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Ca^{2+}-dependent activation of striated muscle involves cooperative interactions of cross-bridges and thin filament regulatory proteins. We investigated how interactions between individual structural regulatory units (RUs; 1 tropomyosin, 1 troponin, 7 actins) influence the level and rate of demembranated (skinned) cardiac muscle force development by exchanging native cardiac troponin (cTn) with different ratio mixtures of wild-type (WT) cTn and cTn containing WT cardiac troponin T/I + cardiac troponin C (cTnC) D65A (a site II inactive cTnC mutant). Maximal Ca^{2+}-activated force (F_{max}) increased in less than a linear manner with WT cTn. This contrasts with results we obtained previously in skeletal fibres (using sTnC D28A, D65A) where F_{max} increased in a greater than linear manner with WT sTnC, and suggests that Ca^{2+} binding to each functional Tn activates < 7 actins of a structural regulatory unit in cardiac muscle and > 7 actins in skeletal muscle. The Ca^{2+} sensitivity of force and rate of force redevelopment (k_{tr}) was leftward shifted by 0.1–0.2 $-\log [Ca^{2+}]$ (pCa) units as WT cTn content was increased, but the slope of the force–pCa relation and maximal k_{tr} were unaffected by loss of near-neighbour RU interactions. Cross-bridge inhibition (with butanedione monoxime) or augmentation (with 2 deoxy-ATP) had no greater effect in cardiac muscle with disruption of near-neighbour RU interactions, in contrast to skeletal muscle fibres where the effect was enhanced. The rate of Ca^{2+} dissociation was found to be $>$ 2-fold faster from whole cardiac Tn compared with skeletal Tn. Together the data suggest that in cardiac (as opposed to skeletal) muscle, Ca^{2+} binding to individual Tn complexes is insufficient to completely activate their corresponding RUs, making thin filament activation level more dependent on concomitant Ca^{2+} binding at neighbouring Tn sites and/or crossbridge feedback effects on Ca^{2+} binding affinity.

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Myocyte contraction is triggered when Ca^{2+} binds to the troponin (Tn) complex and initiates a series of conformational changes through its component proteins. The end result is an increased mobility of tropomyosin (Tm) over the surface of actin strands, allowing for the formation of force-generating cross-bridges between actin and myosin (Farah & Reinach, 1995; Gordon *et al.* 2000). The stoichiometry of thin filament proteins is 1 Tm and 1 Tn complex for every 7 actin monomers (A_7TnTm) such that an individual, 1 μm long actin filament consists of two sets of $\sim$26 A_7TnTm structural regulatory units (RUs) aligned end to end, with one set on each side of the actin helix. This structural arrangement implies that Ca^{2+} binding to a single Tn should initiate cross-bridge binding along a 7 actin span of the thin filament. Within the geometry of the sarcomere, up to 4 myosins heads could possibly interact with each A_7TnTm RU (Gordon *et al.* 2000). However each Tm interacts with neighbouring Tm molecules via head-to-tail overlapping contacts, thereby allowing the possibility that cross-bridge binding can occur along a greater span of the thin filament than the RU. Structural studies indicate that S1 binding to actin influences the position of Tm on the thin filament (Vibert *et al.* 1997; Xu *et al.* 1999). Thus multiple protein–protein interactions are involved in establishing the level of thin filament activation, or number of cross-bridges, during Ca^{2+}-activated force development.

Ca^{2+}-dependent activation of striated muscle force development also involves cooperative mechanisms, as clearly demonstrated by the steep slope of the steady-state force–negative log $[Ca^{2+}]$ (pCa) curve of chemically demembranated (skinned) muscle cells. There are a number of mechanisms thought to be responsible for this cooperativity, including events that could occur within

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an RU or between RUs along thin filaments. These include: (1) coupling between Ca^{2+} binding at N-terminal sites of an individual TnC (in skeletal, but not cardiac muscle) and/or Ca^{2+} binding to the N-terminus of TnC influencing the Ca^{2+} binding of adjacent TnC molecules; (2) strong cross-bridge-mediated enhancement of Ca^{2+} binding to TnC; (3) cross-bridge-mediated stabilization of Tm during Ca^{2+}-dependent activation allowing for exposure of additional strong myosin binding sites within an RU; and (4) Ca^{2+} plus cross-bridge binding in one RU influencing the availability of strong cross-bridge binding in adjacent RUs (i.e. an allosteric spread of activation along thin filaments mediated via head-to-tail interactions of Tm) (Gordon *et al.* 2000).

If activation of one RU influences the activation of an adjacent RU, one possibility is that Ca^{2+} binding to a single Tn makes more than the 7 actins within the RU available for strong myosin binding. We (Regnier *et al.* 2002) recently interrupted interactions between neighbouring RUs of thin filaments in fast skeletal muscle and determined that 10–12 actins are made available for strong myosin binding with Ca^{2+} binding to each Tn. This spread of activation beyond the RU suggests that a functional regulatory unit (FU) is larger than an RU in skeletal muscle, as was also indicated by solution biochemical studies (Greene & Eisenberg, 1980; Geeves & Lehrer, 1994). Our experiments also demonstrated that isolation of individual FUs from one another resulted in loss of most of the slope in the steady-state force–pCa relationship, suggesting that the dominant mechanism of cooperative activation in skeletal muscle involves interactions between adjacent FUs.

Compared to skeletal muscle, the regulation of cardiac muscle contraction appears to be a more graded response to Ca^{2+}-dependent activation. This is demonstrated by a lower Ca^{2+} sensitivity of both steady-state force generation and the rate of force development in skinned muscle cells (Regnier *et al.* 1998*b*, 2002, 2004), as well as cardiac *versus* skeletal muscle differences in the sarcomere length dependence of force generation (Konhilas *et al.* 2002). A possible mechanism for these cardiac *versus* skeletal muscle differences in contractile activation may be the degree to which thin filaments are 'turned on' by the Ca^{2+}-dependent activation of Tn. We (Martyn *et al.* 1999, 2001) and others (Fuchs & Wang, 1991; Wang *et al.* 2001) have demonstrated that Ca^{2+} binding to Tn is enhanced by strong cross-bridges in cardiac, but not skeletal muscle. This suggests one mechanism of cooperative thin filament activation that may be available only to cardiac muscle. We have also demonstrated that cardiac (*versus* skeletal) muscle contractile activation is more dependent on strong cross-bridge binding (Adhikari *et al.* 2004; Regnier *et al.* 2004). Additionally, Butters *et al.* (1997) measured cardiac S1-ATPase activity in the presence of actin reconstituted with various ratios of functional and non-functional cardiac TnC (cTnC), and concluded that less than 7 actins are activated by Ca^{2+} binding to each RU. These results also suggested that individual Ca^{2+}-bound RUs may be more completely activated by Ca^{2+} binding to a neighbouring RU, and that the FU size differs between cardiac and skeletal muscle. However, the size and properties of the FUs in cardiac muscle needs to be examined in cardiomyocytes, where steric constraints imposed by the intact lattice structure of sarcomeres can influence thin–thick filament interactions.

In the current study we used the whole cTn replacement and TnC extraction–reconstitution techniques to study the size and properties of FUs in chemically demembranated rat cardiac muscle. We have previously demonstrated that most or all of the native cTn in preparations of trabeculae can be exchanged (Kohler *et al.* 2003). Native cTn in rat trabeculae was replaced by different ratio mixtures of functional and non-functional cTn. The non-functional cTn (xcTn) contained a cTnC where the single N-terminal Ca^{2+} binding site (site II) was made non-functional by replacement of Asp^{65} with Ala (D65A cTnC (xcTnC)). It was assumed that increasing the proportion of xcTn incorporated into thin filaments increased the probability that one or both cTn surrounding a FU would be non-functional.

We found that reduction of near-neighbour RU interaction did not greatly reduce either the Ca^{2+} sensitivity (pCa_{50}) or slope (n_H, i.e. the apparent cooperatively) of the force–pCa relationship. Additionally, the relative effect of strong cross-bridge inhibition or augmentation on thin filament activation and force production was not influenced by the reduction of near-neighbour RU interactions in cardiac muscle, but was greatly affected in fast skeletal muscle. As such, our data suggest that the length of thin filament activated by Ca^{2+} binding to a Tn may be ≤ 7 actins in cardiac muscle, compared with 10–12 actins in skeletal muscle. If so, the spread of activation along thin filaments with Ca^{2+} binding to a Tn (and subsequent strong cross-bridge binding) may be minimal in cardiac muscle and depend more on concomitant binding of Ca^{2+} to neighbouring RUs and/or cross-bridge-induced increases in Tn–Ca^{2+} binding affinity. This would suggest that the cooperativity of the force–pCa relationship in cardiac muscle results primarily from mechanisms that differ from those found in skeletal muscle (Regnier *et al.* 2002). Preliminary reports of this work have been published previously (Regnier *et al.* 2001; Gillis *et al.* 2005*a*).

