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The role of tropomyosin isoforms and phosphorylation in force generation in thin-filament reconstituted bovine cardiac muscle fibres

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Abstract

The thin filament extraction and reconstitution protocol was used to investigate the functional roles of tropomyosin (Tm) isoforms and phosphorylation in bovine myocardium. The thin filament was extracted by gelsolin, reconstituted with G-actin, and further reconstituted with cardiac troponin together with one of three Tm varieties: phosphorylated α Tm (α Tm.P), dephosphorylated α Tm (α Tm.deP), and dephosphorylated β Tm (β Tm.deP). The effects of Ca, phosphate, MgATP and MgADP concentrations were examined in the reconstituted fibres at pH 7.0 and 25°C. Our data show that Ca^{2+} sensitivity (pCa_{50} : half saturation point) was increased by 0.19 ± 0.07 units when β Tm.deP was used instead of α Tm.deP ($P < 0.05$), and by 0.27 ± 0.06 units when phosphorylated α Tm was used ($P < 0.005$). The cooperativity (Hill factor) decreased (but insignificantly) from 3.2 ± 0.3 (5) to 2.8 ± 0.2 (7) with phosphorylation. The cooperativity decreased significantly from 3.2 ± 0.3 (5) to 2.1 ± 0.2 (9) with isoform change from α Tm.deP to β Tm.deP. There was no significant difference in isometric tension or stiffness between α Tm.P, α Tm.deP, and β Tm.deP muscle fibres at saturating $[\text{Ca}^{2+}]$ or after rigor induction. Based on the six-state cross-bridge model, sinusoidal analysis indicated that the equilibrium constants of elementary steps differed up to 1.7x between α Tm.deP and β Tm.deP, and up to 2.0x between α Tm.deP and α Tm.P. The rate constants differed up to 1.5x between α Tm.deP and β Tm.deP, and up to 2.4x between α Tm.deP and α Tm.P. We conclude that tension and stiffness per cross-bridge are not significantly different among the three muscle models.

Keywords

Muscle; Mechanics; Regulation; Tropomyosin; Isoform; Phosphorylation; Kinetics; Cross-bridge cycle; Elementary steps; Sinusoidal analysis

Introduction

Tropomyosin (Tm) molecules in association with filamentous actin are found in virtually all eukaryotic cells and have numerous roles. Encoded by four genes TPM₁–TPM₄ (α – δ respectively) multiple isoforms arise by alternative transcriptional promoters and by alternative splicing of primary gene transcripts. In skeletal and cardiac muscles these isoforms play a pivotal role in the Ca²⁺ regulation of contraction together with members of the troponin (Tn) complex consisting of the TnT (Tm binding), TnI (inhibitory) and TnC (Ca²⁺ binding); For reviews see Perry (1998, 2001), Smillie (1999), and Gordon et al. (2000). Each Tm molecule is a dimer of two identical or similar α -helical polypeptide chains of 284 amino acid residues configured as a coiled-coil in parallel and in register. Through head-to-tail interaction and interdigitation of 8–9 residues at their N- and C-terminal ends, they form an extended continuum or cable of Tm molecules lying along the actin filament. Spanning seven actin monomers on each strand of F-actin, each Tm molecule also interacts with the Tn complex through the TnT and TnI components. At low levels of Ca²⁺ (10^{−7} M), Tm blocks the myosin binding sites of actin (Haselgrove 1972; Huxley 1972; Chalovich 1992; Tobacman 1996; Cooke 1997). This state presumably corresponds to the “blocked state” described by McKillop and Geeves (1993). If Ca²⁺ concentration exceeds a threshold, it binds to TnC and this signal is transmitted through TnI and TnT to Tm-actin. Tm, in turn, makes an azimuthal shift around the axis of the actin filament, thereby exposing the myosin binding site (Huxley 1972; Kress et al. 1986; Vibert et al. 1997; Xu et al. 1999). This state presumably corresponds to the “closed state” (or “cocked state”) (McKillop and Geeves 1993). Myosin binds to the closed state, possibly altering the actin conformation (Tobacman 1996), thereby increasing the affinity of Tm for actin. This results in a further azimuthal shift of Tm, as determined by cryo EM (Vibert et al. 1997; Rosol et al. 2000) to the “open state” (McKillop and Geeves 1993), in which the actomyosin interaction and concomitant energy transduction are fully active. The interaction of Tm, actin and myosin is mutual and cooperative (Tobacman and Butters 2000). This chain of reactions can be summarized by stating that myosin allosterically affects actin to promote Tm binding, and conversely, Tm allosterically affects actin to promote myosin binding.

From NMR analyses of synthetic peptides a structure for the 8–9 residue overlap of Tm ends has been described (Greenfield et al. 2006). A significantly different X-ray structure at 2.1 Å resolution has been reported more recently (Murakami et al. 2008). This region is an important interaction site for the binding of the TnT component of the Tn complex. Based on antibody, protein chemistry and structural evidence (Ohtsuki 1979; Mak and Smillie 1981; Flicker et al. 1982; Cabral-Lilly et al. 1997) the Tn complex is now known to be highly asymmetrical with a globular portion interacting with Tm in the region of Cys-190 (2/3 of the molecular distance from its N-terminus) and a tail-like portion interacting with and extending beyond the C-terminal/N-terminal overlap. This asymmetry is largely due to the TnT component which, of the three troponin components, plays the major role in anchoring the complex to the overlap region in an antiparallel manner.

The efficiency and properties of the contractile apparatus, including its Ca²⁺ sensitivity, are a function of the isoform levels of the various contractile elements as well as their phosphorylation levels. Tm in adult fast striated muscles is present as two major isoforms, α and β (Cummins and Perry 1973), products of their respective genes. Their relative abundance is variable from α : β ratios of 9:1 as in longissimus dorsi to 1:1 in plantaris muscles (Bronson and Schachat 1982). In adult cardiac tissue of small animals (mouse, rabbit, guinea pig, rat, dog) $\alpha\alpha$ dimer is predominant, and the β isoform is virtually absent. In the slower beating hearts of larger mammals (sheep, pig, bovine, human) the β component constitutes 15–20% of the total (Leger et al. 1976; Ookubo et al. 1975; Rodriguez et al. 2008). The available evidence (Eisenberg and Kielley 1974; Lehrer 1975; Bronson and

Schachat 1982; Brown and Schachat 1985; Muthuchamy et al. 1995) indicates a preferred $\alpha\beta$ species as opposed to the $\alpha\alpha$ or $\beta\beta$ dimers. Thus, in adult skeletal and cardiac tissues where the $\alpha:\beta$ ratio is equal to or greater than 1 only the $\alpha\alpha$ and $\alpha\beta$ dimeric species can be expected to have significant roles. A possible exception is the tongue muscle of rabbit as observed by Bronson and Schachat (1982) for which $\beta\beta$ dimers were observed even though the $\alpha:\beta$ ratio was close to 1:1. The other notable exception is that for fetal tissue as examined in the rabbit and chicken (Amphlett et al. 1976; Roy et al. 1979; Briggs et al. 1990), where $\beta\beta$ dimer is the predominant form until close to birth, replaced by $\alpha\beta$ postnatally, and further replaced by $\alpha\alpha$ and $\alpha\beta$ in the adult. These exceptions may be related to a stabilizing effect on the $\beta\beta$ dimer by coordinately expressed TnT isoforms (Schachat et al. 1985; Briggs et al. 1990).

In a comparison of the rabbit skeletal α Tm and β Tm sequences, Mak et al. (1980) noted that there are only 39 differences out of 284 amino acid residues. It was predicted that because of the conservative nature of the bulk of the substitutions, particularly in *b*, *c* and *f* “outer” positions of the heptad repeat, that they would have little effect on β Tm’s actin binding properties. More recently this presumption has been reinforced by an examination of those amino acids identified as either “proposed” or “consensus” residues for actin interaction (Brown et al. 2005; Singh and Hitchcock-DeGregori 2006). In both cases (α Tm and β Tm) the majority of residues implicated in actin binding are identical with only a small number of highly conserved residue substitutions. More recently, the actin binding affinity of the two isoforms has been measured experimentally and found to be only modestly different (Boussouf et al. 2007).

It was also noted (Mak et al. 1980) that while the 39 amino acid differences are distributed throughout their sequences, a disproportionate number (25) occur in the C-terminal half as compared with 14 in the N-terminal half. Of the former, two charge differences (from α to β : Ser-229-Glu and His-276-Asn) result in a slightly more acidic β Tm molecule. The negative charge at residue 229 at an inner *e* position repeat can be expected to locally destabilize the coiled-coil since Glu occupies the *g* position of the preceding heptad (Glu-224). The replacement of the imidazole side chain of the His residue at position 276 by that of Asn may be related to a possible difference in head-to-tail interaction properties of the two Tms as well as their interaction properties with the T1 portion of TnT. A significantly weaker interaction of the rabbit skeletal TnT1 fragment with β Tm has in fact been demonstrated (Pearlstone and Smillie 1982). Two other more conservative differences in the head-to-tail region of Tm (α to β : Met-281-Ile and Ile-284-Leu) may also contribute to this weaker binding. As indicated above, present evidence suggests that the T2 fragment of TnT in complex with TnI and TnC interacts over an extensive region of α Tm centered at Cys-190, i.e. about two-third of the molecular distance from its N-terminal end and corresponding to constitutive exon 5 (residues 165–188) and alternatively spliced exon 6b (residues 189–213). Interestingly α Tm and β Tm show significant differences in both these regions, residues 172–179 and 185–192. The actin binding, Tn interaction, and functional properties of exon 6b in striated muscle α Tm have been investigated by Hammell and Hitchcock-DeGregori (1997), and Maytum et al. (2004).

Phosphorylation of Tm has been observed in muscles across the phylogenetic tree (Ribolow and Bárány 1977; Mak et al. 1978; O’Connor et al. 1979; Montarras et al. 1981, 1982; Heeley et al. 1982, 1989; Hayley et al. 2008). In embryonic skeletal and cardiac muscles the Tm is highly phosphorylated compared to adult muscle tissues (Heeley et al. 1982) suggesting the possible importance of Tm phosphorylation during development. In both α and β Tms, phosphorylation occurs at Ser-283 (Mak et al. 1978) by a Tm specific kinase (Montgomery and Mak 1984; de Belle and Mak 1987), the penultimate residue at the C-terminus. Not much is known about this kinase except that it requires Mg^{2+} and its MW is

~250 kDa, but it does not require cAMP or Ca-calmodulin (de Belle and Mak 1987). The extra negative charge promotes enhanced head-to-tail interaction of neighbouring skeletal α Tm molecules (Heeley et al. 1989; Sano et al. 2000), and an interaction with skeletal TnT or whole Tn. It also promotes, in the presence of Ca^{2+} , ATPase activity in a reconstituted skeletal-Tm-Tn-actin-S1 system (Heeley 1994). Recently Rao et al. (2009) have provided evidence for increased “long range” myosin activation of thin filaments with phosphorylated Tm but not by dephosphorylated Tm. This may be related to the increased head-to-tail interaction of Tm observed with phosphorylated Tm.

