cases

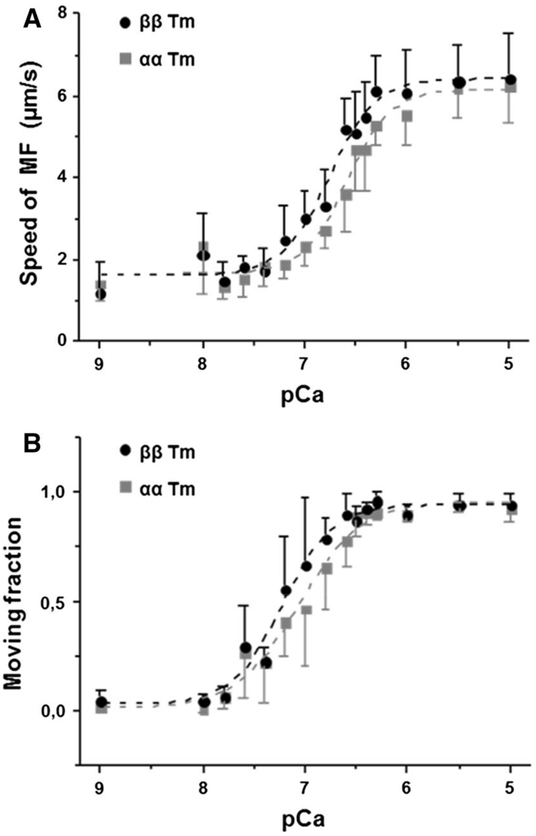


Fig. 5 Ca^{2+} regulation of in vitro motility assays of fully regulated thin filament reconstituted with ααTm or ββTm on fast skeletal HMM. **a** Speed of regulated actin filaments reconstituted with fast skeletal Tn and ααTm (*gray*) or ββTm (*black*) moving on skeletal heavy meromyosin (HMM) coated surfaces at different pCa. *Dashed lines* fit of relationships with Hill equation. *ααTm* pCa_{50} 6.58 ± 0.04 μm/s, *n* 2.03 ± 0.36, *ββTm* pCa_{50} 6.76 ± 0.05 μm/s, *n* 1.90 ± 0.32. **b** Fraction of regulated thin filament moving at different pCa. *Dashed lines* fit of relationships with Hill equation. *ααTm* pCa_{50} 7.06 ± 0.05, *n* 1.43 ± 0.21, *ββTm* pCa_{50} 7.22 ± 0.05, *n* 1.78 ± 0.29. *Symbols* represent mean ± SD. Differences are not significant. All proteins from rabbit fast skeletal muscle

also by a decrease of the speed of contraction and force development (Wolska et al. 1999; for a review of mouse model investigation of Tm function see Jagatheesan et al. 2010).

Results from the present study also show that in skeletal muscle myofibrils at full Ca^{2+} activation, the increase in sarcomeric ββTm content up to about 80 % had no or minor effect on maximal isometric force. The kinetics of force development and redevelopment were essentially preserved in ββTm myofibrils compared to ααTm-enriched or control myofibrils, being the small but significant reduction of k_{ACT} and k_{TR} observed in ββTm myofibrils likely explained by the presence of significant amount of Ca^{2+}-independent tension. A similar decrease in k_{ACT} and k_{TR}, with no change in P_0 and with prolongation of relaxation phase was previously observed in both cardiac (Narolska et al. 2006) and skeletal (Belus et al. 2007) myofibrils in the presence of truncated forms of TnI unable to completely switch-off thin filaments in the absence of Ca^{2+}. Regarding the functional role of Tm isoforms in skeletal muscle, the only information available comes from solution studies of Tm isoforms in the assembly with actin filaments and interacting with myosin heads (Boussouf et al. 2007; for a review see Janco et al. 2012). These studies observed no significant difference in Ca^{2+} sensitivity of skeletal myosin S1 binding to thin filaments reconstituted with skeletal Tn and pure ααTm or ββTm, a condition that simulates the fast skeletal myofibrils of our experiments. Interestingly, Boussouf et al. observed that in the presence of cardiac Tn, the Ca^{2+} sensitivity of thin filaments reconstituted with ββTm was greater (about 0.2 pCa units) compared with thin filaments reconstituted with ααTm, as previously observed in gelsolin treated cardiac fibres (Lu et al. 2010) and TG mouse overexpressing ββTm (for a discussion of this differential effect see Boussouf et al. 2007). Our in vitro motility data and preliminary experiments in rabbit psoas myofibrils replaced with the two Tm isoforms (Scellini et al. 2011) are in agreement with solution studies of skeletal systems, reporting no difference in Ca^{2+} sensitivity with just a trend to an increase in ββTm myofibrils.