Methods

Protein preparation: troponin subunits and complex

Wild-type (WT) rat cTnC and xcTnC were expressed and purified as previously described (Dong *et al.* 1996). For cTn replacement studies, cardiac troponin I (cTnI)

and cardiac troponin T (cTnT) were purified from bovine heart, obtained at a local abattoir, and purified as previously described (Potter, 1982). For the stopped-flow studies of Ca^{2+} dissociation, the subunits of cTn were produced using recombinant methods. cTn complex was reconstituted from isolated (recombinant or native) subunits (1 : 1 : 1 ratio) as previously described (Potter, 1982)

Animal tissue

Male Sprague–Dawley rats (200–250 g) and male New Zealand rabbits were housed in the Department of Comparative Medicine at the University of Washington (UW) and were cared for in accordance with the US National Institutes of Health Policy on Humane Care and Use of Laboratory Animals. All protocols were approved by the UW Animal Care Committee. Rats were killed with sodium pentabarbitol (50 mg kg^{-1}) and hearts were excised and the interior wall of the right ventricle exposed to relaxing solution (see below) containing glycerol (50% v/v) and Triton X-100 (1%) overnight at 5°C. Trabeculae were dissected out of the right ventricular free wall as previously described (Regnier *et al.* 2000) and stored for up to 5 days at 5°C. For further details and solution recipes see Kohler *et al.* (2003). Single rabbit psoas fibre segments were prepared as previously described (Regnier *et al.* 2000). Rabbits were killed with pentobarbital (120 mg kg^{-1}) administered through the marginal ear vein. Isolated fibres were treated with 1% Triton X-100 (v/v) in relaxing solution to remove membranous residue.

Exchange of cTn into permeabilized trabeculae

Endogenous Tn was exchanged with Tn produced from purified proteins as previously described (Kohler *et al.* 2003). In brief, mounted trabeculae were soaked in a rigor solution for 2 h containing a high concentration (3.0–3.5 mg ml^{-1}) of the recombinant Tn and (mM): 3-(*N*-morpholino)propanesulphonic acid (Mops) 20, $MgCl_2$ 5, EGTA 5, KCl 240, dithiothreitol (DTT) 5, butanedione monoxime (BDM) 5 and pepstatin 0.02; pH 6.5 at 10°C.

Following exposure to exchange buffer, trabeculae were exposed to a solution containing bovine serum albumin (1 mg ml^{-1}). The following mixtures, in percentage, of WT cTn : xcTn were made for exchange into trabeculae: 100 : 0, 75 : 25, 50 : 50, 25 : 75 and 0 : 100. Exchanges with 100% xcTn were performed on a periodic basis to validate that the exchange with native cTn was nearly complete.

Extraction of TnC and reconstitution of Tn complexes

In some experiments TnC was selectively extracted from muscle cells as previously described using trifluoperazine (TFP) (Regnier *et al.* 1999, 2002; Moreno-Gonzalez *et al.* 2005). Cardiac trabeculae or rabbit psoas fibres were placed in extracting solution containing 10 mM Mops, 5 mM EDTA and 0.5 mM TFP at pH 6.6 (Metzger *et al.* 1989; Hannon *et al.* 1993). Most muscle preparations were placed in extraction solution for 30 s followed by 10–15 s in relaxing solution (pCa 9.0), and this procedure was repeated five to 10 times. The preparation was then activated at pCa 4.0 to determine the remaining F_{max} and additional extraction was performed until F_{max} no longer decreased. For trabeculae, the remaining F_{max} was 0.26 ± 0.07 of the pre-extracted value. For psoas muscle fibres, extracted values ranged between 0 and 3% of pre-extracted F_{max}.

For trabeculae, cTn complexes were reconstituted by incubation with 100% xcTnC (1 mg ml^{-1}) in pCa 9.2 solution without creatine kinase (CK) or dextran for 15–20 min, with no discernable change in F_{max} from the extracted value. Reconstitution of skeletal Tn (sTn) complexes was achieved by 1–3 min incubations in 1 mg ml^{-1} (total) sTnC in solution at pCa 9.2 as previously described (Regnier *et al.* 2002; Moreno-Gonzalez *et al.* 2005). For skeletal fibres, mixtures of 10–15% sTnC and 85–90% D28A, D63A sTnC (xxsTnC) were used to produce ~20% of the pre-extracted F_{max} for comparisons with trabeculae. Reconstitution was considered complete when force at pCa 4.0 no longer increased with subsequent incubations. We have demonstrated relatively equal binding affinities for sTnC and xxsTnC in the absence of Ca^{2+}. Thus, the procedure for reconstitution with sTnC:xxsTnC mixtures is expected to yield a random distribution of these two TnCs along individual thin filaments throughout the entire fibre diameter (Regnier *et al.* 2002).

Gel and Western blot analysis

Sarcomere protein stoicheometry of trabeculae prior to and following whole Tn exchange was determined from silver-stained sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 2) according to the methods of Giulian *et al.* (1983). Western blots were used to determine the phosphorylaton status of bovine cTnI encorporated into cTn complexes and in rat trabeculae prior to exchange (Fig. 3). For cTn complexes containing WT and D65A cTnC, protein kinase A (PKA) and C (PKC) phosphorylation were performed in a typical reaction mixture containing (mM): Hepes 50 (pH 7.5), $MgCl_2$ 10, $CaCl_2$ 0.5, EGTA 1 and ATP (Tris-salt) 0.1, and 4 μg protein and 3 units of PKA/or 0.13 μg of PKC (Sigma, St Louis, MO, USA). Protein phosphatase 1 (PP1; Sigma) was used to release phosphate groups from phosphorylated serine, threonine and tyrosine residues in buffer containing (mM): Hepes 50, Na_2EDTA 0.1, DTT 5 and $MnCl_2$ 10, and 0.025% Tween 20 at 30°C for 6–12 h. For rat trabecular,

as previously described (Dai *et al.* 2002), samples were homogenized in a solution of 0.1% Triton X-100, 150 mM NaCl, 10 mM Hepes (pH 7.5), 1 mM EDTA, 0.5 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} aprotinin, 10 μg ml^{-1} soybean trypsin inhibitor and 1 μg ml^{-1} pepstatin A. The homogenate was incubated on ice for 5 min and then centrifuged at 2000 *g* for 5 min. The extracts were incubated with goat polyclonal anti-troponin I (Santa Cruz Biotechnology) at 4°C for 1 h, followed by incubation with protein A–sepharose (5 ng ml^{-1}) at 4°C for 30 min, then washed three times with ice-cold lysis solution (see above). All samples were separated on a 12% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were incubated in Tris-buffered saline (TBS) containing 0.05% Tween 20 and 5% blocking powder for 18 h at 4°C. The membranes were then incubated in a 1 : 500 dilution of goat polyclonal anti-phosphoserine (Invitrogen, CA, USA) in TBS with 0.05% Tween 20 and incubated in a 1 : 1000 dilution of horseradish peroxidase-conjugated bovine anti-goat IgG (ICN Biochemicals). Detection was by chemiluminescence, and densitometry was performed on the films.

Solutions for mechanical measurements from trabeculae

Solutions contained (mM): phosphocreatine 15, EGTA 15, Mops at least 40, free Mg^{2+} 1, $Na^+ + K^+$ 135 and DTT 1, 250 units ml^{-1} creatine kinase (Sigma) and 5 mM ATP, 0.5 mM ATP, or 5 mM 2 deoxy-ATP (dATP; Sigma) at pH 7.0 and 15 ± 1°C. Ionic strength was 0.17 M. Affinities of dATP and ATP for Mg^{2+} were assumed to be similar (Regnier *et al.* 1998*a*). For activation solutions, the Ca^{2+} level (expressed as pCa) was varied between pCa 9.0 and pCa 4.0 by adjusting the concentration of calcium propionate.

Mechanical measurements

The ends of trabeculae or rabbit psoas muscle fibres were wrapped with aluminium foil T-clips for attachment to a force transducer (either Model 400A, 2.2 kHz resonant frequency; Cambridge Technology, Watertown, MA, USA or Model AE801, 5 kHz resonant frequency; SensoNor, Horton, Norway) and a servomotor (model 300, Cambridge Technology) tuned for a 300 μs step response. Skeletal fibre segment ends were chemically fixed by focal application of 1% glutaraldehyde in H_2O to minimize compliance (Chase & Kushmerick, 1988). Sarcomere length (L_s) was measured with helium–neon laser diffraction at pCa 9.0. The following measurements were made: steady-state isometric force, the rate of isometric tension redevelopment (k_{tr}) following rapid (< 4 ML s^{-1}) release–restretch (15% L_s) of the preparation (Brenner, 1986) and stiffness determined by small amplitude (~0.1% L_s) sinusoidal length oscillations (1000 Hz).

Analysis

Force–pCa and k_{tr}–pCa relationships are fitted with the Hill equation:

$$F = F_{max}/\left[1 + 10^{n}/\mathrm{pCa} - \mathrm{pCa}_{50}\right]$$

where F_{max} is the force at high [Ca^{2+}] (low pCa), pCa_{50} is the pCa needed to achieve 50% of F_{max} (defined here as the Ca^{2+} sensitivity of force), and n reflects the steepness of the relation. From each Hill fit, the pCa_{50} of force or k_{tr} and the slope (n_H) are determined, and reported values represent the means of the values from the individual fits (± S.E.M). Student's paired t tests were used for comparisons.

Ca^{2+} dissociation rates from whole skeletal and cardiac troponin

A model SX.18 MV stopped-flow instrument (Applied Photophysics Ltd, Leatherhead, UK) was used to measure the Ca^{2+} dissociation rates (k_{off}) from whole Tn complexes using a method modified from that of Tikunova *et al.* 2002). This method uses Quin-2 (Calbiochem) as a fluorescent chelator. Whole Tn (WT skeletal and WT cardiac) was dialysed into a soltion containing (mM): KCl 250, Mops 20, $MgCl_2$ 5 and DTT 1; pH 7.0. The above buffer was used in all stopped-flow experiments. Each complex (6 μM) with 30 μM $CaCl_2$ was rapidly mixed with an equal volume of Quin-2 (150 μM) at 15°C. The samples were excited using a 150 W xenon arc source while emission was monitored through a 510 nm broad band-pass interference filter (Oriel, Stanford, CT, USA). As Quin-2 reports the dissociation of Ca^{2+} from both the N- and C-terminus, a series of reactions with different durations of time (50 ms–20 s) were accumulated for each protein. These were then fitted with either single or double exponentials as appropriate. Once a time scale had been determined over which the fast N-terminal k_{off} went to completion and allowed for the best fit of the data (determined by the residuals), this was used as the time scale for single exponential fits. The k_{off} values presented for the N-terminus of each protein were calculated by summing and fitting the data from three reactions and then repeating this at least four more times with 12 other reactions. The rate presented for the C-terminus represents the second rate from a double exponential fit of data collected over a 20 s period.