In this report, we employed the thin filament extraction and reconstitution technique (Fujita et al. 1996, 2002; reviewed by Kawai and Ishiwata 2006), and the difference in α Tm and β Tm, and the effect of α Tm phosphorylation were studied. With this technique $\geq 90\%$ of Tm can be exchanged, hence it compares favourably to the transgenic approach. The results indicate that the Ca^{2+} sensitivity increases from α Tm to β Tm and with phosphorylation. The thin filament cooperativity is decreased from α Tm to β Tm, but the cooperativity does not change significantly with phosphorylation. In saturating Ca^{2+} , the kinetic constants of the elementary steps of the cross-bridge cycle have the maximum effect of 1.7x from α Tm to β Tm; the maximum effect is 2.4x with phosphorylation. Force and stiffness during full activation and rigor are not significantly affected by isoform change or phosphorylation.

Materials and methods

Chemicals and solutions

The source of chemicals and names of solutions are the same as reported (Lu et al. 2003). In brief, the standard activating solution (5S8P) contained (mM) 6 K_2CaEGTA , 5.8 Na_2MgATP , 1.36 $\text{Na}_2\text{K}_2\text{ATP}$, 15 creatine phosphate (Na_2CP), 8 phosphate (Pi) (mixture of 4 H_2KPO_4 and 4 HK_2PO_4), 1 sodium propionate (NaProp), 73 KProp , 10 NaN_3 , 10 MOPS, and 320 units/ml (0.64 mg/ml) creatine kinase (CK). pCa of this solution is 4.66, $[\text{Mg}^{2+}]$ is 0.5 mM, $[\text{Na}^+]$ is 55 mM, ionic strength 200 mM, and pH adjusted to 7.00. Other activating solutions are variations of this solution by keeping ionic strength at 200 mM, $[\text{Mg}^{2+}]$ at 0.5 mM, and $[\text{Na}^+]$ at 55 mM. All activating solutions include CP and CK, except for the ADP study (0D-3D series), which include 0.2 mM P^1, P^5 -di(adenosine-5') pentaphosphate (A_2P_5) instead. A solution designated 00D contained the same ingredients as 0D with an addition of CP/CK but no A_2P_5 . pCa ($= -\log[\text{Ca}^{2+}]$) was changed in the range of 4.66–7.00 by changing the ratio $[\text{CaEGTA}]:[\text{EGTA}]$ and keeping $[\text{EGTA}]_{\text{total}}$ at 6 mM in the standard activating solution. The pCa 4.00 and 4.30 solutions were made by appropriately adding CaProp_2 and by increasing $[\text{Na}_2\text{K}_2\text{ATP}]$ in the standard activating solution while keeping ionic strength and $[\text{Mg}^{2+}]$ constant by deleting KProp . The relaxing solution (R) contained (mM) 6 EGTA, 2 MgATP , 5 free ATP, 55 KProp , 61 NaProp , 10 NaN_3 , 10 MOPS, and 40 BDM (2,3-butanedione monoxime). The rigor solution (Rg) contained (mM) 8 Pi , 76 NaProp , 103 KProp and 10 MOPS.

Muscle fibres and proteins

Bovine myocardium were obtained and prepared as described (Fujita et al. 2002; Lu et al. 2003). Actin was extracted from acetone powder (a present from Dr. Shin'ichi Ishiwata) (Kondo and Ishiwata 1976) from rabbit fast skeletal muscles according to the method of Spudich and Watt (1971). Purified G-actin was stored at 0°C and used within 1 week after dialysis. Bovine plasma gelsolin was prepared according to the method of Kurokawa et al. (1990).

α Tm was obtained from rabbit cardiac muscles, and β Tm from rabbit back muscles as described (Smillie 1982). The phosphorylated α Tm used in the present study was prepared

by chromatographic methods as described (Heeley et al. 1989) and labeled as α Tm.P. The phosphorylation level of α Tm and β Tm was lowered to <5% either by column chromatography or by treatment with phosphatase (Heeley et al. 1989) and labeled as α Tm.deP and β Tm.deP, respectively. Phosphorylated β Tm was not used because of the scarcity of β Tm, and the low level of phosphorylation of this particular isoform (Mak et al. 1978; Montarras et al. 1981; Heeley et al. 1982). Amino acid sequence analyses have demonstrated that the Tm of rabbit cardiac muscles is purely α -form and is identical to the α -form of rabbit skeletal muscle (Lewis and Smillie 1980). Tn was prepared from rabbit cardiac muscle as described (Potter 1982).

SDS-PAGE

Proteins were dissolved at the concentration of 1 mg/ml. 2 μ l α Tm.deP, 4 μ l rabbit skeletal Tm, and 2 μ l β Tm.deP were loaded in a 15% SDS-acrylamide running gel and 4% stacking gel as reported (Fujita et al. 2002; Kawai et al. 2009). Electrophoresis was performed at 25 mA for 10 min, and then the current was increased to 40 mA until the tracking dye reached the bottom of the gel. Proteins were stained with Coomassie brilliant blue R250.

Alkaline urea PAGE

Proteins were dissolved at the concentration of 1 mg/ml. 2 μ l α Tm.P, 2 μ l α Tm.deP, and a mixture of 1 μ l α Tm.deP and 1 μ l α Tm.P were loaded in a 10% alkaline urea acrylamide gel and electrophoresed for 2 h at 200 V.

Two dimensional PAGE

1 mg/ml α Tm.deP in the Tm and Tn reconstitution buffer without BDM, 1 mg/ml α Tm.P in the buffer without BDM, 1 mg/ml α Tm.P in the buffer with 40 mM BDM, and 1 mg/ml α Tm.deP and 1 mg/ml α Tm.P mixture in the buffer without BDM were incubated on ice for 15 h. 15 μ l of above solutions were loaded on 17 cm, pH 4.7–5.9 IPG (immobilized pH gradients) strips for the first dimension electrophoresis. The IPG strips were put on 12% SDS-acrylamide running gel. The electrophoresis was performed at 40 mA until the tracking dye reached the bottom of the gel. Proteins were stained with Coomassie brilliant blue R250.

Sinusoidal analysis

During an activation, the muscle length was oscillated sinusoidally in 18 frequencies (f) in the range 0.13 Hz and 100 Hz and at a small amplitude (0.125% L_0), and the complex modulus data $Y(f)$ were collected as described (Kawai and Brandt 1980). The complex modulus data were fitted to Eq. 1 with two exponential processes (Kawai et al. 1993; Wannenburg et al. 2000; Fujita et al. 2002), and parameters of exponential processes were obtained.

$$Y(f) = \underset{\text{Process B}}{H - \frac{Bfi}{b+fi}} + \underset{\text{Process C}}{\frac{Cfi}{c+fi}} \quad (1)$$

where $i = \sqrt{-1}$, and H is a constant. Stiffness, which is $Y(f)$ extrapolated to the infinite frequency ($Y_\infty = H - B + C$), is just about the same as the value measured at 100 Hz in cardiac muscles. $2\pi b$ and $2\pi c$ are the apparent rate constants of processes B and C, respectively, and B and C are their respective magnitudes. Process A is not observed in myocardium at temperatures $\leq 25^\circ\text{C}$ (Lu et al. 2006).

Experimental procedure

Myocardium preparations with a diameter of $\sim 100\ \mu\text{m}$ and the length of $\sim 4\ \text{mm}$ were dissected from skinned muscle bundles. One end was attached to a tension transducer and the other end to a length driver with a small amount of nail polish. Preparations were stretched until a small passive tension was detected. At this point, the diameter ($\phi = 103 \pm 4\ \mu\text{m}$; $n = 34$) and the length ($L_0 = 2.14 \pm 0.05\ \text{mm}$; $n = 34$) of the preparations were measured under a dissecting microscope (20 $\times$). The average sarcomere length was in the range 1.9–2.1 μm as detected by confocal microscopy (Fujita et al. 2002). The preparations were further skinned in relaxing solution (Rx) containing 1% Triton X-100 at 25°C for 20 min before each experiment. Triton X-100 was washed out with Rx immediately after the skinning. The extraction and reconstitution procedures were performed at 0–2°C and as reviewed (Kawai and Ishiwata 2006). Activation of fibres was performed at 25°C, and relaxation was performed at 0°C.

Statistical analysis

Data were expressed as means $\pm$ S.E.M. Data were analyzed by using SAS 9.2 software, and one way ANOVA was performed, followed by Bonferoni pairwise comparison between any two groups.

Results

Identification of Tm isoforms

We first determined the purity of αTm and βTm preparations by SDS-PAGE. For this purpose, 15% acrylamide was used for running gel, and 4% for stacking gel. The results are shown in Fig. 1A. Lane 1 is the αTm preparation, and lane 3 is the βTm preparation. Lane 2 is rabbit skeletal Tm which is a mixture of the two, but with more α than β , hence it serves as a convenient marker. From Fig. 1A we conclude that both preparations are 95–100% pure. On SDS, the molecular weight of αTm appeared to be smaller than βTm by 6 kD, which is consistent with earlier results (Muthuchamy et al. 1995, 1998). Although this gel does not have adequate sensitivity to show the level of phosphorylation, neither αTm or βTm preparation used here is phosphorylated.

The level of phosphorylation of αTm

We then determined the level of phosphorylation of αTm with two methods. One was alkaline urea polyacrylamide gel electrophoresis, and the other was two dimensional (2D) gel electrophoresis. Figure 1B shows the result from the alkaline urea PAGE. Lane 1 is phosphorylated αTm ($\alpha\text{Tm.P}$), lane 3 is dephosphorylated αTm ($\alpha\text{Tm.deP}$), and lane 2 is an approximate 1:1 mixture of both. From this figure we conclude that the $\alpha\text{Tm.P}$ preparation is $\sim 100\%$ phosphorylated, and the $\alpha\text{Tm.deP}$ preparation is $\sim 0\%$ phosphorylated.

The other method we used to demonstrate the phosphorylation was 2D gel electrophoresis with pH in the first dimension and SDS (12% acrylamide in the running gel) in the second dimension. The pH range covered was 4.0–7.0. The results are shown in Fig. 2. Figure 2A is the $\alpha\text{Tm.deP}$ preparation, and Fig. 2B is the $\alpha\text{Tm.P}$ preparation. These preparations are the same as those used in Fig. 1B. Figure 2D is a mixture of $\alpha\text{Tm.deP}$ and $\alpha\text{Tm.P}$, but prepared separately from Fig. 1B, lane 2. Figure 2A, B and D were treated with the reconstitution solution in the absence of BDM at 0–2°C for 15 h. Figure 2C is $\alpha\text{Tm.P}$ treated with the reconstitution solution in the presence of 40 mM BDM at 0–2°C for 15 h. This experiment was carried out because there are reports that BDM works as a “chemical phosphatase” and it has a “phosphatase-like activity” (Stapleton et al. 1998; Waurick et al. 1999).