A novel finding of the present work is that in skeletal muscle, ββTm compromises thin filament regulation in resting conditions (pCa 9.0), as suggested by the decrease in resting slack sl, left shift of the sl–passive tension relation and by the prolongation of relaxation (increase in the duration of the slow phase and decrease in the rate of the fast phase). All these effects observed in ββTm-enriched myofibrils suggest an altered switch-off mechanism and partial Ca^{2+}-independent activation of thin filaments. The specific effect of BDM on resting sl and resting length–tension relation strongly suggests the presence of a significant amount of actively cycling cross-bridges in the absence of Ca^{2+} in ββTm myofibrils. Interestingly, the same behaviour was previously observed in skeletal myofibrils showing partial loss of regulation associated with truncated forms of TnI (Belus et al. 2007) or engineered Tms with reduced flexibility (Scellini et al. 2012). As to the prolongation of the slow phase of relaxation and the decrease in fast k_{REL} observed in ββTm myofibrils, they could be due to a reduction of Ca^{2+} dissociation from thin filament and then extended cross-bridges life time (Nixon et al. 2013) and/or to a modification in the equilibria of Ca^{2+} dependent regulation processes of muscle contraction (Scellini et al. 2012). We favour this second explanation because when an engineered TnC with reduced Ca^{2+} dissociation rate (k_{off}) was exchanged in skeletal myofibrils, it led to a prolongation of the slow phase of relaxation without the decrease of fast k_{REL} that was observed here in ββTm myofibrils (Kreutziger et al. 2008). On the other hand, a similar increase in the duration of the slow relaxation phase and decrease in fast k_{REL} was observed in control rabbit psoas myofibrils partially relaxed to Ca^{2+}-activation levels just above the contractile threshold (Tesi et al. 2002a, b) or, in both skeletal and cardiac myofibrils, in the presence of truncated cTnI unable to fully inhibit acto-myosin interactions in the absence of Ca^{2+} (Narolska et al. 2006; Belus et al. 2007). The partial loss of regulation in resting conditions observed here with ββTm cannot be explained by Tm reintroduction failure. The absence of some Tm molecules in the expected position on actin filaments would lead to discontinuous formation of cross-bridge leading to irregular modification of sl along the myofibril and eventually to the disruption of the fragile lattice structure. In addition very small (less than 2 %) reintroduction failure is expected from the gel analysis shown in Fig. 1.

On the other hand, murine TG models overexpressing βTm showed modifications of the regulation state in relaxing conditions and of the relaxation kinetics similar to what observed here in myofibrils after ββTm replacement (Muthuchamy et al. 1995; Jagatheesan et al. 2010).

Within the widely accepted three-state theory of muscle regulation (McKillop and Geeves 1993), the effects observed here in the presence of high levels of ββTm in skeletal sarcomeres could be explained by a reduction of the fraction of “Blocked” states in absence of Ca^{2+} (about 50 % in skeletal muscle—Maytum et al. 2003) and the formation of a “Myosin-induced open state” (Lehrer 2011). This hypothesis is supported by structural studies of the location of Tm on F-actin filaments free of Tn (Lehman et al. 2000) reporting that in the presence of β isoform the molecule lays preferentially on the inner domain of actin in a closed-like “C-state” position away from the blocked state, which is occupied by the ααTm form. Many factors associated with Tm isoforms may change the fractional occupancy of the myosin induced open state (which is low in control conditions). For example, it is possible that the packing of Tm on actin filaments could be altered by the presence of homodimeric ββTm mixed with ααTm and/or that the head–tail overlap of αα with ββTm disrupts the TnT binding site, leading to the partial loss of regulation observed here (see discussion in Lu et al. 2010). This situation may simulate the physiological state of muscles overexpressing TPM2. Interestingly, engineered Tm variants with reduced molecular flexibility induce functional alterations when replaced in myofibrils, which are very similar to what observed here in the presence of increased content of ββTm in sarcomeres (Scellini et al. 2012). The possibility of a difference in molecular flexibility between ααTm and ββTm forms is supported by spectroscopic measurements of rotational dynamics of Tm on actin surface which was decreased in the presence of 20 % β-chains compared to pure αα (Chandy et al. 1999). In conclusion, our study using skeletal muscle myofibrils replaced with Tm isoforms in the homodimeric form suggest that the overexpression of the TPM2 gene product could lead to an incomplete switch-off of thin filaments in the absence of Ca^{2+}. This effect could be further amplified or damped by the matching of the Tn subunit isoforms, by the presence of mutations in the Tm molecule (as those associated with myopathies and cardiomyopathies) or, as recently suggested, by the isoforms of the myosin motor itself (Kopylova et al. 2013).

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Conflict of interest None.

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