Results

Ca^{2+}-activated steady-state force with cTn mixtures

Figure 1*A* shows an example force record demonstrating the ability to exchange native Tn in a trabecula for complexed Tn containing recombinant rat cTnC. This trabecula was first activated with a pCa 4.5 solution to determine maximal Ca^{2+}-activated steady-state force (F_{max}) and rate of force redevelopment (k_{tr}) following the release–restretch protocol (see Methods). The complete loss of force with a 15% L_s release and its redevelopment upon restretch are indicated by the vertical lines in the force record every 5 s. Following maximal activation, the trabecula was relaxed (pCa 9.0) prior to incubation in exchange buffer with 4.2 mg ml^{-1} xcTn for 2 h (first arrow in Fig. 1*A*). After return to relaxing solution, F_{max} was determined to be 10% of the pre-exchanged level. In this example preparation, we then did a second exchange with whole Tn containing 100% WT cTnC (cTn; second arrow in Fig. 1*A*), which resulted in recovery of F_{max} to 77% of the original pre-exchanged value. Absolute values for F_{max} (normalized to cross-sectional area) and k_{tr} are given in the legend to Fig. 1. The exchange protocol was performed periodically with 100% xcTn to verify that complete or near complete replacement of the native cTn was occurring consistently, and a second exchange with 100% cTn was performed a few times to verify that loss of force was due to xcTn (and not preparation degradation). The control experiments with 100% xcTn usually showed more complete loss of F_{max} ($5.5 \pm 1.2\%$, $n = 7$, range, 2.1–10.0%). Single exchanges with 100% cTn generally maintained F_{max} at a greater level ($86 \pm 2\%$ of

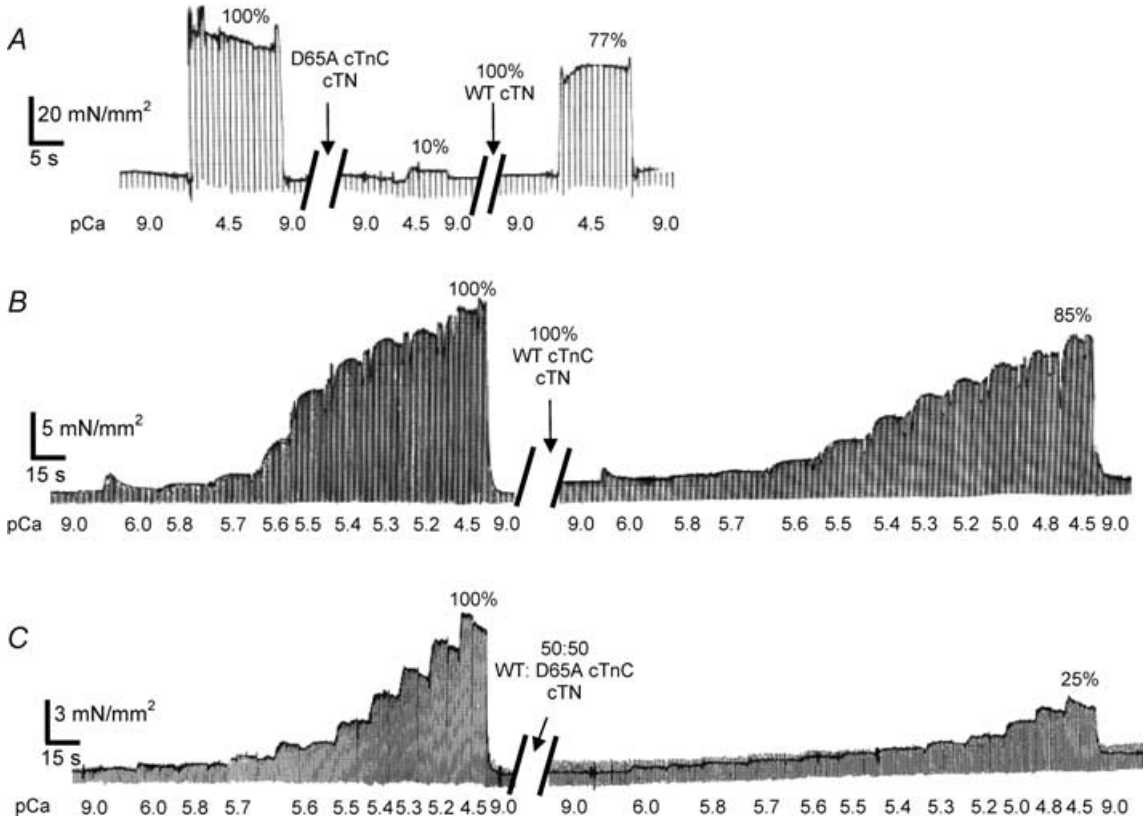


Figure 1. Representative chart recordings showing the effectiveness of whole cardiac troponin (cTn) replacement and the effect of different mixtures of wild-type (WT) cTnC/cTn and non-functional (x) xcTnC/cTn on Ca^{2+}-activated force development in cardiac trabeculae

A, the trabeculae in this experiment was able to produce 59.2 mN mm^{-2} of force at pCa 4.5 (F_{max}) prior to the native cTn being replaced by cTn containing a cTnC unable to be activated by Ca^{2+}. Following this replacement, F_{max} was 6.1 mN mm^{-2} (equal to 10% of pre-replacement F_{max}). The non-functional cTn was then replaced by 100% functional cTn and F_{max} was then found to be 45.8 mM mm^{-2} (equal to 77% of pre-replacement F_{max}). k_{tr} was 11.34 s^{-1} after exchange. *B*, illustrates force–pCa recordings of a trabecula containing native cTnC and then after the native cTn was replaced by 100% WT cTn. F_{max} before replacement was 32.1 mN mm^{-2}, and following replacement was 27.0 mN mm^{-2}. *C*, illustrates force–pCa recordings of a trabecula containing native cTnC and then after the native cTn was replaced by a cTn mixture of 50 : 50 cTn/xTn. F_{max} before replacement was 10.8 mN mm^{-2}, and following replacement was 2.7 mN mm^{-2}.

pre-exchanged F_{max}, $n = 4$) than shown in Fig. 1*A*. This is demonstrated in Fig. 1*B* where force records of the normal experimental protocol are shown for an example 100% cTn exchange. This trabecula was placed in a series of solutions containing increasing Ca^{2+} concentration (indicated as pCa values below the force trace), both before and following the exchange protocol. For the example in Fig. 1*B*, exchange with 100% cTn maintained 86% of pre-exchanged F_{max} and pCa_{50} was 5.38, compared with 5.52 prior to the exchange. Figure 1*C* shows force records for an exchange mixture containing 50% cTn and 50% xcTn and demonstrates a 75% reduction in F_{max} and pCa_{50} of 5.15, compared with 5.38 prior to the exchange.

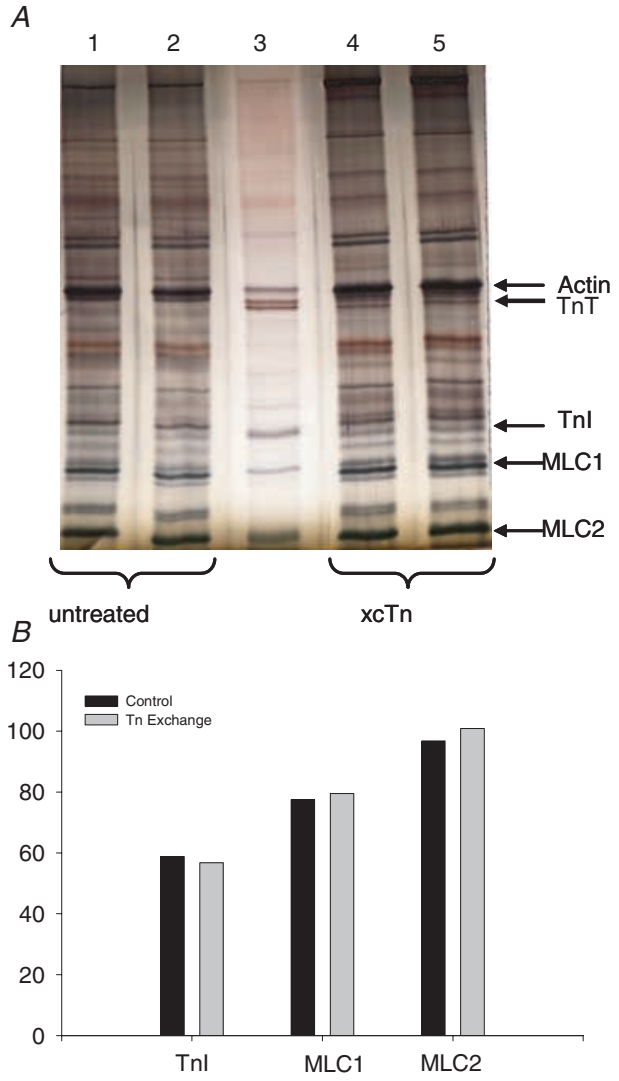


Figure 2. Determination of sarcomere protein stoicheometry
A, silver-stained SDS-PAGE for non-exchanged (lanes 1 and 2) and cTn-exchanged (lanes 4 and 5) trabeculae. Lane 3 is used as a marker lane for protein identification and contains (from top to bottom) actin, troponin (Tn) T, TnI, TnC, myosin light chain (MLC) 1 and MLC2. *B*, densitometric analysis of TnI, MLC 1 and MLC 2. Values are normalized to the percentage of actin to account for any variability in loading conditions. The data demonstrate similar levels of TnI, MLC1 and MLC2 for non-exchanged and cTn-exchanged trabeculae.