As expected, α Tm.P moved closer to the positive pole (to left of the figure) than α Tm.deP (Fig. 2A, B). The isoelectric point (pI) of α Tm.P was ~ 4.81 , pI of α Tm.deP was ~ 4.89 , and their difference was ~ 0.08 , assuming that the distance traveled is linear to pH. From Fig. 2A, B, we conclude that the α Tm.P preparation is $\sim 100\%$ phosphorylated, and the α Tm.deP preparation is $\sim 0\%$ phosphorylated, which is consistent to Fig. 1B. From Fig. 2B, C, we conclude that 40 mM BDM did not work as a chemical phosphatase under our experimental conditions.

Thin filament extraction and reconstitution

Extraction and reconstitution of the thin filament were performed as described (Fujita et al. 1996, 2002; Lu et al. 2003; Kawai and Ishiwata 2006), and the result is shown in Fig. 3A–D. A cardiac muscle fibre was first activated briefly (Fig. 3A) in the standard activating solution at 25°C , followed by thin filament extraction in the solution that contained (mM) 2 K_2CaEGTA , 2.2 $\text{Na}_2\text{K}_2\text{ATP}$, 121 KCl , 4.25 MgCl_2 , 2 leupeptin, 2 diisopropyl fluorophosphates, 40 BDM, 20 MOPS, 0.3 mg/ml gelsolin, and pH 7.00 at 2°C (Fig. 3A, marked G). Extraction was stopped with the relaxing solution when the remaining tension was $\leq 10\%$ of the original tension (Fig. 3B), which took 60–90 min.

The thin filament extracted fibre was then treated with the actin-filament reconstitution solution four times (7 min each) that contained (mM): 4 $\text{K}_2\text{H}_2\text{EGTA}$, 4 Na_2MgATP , 8 KCl , 80 KI , 40 BDM, 20 Pi , 1 mg/ml G-actin, and pH 7.00 at 2°C (Fig. 3B, Ac). After the actin filament reconstitution, the fibre was activated briefly and isometric tension (T_{ac}) was measured in the standard activating solution (Fig. 3C). T_{ac} averaged 13.8 ± 1.5 kPa ($N = 34$), which is usually 50–70% of the initial tension (Fig. 3A) (Fujita et al. 2002). T_{ac} is low, because it was measured in the presence of 8 mM added Pi (Cooke and Pate 1985; Kawai 1986), and in the absence of regulatory proteins (Fujita et al. 2002). All subsequent tension and stiffness data were normalized to T_{ac} to minimize a scatter of the data caused by a possible variation in the length of actin filaments. This method also minimizes the error due to inaccurate estimate of the diameter of fibres.

The actin filament reconstituted fibre was further reconstituted with three kinds of Tm in the presence of rabbit cardiac Tn overnight (12–15 h) at 0 – 2°C in the solution that contained (mM): 6 $\text{K}_2\text{H}_2\text{GTA}$, 2.2 Na_2MgATP , 5 $\text{Na}_2\text{K}_2\text{ATP}$, 8 $\text{K}_{1.5}\text{H}_{1.5}\text{Pi}$, 41 NaProp , 75 Kprop , 10 MOPS, 40 BDM, 1.0 mg/ml Tm, 1.5 mg/ml Tn, and pH 7.00 (Fig. 3C, marked Tm and Tn). Because we used α Tm.P, α Tm.deP and β Tm.deP for thin-filament reconstitution, the reconstituted fibres are called the α Tm.P model, the α Tm.deP model, and the β Tm.deP model, respectively. The effects of MgATP (Fig. 3E), Ca (Fig. 3F), Pi (Fig. 3G), and MgADP (Fig. 3H) were studied with the reconstituted preparations. Rigor tension and stiffness were collected after the final activation (Fig. 3I).

pCa-tension relationship

As Tm is a regulatory protein, it is possible that a small difference in its molecular structure makes a large difference at a partial activation. For this reason, the reconstituted fibres were activated at 25°C with pCa ranging 7.00–4.00 (as in Fig. 3F) in the standard activating solution in which 6 mM K_2CaEGTA was replaced with a proper mixture of K_2CaEGTA and $\text{K}_2\text{H}_2\text{EGTA}$. Tension and stiffness were measured, and the result is shown in Fig. 4. As this figure shows, both tension and stiffness behaved similarly. The α Tm.deP model exhibited the lowest Ca^{2+} sensitivity (curves are shifted to the right), and the Ca^{2+} sensitivity increased in the α Tm.P model and in the β Tm.deP model. The cooperativity, judged by the slope of the pCa-tension plot, may have decreased slightly from the α Tm.deP model to the α Tm.P model, but it decreased significantly from the α Tm.deP model to the β Tm.deP

model. The pCa-tension plots were fitted to the Hill equation (Eq. 2; Brandt et al. 1982) after normalization to the maximum tension.

$$\text{Tension} = \frac{1}{1 + \left(\frac{\text{Ca}_{50}}{[\text{Ca}^{2+}]} \right)^{n_H}} \quad (2)$$

where Ca_{50} is the dissociation constant of Ca^{2+} . pCa_{50} is half saturation point and used for Ca^{2+} sensitivity. The thin filament cooperativity is judged by the Hill factor (n_H), which is $1.737x (=4 \log_{10}e)$ of the slope of the pCa-tension plot at pCa_{50} . These parameters were deduced for each experiment, and averaged for 5–9 experiments and summarized in Table 1. As this table shows, Ca^{2+} sensitivity increased by phosphorylation of αTm by 0.27 ± 0.06 units with error propagation, and from the $\alpha\text{Tm.deP}$ model to the $\beta\text{Tm.deP}$ model by 0.19 ± 0.07 units. The cooperativity decreased from 3.2 ± 0.3 (5) to 2.8 ± 0.2 (7) with phosphorylation, but the change (0.4 ± 0.4) is insignificant. The cooperativity also decreased from 3.2 ± 0.3 (5) to 2.1 ± 0.2 (9) with isoform change from $\alpha\text{Tm.deP}$ to $\beta\text{Tm.deP}$, and this change (1.4 ± 0.4) is significant.

Tension and stiffness of the three muscle models

We then asked whether isometric tension and stiffness at saturating Ca^{2+} concentration are different depending on the Tm model. Three muscle models were activated with the standard activating solution, and tension and stiffness were measured. After normalization by T_{ac} , the data were averaged for 9–10 preparations, and the results are shown in Table 1. Tension increased 1.6–2.0 fold when Tm and Tn were reconstituted and as reported by Fujita and Kawai (2002), Fujita et al. (2002), and Lu et al. (2003). However, there was hardly any difference among three muscle models within the experimental error. The same was true for the stiffness. Consequently, there was hardly any difference in the tension:stiffness ratio.

In the activating condition in the presence of ATP, some cross-bridges are in the detached or weakly attached state, hence tension or stiffness does not represent all cross-bridges. Because we found that the actomyosin interface depends on the type of Tm, which modifies tension and stiffness (Lu et al. 2003; Kawai et al. 2009), it is also desirable to compare three models when all cross-bridges are attached. For this reason, three muscle models were brought to the “high-rigor condition” (Kawai and Brandt 1976) immediately after the standard activation (see Fig. 3I for an example). Initially, tension higher than the standard activation was maintained, then it gradually declined to a steady state in 5–7 min, where rigor tension and stiffness were measured (note oscillation of force in Fig. 3I). The results are shown in Table 2. From the standard activation to rigor, tension increased by 10–50%, and stiffness increased to 2–4 fold. However, within the experimental error, there is hardly any difference in tension, stiffness, or their ratio among the three muscle models examined. This result is consistent with those during activation (Table 1). These results suggest that once fully activated with Ca^{2+} and ATP, or in the absence of ATP (rigor), there is little difference in tension and stiffness between αTm and βTm models, or the phosphorylation status of αTm .

Cross-bridge kinetics in the standard activating solution

The complex modulus data during the standard activation are compared in Fig. 5 for three muscle models. They appear to be similar in shape: they have the general appearance of myocardium and slow twitch skeletal muscle fibres (Kawai et al. 1993; Wang and Kawai 2001; Wannenbourg et al. 2000; Fujita et al. 2002), although some difference in the position

of peaks can be noticed in Fig. 5A, B. The complex modulus data were fitted to Eq. 2, the fitted parameters were averaged for 9–10 experiments, and results are summarized in Table 1. Also included is $f_{\min}$, that is the frequency at which the dynamic modulus assumes a minimum. This value is close to the characteristic frequency b in myocardium (Table 1). Table 1 demonstrates that, under the control activating condition, all parameters shown are not significantly different among the three muscle models we have examined.

Elementary steps of the cross-bridge cycle

The control activation is under the one particular condition, which may not show a difference in cross-bridge kinetics. For this reason, the Pi concentration (Fig. 3G), the MgATP concentration (Fig. 3E), and the MgADP concentration (Fig. 3H) were changed at saturating $[Ca^{2+}]$ (pCa 4.66), and elementary steps of the cross-bridge cycles (Scheme 1) were characterized. In Scheme 1, A = actin, M = myosin, D = MgADP, S = MgATP, and P = Pi = phosphate. The uppercase letter K indicates the association or equilibrium constant, and the lowercase letter k indicates the rate constant. These are as a whole called the kinetic constants.

The force generation step 4 and the Pi release step 5 can be characterized by studying the effect of Pi (Fig. 3G). Figure 6A, B respectively plot tension and stiffness obtained from the Pi study. Tension and stiffness decreased and approached saturation with an increase in the Pi concentration in all 3 models, which is consistent with earlier reports on skeletal muscle fibres (Cooke and Pate 1985; Kawai 1986; Bowater and Sleep 1988; Stienen et al. 1990), on skeletal myofibrils (Tesi et al. 2002), as well as on myocardium (Herzig et al. 1982; Kawai et al. 1993; Fujita et al. 2002). In our study, both tension and stiffness had no significant differences among the three models as the Pi concentration was changed. The kinetic constants of steps 4 and 5 can be characterized by plotting the sum (Fig. 6C) and the product (Fig. 6D) of $2\pi b$ and $2\pi c$ (Kawai et al. 1993; Lu et al. 2005). Both plots are increasing functions of $[Pi]$ with concave downwards and can be approximated by hyperbolic functions. From these plots, we derived k_4 , k_{-4} , and K_5 , which are listed in Table 3.

The steps associated with ATP binding (step 1) and the cross-bridge detachment (step 2) that ensue the binding can be characterized by studying the effect of MgATP (Fig. 3E). Figure 7A, B respectively plot tension and stiffness obtained from the MgATP study. Tension and stiffness appeared to have a peak at 0.1–0.2 mM MgATP, but otherwise they did not change significantly with MgATP in all 3 models, which is consistent with earlier results on skeletal muscle fibres (Kawai and Brandt 1977; Ferenczi et al. 1982; Kawai and Halvorson 1989) as well as on myocardium (Kawai et al. 1993; Lu et al. 2005). As in the Pi study, both tension and stiffness were not significantly different among three muscle models. The kinetic constants of steps 1 and 2 can be characterized by plotting the sum (Fig. 7C) and the product (Fig. 7D) of $2\pi b$ and $2\pi c$ (Kawai et al. 1993; Lu et al. 2005). Both plots are increasing functions of $[MgATP]$ with concave downwards and can be approximated by hyperbolic functions. From these plots, we derived K_1 , k_2 , and k_{-2} , which are listed in Table 3.

The ADP binding step 0 can be characterized by studying the effect of MgADP (0–3 mM) (Fig. 3H) on $2\pi c$ at a constant $[Na_2MgATP]$ (2 mM) and $[Pi]$ (8 mM) (Kawai and Halvorson 1989). The resulting tension is plotted in Fig. 8A, stiffness in Fig. 8B, and the rate constant $2\pi c$ in Fig. 8C. As is seen in Fig. 8C, rate constant plots are decreasing functions of MgADP with concave upwards and can be approximated by hyperbolic functions. The extra solution (00D), which has CP and CK but otherwise is identical to the 0D solution (no added ADP), was tested and the apparent rate constant $2\pi c$ was placed on the respective hyperbolic curves (filled symbols). Their negative displacement from the origin along the abscissa gives contaminating MgADP in the 0D solution. This value was found to be in the range of 0.05–0.13 mM for three muscle models. Tension and stiffness increased and approached

saturation with the increase in the MgADP concentration. This result is consistent with other reports (Cooke and Pate 1985; Kawai and Halvorson 1989; Kawai and Zhao 1993). The tension and stiffness in the 00D solution were almost the same as the 0D solution (Fig. 8A, B). This is expected, because the contaminating ADP in the 0D solution is only 0.05–0.13 mM (above) and small. This result is consistent with our previous results (Lu et al. 2003; Lu et al. 2005). As in the Pi and the ATP studies, both tension and stiffness were not significantly different among three muscle models examined.

From the studies of Pi, MgATP, and MgADP, all kinetic constants that characterize the cross-bridge scheme 1 were deduced as described (Kawai et al. 1993; Lu et al. 2005), and the results are listed in Table 3. As this Table demonstrates, there were some variations among the three muscle models. With phosphorylation, the kinetic constants either stayed the same (k_2 , k_4 , k_{-4} , K_5) increased (K_0 , K_1) by 1.4–1.99x, or decreased (k_{-2}) by 2.4x. When isoforms were compared from α Tm to β Tm, the kinetic constants either stayed the same (k_2 , k_{-2} , k_{-4} , K_5), or increased (K_0 , K_1 , k_4) by 1.3–1.7x.

To examine whether the population of strongly attached cross-bridges (AM, AMD, AMS, AM*DP, AM*D) are different among the three muscle models, this was calculated for the standard activating condition ($[MgATP] = 5$ mM, $[Pi] = 8$ mM, $[MgADP] = 0.02$ mM) based on equations 8–14 of Zhao and Kawai (1996) and the kinetic constants listed in Table 3. The results demonstrate that the population is $35 \pm 8\%$ (α Tm.P), $50 \pm 6\%$ (α Tm.deP), and $53 \pm 3\%$ (β Tm.deP) with error propagation. To deduce force/cross-bridge, the tension values in Table 1 were divided by respective population to result in $4.5 \pm 1.1 T_{ac}$ (α Tm.P), $4.0 \pm 0.6 T_{ac}$ (α Tm.deP), and $3.2 \pm 0.3 T_{ac}$ (β Tm.deP). These values are not significantly different, indicating that the tension/cross-bridge is not significantly different among three muscle models.

Discussion

The purpose of our experiments was to determine how unphosphorylated α Tm and β Tm differ functionally, and how α Tm phosphorylation affects activation of the regulatory system, the amount of force generated, and the elementary steps of the cross-bridge cycle. Our results indicate that the differences are most pronounced under conditions of partial Ca^{2+} activation (Table 1; Fig. 4). At full Ca^{2+} activation, neither tension nor stiffness differs significantly regardless of which Tm form is present (Table 1), although the kinetic constants do differ to some extent (Table 3).

Ca^{2+} sensitivity

We found that the Ca^{2+} sensitivity (pCa_{50}) of phosphorylated α Tm was 0.27 ± 0.06 greater than that of the unphosphorylated form (Table 1). We also found that pCa_{50} is 0.19 ± 0.07 greater in β Tm.deP than in α Tm.deP, which is consistent with Palmiter et al. (1996). Thus, ordering the muscle models based on pCa_{50} results in α Tm.P > β Tm.deP > α Tm.deP. This indicates that β Tm.deP may have a similar, but weaker role of α Tm.P. The phosphorylation takes place at Ser-283. Also of interest is His-276 of α Tm, which is Asn in β Tm, hence there is a charge change. According to the NMR solution structure, His-276 does not participate in the head-to-tail interaction among α Tm molecules, and is largely exposed to the solution (Greenfield et al. 2006); hence it could interact with TnT. Ser-283, however, is involved in interchain interactions among Tm molecules, binding to the Lys-7 and Lys-12 of the next Tm (Greenfield et al. 2006; Johnson and Smillie 1977). In the crystal structure, His-276 of the α Tm L-arm interacts with TnT Ala-80 and Asp-83, which in turn interact with Lys-12 of the next Tm (Murakami et al. 2008). His-276 also abuts TnT Phe-86, suggesting that β Tm Asn-276 can interact hydrophobically with TnT Phe-86. In the crystal structure, Ser-283 on each Tm arm is likewise exposed to the solution. The fact that the Ca^{2+} sensitivity changes

with phosphorylation implies that either Ser-283 interacts directly with TnT, or the phosphorylation signal is transmitted through the coiled-coil structure of Tm to modify the Tm–TnT interaction elsewhere, just as a signal is transmitted through the coiled-coil of Tm (Farah and Reinach 1999) or that of dynein's stalk (Kon et al. 2009). The fact that phosphorylated Ser-283 has an average charge of -1.5 at pH 7.0 lead us to hypothesize that disruption of the C-terminal charge plays a role in the Ca^{2+} sensitivity (Gaffin et al. 2004). Because the charge at His-276 (αTm) approaches $+1$ ($\text{pK}_a = 7.27\text{--}7.98$ was reported in the presence of TnT1: Brisson et al. 1986), the net charge at pH 7.0 for residues 274 through 284 is about -2.5 for $\alpha\text{Tm.P}$, -2.0 for $\beta\text{Tm.deP}$, and -1.0 for $\alpha\text{Tm.deP}$, which is the same order as that of pCa_{50} . We infer from this analysis that an increase in the negative charge at the C-terminus of the Tm molecules results in an increase in Ca^{2+} sensitivity. Evidence for and against this hypothesis is as follows.

A series of reports on studies of transgenic mice has described the effects of expressing βTm and chimeric $\alpha\beta$ Tm in the murine heart, an organ in which the level of βTm is normally very low ($\sim 2\%$). These studies demonstrated that an elevation in βTm expression is accompanied by a reduction in αTm expression to maintain the total Tm content, which must be a consequence of translational control. When the high βTm expression level (75%) was examined, the effect was severe, and resulted in death at 10–14 postnatal days (Muthuchamy et al. 1998). Pathological abnormalities included thrombus formation, atrial enlargement and fibrosis, as well as impairment of contraction and relaxation. It is possible that the increase in Ca^{2+} sensitivity and the decrease in cooperativity in this scenario are the cause of malfunction. Our result demonstrates that βTm leads to increases in the nucleotide binding (K_0 and K_1) and the rate of force generation (k_4) compared to those of αTm (Table 3), which leads to a change in the contraction and relaxation kinetics. When βTm expression was intermediate (50–65%), deleterious effects were less serious, with the rates of maximal contraction and relaxation reduced, the pCa_{50} of the myofibrillar ATP hydrolysis rate and fibre force increased, and a reduction in maximal force at saturating Ca^{2+} (Wolska et al. 1999).

A further consideration in the above transgene studies was the influence of Tn isoforms. Since βTm is virtually absent in adult mouse hearts, and Tm and TnT isoforms are coordinately expressed throughout development, the introduction of βTm by transgenesis as a replacement for αTm may well lead to a Tm–Tn mismatch with unpredictable consequences. Experimental evidence supporting the notion that such a mismatch influences Ca^{2+} sensitivity was provided by a recent report (Boussouf et al. 2007). In this solution study, when actin filament was reconstituted with skeletal Tn, pCa_{50} of the rate constant measuring pyrene fluorescence in the stopped flow method was the same regardless of whether α or βTm was used. In contrast, when reconstituted with cardiac Tn, pCa^{50} was 0.2 units higher in βTm than αTm . Although this reservation concerning the potential effects of a mismatch between βTm and rabbit cardiac Tn applies to the present report, it is not relevant to the effects of phosphorylation of adult rabbit cardiac αTm and cardiac Tn, because the sequences of the rabbit skeletal Tm and cardiac Tm are identical and encoded by the same gene (Lewis and Smillie 1980). We also note that the whole rabbit cardiac Tn employed in the present study is isolated from adult rabbit.

Heeley (1994) reported that pCa_{50} of the steady state ATP hydrolysis rate, as measured in the solution system using rabbit skeletal muscle proteins, did not differ with the phosphorylation of Tm. This does not necessarily contradict our finding that pCa^{50} increased with phosphorylation, because the two reports differ in several aspects: (1) experimental model (isolated proteins vs. structured muscle fibres), (2) ionic strength (50 mM vs. 200 mM), (3) the rate-limiting step (step 3 vs. step 6), (4) the parameters measured (ATP hydrolysis rate vs. isometric tension and kinetic constants), and (5) the tissue subtype

(skeletal vs. cardiac). In particular, the steady state ATP hydrolysis rate measures the rate-limiting step (the slowest forward step) in the cross-bridge cycle. If step 3 is the rate-limiting step as in the solution system, then a change in the Ca^{2+} -sensitive step 4 has little effect on the ATP hydrolysis rate. In fibre studies, the kinetic constants measure fast steps. The primary states that bear force are the AM*DP and AM*D states (Scheme 1), and the rate-limiting step is step 6 rather than step 3; hence a small change in the Ca^{2+} sensitivity (step 4) is readily reflected in the force measurement. In the case of proteins alone, electrostatic interactions are enhanced at low ionic strength (as in solution studies) but decreased at high ionic strength (as in fibres), yet the hydrophobic interaction is not affected. Additional differences may arise from large scale cooperativity, which is present in fibres, but not present in the solution system. Finally, the use of skeletal muscle proteins in the Heeley study may have resulted in differences from our results on cardiac muscles.