To determine whether the exchange protocol influenced the stoichiometry of contractile proteins, silver stain SDS gels were compared for non-exchanged trabeculae *versus* trabeculae exchanged with 100% xcTn (Fig. 2*A*). Densitometric analysis was performed with values normalized to actin content for each trabeculae. Figure 2*B* shows this analysis for the SDS gels of trabeculae in Fig. 2*A*, and demonstrates that cardiac TnI, myosin light chain (MLC) 1 and MLC2 content were not affected by the exchange protocol. Thus, while we did not do an exhaustive analysis of sarcomeric proteins, these data suggest that the stoichiometric ratio of contractile and thin filament regulatory proteins was unaffected by whole Tn exchanges in cardiac muscle. An additional consideration for these experiments is that Tn complexes contained purified bovine cTnI and cTnT, both of which have multiple phosphorylation sites that can influence the Ca^{2+} dependence of force development. Therefore we assessed the phosphorylation state of cTn and xcTn complexes used for exchange in trabeculae. Figure 3*A* shows a Coomassie blue-stained SDS gel for the cTn and xcTn that was untreated (lanes 2 and 3), incubated with PKA + PKC to achieve maximal Tn phosphorylation (lanes 4 and 5) or incubated with protein phosphatase (PP1) to dephosphorylate troponin complexes (lanes 6 and 7). A Western blot of cTnI for serine phosphorylation is shown in the bottom row (see Methods). Densitometry analysis, normalized to phosphorylation levels of untreated cTn or xcTn to correct for any differences in protein loading, suggests that > 70% of the bovine cTnI ser-phosphorylation sites (Ser 23, 24, 43 and 45) of Tn complexes were phosphorylated (Fig. 3*B*) and that PP1 can almost completely de-phosphorylate these sites for both complexes. The gel band for cTnI (lane 3) is located between molecular weight marker bands of 29 and 19 kDa (lane 1) and shows significant phosphorylation with Western blot analysis. Figure 3*C* demonstrates that significant cTnI phosphorylation also occurs in rat trabeculae used for whole cTn exchange experiments. These data suggest that (1) the exchange protocol did not significantly alter contractile or regulatory protein composition, other than the intended exchange and (2) that the phosphorylation state of cTnI for endogenous Tn and exchanged Tn was probably similar.

The steady-state force–pCa relationship was measured for different ratio mixtures of cTn:xcTn in the exchange solution. Butters *et al.* (1997) demonstrated that the affinity of cTn and xcTn for thin filaments was similar in the low [Ca^{2+}] exchange solutions, as determined using the method of Huynh *et al.* (1996). This suggests that the amount of each Tn exchanged into the thin filament of the trabeculae was similar to the proportions in the exchange solutions. The data are summarized in Figs 2

and 3 and the Hill fit parameters F_{max}, pCa_{50} and apparent cooperativity of activation (n_H) are summarized in Table 1. Data are normalized to F_{max} following exchange with 100% cTn for ease of comparison. Figure 4 shows that decreasing the fraction of functional cTn in the exchange mixture reduced F_{max} in a more than proportional manner. The concave (up) nature of the F_{max} *versus* fraction of function cTn relationship is similar to that found by Butters *et al.* (1997) for the relationship myosin S1-thin filament MgATPase *versus* fraction of function cTn. Our data regarding the organized structure of the sarcomere supports their conclusions from protein solution studies that positive cooperativity between RUs must occur to achieve the activation state found with saturating levels of Ca^{2+}. It also implies that < 7 actins may become available for strong cross-bridge formation with Ca^{2+} binding an individual cTn. These data contrast with our findings in rabbit (psoas) skeletal muscle fibres (Regnier *et al.* 2002) where F_{max} was reduced by a less than proportional manner with decreasing functional Tn. The best fit line for the skeletal muscle fibre data (Fig. 4 of Regnier *et al.* 2002) is redrawn in Fig. 4 for comparison (dotted line). From these experiments we concluded that 10–12 actins were activated by Ca^{2+} binding the Tn of each FU. This comparison demonstrates a distinct difference between cardiac and skeletal muscle in the extent that activation spreads along thin filaments with Ca^{2+} binding.

Figure 5 summarizes the Ca^{2+} dependence of force development for the trabeculae containing different numbers of FUs. Ca^{2+} sensitivity of force (pCa_{50}) was reduced by 0.1–0.2 pCa units and n_H was little affected for trabeculae where exchange mixtures contained < 50% functional Tn (Table 1). For trabeculae where exchange

Table 1. Hill fit parameters for trabeculae containing different mixtures of WT cTn:xcTn

cTn : xcTn	pCa_{50}	Slope (n_H)	F_{max}
100 : 0 (control)	5.43 ± 0.02	3.4 ± 0.3	100 ($n = 5$)
75 : 25	5.39 ± 0.02	3.5 ± 0.3	67 ± 3 ($n = 5$)
50 : 50	5.23 ± 0.03	2.8 ± 0.4	27 ± 4 ($n = 9$)
25 : 75	5.32 ± 0.02	4.0 ± 0.4	16 ± 2 ($n = 6$)

cTn : xcTn, cardiac troponin : non-functional cardiac troponin; pCa_{50}, Ca^{2+} sensitivity; F_{max}, maximal Ca^{2+} activated force.

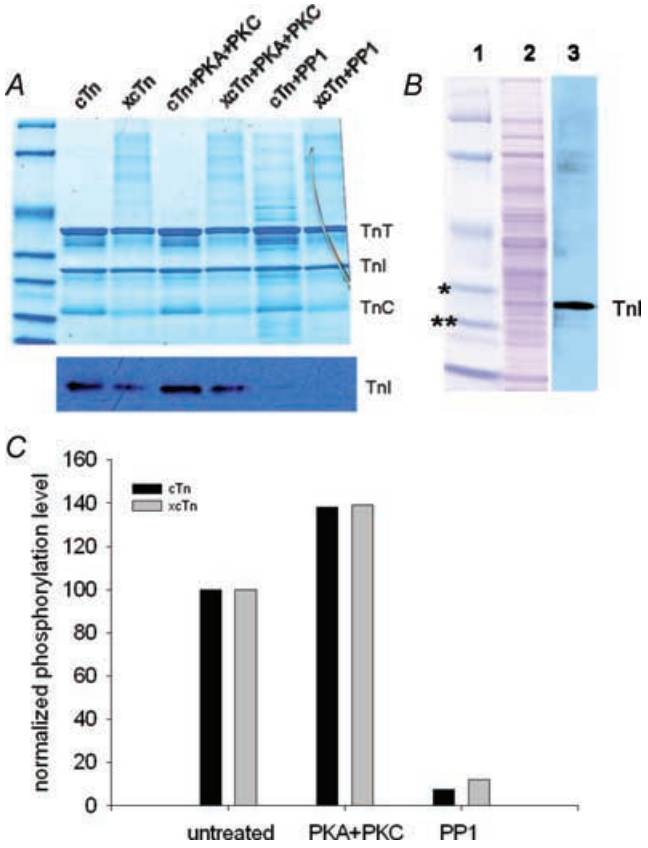


Figure 3. Analysis of cardiac troponin (cTn) I phosphorylation for cTn complexes and a pre-exchanged rat trabecula
A, top part of panel shows a Coommassie blue-stained SDS-PAGE of untreated cTn (lane 2), untreated xcTn (lane 3), cTn treated with PKA + PKC (lane 4), xcTn treated with PKA + PKC (lane 5), cTn treated with PP1 (lane 6) and xcTn treated with PP1 (lane 7). Lane 1 is a marker lane for verification of cTn subunit molecular weights. The bottom row is the Western blot for lanes 2–7, to determine the level of cTnI phosphorylation. Details are provided in the text (see Methods and Results). *B*, contains three lanes: 1, a marker lane showing 29 kDa (*) and 19 kDa (**) molecular weight standards; 2, a Coommassie blue-stained gel of an untreated rat trabecula; and 3, the Western blot for phospho-serine cTnI. *C*, is a densitometric analysis of the Western blot in *A*, with values normalized to those obtained for no treatment. The Western blot demonstrates a significant level of cTnI phosphorylation in the trabeculae used for experiments, similar to the levels in the cTn complexes used for exchanges.