Cooperativity

Large-scale cooperativity could take place in the following interactions: (1) Tn–Tm, (2) Tm–Tm, (3) Tm–actin, and (4) actin–myosin. The cooperativity, as deduced from the Hill factor (n_H) of the pCa-tension curve, may be influenced by contributions from all of them. In terms of the Tn–Tm interaction (1), the weaker interaction of Tn with β Tm than with α Tm (Mak et al. 1980; Pearlstone and Smillie 1982), and the decrease in n_H from α Tm to β Tm (Table 1), are consistent with each other. In terms of the Tm–Tm interaction (2), there is evidence suggesting that Tm phosphorylation increases the head-to-tail association of Tm molecules (Heeley et al. 1989; Sano et al. 2000), and interactions between neighboring Tm molecules are known to increase n_H (Ding et al. 2002). Therefore, the fact that n_H is lower for α Tm.deP than for β Tm.deP (Table 1) may mean that the effect of phosphorylation is eclipsed by the Tn–Tm interaction (1). In terms of the Tm–actin interaction (3), Tm (and Tn) is known to enhance the actin–myosin interaction (Murray et al. 1982; Chalovich 1992; Tobacman 1996; Fujita et al. 2002, 2004). In terms of the actin–myosin interaction (4), the presence of cycling cross-bridges is known to contribute significantly to the steep Ca^{2+} response (Güth and Potter 1987) and to the increase in thin-filament activation (McKillop and Geeves 1993).

Kinetic constants of elementary steps

Interestingly, our results on the kinetic constants (Table 3) indicate that the effects of a change from α Tm to β Tm and those of α Tm phosphorylation are in the same direction for steps 0–2, but in opposite directions for steps 4–5. This observation implies that a change in Tm affects the cross-bridge interaction between myosin and actin differently depending on the status of nucleotide, i.e. before (steps 0–2) or after (steps 4–5) cleavage of the ATP's γ -phosphate. This is reasonable because the conformation of myosin changes dramatically with the cleavage of ATP (Wakabayashi et al. 1992), which results in a recovery stroke of the lever arm.

Isometric tension

Force generation occurs at step 4 (isomerization of the AM.ADP.Pi complex), and the same force is maintained at step 5 (Pi release) as demonstrated in rabbit psoas muscle fibres (Kawai and Halvorson 1991; Fortune et al. 1991; Dantzig et al. 1992; Kawai and Zhao 1993; Takagi et al. 2004) and in myofibrils (Tesi et al. 2002). Our current results extend our earlier finding that adding Tm and Tn to actin filament-reconstituted muscle fibres augments the force ($T > T_{ac}$ in Table 1) generated by each cross-bridge (Fujita and Kawai 2002; Fujita et al. 2002). They demonstrate that the degree of force augmentation does not differ between α Tm and β Tm, or does not depend on the phosphorylation status of α Tm. Our further analysis indicates that force/cross-bridge or force augmentation/cross-bridge is not significantly different among three muscle models. This force augmentation is presumably

due to the fact that the actomyosin interaction surface increases as actin is polymerized and regulatory proteins are added, resulting in a more stable actin–myosin linkage (Fujita and Kawai 2002; Fujita et al. 2002; Lu et al. 2003; 2006). Similarly, rigor tension and stiffness did not differ significantly with a change in the Tm isoform or its phosphorylation (Table 2). From this observation we infer that the rigor state is not influenced by the Tm isoform or its phosphorylation. In other words, once rigor is achieved, the quality of the actin–myosin interface does not differ among three muscle models studied. This conclusion is consistent with those presented in our recent report showing that rigor stiffness is not affected when truncated Tm mutants are introduced to the thin filament (Kawai et al. 2009).

Our tension result for Tm phosphorylation is at variance with the findings presented in a report by Vahebi et al. (2007), who also studied heart muscle from transgenic mice. This group reported that tension decreased by ~26% when the level of Tm-pseudophosphorylation was decreased from 40% to ~20%. A linear extrapolation of their result suggests that an up to 3.7 $[(1 + 0.26 \times 3)/(1 - 0.26 \times 2)]$ -fold increase in tension would be observed if the level of Tm phosphorylation were increased from 0% to 100%. While we are aware that the relationship may not be linear, this calculation nevertheless suggests that phosphorylation should have a significant effect on tension. In no case did we observe such a large increase in tension when comparing α Tm.deP and α Tm.P. The difference in the two reports may be a consequence of the methods used. Vahebi et al. (2007) used MAP kinase to increase phosphorylation, which may have resulted in the phosphorylation/dephosphorylation of multiple proteins, whereas the current study altered the level of phosphorylation of Tm only (from 0% to 100%).

The mechanism underlying the macromolecular interactions

In order for Tm to modify the elementary steps of the cross-bridge cycle, it must affect actin and/or myosin. The fact that the binding of both ATP and ADP increases when Tm is phosphorylated (Table 3) implies that a change in the charge of the Tm C-terminus modifies the conformation of the nucleotide-binding pocket of myosin. The pathway of this information flow is Tm $\rightarrow$ actin $\rightarrow$ myosin; i.e., the state of Tm allosterically affects the conformation of actin, which in turn affects the conformation of myosin (Tobacman and Butters 2000; Fujita et al. 2002; Lu et al. 2003, 2006). Tm is not likely to have a direct allosteric effect on myosin because no strong interaction between these two molecules has been demonstrated. We pointed out the possibility that the positively charged loop 1 of myosin may serve as a carrier of negatively charged MgATP^{2-} ; hence, the position of loop 1 may determine the nucleotide affinity (Wang and Kawai 2001). This direction of information flow would be the reversal of that proposed by other investigators, with the binding of S1 to actin affecting the conformation of actin (Hill et al. 1980; Greene and Eisenberg 1988) and this, in turn, increasing the affinity of Tm for actin (Chalovich 1992); i.e., the direction is: myosin $\rightarrow$ actin $\rightarrow$ Tm. Taken together, these results lead us to conclude that the information flow is bidirectional, and that the two sets of studies may be seeing the same phenomenon from two opposing ends. The fact that the ATP association constant (K_1) increases by 1.9x with Tm phosphorylation (Table 3) implies that the contour of the ATP binding pocket is also affected by the status of Tm, supporting the hypothesis that information flows from Tm to actin, and from actin to myosin.

Conclusion

We have demonstrated that Ca^{2+} sensitivity increases when β Tm is used instead of α Tm, and when α Tm is phosphorylated. These observations imply that the electrostatic force may stabilize the TnT1 interaction with the C-terminal domain of Tm. In saturating Ca^{2+} , the maximum effect of the isoform switch on the kinetic constants of the elementary steps of the cross-bridge cycle is a 1.7x increase, and that of a change to the phosphorylated state is an

increase of 2.4x. These results suggest that Tm allosterically affects the conformation of actin, which in turn affects the actomyosin interaction. Force and stiffness during activation or rigor induction do not differ much among the three muscle models.

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References

- Amphlett GM, Syska H, Perry SV. The polymorphic forms of tropomyosin and troponin I in developing rabbit skeletal muscle. *FEBS Lett.* 1976; 63:22–26. [PubMed: 1261686]
- Boussouf SE, Maytum R, Jaquet K, Geeves MA. Role of tropomyosin isoforms in the calcium sensitivity of striated muscle thin filaments. *J Muscle Res Cell Motil.* 2007; 28:49–58. [PubMed: 17436057]
- Bowater R, Sleep J. Demembranated muscle fibers catalyze a more rapid exchange between phosphate and adenosine triphosphate than actomyosin subfragment 1. *Biochemistry.* 1988; 27:5314–5323. [PubMed: 3167048]
- Brandt PW, Cox RN, Kawai M, Robinson T. Effect of cross-bridge kinetics on apparent Ca^{2+} sensitivity. *J Gen Physiol.* 1982; 79:997–1016. [PubMed: 6980967]
- Briggs MM, McGinnis HD, Schachat F. Transitions from fetal to fast troponin T isoforms are coordinated with changes in tropomyosin and α -actinin isoforms in developing rabbit skeletal muscle. *Dev Biol.* 1990; 140:253–260. [PubMed: 2373251]
- Brisson JR, Golosinska K, Smillie LB, Sykes BD. Interaction of tropomyosin and troponin T: a proton nuclear magnetic resonance study. *Biochemistry.* 1986; 25:4548–4555. [PubMed: 3768297]
- Bronson DD, Schachat FH. Heterogeneity of contractile proteins: differences in tropomyosin in a fast, mixed and a slow skeletal muscles of the rabbit. *J Biol Chem.* 1982; 257:3937–3944. [PubMed: 7061518]
- Brown HR, Schachat FH. Renaturation of skeletal muscle tropomyosin: implications for in vivo assembly. *Proc Nat Acad Sci USA.* 1985; 82:2359–2363. [PubMed: 3857586]
- Brown JH, Zhou Z, Reshetnikova L, Robinson H, Yammani RD, Tobacman LS, Cohen C. Structure of the mid-region of tropomyosin: bending and binding sites for actin. *Proc Nat Acad Sci USA.* 2005; 102:18878–18883. [PubMed: 16365313]
- Cabral-Lilly D, Tobacman LS, Mehegan JP, Cohen C. Molecular polarity in tropomyosin–troponin T co-crystals. *Biophys J.* 1997; 73:1763–1770. [PubMed: 9336171]
- Chalovich JM. Actin mediated regulation of muscle contraction. *Pharmacol Ther.* 1992; 55:95–148.
- Cooke R. Actomyosin interaction in striated muscle. *Physiol Rev.* 1997; 77:671–697. [PubMed: 9234962]
- Cooke R, Pate E. The effects of ADP and phosphate on the contraction of muscle fibers. *Biophys J.* 1985; 48:789–798. [PubMed: 3878160]
- Cummins P, Perry SV. The subunits and biological activity of polymorphic forms of tropomyosin. *Biochem J.* 1973; 133:765–777. [PubMed: 4270662]
- Dantzig J, Goldman Y, Millar NC, Laktis J, Homsher E. Reversal of the cross-bridge force-generating transition by the photogeneration of phosphate in rabbit psoas muscle fibres. *J Physiol.* 1992; 451:247–278. [PubMed: 1403812]
- de Belle I, Mak AS. Isolation and characterization of tropomyosin kinase from chicken embryo. *Biochim Biophys Acta.* 1987; 925:17–26. [PubMed: 3593768]
- Ding W, Fujita H, Kawai M. The length of cooperative units on the thin filament in rabbit psoas muscle fibres. *Exp Physiol.* 2002; 87:691–697. [PubMed: 12447449]