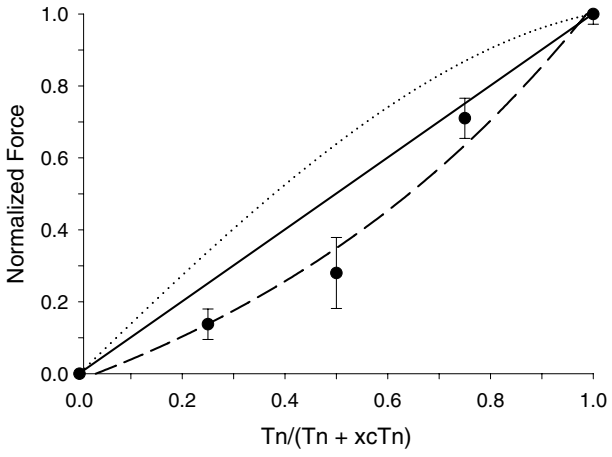


Figure 4. Effect of the proportion of functional cardiac troponin (cTn) on maximum force generation (pCa 4.0) in rat cardiac trabeculae (●)
For all mixture ratios > 0.25, F_{max} was less than proportionality between force and cTnC content (indicated by continuous line). Data from Regnier *et al.* (2001) for skeletal muscle containing different proportions of functional sTnC has been added to the figure (dotted line) to enable comparison.

mixtures contained only 25% cTn, while F_{max} was greatly reduced (0.16 of control F_{max}), pCa_{50} was decreased by only 0.11 units and n_H was unchanged from control. In comparison, we previously reported (Regnier *et al.* 2002) that reconstitution of skeletal fibre Tn complexes with sTnC + xxsTnC mixtures resulting in similar F_{max} (~0.15 of pre-extracted F_{max}), a 0.45 pCa unit shift to decreased Ca^{2+} sensitivity and significant reduction of n_H (1.7 *versus* 3.8 for control). This comparison demonstrates that the loss of near-neighbour FUs has much less effect on the pCa_{50} and n_H of force development in cardiac *versus* skeletal muscle.

The most frequently used method to replace native skeletal or cardiac TnC with recombinant TnC mutants has been the TnC extraction method (Gulati *et al.* 1991; Martyn & Gordon, 2001; Gillis *et al.* 2005*b*). This method works quite well for skeletal muscle, where virtually all of the native TnC can be extracted, providing for full reconstitution of Tn complexes with the TnC of choice. However, it has generally been less effective for cardiac muscle cell preparations, with most investigators reporting between ~11–24% remaining force (with saturating $[Ca^{2+}]$) following the extraction procedure. To determine how our results with the whole Tn exchange protocol compare with this method, the TnC extraction protocol (see Methods) was used on a small group of trabeculae from normal rats (containing mostly α-myosin) or PTU-treated rats (containing β-myosin), followed by incubation with 100% xcTnC. The results are summarized in Table 2. For these trabeculae the reconstituted F_{max} was 32 ± 3% and 20 ± 2% of pre-extracted values for normal and PTU-treated rats, respectively, comparing most closely

Table 2. Hill fit parameters for trabeculae containing either myosin isoform V1 or V3 following reconstitution with different cTn:xcTn mixtures

Myosin type	Treatment	F_{max}	pCa_{50}	n_H
V3 ($n = 5$)	Control	1.00	5.61 ± 0.03	3.3 ± 0.2
	IRUs	0.20 ± 0.02	5.27 ± 0.04	2.9 ± 0.3
V1 ($n = 4$)	Control	1.00	5.46 ± 0.02	4.7 ± 1.0
	IRUs	0.32 ± 0.03	5.17 ± 0.05	3.4 ± 0.5

Myosin V3 was expressed in rat hearts as a result of propylthiouracil (PTU) administered through drinking water (0.8%) for 4–8 weeks before the animals were killed. IRU, isolated regulatory unit; F_{max}, maximal Ca^{2+} activated force; pCa_{50}, Ca^{2+} sensitivity; n_H, apparent cooperatively.

to the values obtained for cTn exchanges with 50 : 50 and 25 : 75 mixtures of cTn:xcTn (Table 1), respectively. The decrease in Ca^{2+} sensitivity was ~0.3 pCa units for each group of trabeculae, and n_H was not significantly reduced (similar to the results of the cTn exchange protocol). However, in trabeculae reconstituted with 100% purified rat cTnC, we find an approximate 0.1–0.15 pCa unit decrease in Ca^{2+} sensitivity with similar or slightly reduced n_H compared with pre-extracted values (data not shown), suggesting that changes in these parameters are similar to those using the whole cTn exchange protocol. Thus it appears that either method of reducing the level of functional cTn has little or no effect on the apparent cooperativity (n_H) of cardiac thin filament activation and force development.

Because loss of near-neighbour RU interactions does not greatly influence n_H, the question is: what does? Fuchs & Wang (1991) and Martyn *et al.* (2001) have demonstrated that strong cross-bridge binding increases Ca^{2+} binding

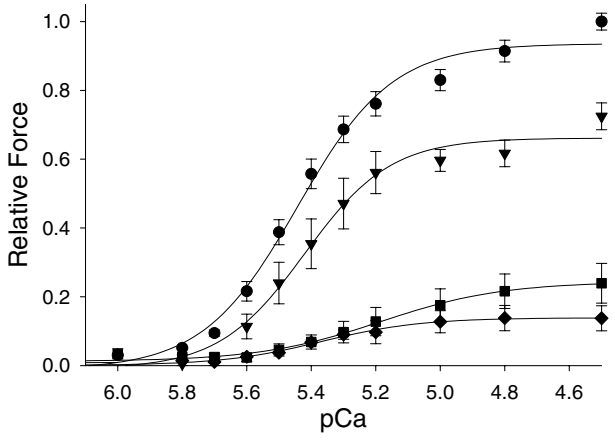


Figure 5. Force–pCa relations of cardiac trabeculae after replacement of endogenous cardiac troponin (cTn) with different proportions of functional/non-functional cTn/xcTn
A, endogenous cTn was replaced with mixtures containing: 25 : 75, cTn:xcTn (♦); 50 : 50 cTn:xcTn (■); 75 : 25, cTn:xcTn (▼); and 100 : 0, cTn:xcTn (●). *B*, replacement was with a 50 : 50 mixture of cTn/xcTn for three trabeculae where the exchanged F_{max} was 27 ± 4% of the pre-exchanged value.

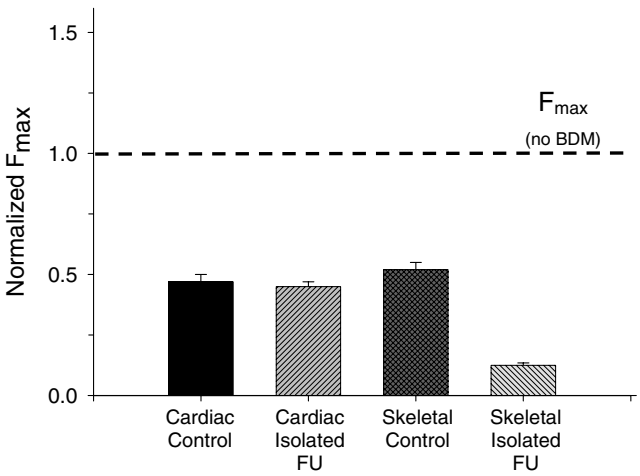


Figure 6. Effect of BDM on F_{max} (pCa 4.0) in skeletal and cardiac muscle for intact and isolated functional unit (FU) preparations
Force values are normalized to values obtained in the absence of BDM (dashed line) and F_{max} in isolated FU preparations was ~0.2 pre-extracted F_{max} prior to BDM treatment.

in cardiac muscle, but not skeletal muscle. Therefore we studied the effects of strong cross-bridge augmentation or inhibition on Ca^{2+}-activated force development in trabeculae with isolated FUs. Figure 6 demonstrates that F_{max} was inhibited by ~50% with addition of 10 mM BDM to the pCa 4.0 activation solution under control (pre-extraction) conditions. Following the reduction of FUs to obtain ~0.20 F_{max} the relative level of inhibition by BDM was not changed. Similar experiments were done in rabbit psoas skeletal fibres for comparison (Fig. 6). Under control conditions 10 mM BDM reduced F_{max} by ~50%, as in cardiac muscle. However, in contrast to cardiac muscle, the relative level of force inhibition following FU reduction to produce ~0.2 F_{max} was much greater (~85% reduction). Figure 7*A* shows the converse experiment, where augmenting the number of strong cross-bridges (via replacement of ATP with dATP) increased F_{max} both in pre-extraction (control) conditions and following the exchange protocol to produce ~0.2 F_{max} with isolated FUs in cardiac trabeculae (Regnier *et al.* 2002, 2004). By contrast, when this experiment was repeated using either rabbit psoas (fast) or soleus (slow) skeletal muscle, dATP did not increase F_{max} in control measurements but increased F_{max} by 40% when FUs were isolated in fast skeletal muscle (Fig. 7*B*). Together these experiments with BDM and dATP demonstrate that maximal activation of skeletal muscle fibres becomes more dependent on strong cross-bridge binding when the number of FUs is greatly reduced, but there is no increased dependence of maximal activation on strong cross-bridge formation in cardiac muscle with reduced numbers of FUs. The combined data suggest that near-neighbour FU interactions are more important for activation in skeletal (*versus* cardiac) muscle, and that the predominant form of apparent cooperativity in thin filament activation probably differs between the two striated muscle types.

Ca^{2+}-activated rate of force redevelopment (k_{tr}) with cTn mixtures

To determine how loss of near-neighbour RU interactions affects the maximal rate of force development, k_{tr} was

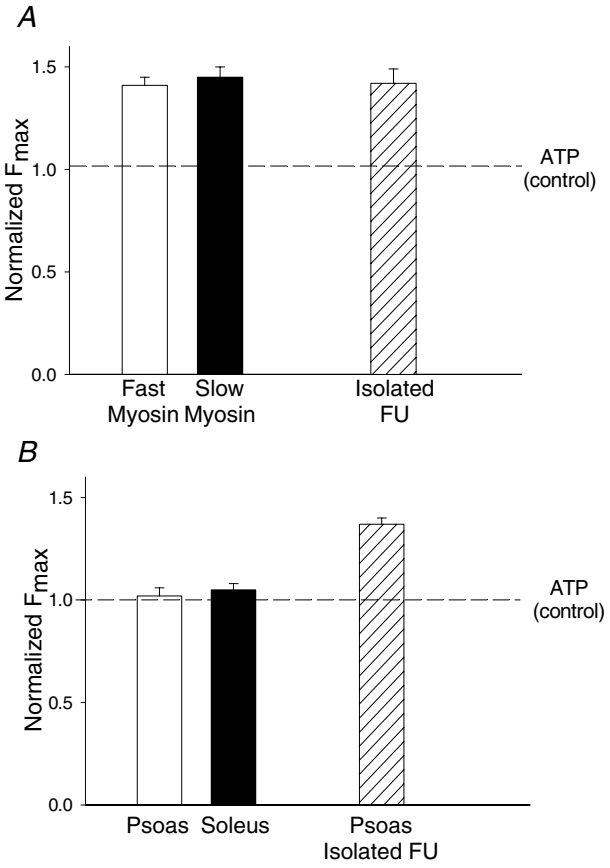


Figure 7. Effect of deoxy-ATP (dATP) on maximal force (F_{max}, pCa 4.0) in skeletal (*A*) and cardiac muscle (*B*) with intact and isolated regulatory units (RUs)
Reconstituted F_{max} for both skeletal and cardiac preparations with isolated FUs was ~0.2 F_{max}.

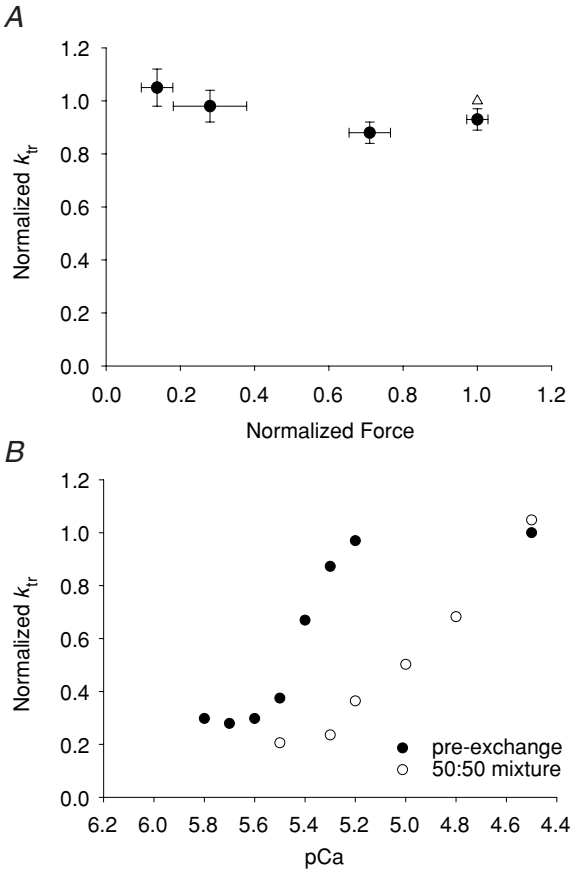


Figure 8. Effect of functional unit (FU) isolation on the maximum rate of force redevelopment (k_{tr}) (pCa 4.0; ●) (*A*) and the rate of force redevelopment over a range of pCa values for a single example trabeculae (*B*)
Data from measurements made prior to extraction (△) are added to the figure for comparison in *A*.

measured in solution of pCa 4.0 in all trabeculae for each of the different Tn mixtures. The data, summarized in Fig. 8*A*, clearly demonstrate that maximal k_{tr} is not dependent on either the number of FUs or the absolute level of isometric force production in cardiac muscle. We (Moreno-Gonzalez *et al.* 2003, 2007) and others (Morris *et al.* 2001) have demonstrated that this is also the case for skeletal muscle fibres reconstituted with mixtures of sTnC:xxsTnC or cTnC:xcTnC, respectively.

To determine whether loss of near-neighbour FU interactions alters the Ca^{2+} dependence of the rate of force development, we measured k_{tr} in a subset of trabeculae using a 50 : 50 cTn:xcTn exchange mixture that produced $21 \pm 4\%$ of the pre-exchange F_{max}. This level of exchange allowed for accurate measures over the range of pCa used to determine minimal and maximal k_{tr} values. The data for an example trabecula is shown in Fig. 8*B*, with values normalized to k_{tr} at pCa 4.0 prior to the whole cTn exchange protocol. The data demonstrate that the Ca^{2+} sensitivity of k_{tr} is right-shifted (less sensitivity) following the exchange protocol, by a similar extent as for steady-state force (Fig. 5 and Table 1). However, the Ca^{2+} dependence (maximum–minimum values) of k_{tr} appears to be unaffected by reductions in near-neighbour RU interactions. The data for three trabeculae were similar, suggesting that the mechanism(s) determining the Ca^{2+} dependence of k_{tr} is likely to reside within individual FUs with little influence of RU–RU interactions.

Ca^{2+} dissociation rates (k_{off}) from cTn and sTn

So why would cardiac muscle thin filament activation be more dependent on strong cross-bridge binding than skeletal muscle? One possible reason could be a less effective transmission of the Ca^{2+} signal for contractile activation, perhaps due to the different isoforms of Tn and/or Tm. For example, if the time Ca^{2+} stays bound to Tn is shorter in cardiac than in skeletal muscle, it may decrease the probability of strong cross-bridge binding in cardiac muscle. Therefore, to characterize kinetic differences between the Ca^{2+}-dependent activation of cardiac Tn and fast skeletal Tn, the k_{off} values of these two protein complexes were measured. The results illustrate that the rate of Ca^{2+} dissociation from the N-terminus of cTn ($66.0 \pm 1.0\ s^{-1}$) is 2.5-fold greater than that of sTn ($25.8 \pm 0.1\ s^{-1}$) at 15°C (Fig. 9). Ca^{2+} dissociation from the C-terminus was at least two orders of magnitude slower for cTn and sTn ($0.67 \pm 0.01\ s^{-1}$ and $0.15 \pm 0.01\ s^{-1}$, respectively) and did not influence the N-terminal measurements. If it is assumed that Ca^{2+} binding affinity of the TnC Ca^{2+} trigger sites (N-terminus) is determined primarily by k_{off} (Johnson *et al.* 1994), our results support a hypothesis that shorter interaction time of cTnC with cTnI, compared with the interaction of sTnC with sTnI, could effectively provide a lower level of thin filament activation at any given $[Ca^{2+}]$.

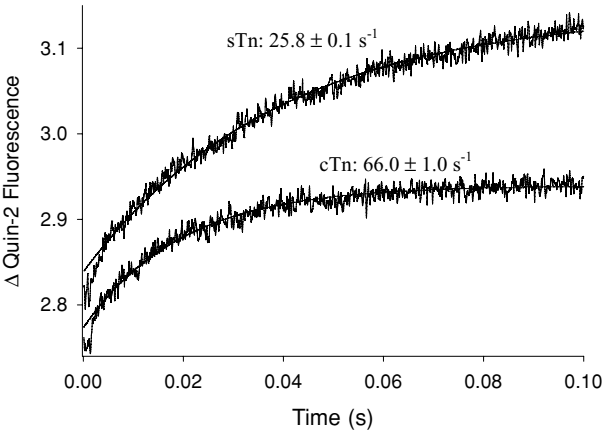


Figure 9. Comparison of the rates of Ca^{2+} dissociation from the N-terminus of cadiac and skeletal troponin at 15°C
The troponin complexes, in the presence of 30 μM Ca^{2+}, were mixed rapidly with 150 μM Quin-2. Fluorescence was monitored through a 510 nm broad band-pass interference filter. The traces have been displaced vertically to allow comparison. Ca^{2+} dissociation from the C-terminus of cTn and sTn at 15°C was $0.67 \pm 0.01\ s^{-1}$ and $0.15 \pm 0.01\ s^{-1}$, respectively.

Discussion

The goal of this study was to investigate how the interaction of Tn complexes along thin filaments influences the level and rate of force development in rat cardiac muscle during isometric contraction and to study the activation properties of thin filament RUs when these interactions are greatly reduced. The main conclusions from this work are that (1) Ca^{2+} binding to each cTn activates a length of thin filament less than or similar to that of a structural RU (A_7TmTn; i.e. the functional regulatory unit (FU) is equal to the RU), (2) the Ca^{2+} sensitivity (pCa_{50}) of thin filament activation (as measured by steady-state force) is determined predominantly within the FU and (3) the Ca^{2+} dependence and maximal rate of force development (as determined from k_{tr}) is determined by the properties of individual FUs. Additionally, the limited spread of activation along thin filaments may be due, at least in part, to the characteristic properties of cTn following Ca^{2+} binding. Integration of the results with similar studies using skeletal muscle fibres (both here and from other studies) provide clues to the unique activation properties of cardiac thin filaments that allow the fine tuning of cardiac contractility at the cellular level that is required on a beat-to-beat basis.