- Eisenberg E, Kielley WW. Troponin–tropomyosin complex. Column chromatographic separation and activity of the three active troponin components with and without tropomyosin present. *J Biol Chem.* 1974; 249:4742–4748. [PubMed: 4276966]
- Farah CS, Reinach FC. Regulatory properties of recombinant tropomyosins containing 5-hydroxytryptophan: Ca^{2+} -binding to troponin results in a conformational change in a region of tropomyosin outside the troponin binding site. *Biochemistry.* 1999; 38:10543–10551. [PubMed: 10441151]
- Ferenczi MA, Simmons RM, Sleep JA. General considerations of cross-bridge models in relation to the dependence on MgATP concentration of mechanical parameters of skinned fibers from frog muscles. *Soc Gen Physiol Ser.* 1982; 37:91–107. [PubMed: 6216600]
- Flicker PF, Phillips GN Jr, Cohen. Troponin and its interactions with tropomyosin. An electron microscope study. *J Mol Biol.* 1982; 162:495–501. [PubMed: 7161805]
- Fortune NS, Geeves MA, Ranatunga KW. Tension responses to rapid pressure release in glycerinated rabbit muscle fibers. *Proc Natl Acad Sci (USA).* 1991; 88:7323–7327. [PubMed: 1871140]
- Fujita H, Kawai M. Temperature effect on isometric tension is mediated by regulatory proteins tropomyosin and troponin in bovine myocardium. *J Physiol.* 2002; 539:267–276. [PubMed: 11850518]
- Fujita H, Yasuda K, Niitsu S, Funatsu T, Ishiwata S. Structural and functional reconstitution of thin filaments in the contractile apparatus of cardiac muscle. *Biophys J.* 1996; 71:2307–2318. [PubMed: 8913572]
- Fujita H, Sasaki D, Ishiwata S, Kawai M. Elementary steps of the cross-bridge cycle in bovine myocardium with and without regulatory proteins. *Biophys J.* 2002; 82:915–928. [PubMed: 11806933]
- Fujita H, Lu X, Suzuki M, Ishiwata S, Kawai M. The effect of tropomyosin on force and elementary steps of the cross-bridge cycle in reconstituted bovine myocardium. *J Physiol.* 2004; 556:637–649. [PubMed: 14742733]
- Gaffin RD, Gokulan K, Sacchettini JC, Hewett T, Klevitsky R, Robbins J, Muthuchamy M. Charged residue changes in the carboxy-terminus of alpha-tropomyosin alter mouse cardiac muscle contractility. *J Physiol.* 2004; 556.2:531–543. [PubMed: 14766940]
- Gordon AM, Homsher E, Regnier M. Regulation of contraction in striated muscle. *Physiol Rev.* 2000; 80:853–924. [PubMed: 10747208]
- Greene LE, Eisenberg E. Relationship between regulated actomyosin ATPase activity and cooperative binding of myosin to regulated actin. *Cell Biophys.* 1988; 12:59–71. [PubMed: 2453286]
- Greenfield NJ, Huang YJ, Swapna GV, Bhattacharya A, Rapp B, Singh A, Montelione GT, Hitchcock-DeGregori SE. Solution NMR structure of the junction between tropomyosin molecules: implications for actin binding and regulation. *J Mol Biol.* 2006; 364:80–96. [PubMed: 16999976]
- Güth K, Potter JD. Effect of rigor and cycling cross-bridges on the structure of troponin C and on the Ca^{2+} affinity of the Ca^{2+} -specific regulatory sites in skinned rabbit psoas fibers. *J Biol Chem.* 1987; 262:13627–13635. [PubMed: 3654633]
- Hammell RL, Hitchcock-DeGregori SE. The sequence of the alternatively spliced sixth exon of α -tropomyosin is critical for cooperative actin binding but not for interaction with troponin. *J Biol Chem.* 1997; 272:22409–22416. [PubMed: 9278391]
- Haselgrove JC. X-ray evidence for a conformational change in the actin-containing filaments of vertebrate striated muscle. *Cold Spring Harb Symp Quant Biol.* 1972; 37:341–352.
- Hayley M, Chevaldina T, Mudalige WA, Jackman DM, Dobbin AD, Heeley DH. Shark skeletal muscle tropomyosin is a phosphoprotein. *J Muscle Res Cell Motil.* 2008; 29:101–107. [PubMed: 18763042]
- Heeley DH. Investigation of the effects of phosphorylation of rabbit striated muscle $\alpha\alpha$ -tropomyosin and rabbit skeletal muscle troponin T. *Eur J Biochem.* 1994; 221:129–137. [PubMed: 8168502]
- Heeley DH, Moir AJ, Perry SV. Phosphorylation of tropomyosin during development in mammalian striated muscle. *FEBS Lett.* 1982; 146:115–118. [PubMed: 7140972]
- Heeley DH, Watson MH, Mak AS, Dubord P, Smillie LB. Effect of phosphorylation on the interaction and functional properties of rabbit striated muscle $\alpha\alpha$ -tropomyosin. *J Biol Chem.* 1989; 264:2424–2430. [PubMed: 2521628]

- Herzig JW, Peterson JW, Solaro RJ, Ruegg JC. Phosphate and vanadate reduce the efficiency of the chemo-mechanical energy transformation in cardiac muscle. *Adv Exp Med Biol.* 1982; 151:267–281. [PubMed: 6217726]
- Hill TL, Eisenberg E, Greene L. Theoretical model for the cooperative equilibrium binding of myosin subfragment 1 to the actin–troponin–tropomyosin complex. *Proc Natl Acad Sci USA.* 1980; 77:3186–3190. [PubMed: 10627230]
- Huxley HE. Structural changes in actin- and myosin-containing filaments during contraction. *Cold Spring Harbor Symp Quant Biol.* 1972; 37:361–376.
- Johnson P, Smillie LB. Polymerizability of rabbit skeletal tropomyosin: effects of enzymic and chemical modifications. *Biochemistry.* 1977; 16(10):2264–2269. [PubMed: 861209]
- Kawai M. The role of orthophosphate in crossbridge kinetics in chemically skinned rabbit psoas fibers as detected with sinusoidal and step length alterations. *J Muscle Res Cell Motil.* 1986; 7:421–434. [PubMed: 3491834]
- Kawai M, Brandt PW. Two rigor states in skinned crayfish single muscle fibers. *J Gen Physiol.* 1976; 68:267–280. [PubMed: 821913]
- Kawai M, Brandt PW. Effect of MgATP on stiffness measured at two frequencies in Ca^{2+} -activated muscle fibers. *Proc Natl Acad Sci USA.* 1977; 74:4073–4075. [PubMed: 269452]
- Kawai M, Brandt PW. Sinusoidal analysis: a high resolution method for correlating biochemical reactions with physiological processes in activated skeletal muscles of rabbit, frog and crayfish. *J Muscle Res Cell Motil.* 1980; 1:279–303. [PubMed: 6971874]
- Kawai M, Halvorson HR. Role of MgATP and MgADP in the cross-bridge kinetics in chemically skinned rabbit psoas fibers. Study of a fast exponential process (C). *Biophys J.* 1989; 55:595–603. [PubMed: 2785822]
- Kawai M, Halvorson HR. Two step mechanism of phosphate release and the mechanism of force generation in chemically skinned fibers of rabbit psoas. *Biophys J.* 1991; 59:329–342. [PubMed: 2009356]
- Kawai M, Ishiwata S. Use of thin filament reconstituted muscle fibres to probe the mechanism of force generation. *J Muscle Res Cell Motil.* 2006; 27:455–468. [PubMed: 16909198]
- Kawai M, Zhao Y. Cross-bridge scheme and force per cross-bridge state in skinned rabbit psoas muscle fibers. *Biophys J.* 1993; 65:638–651. [PubMed: 8218893]
- Kawai M, Saeki Y, Zhao Y. Cross-bridge scheme and the kinetic constants of elementary steps deduced from chemically skinned papillary and trabecular muscles of the ferret. *Circ Res.* 1993; 73:35–50. [PubMed: 8508533]
- Kawai M, Lu X, Hitchcock-DeGregori SE, Stanton KJ, Wandling MW. Tropomyosin period 3 is essential for enhancement of isometric tension in thin filament-reconstituted bovine myocardium. *J Biophysics.* 2009; 2009:1–17.
- Kon T, Imamura K, Roberts AJ, Ohkura R, Knight PJ, Gibbons IR, Burgess SA, Sutoh K. Helix sliding in the stalk coiled coil of dynein couples ATPase and microtubule binding. *Nature Struct Mol Biol.* 2009; 16(3):326–333.
- Kondo H, Ishiwata S. Uni-directional growth of F-actin. *J Biochem.* 1976; 79:159–171. [PubMed: 780347]
- Kress M, Huxley HE, Faruqi AR, Hendrix J. Structural changes during activation of frog muscle studied by time-resolved X-ray diffraction. *J Mol Biol.* 1986; 188:325–342. [PubMed: 3735425]
- Kurokawa H, Fujii W, Ohmi K, Sakurai T, Nonomura Y. Simple and rapid purification of brevin. *Biochem Biophys Res Commun.* 1990; 168:451–457. [PubMed: 2334416]
- Leger J, Bouvert P, Schwartz K, Swynghedauw B. A comparative study of skeletal and cardiac tropomyosins; subunits, thiol group content and biological activities. *Pflugers Archiv.* 1976; 362:271–277. [PubMed: 131298]
- Lehrer SS. Intramolecular crosslinking of tropomyosin via disulfide bond formation: evidence for chain register. *Proc Nat Acad Sci USA.* 1975; 72:3377–3381. [PubMed: 1059125]
- Lewis WG, Smillie LB. The amino acid sequence of rabbit cardiac tropomyosin. *J Biol Chem.* 1980; 255:6854–6859. [PubMed: 6993480]