Our interpretation of the results in this study relies on the assumption that the recombinant cTn exchanged into the trabeculae binds along the entire length of the thin filament and that the binding affinity of cTn and xcTn complexes are similar. We have previously demonstrated through colocalization studies of phalloidin

green-labelled actin and rhodamine-labelled exchanged into trabeculae that there is uniform distribution of the exchanged xcTn along the thin filament (Kohler *et al.* 2003). Comparison of the fluorescence intensity scans of the labelled xcTn along the trabeculae with that of actin illustrated that the two labelled molecules are present in the same location throughout the preparation. Additionally, xcTn and cTn bind to the thin filament with similar affinity in low Ca^{2+} solutions, as determined by Butters *et al.* (1997). These results, together with our demonstration that exchange with xcTn can almost eliminate (up to ∼98%) Ca^{2+}-activated force production, suggest that cTn is uniformly and effectively replaced throughout the thin filament. Another potential concern is that cTn complexes used for exchange were composed of cTnI and cTnT purified from bovine heart and recombinant rat cTnC (WT or xcTnC). The amino acid sequence of rat cTnI is 89% similar to bovine cTnI and rat cTnT is 87% similar to bovine cTnT. The use of 'chimeric' cTn in this replacement could result in a difference in how the subunits interact (compared with all rat proteins) as a result of the sequence differences. However, the control measurements were made using 100% chimeric cTn (or xcTn). There was a small decrease in the Ca^{2+} sensitivity of force following exchange of native cTn with WT cTnC-cTn. This decrease does not appear to result from non-specific extraction of other sarcomere contractile proteins, as demonstrated in Fig. 2. It could result from a different phosphorylation state of the exchanged WT cTnC-cTn, as Fig. 3 shows the bovine cTnI used was phosphorylated. However, Fig. 3 also demonstrates cTnI phosphorylation of the native rat cTnI. Thus, we believe our results were not due to loss of sarcomeric proteins and are at least relatively independent of phosphorylation status.

Cooperative thin filament activation in cardiac *versus* skeletal muscle

A primary purpose of the present study was to compare experiments in cardiac muscle with similar data we have obtained in fast skeletal muscle here and in previous work (Regnier *et al.* 2002; Moreno-Gonzalez *et al.* 2005). The results of the current study suggest that while cardiac and skeletal muscle myofilament structure is similar and they contain the same compliment of thin and thick filament proteins, the underlying mechanisms that regulate thin filament activation and steady-state force development in chemically demembranated preparations differ. Here we characterized the role of individual FUs compared with near-neighbour FU interactions along the thin filament during contractile activation by replacing endogenous Tn with different mixtures of 'functional' and 'non-functional' Tn to increase isolation of FUs in cardiac trabeculae. In skeletal muscle, we were able to accomplish this by extraction of endogenous sTnC and replacement with mixtures of 'functional' and 'non-functional' (D28A and D63A) sTnC. Interestingly, our F_{max} *versus* the fraction of functional cTn data suggest that the size of the FU in cardiac muscle (7 actins) is less than that found with similar measurements in skeletal muscle (10–12 actins; Regnier *et al.* 2002) when Ca^{2+} binds to an individual Tn (Fig. 4). This result implies that the activation of a single Tn complex by Ca^{2+} binding in cardiac muscle 'turns on' a smaller length of the thin filament than in skeletal muscle. A potential implication of this is that smaller FU size may result in less near-neighbour FU interaction in cardiac (*versus* skeletal) muscle and, consequently, a greater reliance on local (within each FU) cooperative feedback mechanisms for contractile activation.

The definition of FU size that we have presented here is the length of the thin filament (i.e. the number of actins) activated by Ca^{2+} binding to a single Tn. However, if the FU is defined as the conditions that are required to most completely activate an RU (A_7TmTn), then it may be that an FU contains two or three RUs if Ca^{2+} binding to neighbouring Tn complexes can more completely activate an RU. This conclusion was reached from the solution studies of Butters *et al.* (1997), who used similar methods to vary the fraction of FUs in reconstituted cardiac thin filaments. They found a less than proportional increase in maximal Ca^{2+}-activated cardiac S1-thin filament ATPase as the fraction of functional RUs was increased, with the shape of the curve being quite similar to what we found for F_{max} *versus* the fraction of functional Tn (Fig. 4). Their interpretation of the data was that individual RUs were fully activated only when neighbouring RUs are simultaneously activated by Ca^{2+}. As such, both solution and cellular mechanical data indicate that the FU size (with Ca^{2+} binding to a single Tn) is smaller in cardiac muscle. Additionally, both definitions of FU size could explain the moderate shift in the force–pCa curve (Table 1) as cTn content in trabeculae is increased. However, whether Ca^{2+} binding to neighbouring Tn complexes on cardiac thin filaments contributes significantly to cooperative activation remains an open question. This is because, if we achieved isolation of individual RUs (i.e. each functional Tn is surrounded by non-functional Tn on each side) it is more difficult to determine how loss of near-neighbour RU interactions has little influence on n_H unless a primary form of cooperativity in thin filament activation resides within the individual RU. Alternatively, if Ca^{2+}-dependent activation of an RU requires activation of neighbouring RUs, these completely isolated RUs may not be functional. It may be that the only RUs that can produce force are ones with neighbouring RUs that can bind Ca^{2+} and activate, as suggested by Butters *et al.* (1997). If this is the case, n_H may be less dependent on the fraction of functional cTn in thin filaments. The present work does not allow us to confidently distinguish between these two possibilities.

Additional support for the hypothesis of minimal interaction between FUs comes from the force–pCa relationship. In skeletal muscle we found that isolating FUs greatly reduced n_H and pCa_{50} of the force–pCa relationship (Regnier *et al.* 2002), suggesting that spread of activation beyond the boundaries of an RU with Ca^{2+} binding into near-neighbour RUs, appear to be the primary determinant of cooperative activation in skeletal muscle. In agreement with our study, Moss *et al.* (1985) showed that partial extraction of sTnC resulted in a reduced F_{max} and a right-shift of the relative force–pCa relationship. These authors suggested that the Ca^{2+} sensitivity of an RU within the thin filament may vary with the state of activation of adjacent RUs. Our finding that isolation of FUs in cardiac muscle has little effect on n_H (Table 1) indicates that the steepness of the force–pCa curve probably involves cooperative mechanisms within individual RUs and that interactions between neighbouring RUs has less influence (at least at submaximal levels of Ca^{2+}) in cardiac muscle. The decrease in pCa_{50} was also much less than in skeletal muscle, but may reflect at least some near-neighbour interactions. Additionally, in experiments where native cTnC was extracted until the remaining F_{max} was 20–30% of pre-extracted values, we found similar results to when the whole cTn exchange protocol was used. Whether or not the cTnC of these preparations was randomly extracted from thin filaments, it supports our conclusion that reducing near-neighbour FU interactions has little effect on n_H in cardiac muscle. Therefore, our current results in cardiac muscle illustrate a difference in the mechanisms that determine the highly cooperative Ca^{2+} dependence of force development in the two muscle types (see below).

Cooperative events within individual FUs

In a recent study we (Moreno-Gonzalez *et al.* 2005) found that when skeletal fibre near-neighbour FU interactions are reduced, the level of thin filament activation at lower $[Ca^{2+}]$ is determined primarily by the direct effects of Ca^{2+} on regulatory protein mobility, while at higher $[Ca^{2+}]$ the final level of thin filament activation is primarily determined by strong cycling cross-bridges. Work by Fitzsimons *et al.* (2001) also supports the role of strong cycling cross-bridges in fully activating the skeletal thin filament. In cardiac muscle, the cross-bridge-dependent component controlling the force–pCa curve may predominate under normal conditions as well as when near-neighbour FU interactions are minimized. This would explain the relative insensitivity of cardiac pCa_{50} to loss of near-neighbour FU interactions (~0.1–0.2 pCa units; Table 1) compared with skeletal muscle (~0.5 pCa units; Regnier *et al.* 2002). It could also explain the interesting difference between these two muscle types, namely that loss of near-neighbour FU interactions greatly reduced n_H in skeletal (Regnier *et al.* 2002) but not cardiac muscle (Table 1). As mentioned above, this suggests that cooperative activation mechanisms are controlled locally in cardiac muscle, at the level of individual FUs.

One 'local' form of positive cooperative events is strong crossbridge feedback to increase Ca^{2+} binding to Tn. Both $^{45}Ca^{2+}$ binding (Fuchs & Wang, 1991; Wang *et al.* 2001) and TnC structural changes associated with increased TnC–TnI interaction (Martyn *et al.* 1999, 2001) are enhanced by strong cross-bridges in cardiac, but not skeletal muscle. As such, cross-bridge-induced increases in cTn Ca^{2+} binding may be a significant mechanism of cooperative activation in cardiac muscle (see below).

A potential consequence of smaller FU size may be that Ca^{2+} binding to cTn is less effective in activating cardiac thin filaments. The formation of strong cross-bridges in the cardiac thin filament may act to stabilize the interaction of cTnC with cTnI and therefore might be required to fully activate the thin filaments (or maintain activation). Several lines of evidence support this hypothesis. Using preparations with isolated FUs, we have shown that both inhibition (Fig. 6) and augmentation (Fig. 7) of strong cross-bridge binding greatly influences F_{max} in skeletal, but not cardiac muscle. BDM, which inhibits cross-bridge transition from weak to force-generating states (Regnier *et al.* 1995) had a much larger effect on F_{max} of rabbit psoas skeletal muscle fibres after reconstitution with only small fractions of functional sTnC. We concluded that this was due to the loss of near-neighbour FU interactions (spread of activation along thin filaments) in psoas fibres. Conversely, strong cross-bridge inhibition by BDM had no greater (relative) effect on F_{max} with the loss of near-neighbour FU interactions in rat trabeculae (Fig. 6), suggesting that Ca^{2+}-mediated strong cross-bridge binding and force development are controlled mainly at the level of individual FUs. Additionally, in the present study (Fig. 7) and in previous work, we have provided evidence that thin filaments of normal cardiac muscle may not be completely activated even with saturating levels of Ca^{2+}. This is suggested by the fact that augmenting strong cross-bridge binding (as determined from stiffness measurements) with dATP substantially increases both F_{max} and maximal k_{tr} (Regnier *et al.* 2000, 2004) in cardiac muscle. Indeed, the increase in F_{max} for control trabeculae is similar to that seen in preparations with isolated FUs (Fig. 8*A*). This is not due to a dATP-induced increase in the rate of cross-bridge cycling *per se*, because the enhancement of steady-state force and force development kinetics was independent of cardiac myosin isoform (α versus β myosin heavy chain). Also shown in Fig. 7, the augmentation is particular to cardiac muscle, as dATP does not increase F_{max} of either fast or slow twitch rabbit skeletal muscle fibres. As soleus muscle contains cardiac TnC, it suggests that the augmentation of F_{max} in cardiac muscle is not due specifically to TnC isoform either, but could be due to TnC interaction with

other Tn subunits (see below). It is interesting that in skeletal muscle fibres with isolated FUs, dATP increased F_{max} by approximately 40% (Fig. 7*B*), similar to what is seen in cardiac muscle. Taken together the data suggest that strong interaction between near-neighbour RUs and/or the extent of spread in activation along thin filaments are/is an important determinant of the amount of cross-bridge binding and force generation, and that this is more limited in cardiac (*versus* skeletal) muscle.

Maximal and submaximal k_{tr} are determined by properties of individual FUs

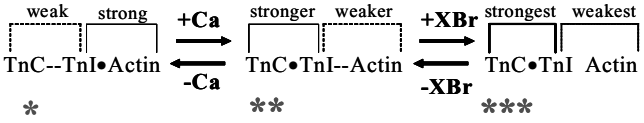
Similar to steady-state force, k_{tr} appears to be controlled at the level of individual FUs in cardiac muscle. We found no influence of near-neighbour FU interactions on maximal k_{tr} (Fig. 8*A*). Similarly, we recently reported that loss of near-neighbour FU interactions has minimal effect on maximal k_{tr} in skeletal fibres (Moreno-Gonzalez *et al.* 2003, 2007). Additionally, Morris *et al.* (2001) found similar results in skeletal muscle fibres when endogenous TnC was extracted and Tn complexes were reconstituted with varying mixtures of functional and non-functional cardiac TnC. We also found that decreasing the number of FUs has little or no effect on the Ca^{2+} dependence of k_{tr} in cardiac muscle (Fig. 8*B*). We previously reported minimal effect of decreasing FUs on the Ca^{2+} dependence of k_{tr} in skeletal fibres (Moreno-Gonzalez *et al.* 2003, 2007). However, when native TnC of skeletal muscle fibres was replaced with cTnC or an sTnC mutant, both of which had lower Ca^{2+} binding affinity, maximal k_{tr} and the Ca^{2+} dependence of k_{tr} were greatly affected independent of force. Thus, it appears that k_{tr} is controlled by the properties of regulatory proteins and by mechanisms that work at the level of individual FUs in both cardiac and skeletal muscle.

The role of troponin in cooperative activation

One possible explanation for why strong cross-bridges enhance Ca^{2+} binding to cTn in cardiac muscle is that they may strengthen the interaction between cTnI and the N-terminus of cTnC (cNTnC) during activation. Solution NMR studies by Li *et al.* (1999) demonstrated that the strength of interaction between the hydrophobic patch of cNTnC (exposed upon Ca^{2+}-dependent activation) and the cTnI peptide 147–163 is six times less than that between sNTnC and the corresponding sTnI peptide. It is this interaction that is thought to pull cTnI away from an inhibitory position on the actin monomers of the thin filament. A likely reason for this lower strength of interaction between cardiac proteins is that the size of the hydrophobic patch exposed on cNTnC with Ca^{2+}-dependent activation is approximately one-third the size of that exposed on sNTnC (162 Å^2 *versus* 500 Å^2) (Gagne *et al.* 1995; Li *et al.* 1999). There are two possible consequences of the lower strength of interaction between cTnC and cTnI during Ca^{2+}-dependent activation. The first is that it is harder for cTnC to 'pull' cTnI away from interacting with actin and assume the 'activated' conformation. The second is that it may be easier for activated cTn to become deactivated by the cTnI re-establishing a strong interaction with actin. There is also a measurable delay between Ca^{2+} binding or dissociation and the associated confirmation changes in isolated cTnC or as part of the Tn complex, as measured by extrinsic fluorescent probes in solution (Dong & Cheung, 1996; Dong *et al.* 1997; Hazard *et al.* 1998). Furthermore, the conformational changes for saturated Ca^{2+} binding are not complete, indicating that not all cTnC is in the activated conformation (Dong & Cheung, 1996; Dong *et al.* 1997). This could have great significance in terms of thin filament activation in cardiac muscle.

In the current study, the k_{off} of Ca^{2+} from the N-terminus of cTn was 2.5-fold faster than that from sTn. This measurement represents Ca^{2+} dissociation from cTnC site II while the protein complex is in the activated state. As a result, the protein complex would become deactivated as the hydrophobic patch on the N-terminus of cTnC loses contact with the switch region of cTnI (Li *et al.* 2004). The difference in k_{off} between cTn and sTn suggests that, under identical conditions, cTn may deactivate at a faster rate and would therefore be in the activated state for a shorter period of time than sTn. This could be due, at least in part, to site II of cTnC having a lower Ca^{2+} affinity than the N-terminus of sTnC (sites I and II). An additional factor could be a lower affinity of cNTnC for the C-terminus of cTnI during activation. The k_{off} data (Fig. 9) suggest there may be fundamental differences in the functional characteristics of sTn and cTn, such that the kinetics of Tn subunit interactions can influence initial strong cross-bridge binding and subsequent cTn-mediated thin filament activation.

The proposed interdependence of Ca^{2+} and cross-bridge binding in thin filament activation is illustrated in Scheme 1, which describes the relative binding affinity of cTnI for cTnC *versus* actin, in the absence and presence of Ca^{2+} and cross-bridges. In the absence of Ca^{2+} and cross-bridges, the strength of cTnI interaction with cNTnC is weak whereas cTnI interaction with actin monomers is strong (*). When Ca^{2+} binds to cTnC, the interaction with cTnI strengthens and cTnI interaction with actin weakens (**). Subsequent strong cross-bridges further weaken cTnI interaction with actin



and stabilize the interaction with cTnC, as demonstrated by Robinson *et al.* (2004).

The role of Tm in cooperative activation

Above, we discussed possible mechanistic differences between cardiac and skeletal muscle Tn in cooperative activation of thin filaments and force generation. These mechanistic differences are likely to occur 'locally' (i.e. within the confines of an FU or between two neighbouring RUs). However, the biophysical nature of Tm may be an important determinant of the size of FUs. In other words, one significant mechanistic difference between cardiac and skeletal muscle may be the role that Tm plays in transmitting the activation signal along the thin filament via end-to-end Tm interactions in neighbouring RUs. Chandy *et al.* (1999) demonstrated that cardiac Tm is more mobile than skeletal Tm. This greater mobility could result in greater hysteresis of cardiac Tm movement across the surface of actin with Ca^{2+}-dependent activation, compared with skeletal Tm. A 'stiffer' Tm could facilitate exposure of additional myosin binding sites beyond the boundaries of a Ca^{2+}-activated RU, into neighbouring RUs along the thin filament. In cardiac muscle the more flexible or 'floppy' Tm would limit the number of actins made available for myosin binding with the activation of a single FU. The importance of strong cross-bridge binding and facilitating exposure of a maximal number of myosin binding sites may therefore be more critical in allowing cardiac (*versus* skeletal) muscle to be fully activated. Studies by Lehman *et al.* (2000) using electron microscopy and image reconstruction have demonstrated that cardiac Tm is in a more inhibitory position on the thin filament than skeletal Tm at low $[Ca^{2+}]$. This implies that cardiac Tm would have to move further to expose the same number of myosin binding sites during the Ca^{2+}-dependent activation compared with skeletal Tm. Additionally, if the Ca^{2+}–Tn interaction time is shorter in cardiac muscle (as indicated from a faster k_{off}; Fig. 9), TnC–TnI interaction time may be less as well, potentially reducing the time that Tm is mobile. This could result in fewer cross-bridge binding sites exposed and/or a reduced time of exposure at the same level of Ca^{2+}. Finally, if TnC–TnI interaction is weaker/shorter in cardiac muscle, the resulting stronger TnI–actin interaction could further reduce Tm mobility and/or the time that Tm is mobile.

Significance

An important consequence of cardiac thin filaments not being fully activated by Ca^{2+} alone may be that the level of activation can be more affected by increases/decreases in strong cross-bridge binding. In other words, Ca^{2+} binding to cTn enables initial cross-bridge binding, but the activation level is determined and maintained more directly by the number of strong cross-bridges that are bound. This would provide cardiac muscle with a way to finely control activation and the level of force generation in individual sarcomeres. Such fine control at the cellular (sarcomere) level is important because all cardiomyocytes are activated during systole, in contrast to skeletal muscle where a main mechanism of varying force is via the number of fibres recruited. The greater dependence of cardiac muscle activation on strong cross-bridges may also explain the steeper length dependence of force in cardiac *versus* skeletal muscle and provide a mechanism for rapid deactivation at the end of systole at shorter sarcomere lengths.

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Investigation of thin filament near-neighbour regulatory unit interactions during force development in skinned cardiac and skeleta muscle

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