- Lu X, Tobacman LS, Kawai M. Effects of tropomyosin internal deletion $\Delta 23\text{Tm}$ on isometric tension and the cross-bridge kinetics in bovine myocardium. *J Physiol (Lond)*. 2003; 553:457–471. [PubMed: 14500764]
- Lu X, Bryant MK, Bryan KE, Rubenstein PA, Kawai M. Role of the N-terminal negative charges of actin in force generation and cross-bridge kinetics in reconstituted bovine cardiac muscle fibres. *J Physiol*. 2005; 564:65–82. [PubMed: 15649975]
- Lu X, Tobacman LS, Kawai M. Temperature-dependence of isometric tension and cross-bridge kinetics of cardiac muscle fibers reconstituted with a tropomyosin internal deletion mutant. *Biophys J*. 2006; 91:4230–4240. [PubMed: 16980359]
- Mak AS, Smillie LB. Structural interpretation of the two-site binding of troponin on the muscle thin filament. *J Mol Biol*. 1981; 149:541–550. [PubMed: 7310890]
- Mak A, Smillie LB, Bárány M. Specific phosphorylation at serine-283 of alpha tropomyosin from frog skeletal and rabbit skeletal and cardiac muscle. *Proc Natl Acad Sci (USA)*. 1978; 75:3588–3592. [PubMed: 278975]
- Mak AS, Smillie LB, Stewart GR. A comparison of the amino acid sequences of rabbit skeletal muscle α - and β -tropomyosins. *J Biol Chem*. 1980; 255:3647–3655. [PubMed: 7364764]
- Maytum R, Bathe F, Konrad M, Geeves MA. Tropomyosin exon 6b is troponin-specific and required for correct acto-myosin regulation. *J Biol Chem*. 2004; 279:18203–18209. [PubMed: 14752114]
- McKillop DFA, Geeves MA. Regulation of the interaction between actin and myosin subfragment 1: evidence for three states of the thin filament. *Biphs J*. 1993; 65:693–701.
- Montarras D, Fiszman MY, Gros F. Characterization of the tropomyosin present in various chick embryo muscle types and in muscle cells differentiated in vitro. *J Biol Chem*. 1981; 256:4081–4086. [PubMed: 7217070]
- Montarras D, Fiszman MY, Gros F. Changes in tropomyosin during development of chick embryonic skeletal muscles in vivo and during differentiation of chick muscle cells in vitro. *J Biol Chem*. 1982; 257(1):545–548. [PubMed: 7053384]
- Montgomery K, Mak AS. In vitro phosphorylation of tropomyosin by a kinase from chicken embryo. *J Biol Chem*. 1984; 259:5555–5560. [PubMed: 6325440]
- Murakami K, Stewart M, Nozawa K, Tomii K, Kudou N, Igarashi N, Shirakihara Y, Wakatsuki S, Yasunaga T, Wakabayashi T. Structural basis for tropomyosin overlap in thin (actin) filaments and the generation of a molecular swivel by troponin T. *Proc Nat Acad Sci USA*. 2008; 105:7200–7205. [PubMed: 18483193]
- Murray JM, Knox MK, Trueblood CE, Weber A. Potentiated state of the tropomyosin actin filament and nucleotide-containing myosin subfragment 1. *Biochemistry*. 1982; 21:906–915. [PubMed: 6462176]
- Muthuchamy M, Gripp IL, Grupp G, O'Toole BA, Kiert AB, Boivin GP, Neumann J, Wieczorek DF. Molecular and physiological effects of overexpressing striated muscle β -tropomyosin in the adult murine heart. *J Biol Chem*. 1995; 270:30593–30603. [PubMed: 8530495]
- Muthuchamy M, Boivin GP, Grupp IL, Wieczorek DF. Beta-tropomyosin overexpression induces severe cardiac abnormalities. *J Mol Cell Cardiol*. 1998; 30:1545–1557. [PubMed: 9737941]
- O'Connor CM, Balzer DR, Lazarides E. Phosphorylation of subunit proteins of intermediate filaments from chicken muscle and nonmuscle cells. *Proc Nat Acad Sci USA*. 1979; 76:819–823. [PubMed: 284403]
- Ohtsuki I. Molecular arrangement of troponin-T in the thin filament. *J Biochem*. 1979; 86:491–497. [PubMed: 479140]
- Ookubo N, Ueno H, Ooi T. Similarities and differences of the α and β components of tropomyosin. *J Biochem*. 1975; 78:739–747. [PubMed: 1240098]
- Palmiter KA, Kitada Y, Muthuchamy M, Wieczorek DF, Solaro RJ. Exchange of beta- for alpha-tropomyosin in hearts of transgenic mice induces changes in thin filament response to Ca^{2+} , strong cross-bridge binding, and protein phosphorylation. *J Biol Chem*. 1996; 271:11611–11614. [PubMed: 8662805]
- Pearlstone JR, Smillie LB. Binding of troponin-T fragments to several types of tropomyosin. Sensitivity to Ca^{2+} in the presence of troponin-C. *J Biol Chem*. 1982; 257:10587–10592. [PubMed: 7107628]

- Perry SV. Troponin T: genetics, properties and function. *J Muscle Res Cell Motil.* 1998; 19:575–602. [PubMed: 9742444]
- Perry SV. Vertebrate tropomyosin: distribution, properties and function. *J Muscle Res Cell Motil.* 2001; 22:5–49. [PubMed: 11563548]
- Potter JD. Preparation of troponin and its subunits. *Methods Enzymol.* 1982; 85(Pt B):241–263. [PubMed: 7121270]
- Rao VS, Marongelli EN, Guilford WH. Phosphorylation of tropomyosin extends cooperative binding of myosin beyond a single regulatory unit. *Cell Motil Cytoskeleton.* 2009; 66:10–23. [PubMed: 18985725]
- Ribolow H, Bárány M. Phosphorylation of tropomyosin in live frog muscle. *Arch Biochem Biophys.* 1977; 179:718–720. [PubMed: 851366]
- Rodríguez AG, Flint GV, Regnier M. Effect of tropomyosin isoforms on actomyosin interaction in an in vitro motility assay. *Biophys J.* 2008; 94:232A.
- Rosol M, Lehman W, Craig R, Landis C, Butters C, Tobacman LS. Three-dimensional reconstruction of thin filaments containing mutant tropomyosin. *Biophys J.* 2000; 78:908–917. [PubMed: 10653803]
- Roy RK, Sreter FA, Sarkar S. Changes in tropomyosin subunits and myosin light chains during development of chicken and rabbit striated muscles. *Dev Biol.* 1979; 69:15–30. [PubMed: 446888]
- Sano K, Maeda K, Oda T, Maeda Y. The effect of single residue substitutions of serine 283 on the strength of head to tail interaction and actin binding properties of rabbit skeletal muscle α -tropomyosin. *J Biochem (Tokyo).* 2000; 127:1095–1102. [PubMed: 10833280]
- Schachat FH, Bronson DD, McDonald OB. Heterogeneity of contractile proteins. A continuum of troponin-tropomyosin expression in mammalian skeletal muscle. *J Biol Chem.* 1985; 260:1108–1113. [PubMed: 3968057]
- Singh A, Hitchcock-DeGregori S. Dual requirement for flexibility and specificity for binding of the coiled-coil tropomyosin to its target, actin. *Structure.* 2006; 14:43–50. [PubMed: 16407064]
- Smillie LB. Preparation and identification of α - and β -tropomyosins. *Methods Enzymol.* 1982; 85(Pt B):234–241. [PubMed: 6289041]
- Smillie, L. Tropomyosins. In: Kreis, T.; Vale, R., editors. *Guidebook to the cytoskeletal and motor proteins.* 2. Oxford University Press; Oxford: 1999. p. 159-164.
- Spudich JA, Watt S. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J Biol Chem.* 1971; 246:4866–4871. [PubMed: 4254541]
- Stapleton MT, Fuchsbauer CM, Allshire AP. BDM drives protein dephosphorylation and inhibits adenine nucleotide exchange in cardiomyocytes. *Am J Physiol.* 1998; 275:H1260–H1266. [PubMed: 9746474]
- Stienen GJ, Roosemalen MC, Wilson MG, Elzinga G. Depression of force by phosphate in skinned skeletal muscle fibers of the frog. *Am J Physiol.* 1990; 259:C349–C357. [PubMed: 2143356]
- Takagi Y, Shuman H, Goldman YE. Coupling between phosphate release and force generation in muscle actomyosin. *Philos Trans R Soc Lond B Biol Sci.* 2004; 359:1913–1920. [PubMed: 15647167]
- Tesi C, Colomo F, Piroddi N, Poggesi C. Characterization of the cross-bridge force-generating step using inorganic phosphate and BDM in myofibrils from rabbit skeletal muscles. *J Physiol (London).* 2002; 541:187–199. [PubMed: 12015429]
- Tobacman LS. Thin filament-mediated regulation of cardiac contraction. *Annu Rev Physiol.* 1996; 58:447–481. [PubMed: 8815803]
- Tobacman LS, Butters CA. A new model of cooperative myosin-thin filament binding. *J Biol Chem.* 2000; 275:27587–27593. [PubMed: 10864931]
- Vahebi S, Ota A, Li M, Warren CM, de Tombe PP, Wang Y, Solaro RJ. p38-MAPK induced dephosphorylation of α -tropomyosin is associated with depression of myocardial sarcomeric tension and ATPase activity. *Circ Res.* 2007; 100:408–415. [PubMed: 17234967]
- Vibert P, Craig R, Lehman W. Steric-model for activation of muscle thin filaments. *J Mol Biol.* 1997; 266:8–14. [PubMed: 9054965]

- Wakabayashi K, Tokunaga M, Kohno I, Sugimoto Y, Hamanaka T, Takezawa Y, Wakabayashi T, Amemiya Y. Small-angle synchrotron x-ray scattering reveals distinct shape changes of the myosin head during hydrolysis of ATP. *Science*. 1992; 258:443–447. [PubMed: 1411537]
- Wang G, Kawai M. Effect of temperature on elementary steps of the cross-bridge cycle in rabbit soleus slow-twitch muscle fibres. *J Physiol (Lond)*. 2001; 531:219–234. [PubMed: 11179405]
- Wannenburg T, Heijne GH, Geerdink JH, Van Den Dool HW, Janssen PM, De Tombe PP, Wannenburg, et al. Cross-bridge kinetics in rat myocardium: effect of sarcomere length and calcium activation. *Am J Physiol Heart Circ Physiol*. 2000; 279:H779–H790. [PubMed: 10924078]
- Waurick R, Knapp J, Van Aken H, Boknik P, Neumann J, Schmitz W. Effect of 2, 3-butanedione monoxime on force of contraction and protein phosphorylation in bovine smooth muscle. *Naunyn Schmiedebergs Arch Pharmacol*. 1999; 359:484–492. [PubMed: 10431760]
- Wolska BM, Keller RS, Evans CC, Palmiter KA, Phillips RM, Muthuchamy M, Oehlenschlaeger J, Wieczorek DF, de Tombe PP, Solaro RJ. Correlation between myofilament response to Ca^{2+} and altered dynamics of contraction and relaxation in transgenic cardiac cells that express β -tropomyosin. *Circ Res*. 1999; 84:745–751. [PubMed: 10205142]
- Xu C, Craig R, Tobacman L, Horowitz R, Lehman W. Tropomyosin positions in regulated thin filaments revealed by cryoelectron microscopy. *Biophys J*. 1999; 77:985–992. [PubMed: 10423443]
- Zhao Y, Kawai M. Inotropic agent EMD-53998 weakens nucleotide and phosphate binding to cross-bridges in porcine myocardium. *Am J Physiol Heart Circ Physiol* 40. 1996; 271:H1394–H1406.

Fig. 1.

A SDS-PAGE of dephosphorylated α Tm and β Tm. Lane 1: α Tm.deP. Lane 2: skeletal Tm. Lane 3: β Tm.deP. **B** Alkaline urea PAGE showing the phosphorylation status of α Tm. Lane 1: α Tm.P. Lane 2: approximate 1:1 mixture of α Tm.P and α Tm.deP. Lane 3: α Tm.deP

Fig. 2.

Two dimensional gel electrophoresis of α Tm showing phosphorylation. (A) is α Tm.deP treated with reconstitution buffer without BDM. (B) is α Tm.P treated with reconstitution buffer without BDM. (C) is α Tm.P treated with reconstitution buffer with 40 mM BDM. (D) is a mixture of α Tm.P and α Tm.deP treated with reconstitution buffer without BDM. All treatments were over night at 0–2°C

Fig. 3.

Slow pen trace of force records demonstrating (A–D) removal and reconstitution of the thin filament, (E) MgATP study, (F) pCa study, (G) Pi study, (H) MgADP study, and (I) standard activation and rigor. (A) Control myocardium; (B) After gelsolin treatment (G) for 50 min; (C) After actin filament-reconstitution (Ac); (D) After thin filament-reconstitution (Tm & Tn) for 12 h. β Tm.deP was used for this particular experiment. Activations A–D were performed in the standard activating solution (5S8P) by elevating the temperature from 2–25°C, and all relaxations (R) were performed at 2°C in the relaxing solution that includes 6 mM EGTA and 40 mM BDM. Numbers above tension trace indicate the mM concentration of (E) MgATP, (G) Pi, (H) MgADP, or (F) the pCa value. In H, the fibre was first activated at 25°C in the 00D solution that included CP and CK, but no ADP. It was then transferred to the 0D solution that did not include CP, CK, or ADP at 2°C, which caused partial relaxation. The temperature was then elevated to 25°C to result in activation. Thereafter, the MgADP concentration was increased stepwise as indicated. In I, the fibre was first activated in the standard activating solution, followed by wash with the rigor solution (Rg) twice at 25°C. In D–I, sinusoidal analysis was performed at each condition taking ~35 s, which is most prominent as force oscillation; the high frequency component was filtered by a 2nd order low pass filter with 10 Hz cut off frequency. Sharp vertical transients in force was caused by stopping the pen recorder, except that at the end (Cut), which was caused by cutting the preparation. The entire experiment is based on one myocardial preparation

Fig. 4. pCa-tension (in **A**) and pCa-stiffness (in **B**) relationships. Tension and stiffness were normalized to their maximum. α Tm.P (*square*), α Tm.deP (*circle*), and β Tm.deP (*triangle*). Experiments were carried out in the presence of 5 mM MgATP and 8 mM Pi. Curved lines are theoretical projections based on Eq. 2. The means of 5–9 experiments with SEM error bar are shown

Fig. 5.

The complex modulus data of thin filament-reconstituted myocardium during standard activation. The plots of the complex modulus $Y(f)$ of thin filament-reconstituted myocardium by using α Tm.P (square), α Tm.deP (circle), and β Tm.deP (triangle). Elastic modulus (in **A**) and viscous modulus (in **B**) are plotted against frequency. Viscous modulus is plotted against elastic modulus (Nyquist plot) in **C**. The unit of both elastic and viscous moduli is kPa. These data were obtained in the standard activating solution (5S8P, pCa 4.66). The complex modulus data are based on the average of 10–13 experiments. Frequencies used are (Hz): 0.13, 0.25, 0.35, 0.50, 0.70, 1.0, 1.4, 2.0, 3.1, 5.0, 7.1, 11, 17, 25, 35, 50, 70, and 100. These points are represented in the clock-wise direction in *c*

Fig. 6.

The effect of phosphate on isometric tension (in **A**), stiffness (in **B**), and the sum (in **C**) and the product (in **D**) of $2\pi b$ and $2\pi c$. Experiments were carried out in the presence of 5 mM MgATP at pCa 4.66. Tension and stiffness were normalized to the actin tension ($T_{ac} = 13.8 \pm 1.5$ kPa, $n = 34$) in the standard activating solution. The mean and SEM are shown. (square) α Tm.P model; (circle) α Tm.deP model; (triangle) β Tm.deP model. In **a** and **b**, $n = 10$ (α Tm.P), 11 (α Tm.deP), and 13 (β Tm.deP). In **C** and **D**, continuous curves are based on Eqs. 2 and 3 of Lu et al. (2005) with best fit parameters. $n = 5$ (α Tm.P), 10 (α Tm.deP), and 9 (β Tm.deP)

Fig. 7.

The effect of MgATP on isometric tension (in **A**) and stiffness (in **B**); the sum (in **C**) and the product (in **D**) of $2\pi b$ and $2\pi c$. Experiments were carried out in the presence of 8 mM Pi at pCa 4.66. Tension and stiffness were normalized to T_{ac} . The mean and SEM are shown. (*square*) α Tm.P muscle model; (*circle*) α Tm.deP model; (*triangle*) β Tm.deP model. In *a* and *b*, $n = 10$ (α Tm.P), 11 (α Tm.deP), and 13 (β Tm.deP). In **C** and **D**, continuous curves are based on Eqs. 2 and 3 of Lu et al. (2005) with best fit parameters. $n = 7$ for all Tm models

Fig. 8.

The effect of MgADP on isometric tension (in **A**), stiffness (in **B**), and the rate constant $2\pi c$ (in **C**). Experiments were carried out in the presence of 8 mM Pi and 2 mM MgATP at pCa 4.46–4.56. Tension and stiffness were normalized to T_{ac} . The mean and SEM are shown. (*square*) α Tm.P muscle model; (*circle*) α Tm.deP model; (*triangle*) β Tm.deP model. In **A** and **B**, $n = 10$ (α Tm.P), 11 (α Tm.deP), and 13 (β Tm.deP). In **C**, continuous curves are based on Eq. 5 of Lu et al. (2003) with best fit parameters. Filled symbols are extrapolation to true zero ADP concentration (0D solution). Extrapolated ADP concentration at 0D solution is 0.13 ± 0.04 mM ($n = 5$) (α Tm.P), 0.05 ± 0.02 mM ($n = 11$) (α Tm.deP), and 0.10 ± 0.02 mM ($n = 10$) (β Tm.deP).

Scheme 1.

Cross-bridge model used to analyze the kinetic data

Table 1

Ca sensitivity, cooperativity (Hill factor), tension, stiffness, and parameters of exponential processes of thin filament reconstituted bovine myocardium in the standard activating solution at 25°C are shown

Parameters	α Tm.P	α Tm.deP	β Tm.deP	Units
Ca sensitivity (pCa ₅₀)	5.56 ± 0.05 (7)*	5.29 ± 0.04 (5)	5.48 ± 0.06 (9)*	−log ₁₀
Cooperativity (n_H)	2.8 ± 0.2 (7)	3.2 ± 0.3 (5)	2.1 ± 0.2 (9)*	–
T (Tension)	1.59 ± 0.18 (10)	2.02 ± 0.20 (10)	1.71 ± 0.11 (9)	T_{ac}
$2\pi b$	9.2 ± 0.6 (10)	11.2 ± 0.9 (10)	9.8 ± 0.7 (9)	s ^{−1}
$2\pi c$	42 ± 3 (10)	48 ± 2 (10)	50 ± 4 (9)	s ^{−1}
$2\pi f_{min}$	11.4 ± 0.5 (10)	11.5 ± 0.7 (10)	9.6 ± 0.5 (9)	s ^{−1}
H	44 ± 9 (10)	40 ± 7 (10)	25 ± 4 (9)	T_{ac}
B	56 ± 7 (10)	53 ± 5 (10)	44 ± 5 (9)	T_{ac}
C	127 ± 16 (10)	138 ± 11 (10)	119 ± 12 (9)	T_{ac}
Y_{∞} (Stiffness)	115 ± 17 (10)	124 ± 13 (10)	100 ± 11 (9)	T_{ac}
Ratio ($T:Y_{\infty}$)	1.48 ± 0.13 (10)	1.70 ± 0.14 (10)	1.82 ± 0.15 (9)	%L ₀

± Represents SEM. Tension and moduli (H , B , C , Y_{∞}) are normalized to the tension developed when the actin filament was reconstituted and activated at the same condition ($T_{ac} = 13.8 \pm 1.5$ kPa, $n = 34$). f_{min} is the frequency at which the dynamic modulus becomes minimum, and

* $P < 0.05$ compared to the α Tm.deP model

Table 2

Rigor tension and stiffness of thin filament reconstituted bovine myocardium. Stiffness during rigor induction was measured at 100 Hz

Parameters	α Tm.P	α Tm.deP	β Tm.deP	Units
Tension	2.0 ± 0.3	2.2 ± 0.3	2.6 ± 0.4	T_{ac}
Stiffness	320 ± 60	320 ± 40	400 ± 90	T_{ac}
Ratio	0.62 ± 0.06	0.70 ± 0.08	0.85 ± 0.13	%L ₀
<i>N</i>	10	10	10	

Tension and stiffness were normalized to T_{ac} ($T_{ac} = 15.6 \pm 1.9$ kPa, $n = 30$; This T_{ac} is not significantly different from T_{ac} used in Table 1). There is no significant difference in rigor tension or stiffness between α Tm.P and α Tm.deP muscle models, and between α Tm.deP and β Tm.deP muscle models ($P > 0.05$)

Table 3

Kinetic constants of the elementary steps of the cross-bridge cycle (Scheme 1) in the thin filament reconstituted bovine myocardium

Kinetic constants	α Tm.P	α Tm.deP	β Tm.deP	Units
K_0	125 ± 20 (5)	89 ± 5 (11)	116 ± 13 (10)	mM^{-1}
K_1	6.3 ± 1.3 (7)	3.4 ± 0.6 (7)	5.8 ± 1.2 (7)	mM^{-1}
k_2	34 ± 4 (7)	29 ± 5 (7)	36 ± 3 (7)	s^{-1}
k_{-2}	10 ± 2 (7)	24 ± 6 (7)	17 ± 5 (7)	s^{-1}
K_2	4.7 ± 1.5 (7)	2.3 ± 1.0 (7)	3.3 ± 0.8 (7)	–
$K_1 k_2$	230 ± 60 (7)	111 ± 36 (7)	220 ± 60 (7)	$\text{mM}^{-1} \text{s}^{-1}$
k_4	13.2 ± 5.2 (5)	14.5 ± 1.4 (10)	22.3 ± 3.2 (9)	s^{-1}
k_{-4}	131 ± 28 (5)	118 ± 12 (10)	111 ± 8 (9)	s^{-1}
K_4	0.10 ± 0.04 (5)	0.13 ± 0.01 (10)	0.21 ± 0.03 (9)	–
K_5	0.060 ± 0.038 (5)	0.043 ± 0.006 (10)	0.045 ± 0.005 (9)	mM^{-1}
k_4/K_5	710 ± 330 (5)	480 ± 140 (10)	530 ± 70 (9)	$\text{mM}^{-1} \text{s}^{-1}$
K_4/K_5	4.4 ± 1.8 (5)	3.6 ± 0.7 (10)	4.7 ± 0.5 (9)	mM^{-1}